

University of Nevada, Reno

**Separation and Purification of a Monoclonal Antibody for use in Patients Suffering from  
Wet Macular Degeneration**

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

Bachelor of Science in Chemical Engineering and the Honors Program

by

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**ABSTRACT**

Novel process designs were created in an effort to optimize the efficiency of monoclonal antibody separation and purification for an industrial process. A base case was established that utilized common protein separation techniques such as cation exchange chromatography, anion exchange chromatography, and hydrophobic interaction chromatography (HIC). Alternate process designs were also made that utilized immobilized metal affinity chromatography (IMAC) and various multimodal chromatography mediums. Experiments were conducted to determine the most effective mediums that could be used in the process design. To better analyze the new alternate plans, an economic analysis was conducted which determined what alternate plan would be more cost effective. From analysis, alternate design 1 and alternate design 2a were determined to be the preferred design due to enhanced performance and lesser overall capital cost.

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## **INTRODUCTION**

The objective of this project is to analyze and design a process in which a monoclonal antibody fragment is separated and purified for use in medicine. The monoclonal antibody is an antigen binding fragment (Fab) that mimics Lucentis (Ranibizumab). The senior design team works in conjunction with the pharmaceutical company, Genentech, to design this purification process. In an effort to have a rational design, Genentech has provided various parameters to better guide the process. To start off, it is assumed that the design will have to accommodate 1000L of fermented culture in which the original Fab concentration is 5 g/L. Within the 1000L fermented culture, 20% to 25% is assumed to be solid cell content which includes cells and cellular debris. Within the design process, a maximum of eight unit operations can be used from start to finish. The maximum allotted runtime for each unit operation is 24 hours. It must be assumed that each unit operation is at least 90% efficient with respect to its operation and performance. An overall industrial yield of 65%-75% is desired with a purity of 99%.

Ranibizumab is a humanized monoclonal antibody that is derived from the Immunoglobulin G (IgG) class <sup>1</sup>. The molecular weight of the Fab is 100 kDa and its isoelectric point (pI) is 7.15. Once purified, Ranibizumab is used to treat ailments such as macular degeneration, which plagues 11-12 million people in the United States alone <sup>2</sup>. Macular degeneration typically occurs late in life (retirement age) and is a condition in which excessive vascular growth near the macula causes tears within the retinal epithelial lining. Vascular growth is caused by Vascular Endothelial Growth Factors (VEGF) which bind onto the blood vessel lining. Once attached to receptors located on the blood vessel lining, the VEGF induces incomplete growth. That is, the blood vessels grow but

oftentimes leak blood and other fluids. The fluids then fill up around the macula which causes swelling in the area. This swelling oftentimes leads to small tears within the retina and a severe distortion in vision. Ranibizumab works by binding onto the VEGF proteins so that it cannot attach to the blood vessel receptors; thus stopping growth and fluid leakage.

Now that the baby boomer generation is in the transition to retirement, it is imperative to create more efficient processes that can effectively separate and purify Ranibizumab since the need is expected to increase dramatically. Consequently, this project aims to create process designs which increase the efficiency of separation and purification of the monoclonal antibody, Ranibizumab, for intraocular use in patients suffering from ailments such as macular degeneration.

## **THEORY**

To better design a process, it is imperative to have some point of reference from which one can deviate from. As a result, a base case is made. This base case is assumed to be the current process that is utilized by Genentech to purify and separate the Fab from solution. One obstacle to the creation of a base case is that the actual process used by Genentech is not known. Since this is proprietary information, Genentech does not openly disclose their process. As a result, pieces of information obtained from feedback throughout the years are used to make a base case. Since it was encouraged to work off of previous years' work, a base case was made and is shown in Figure 1.

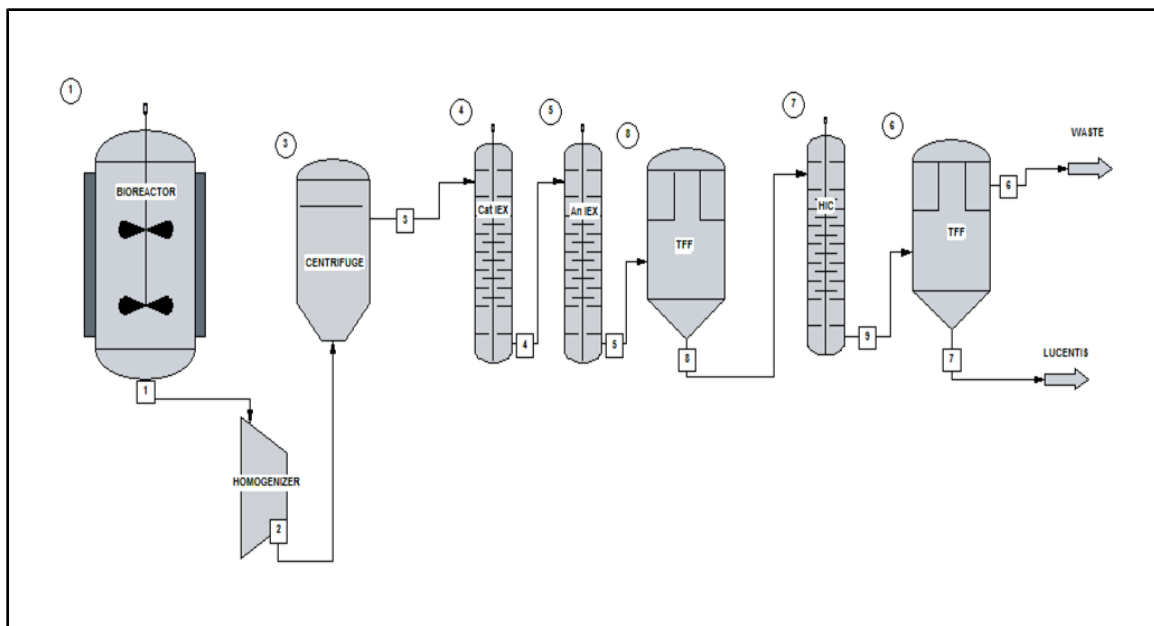


Figure 1: Block flow diagram which illustrates the base case designed.

From figure 1, it is seen that the first unit operation is the bioreactor which will be used to grow and ferment the E. Coli. The cell culture will be used to express the Fab which has been genetically inserted beforehand. Once it is time to harvest the protein, the 1000L fermented batch will be passed through a high pressure homogenizer that will lyse the E. Coli and cause the Fab to be released into the solution. Once all the cells are properly homogenized, the batch will be sent to a disk centrifuge which will remove the solid content from solution. The batch will then be sent through a cation exchange chromatography column which will be the first step of protein separation. This is followed by an anion exchange chromatography column in which the proteins are further separated. Following the two chromatography unit operations is a tangential flow filter operation which further purifies and concentrates the Fab. The last separation unit operation is HIC which will be used as a polishing step. The final unit operation will be

the tangential flow filter which is used to return the Fab to proper pH and physiological conditions. A better explanation of each unit operation is given below.

### Bioreactor

Since most Fab proteins are still made in vivo, a bioreactor is necessary to grow a cell culture that produces a protein of interest. There are various parameters that must be maintained throughout the bioreactor operation, these parameters include temperature, stir speed, pH, and dissolved oxygen/nutrient concentration. Oxygen will also need to be bubbled up through the solution to ensure uniform concentration. Additionally, nutrients such as glucose/sucrose will be introduced to promote cell growth by the following equation:

$$\frac{dX}{dt} = \mu_{max} \frac{S}{K_s + S} X - \frac{F}{V} X \quad \text{Eqn. 1}$$

Equation 1 represents a mass balance around the substrate entering the reactor. X is the amount of biomass in the reactor, where dX/dt is the change in biomass per unit time.  $\mu_{max}$  is the maximum cell growth rate during the exponential phase of growth within the reactor. S represents the substrate, while  $K_s$  is the growth constant, F is the feed volume, and V is the reactor volume, respectively<sup>3</sup>.

The four growth regions for E. Coli growth include the lag phase, exponential phase, and stationary phase, followed by the death phase. The lag phase involves minimal increase in cell density and a period of adaptation of the cells. Cells divide at a constant rate during the exponential phase, and the biomass can be expressed as a function of the growth and death rate:

$$\frac{dX}{dt} = (\mu - k_d)X \quad \text{Eqn. 2}$$

Where  $X$  is the biomass,  $\mu$  is the growth rate, and  $K_d$  is the cell death rate. The stationary phase represents equilibrium in the growth and death rate of the cells. The death phase occurs when the death rate of cells exceeds the growth rate. This rate is illustrated by equation 3:

$$\frac{dX}{dt} = -K_d X \quad \text{Eqn. 3}$$

A jacketed bioreactor is preferred so that heat generated by cell growth can be dissipated more efficiently. A jacketed bioreactor will also allow for constant temperature maintenance at 37 °C. The pH will be controlled at 7 (neutral) and the agitator will be used to maintain a homogeneous mixture.

Oxygen must be continuously supplied in aerobic processes in order to maximize productivity. Oxygen transfer rate and oxygen consumption by the cell must be well understood for this to be achieved. Often oxygen becomes the factor governing metabolic pathways in microbial cells, therefore dissolved oxygen in the broth must be closely monitored. Oxygen uptake rate (OUR) is most commonly calculated from inlet and outlet gas phase concentrations used in the following mass balance <sup>4</sup>:

$$OUR = K_L a (C^* - C_L) \quad \text{Eqn. 4}$$

Where  $K_L a$  is the volumetric oxygen mass transfer coefficient,  $C^*$  is the saturated dissolved oxygen concentration, and  $C_L$  represents the actual dissolved  $O_2$  concentration in the bulk phase. From equation 4, the following relationship describing  $C^*$  can be derived:

$$C^* = e^{-K_L a t} + C_L \quad \text{Eqn. 5}$$

It is important to note that  $K_L a$  for stirred tanks can be calculated based on equation 6, knowing the empirical constant ( $k$ ), the power requirement ( $P_g$ ), bioreactor volume ( $V_R$ ),

superficial gas exit speed ( $v_s$ ), volumetric air flow rate ( $F_a$ ), bioreactor cross-sectional area ( $A$ ), and impeller rotation speed ( $N$ ):

$$K_L a = k \left( \frac{P_g}{V_R} \right)^{0.4} (v_s)^{0.5} (N)^{0.5} \quad \text{Eqn. 6}$$

These units depend on correlation data and experimentally determined  $K_L a$  values are preferred.

Heat generation from microbial cell metabolism is also an important factor to consider when it comes to bioreactor design. Total heat evolved ( $Q_{GR}$ ), then, depends primarily on the density of the culture, volume of the culture, and growth rate of the culture, and can be described by the following relationship:

$$Q_{GR} = V_L \mu X Y_H \quad \text{Eqn. 7}$$

Where  $V_L$  is the volume of the cell culture and  $Y_H$  is the metabolic heat evolved per gram of cell produced.  $Y_H$  depends upon the degree of oxidation of the substrate. The value of  $Y_H$  can vary from 2.4 kcal/g to 16.4 kcal/g depending upon the substrate.  $Q_{GR}$  can be calculated as the product of OUR for aerobic fermentations as seen below.

$$Q_{GR} = 0.12 OUR \quad \text{Eqn. 8}$$

#### Homogenizer

The homogenizer is the first step in the purification and separation process since the bioreactor grows the antibody of interest. Cells are killed by a high pressure buildup across the homogenization valve, which puts shear stress on the cell wall that causes cell lysis<sup>5</sup>. This unit operation starts with a fermented feed from the bioreactor that enters the valve chamber in periodic variations (or pulsatile flow). These periods cause the valves to close to create cell suspensions against an impact ring of hard material that serves as the inner wall for the chamber. The homogenizer forces cell suspensions through narrow

channels under high-pressure at a high velocity on a hard-impact ring. The valve then opens which causes a severe drop in pressure. This causes the cells to lyse. The lysate can either exit or be passed through the homogenizer again for further processing. Typical high pressure homogenizers operate at 15,000psi to 20,000psi to lyse E. Coli <sup>6</sup>. It is imperative that the operating pressure is not excessively high; for higher pressure drops will correspond to more efficient cell lysing but may also denature the antibody of interest.

Since the pressure drop is the most important aspect of the high pressure homogenizer, empirical equations have been developed to better determine the difference in pressure as a function of flow as seen below. The following equation is the basis for determining how many passes are needed for cell lysis in a high pressure homogenizer:

$$\log\left(\frac{R_m}{R_m-R}\right) = KN \quad \text{Eqn. 9}$$

Where R is the amount of soluble protein released after an N number of cycles through the homogenizer,  $R_m$  is the maximum release of the protein, K is a temperature dependent first-order rate constant denoted as  $kP^x[\text{fn}(T)]$  with k and x depending on the E-coli grown in the bioreactor and P being the operating pressure. This equation is important to know because it gives an idea about how many passes are required to achieve the desired yield of protein for further purification steps, what is the temperature of the E. Coli at the inlet and outlet, how much cooling will be needed to compensate the rise in temperature from the rising operating pressure in the fixed volume of the unit operation.

An increase in the inlet flow rate and operating pressure will cause a temperature increase that must be taken into account when adding a cooling system in between

passes. The temperature increase is very important to consider since without appropriate cooling this could lead to the inactivity of the desired protein from the bioreactor step that can be caused primarily by excessive shear stresses. Figure 2 below shows how additional parameters are necessary to keep the process continuous and take into account various mass and energy balances.

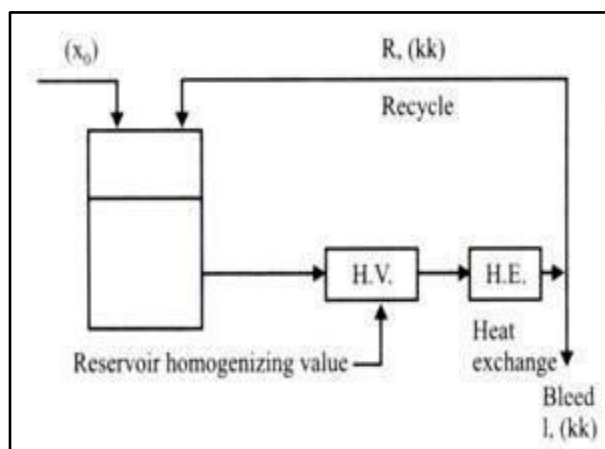


Figure 2: Example of a high-pressure homogenizer operated continuously<sup>7</sup>.

#### Disk Centrifuge

A disk centrifuge is a device used to separate solid particulates from solution. This unit operation is necessary since there are solid particulates within the fermented culture post homogenization. The lysing of E. Coli causes small solid particles to be suspended in fermented solution. This presents a lot of downstream problems if left untreated. Homogenization utilizes rotation a means of separation from cytoplasmic solution and cellular debris. Disk centrifuges work by introducing feed through the middle of the unit operation while rotating. Through time, the solid particulates are deposited along the side of the bowl and the purified liquid is pumped through the top of

the module. Since there is solid material accumulating along the side of the bowl, the bowl occasionally opens up to allow the material to exit as a waste stream. Since solid particulates separate naturally from solution via gravity, rate at which settling occurs is the settling velocity as shown by equation 10.

$$v_s = \frac{(\rho_{cell} - \rho_{solution})gd^2}{18\mu} \quad \text{Eqn. 10}$$

Where  $\rho_{cell}$  is the density of the cellular debris,  $\rho_{solution}$  is the density of the solution,  $g$  is the force of gravity,  $d$  is the average particulate diameter, and  $\mu$  in this case is the viscosity of the solution<sup>8</sup>. The settling velocity can then be used to determine the recommended flow throughout the centrifuge as seen in equation 11.

$$Q = \frac{4\pi\omega^2 v_s (N-1)(r_2^3 - r_1^3)}{3g \tan \theta} \quad \text{Eqn. 11}$$

Where  $\omega$  is the angular velocity,  $N$  is the number of disks within the centrifuge,  $r_2$  is the radius of the disks,  $r_1$  is the radius of the feed pipe, and  $\theta$  is the angle made by the intersection of the disk and the feed line<sup>9</sup>. Figure 3 illustrates the disk centrifuge as well as all of the dimensions needed for equation 11.

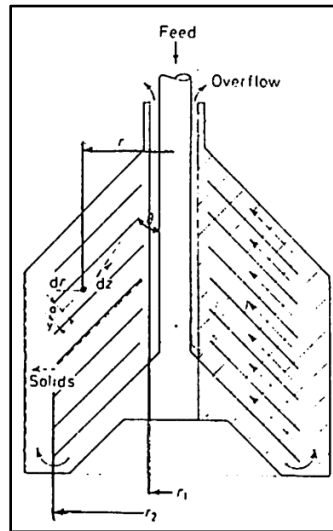


Figure 3: Basic schematic of a disk centrifuge<sup>10</sup>.

## Ion Exchange Chromatography

A method for separating molecules dissolved in solution is by adsorption of those molecules into a charged matrix. While some molecules are held up in the matrix (also called resin or media), other molecules without a selectable trait elute through the column. The use of resin in a chromatography column that can interact with molecule charge is called ion exchange chromatography (IEX). The idea is that the molecule can be manipulated into having a selectable trait that will bind into the resin. The isoelectric point (pI) is the parameter of a chemical species that is important for manipulating the molecule, which is the pH that a molecule will have a neutral charge in solution. In acidic conditions, the protein will be positively charged. Conversely, in basic conditions the protein will be negatively charged.

There are two types of IEX resins. The first is cation exchange chromatography in which negatively charged resin particles allow positively charged proteins to bind onto the medium. The other type of IEX is anion exchange which uses a positively charged ligand to exchange anions between the resin and solution. Figure 4 illustrates the binding mechanism for both types of IEX.

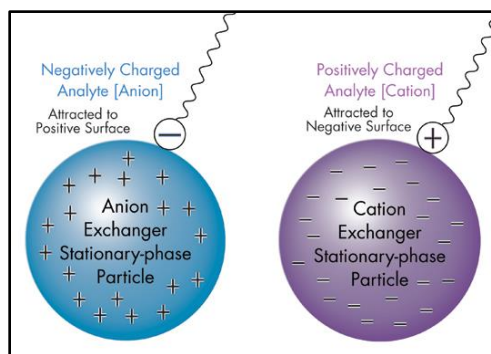


Figure 4: Depiction of how cation or anion exchange resins interact with molecules in solution <sup>11</sup>.

When designing a chromatography column the bed height is a design parameter to consider at the lab scale in order to scale-up to an industrial scale. A higher bed column can result in greater yield and purity. An issue of increasing bed height is that it may decrease the linear velocity of the fluid which corresponds to higher residence times. Although the column can become pressurized, this comes with additional equipment and operating costs. Also the column cannot be too long, have an excessively long column (greater than 30 cm) will result in higher pressure requirements to maintain flow rates.

Other operating parameters include conductivity and pH. General behavior of dynamic binding capacity of a protein on IEX resins decrease with increasing conductivity and decreasing protein charge<sup>12</sup>. First, as the salt concentration and conductivity increase there is a greater supply of counter-ions which will bind on the resin instead of the protein. Second, as the pH approaches the pI then the dynamic binding capacity decreases, so it is best to capture the protein of interest at a pH that is far from the pI to increase process yield.

Over time the dynamic binding capacities decrease due to fouling of the exchange media. One method of determining the dynamic binding capacity is by constructing an experiment to explore the Langmuir isotherm in equation 12<sup>13</sup>.

$$q = q_m \left( \frac{c}{K+c} \right) \quad \text{Eqn. 12}$$

Where  $q$  is the dynamic binding capacity,  $c$  is concentration of protein, and  $q_{\max}$  is the binding capacity of the protein under optimum conditions or initial conditions. Manufacturers test the maximum dynamic binding capacity for a protein by measuring the concentration of protein at 5% breakthrough of protein in the outlet. By performing

this experiment on a lab-scale before, during, or after each cycle will allow a performance tracking of the resin to model the number of cycles before the resin will need to be replaced due to fouling. Thus number of runs that each resin can go through is dependent upon the desired efficiency. Since 90% is required, any percentage below that will indicate that it is time to replace the resin.

One aspect of overall chromatography column design is its height. Although, in theory, column height can be as high as needed to get an effective separation, the pressure and volumetric requirements make long columns unfeasible. Consequently, the height of the theoretical plate (HETP) can be determined so that the overall column design may be optimized. The Van Deemter equation for the HETP is shown below <sup>14</sup>.

$$H = \frac{2\gamma}{v} + 2\lambda + Cv \quad \text{Eqn. 13}$$

Where  $\gamma$  is the external obstruction factor,  $\lambda$  is eddy dispersion coefficient,  $C$  is the overall solid-liquid mass transfer coefficient, and  $v$  is the reduced velocity. The Van Deemter can be plotted as a function of the reduced velocity to obtain profiles as seen in Figure 5.

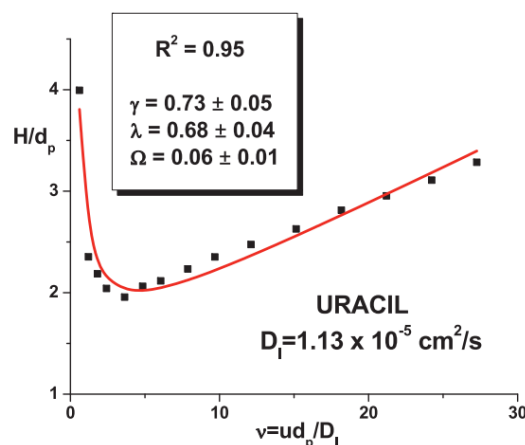


Figure 5: Van Deemter plot of nitrogenous base uracil demonstrates typical curves <sup>14</sup>.

### Tangential Flow Filtration

Tangential Flow Filtration is a purification step used to separate particles by molecular size. This method of filtration is different than normal filtration because instead of having the membrane be perpendicular to flow, the membrane is parallel to flow. Tangential flow filtration is more efficient than conventional forms of filtration since there is no bottleneck effect seen. This results in less resistance to mass transfer and makes for an optimized unit operation. Having the membrane parallel to flow has three distinct advantages. First, clogging of membrane pores is almost eliminated due to parallel flow. Although a gel layer is eventually formed, it takes longer to form since any particles that may clog the filter pores are swept away by flow of the inlet feed. Second, the surface area of membrane is increased since particles permeate in a radial orientation instead of having one membrane in the path of flow. Instead of having a filter that is size of the pipe's cross sectional area, the filter is wrapped around the pipe similar to a French drain pipe. This greatly increases the filters surface area and consequently allows for higher flowrates than other types of filters. Third, resistance to permeate mass transfer is minimized since flow is not perpendicular to membrane. Although this is synonymous to the first point, it is important to emphasize that the resistance to mass transfer is still smaller than conventional filtration assuming similar run times.

Another advantage of tangential flow filtration is the fact that buffer exchange can occur if needed. This is excellent since other types of chromatography may require the protein to be in a different buffer from what it may elute from. Since what permeates out from the filter is smaller proteins and buffer, different buffer may be added so that the exchange can occur. Tangential flow filtration also has the ability to concentrate the

protein of interest which is why it is chosen to be the final unit operation. Since buffer permeates out the filter while allowing for protein retention, finite volumes may be permeated out so that sufficient protein concentration can occur. For full protein retention, it is recommended that the pore size be at least three times smaller than the actual protein of interest<sup>15</sup>.

Since a filter is utilized throughout this unit operation, it is imperative to know volumetric permeate flux which is given below<sup>16</sup>.

$$J = \frac{\Delta P_{Membrane}}{\mu(R_c + R_M)} \quad \text{Eqn. 13}$$

Where J is volumetric permeate flux,  $\Delta P_{membrane}$  is the transmembrane pressure,  $R_M$  is the resistance of the filter medium, and  $R_c$  is the resistance of the cake (feed permeate). Assuming that the filter surface area is the same throughout the runtime, equation 13 can be modified so that the flowrate is a function of the transmembrane pressure as seen in equation 14.

$$Q = \frac{\Delta P_{Membrane}}{\mu(R_c + R_M)} A \quad \text{Eqn. 14}$$

Where Q is the volumetric permeate flow rate and A is the effective surface area of the filter. The transmembrane pressure is given by the following equation<sup>17</sup>.

$$\Delta P_{Membrane} = \frac{P_{Retentate} + P_{Feed}}{2} + P_{Permeate} \quad \text{Eqn. 15}$$

Where  $P_{retentate}$  is the pressure of the retained product strain,  $P_{Feed}$  is the pressure of the inlet feed, and  $P_{Permeate}$  is the pressure of the filtered out stream (assumed to be zero since it is usually an open line).

### Hydrophobic Interaction Chromatography (HIC)

Since most proteins are dissolved in an aqueous solvent, their hydrophobic regions are localized inside themselves. That is, the hydrophilic regions of protein face outward toward the water and protects the hydrophobic regions. This, however is reversed when the same protein is dissolved in a solution that caters to hydrophobic characteristics. Antichotropic salts such as ammonium sulphate aid in the augmentation of these hydrophobic characteristics when the salt concentration high enough. This also allows for the protein to bind onto hydrophobic resins which is why HIC allows different way of protein separation. Salt concentrations in buffers must be higher than normal buffers to get effective binding onto the HIC resin (values from 1.2M to 2.0M) <sup>18</sup>. To elute out proteins, the salt concentration is decreased until they hydrophobic sections of the protein can no longer bind onto the resin. Once the proteins cannot bind to the resin, they elute out.

## **EXPERIMENTATION**

To get better acquainted with the overall process, various experiments were conducted. These experiments include operating a bioreactor (for the first time in the unit operations laboratory), cation exchange chromatography experiment, and a tangential flow filter experiment. All procedures and results are detailed further below

### Bioreactor

The design of a bioreactor unit operation has a very large impact on the overall yield of finished product. This step is the first step in any bio-production process. The bioreactor unit operation has many components such as the growth of colonies, the growth of inoculation or starter cultures, and the bioreactor operation itself. With the

main emphasis (for engineers) being the design and optimization of the latter bioreactor operation. The optimization of a bioreactor will include the use of many different continual real time sensors. Some sensors to be considered will include: pH, temperature, electrical conductivity, and dissolved oxygen content. Varying these parameters will have great impact and implications towards the overall yield of product, biomass concentration, and success of the bioreactor operation. The investigation of the impact that these parameters have on the operation of the bioreactor will help to develop an optimization scheme, and consequently profitability of the operation.

To initialize bioreactor operation, a suitable *E. coli* strain with a gene of interest and antibiotic resistance was grown in vitro. The cell culture was grown on a petri dish with an antibiotic specific to the resistance of the *E. coli* strain. The strain was then inoculated in a 5mL beaker. This was repeated two more times so that there were sufficient starter cultures for the bioreactor operation. The starter cultures were then grown in a larger beaker on top of a shake table for 12-18 hours at 37°C. Following sufficient shaking, the beaker in which the starter cultures were grown displayed a rather cloudy solution, which is normal. After sterilization of the bioreactor, the starter culture was poured with nutrient medium (terrific broth) into a 1 liter jacketed bioreactor vessel until 75% of the total volume is occupied. One-fourth of the volume was left open to accommodate any fouling during bioreactor operation. Control operations were initialized with a desired temperature of 37 °C (before starter cultures were poured); other monitored parameters were electrical conductivity and pH. For every 30 minute interval, 3mL aliquots of the bioreactor medium were taken for absorbance measurements, the electrical conductivity and pH were also noted when retrieving the aliquots.

Measurements were noted until the cell culture was well into the stationary phase. To finish the bioreactor operation, all components were sterilized with a 10% bleach solution.

The bioreactor was run for a total of 11.5 hours. The pH, electrical conductivity, and absorbance were monitored and recorded every 30 minutes. Figure 6 shows the electrical conductivity through the duration of the experiment. The next important variable that was monitored over the course of the experiment was pH as can be seen in figure 7 below. The spectral absorbance was also investigated over the course of the experiment. When the absorbance was reporting above 1.5 the solution taken from the bioreactor was diluted by half and the resulting absorbance was doubled. When halving the solution produced absorbance values above 1.5 the solution then was diluted to a third of its concentration. All samples were blanked against the medium used in the bioreactor, and all samples were diluted with the same medium as well. The results of the absorbance spectra and be seen below in figure 8.

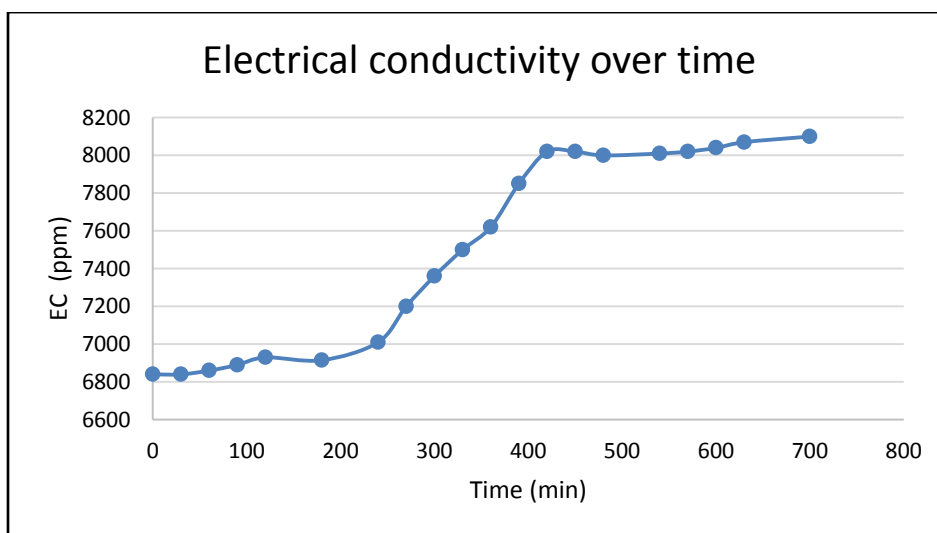


Figure 6: Electrical conductivity throughout time displays typical E. Coli growth.

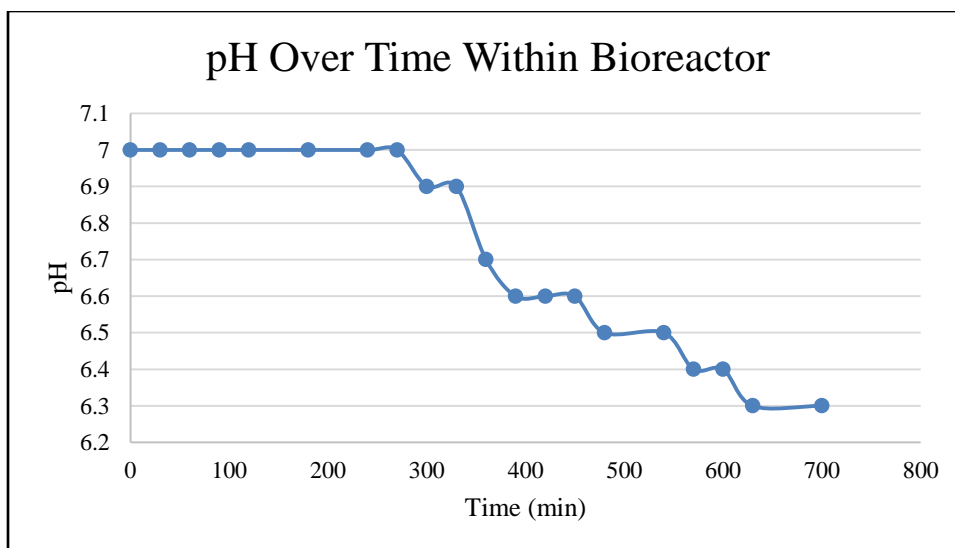


Figure 7: The pH of the bioreactor over the course of the experiment.

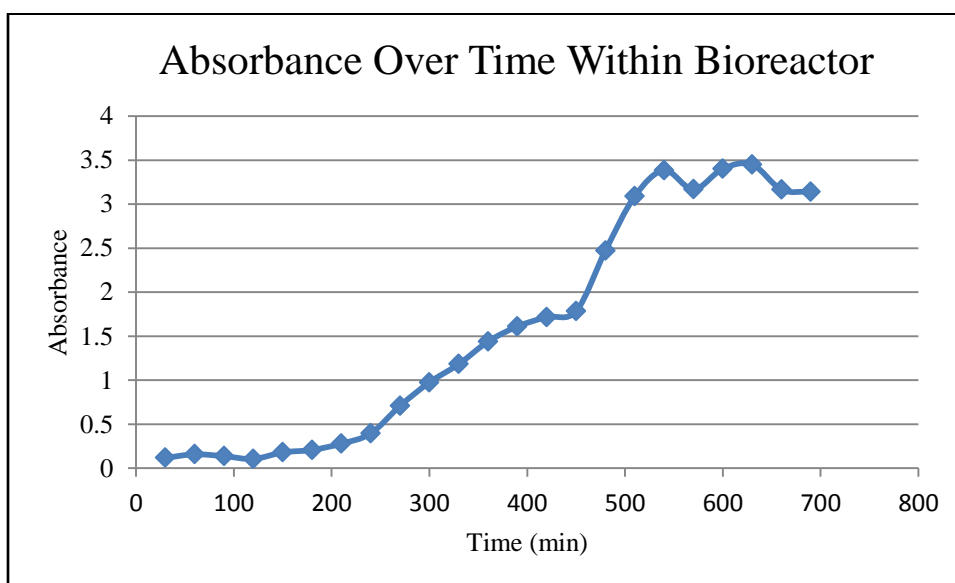


Figure 8: The absorbance of the bioreactor medium over the course of the experiment.

The bioreactor operation used an *E. Coli* that had a plasmid in use that had an ampicillin resistance gene, and was able to produce a lac operon expressed experimental muscular dystrophy therapy drug that is currently in the mouse phase of development. Although the gene of interest in this cell culture was not expressed during bioreactor

operation, it could have been expressed with the use of IPTG (Isopropyl  $\beta$ -D-1-thiogalactopyranoside) which induces gene expression. The use of IPTG is now an industry standard, where before feeding with lactose instead of glucose was used. The medium or broth that was used was terrific broth with ampicillin at a concentration of 1mL/L of broth. The antibiotic resistance in the plasmid in the E. coli was specific to ampicillin. As seen in figure 6, the electrical conductivity increased during the course of the experiment. Electrical conductivity is a measure of the ionic strength of the medium, or it is a measure of the amount of ions contained in the mixture. Figure 6 follows a normal growth curve where from 0 minutes to 200 minutes the lag phase is observed, from 200 minutes to 450 minutes the exponential phase is observed, and the last part of the curve is represented by the stationary phase. This trend is also observed in figure 8, but the exponential phase is not as exponential and is more linear. After discussion with the Genentech professionals, it was concluded that there may have been a substrate limitation which is why the growth was more linear than expected. This limitation is most likely due to the oxygen amount that was able to dissolve into the solution since an air diffuser was not used. An air diffuser allows for enhanced oxygen dissolution and decreased resistance to mass transfer. From figure 7 it can be seen that the pH decreased during the course of this experiment from a starting value of 7 to 6.3 at the end. The evidence that these two figures provides is that there was a growth associated product being formed that must have been an acid. After further analysis, it was found that the growth associated product was most likely acetic acid<sup>3,19</sup>.

For future runs of this experiment the first piece of equipment that should be included should be an air diffuser. Along with the air diffuser a HEPA filter should be

added to the air inlet to avoid any contamination of the medium. To measure the amount of oxygen in the medium a dissolved oxygen sensor should be added. To measure the cell mass concentration within the bioreactor during the course of the experiment, a centrifuge should be used to separate out the cells from the medium, which can then be dried and weighed. The pH of the solution should also be held constant throughout the experiment with the addition of pH 7 buffer as needed. Since Genentech uses its bioreactors in fed-batch mode a feed to the bioreactor should be included to maximize the cell mass concentration of the bioreactor. Finally using an E. Coli strain that has a plasmid that is made to express a monoclonal antibody fragment will allow for the Genentech senior design project to make the project self-sustaining.

#### Cation Exchange Chromatography

Cation exchange chromatography (CEX) plays the role of capturing desired proteins from a large mixture of cellular debris. The captured proteins are then sent on to several polishing steps to extract only one specific protein. CEX uses a pH gradient to gently remove a desired antibody from the resin based off the pI of that antibody. The Lucentis like protein has a pI of 7.15, which means a pH slightly above 7.15 provides sufficient ionic strength to separate the bound antibody from the negatively charged resin.

To start out, a column with a height of 30cm and a diameter of .7cm held the resin. The column was filled to a height of 10cm with Capto S ImpAct resin, provided by GE Life Sciences. This equates to about  $3.8\text{cm}^3$  of column volume. A pH gradient was used during the elution process to remove the desired antibody from the column resin. The pI of the antibody is 7.15, so a pH range of 6.6 to 7.4 was used to extract the

antibody. Once the resin settled, four column volumes of starting buffer were rinsed through the column. The starting buffer contains .02M sodium phosphate with a pH of 6.6. Next, a 22mL sample of stock protein solution, provided by Genentech, was loaded on top of the column resin. Four column volumes of starting buffer were used to wash the stock protein sample through the column. The purpose of this step was to rinse out proteins that failed to bind. The pH gradient was then set up to elute the desired protein from the resin. After the starting buffer wash, three different mixtures were used to elute the column; buffer one, buffer two, buffer three, and the elution buffer. Buffer one contains 75% starting buffer and 25% elution buffer with an overall pH of 6.9. Buffer two is a 50/50 mixture of starting and elution buffer with a pH of 7.1. Buffer three is composed of 25% starting buffer and 75% elution buffer with a pH of 7.3. Finally, the elution buffer of pH 7.4 was used to strip any remaining antibody from the resin. Four column volumes of each buffer were used while 2mL samples were constantly taken from the outlet stream, and then analyzed on a spectrophotometer at a wavelength of 280nm. Figure 8 displays the absorbance as a function of a 2mL sample trial along with the buffer concentration.

From figure 9, it is seen that during the start and first elution buffer, a lot of proteins elute out. When considering that the pI of the antibody is 7.15 and the pH of these buffers were less than 7.00, it is safe to conclude that any proteins that eluted out during the use of these buffers are unwanted and can be discarded. Proteins continue to elute out until the third elution buffer where the pH crosses the pI. Consequently a spike in absorbance is seen right after the use of the third elution buffer. The sample was isolated and taken to the University Of Nevada School Of Medicine for further analysis

of content using gel electrophoresis. Figure 10 illustrates the results of the gel electrophoresis.

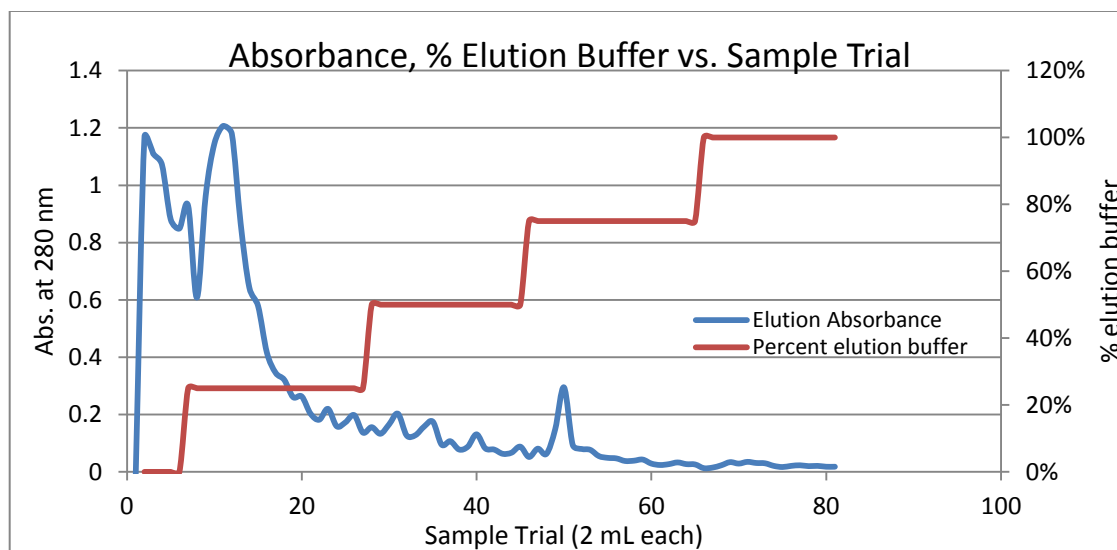


Figure 9: absorbance as a function of a 2mL sample trial along with the buffer concentration.

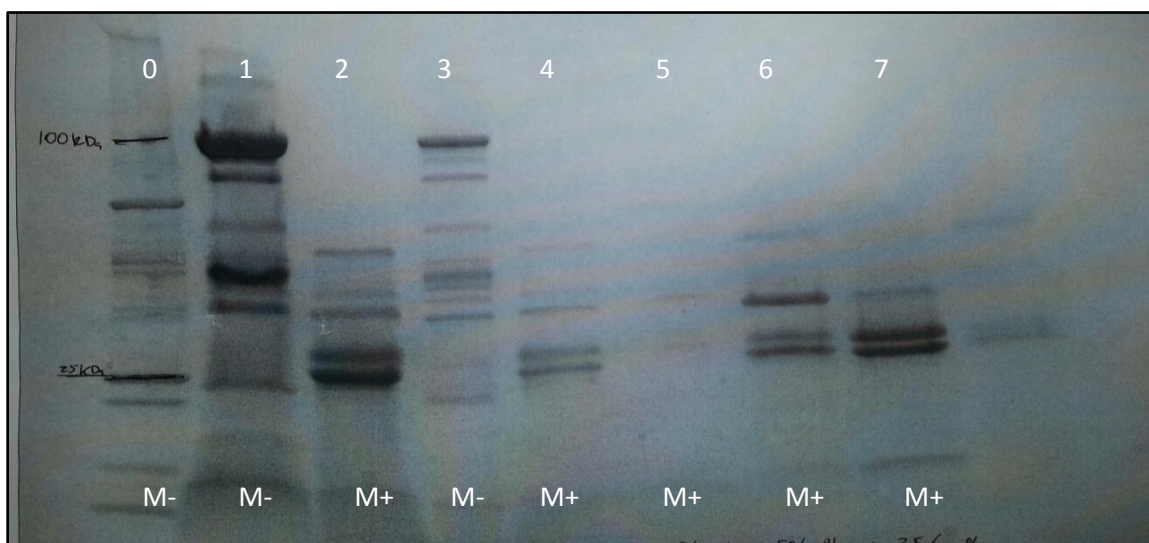


Figure 10: Results of gel electrophoresis from the CEX experiment.

To better determine if the antibody of interest was isolated, mercaptoethanol was added to some gel wells (denoted by M+). Mercaptoethanol is well known to break disulfide bridges within proteins. When a Fab interacts with mercaptoethanol, it is split

into 20kDa to 25 kDa pieces (with the original size at 100 kDa). This allows for easy monitoring of the antibody throughout the gel electrophoresis process. Within figure 10, well 0 is the standard in which proteins of known molecular weight are processed, this serves as a sort of “line” from which other proteins can be analyzed. Wells 1-4 are of the original stock solution; wells 3 and 4 are diluted 6 fold from the original stock solution. In addition to this, wells 2 and 4 have mercaptoethanol added, that is why there are bands showing up around the 20 kDa to 25 kDa range. Well 5 is the sample taken when the composition of the buffer was 75% start buffer and 25% elution buffer. Well 6 is the sample taken when the composition of the buffer was 50% start buffer and 50% elution buffer. Well 7 is the sample taken when the composition of the buffer was 25% start buffer and 75% elution buffer. Well 7 seems to have the antibody of interest as seen with the apparent bands around the 20 kDa to 25 kDa range. This confirms that the antibody of interest was successfully isolated.

#### Tangential Flow Filtration

The tangential flow filtration (TFF) experiment was conducted by operating a lab scale Pellicon XL 30 kDa membrane. The buffer used for this experiment was 2M ammonium sulphate at room temperature of 25 °C. The first step of the experiment was to determine the initial operating parameters before the insertion of the sample into the system. Parameters determined at the beginning were permeate flux and transmembrane pressure. To determine such parameters, the buffer was pumped through the system and samples of approximately 10 mL with noted time. The initial operating parameters of permeate flow and transmembrane pressure were set as 29.2 mL/min and 20 psi,

respectively. Before the insertion of the protein into the system, it was necessary to prepare it to adequate conditions required by the filter membrane. The preparation of the protein sample was performed by diluting 20 mL of the isolated antibody sample in 80 mL of 2M ammonium sulphate.

The system composed of one feed stream, a permeate stream and a retentate stream. The flux of the system was closely monitored and after 80 mL of the initial solution had been transferred to the permeate stream, 100 mL of pure buffer was incorporated to the system. This process was repeated until the retentate presented a pH equal to the pure buffer which was 6.65. The pH data collected was plotted at each diavolume and is represented by figure 11. The permeate flux was also monitored and is represented by figure 12 shown below.

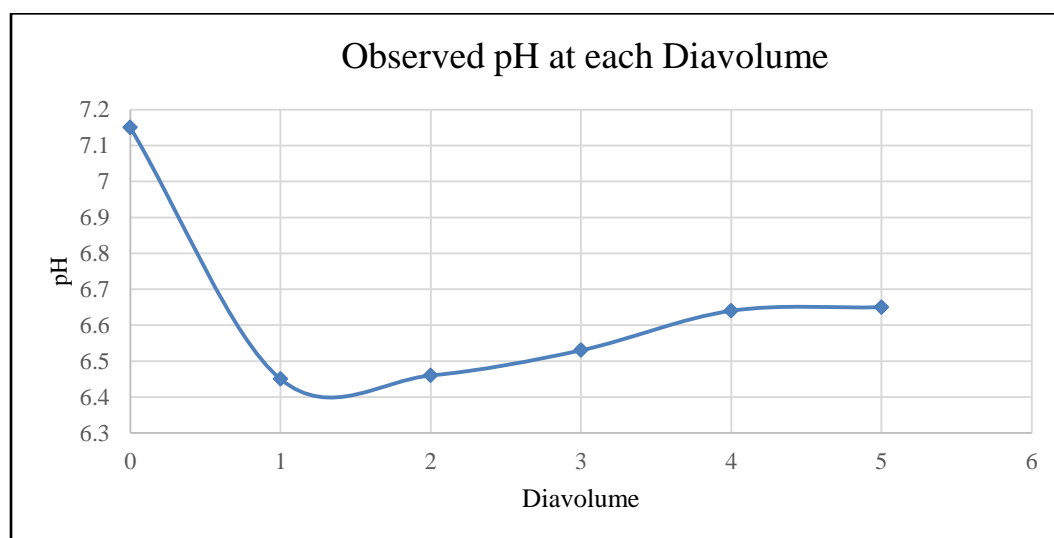


Figure 11: pH change at each diavolume throughout the experiment.

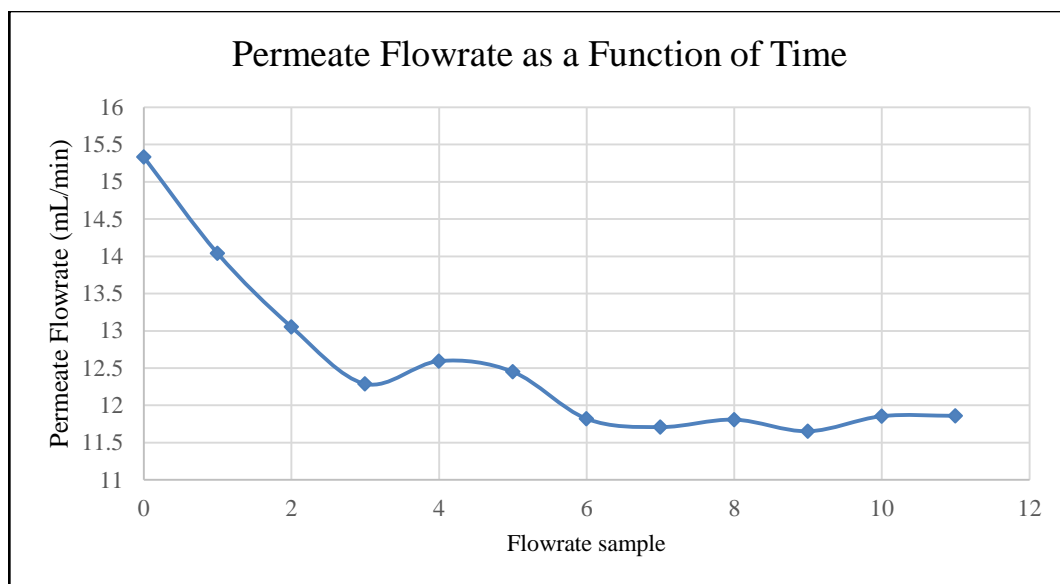


Figure 12: Retentate flow rate during the antibody processing.

A decrease in flowrate is noticed throughout the duration of the experiment. This fact can be explained by the increase in mass transfer resistance caused by a gel layer formed within the membrane pores. After a period of time, the flowrate stabilizes around 11.7 mL/min. This occurs due to the fact that the gel layer reaches a constant thickness and the mass transfer coefficient no longer changes. This information is crucial because the overall resistance coefficient can be experimentally determined once the TFF has reached steady state. The resistance coefficient can then be used for scale up purposes in future design projects.

### **SCALE UP**

A full scale process of the base case has been designed. Several proposed alternate cases can deviate from the base case as will be shown in later sections.

### Bioreactor

For the bioreactor, Sartorius Stedim was contacted to obtain more information regarding bioreactor design. The technical representatives recommended using a 1300L jacketed vessel made out of stainless steel. The reason for making the vessel larger than the necessary working volume is so that there is enough flexibility to accommodate changes in the process. The volume is also made larger so that the system can account for fouling. The desired air flow rate into the bioreactor will be approximately 65 L/min. The operating pH range will be 5.5-8.5 with the desired pH at 7.00. The desired temperature for the bioreactor while in operation will be 37 °C. Although the width and length can be changed if needed, the desired width of the bioreactor will be 0.943m with the height of the bioreactor at 1.87m. Assuming a perfect circle cross section, the total volume with these dimensions will be approximately 1306L. The cost of the bioreactor will range from \$1.0 million to \$1.2 million.

### Homogenizer

The homogenizer that will be utilized in this process will need to have a pressure drop of 15,000 psi to 20,000 psi. This range is given so that, through experimentation, the optimum pressure drop can be found. Although there are homogenizers that exceed this pressure requirement, it is imperative to run this unit operation at a pressure that is high enough to lyse the E. Coli cells but not too high so that the protein denatures due to shear stress. For this process the DBEE 4000 High Pressure homogenizer manufactured by BEE International was chosen. This high pressure homogenizer has the ability to safely process 360 L/hr of E. Coli stream at 20,000 psi. The fast flowrate going through the homogenizer will allow multiple runs of the stream to go through if needed. For the sake

of this process, the broth solution is planned to pass through the homogenizer two times but can run more times if need be. More experimentation will need to be done in order to determine the optimum number of runs needed for 98% efficiency with respect to cell lysis. Figure 13 displays the DBEE 4000 which has a touchscreen monitor for better process control. The quoted cost for this model was \$250,000.



Figure 13: The DBEE 4000 high pressure homogenizer in operation <sup>20</sup>.

### Centrifuge

The disk centrifuge that will be used must be able to separate the solid particulates from solution with at least 95% efficiency. The disk centrifuge chosen is the Alfa Laval BTUX 305. Although this model has the ability to process 2500 L/hr of lysed E. Coli solution, the desired flowrate though this unit operations will be 360 L/hr; the same as the

homogenizer. This will provide the ability to have multiple runs through the centrifuge if need be. It is estimated that 237.5L to 245L of solid particulates and liquid will be separated from solution. The centrifuge will be operated around the range of 3500 rpm to 5500 rpm; more experimentation will be needed to determine the best rpm for operation. Figure 14 illustrates the BTUX 305 with the bowl and the motor that powers the unit operation. The quoted cost of this unit operation was \$650,000<sup>21</sup>.



Figure 14: The BTUX 305 centrifuge.

#### Cation Exchange Chromatography

The cation exchange resin that will be used in this process is Capto S ImpAct manufactured by GE Healthcare Life Sciences. This resin is particularly made for antibody separation with a high binding capacity at 100 mg/mL. The binding ligand used in the Capto S ImpAct is the sulfonate ligand which attracts positively charged protein regions<sup>22</sup>. At a binding efficiency of 20%, it is estimated that 207.5L of the resin will be needed to successfully capture the antibody of interest. The resin will not be placed in

one large chromatography column; instead, the resin will be split and poured into three chromatography columns with a maximum height of 30 cm. Table 1 displays the dimensions for the cation exchange chromatography.

Table 1: Column conditions for cation exchange chromatography.

Height of Resin (cm)	26.1
Volume of Resin in each column (L)	73.7
Column Diameter (cm)	60
Number of Required columns	3
Starting pH	6.6
Finishing pH	7.4
Buffer recommended	50 mM NaH <sub>2</sub> PO <sub>4</sub> , 300mM NaCl

#### Anion Exchange Chromatography

The anion exchange chromatography resin that will be used is Capto Q ImpRes which is also manufactured by GE Healthcare Life Sciences. Capto Q ImpRes is a strong anion exchange resin that utilizes a positively charged quaternary amine to bind to negatively charged regions of protein. It has a binding capacity of 55 mg/mL which is far less than the 100 mg/mL<sup>23</sup>. This is why this resin is used as an intermediate chromatography step and not as a capturing step. It was determined that 206.1L of the resin will be needed to achieve a successful intermediate separation. Table 2 displays the dimensions needed for the anion exchange chromatography.

Table 2: Column conditions for anion exchange chromatography.

Height of Resin (cm)	25.9
Volume of Resin in each column (L)	73.4
Column Diameter (cm)	60
Number of Required columns	3
Starting pH	8.0
Finishing pH	6.5
Buffer recommended	50 mM NaH <sub>2</sub> PO <sub>4</sub> , 300mM NaCl

### Tangential Flow Filtration

The TFF module that will be used for this process will be the EMD Millipore Pellicon 2 Maxi Ultrafiltration Module. The flowrate through the system will be 7 L/min, the surface area of the filter will be set at 2.5 m<sup>2</sup> but can be doubled to 5.0 m<sup>2</sup> since the apparatus can hold two filters. The operating pressure desired will be 20 psi since the maximum operating pressure is 30 psi<sup>24</sup>. The pore size of the filter will be 30 kDa since that is more than three times smaller than the size of the antibody that must be retained (100 kDa). There will also need to be three vessels, one vessel will house the feed (solution with the antibody), another vessel that will house the buffer used in buffer exchange, and the last vessel will need to house the permeate that will transfer through the TFF. The size of the buffer vessel will need to be 4000L so that it can contain 3500L buffer. The feed vessel will need to be 1000L so that 700L to 750L of the feed solution can be housed. The permeate vessel will also need to be at least 4000L so that it can house as much permeate as possible; this size can be increased or decreased depending upon the desired rate of discard.

In addition to the vessels, a modified EMD Millipore Cogent M1 TFF Control System will be used as a means of having some sort of process control. Although only used for lab scale models, the Cogent M1 system can be modified so that it can control process scale material. This system has pumps and gauges which allow the operator to know at what conditions the TFF is operating at. This allows the process engineer more control over the operation. It also has temperature gauges which give the process engineer more information regarding its operation<sup>25</sup>. The Cogent M1 TFF control system will allow for purification, buffer exchange and concentration of the antibody solution.

### Hydrophobic Interaction Chromatography

The hydrophobic interaction chromatography resin that will be used is Capto Phenyl ImpRes which is manufactured by GE Healthcare Life Sciences. Capto Phenyl ImpRes utilizes a phenyl group to bind to hydrophobic regions of protein. It has a binding capacity of 19 mg/mL<sup>26</sup>. It was determined that 250.7L of the resin will be needed to achieve a successful intermediate separation. The high volumetric amount was a result of the low binding capacity of the resin. Table 3 displays the dimensions needed for the hydrophobic interaction chromatography.

Table 3: Column conditions for hydrophobic interaction chromatography.

Height of Resin (cm)	29.7
Volume of Resin in each column (L)	84.0
Column Diameter (cm)	60
Number of Required columns	3
Starting pH	6.5
Finishing pH	6.5
Buffer recommended	1.2M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>

### PRESENTATION OF ALTERNATE CASES

Now that the base case has been firmly established, the following sections will have the alternate cases presented. The changes to the base case were made in order to streamline the process or to make the purification easier. Alternate case 1 will introduce the concept of depth filtration which will be used to filter out small particulates that escape the centrifuge. Alternate case 2 will make the case that changing the antibody on the genetic level will allow for easier and more efficient purification. Alternate case 3 will introduce monolithic chromatography as a means of polishing off the chromatography process. The alternate cases will be followed by economic analysis

which will demonstrate which process designs are recommended for further research and development.

### Alternate Case 1

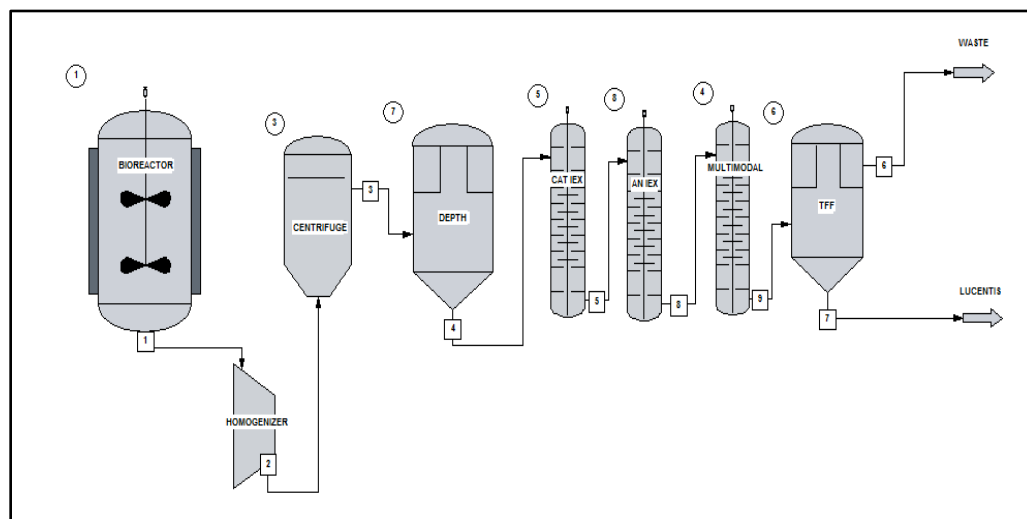


Figure 15: Block flow diagram of Alternate Case 1.

One proposed separation process is the use depth filtration in upstream separation and purification. New advances made in this field that allow for excellent filtration before chromatography and has the potential to replace tangential flow filtration, chromatography separation, and a disk centrifuge. Depth filtration functions on the principle of adsorption, particles are retained throughout the medium instead of only being trapped on the surface, as in membrane-only separation. Depth filters can retain a high amount of particles before becoming clogged in comparison to other filter types. Depth filters are ideal for filtering fluid systems that contain solid particulates. The self-contained filters also protect lab technicians from being exposed to any biohazards present in the system <sup>27</sup>. This idea was initially proposed because of the fact that small

solid particulates would clog the chromatography resin, critically fouling and adding resistance to the overall separation.

The most recent advance is called lenticular pod depth filtration, which uses inexpensive, disposable filters with incrementally decreasing pore size to filter the lysed cell broth. The finest filter is a 0.2 $\mu\text{m}$  membrane filter, with a maximum pore size of 10 $\mu\text{m}$ <sup>28</sup>. Optimization of pore size, surface area, flux, and filter type can be done at lab scale and is linearly scalable to pilot scale and beyond. Three pod stages are usually employed. Premature plugging of filters is avoided by using steps of finer and finer filter material progressively. The greatest challenge to harvest is dependent on cell viability and cell density throughout the process. High cell densities and low cell viabilities present the greatest challenge. Design parameters and desired values are present in Table 4. These values are primarily based on available product information from the manufacturer<sup>29</sup>.

Table 4: Design parameters and recommended values for depth filtration design

Pressure (psi)	15
Volumetric Flux ( $\text{L m}^{-2} \text{hr}^{-1}$ )	150
Filter Area ( $\text{m}^2$ )	1.1
Run Time (hours)	2.3

As an adjustment to the base case, a lenticular pod depth filter would be placed immediately following the centrifuge in the process. The columns should report higher yield of product since less material is present in the lysed solution. Depth filtration in this application has several advantages over TFF. First, depth filtration is inexpensive relative to TFF. Even with replacing disposable cartridges on a regular basis, the initial

investment cost is extremely low. The disposable cartridges remove the need for steam-in-place (SIP) and clean-in-place (CIP), which greatly reduce utility costs. A cost comparison between the two systems is available in the economics section to follow. Another advantage is that depth filtration systems are rather compact. The disk pods are stackable and held together by a simple steel frame. Required floor space is greatly reduced, taking up approximately 0.5 cubic feet per pod at lab scale.

EMD Millipore, the primary industrial provider of general pod systems, and also lenticular pod systems specifically, manufacture several different models of filters, several of which are specifically designed for antibody separation from a protein solution. The selected filter for this case is the Milistak+ B1HC media, which has been determined to be most effective for processing our solution based upon product application to pharmaceutical-specific processes<sup>29</sup>. The base product has been quoted to cost \$12,950 and replacement filters for the selected product are available \$648 each. The final apparatus would require 2 filters per day.

The other significant change seen in figure 15 is the use of multimodal chromatography in lieu of HIC. Multimodal chromatography has several advantages over HIC. First, it has a higher binding capacity than the selected HIC resin<sup>30</sup>. This allows for fewer amounts of multimodal resin to be used relative to HIC. Second, the buffers used for multimodal chromatography is very similar to the buffers used in the cation and anion exchange chromatography resins. This streamlines the overall process and requires no buffer exchange. However a TFF is the final unit operation so that the antibody environment returns to physiological conditions.

The multimodal chromatography resin that will be used is Capto MMC ImpRes which is manufactured by GE Healthcare Life Sciences. Capto MMC ImpRes is a strong cation multimodal resin that utilizes a phenyl group, carbonyl group, carboxyl group, secondary amine, and a thiophilic group to bind to various regions of protein. It has a binding capacity of 75 mg/mL which is higher than the 19 mg/mL afforded by the replaced HIC resin <sup>30</sup>. From calculations, it was determined that 62.2L of the resin is needed to achieve a successful intermediate separation. The low resin requirement is the product of a highly efficient binding capacity. Table 5 displays the dimensions needed for the multimodal chromatography.

Table 5: Column conditions for multimodal chromatography.

Height of Resin (cm)	17.7
Volume of Resin in each column (L)	62.3
Column Diameter (cm)	60
Number of Required columns	1
Starting pH	6.5
Finishing pH	6.5
Buffer recommended	25 mM NaH <sub>2</sub> PO <sub>4</sub> , 100mM NaCl to 25 mM NaH <sub>2</sub> PO <sub>4</sub> , 1M NaCl

### Alternate Case 2

For this alternate case, changes to the protein are made on the genetic level. Literature has demonstrated that inserting a gene for a histidine marker (His-tag) makes it easier to separate and purify the protein of interest <sup>31</sup>. A His-tag is a cluster of histidine residues that are attached to the N-terminus or C-terminus of a desired protein, in this case the antibody. Attaching the His-tag to one end of the protein typically does not affect

its performance although it is possible. The His-tag increases the overall molecular weight of the protein by one kDa. The objective of the His-tag is to use it as a means for separation instead of the desired protein itself. Conventional methods of purification require the interaction with some chromatography resin directly with the protein. Direct protein interaction may result in decreased performance since the composition has been altered with. Installing a His-tag will allow for purification to focus on the marker rather than the protein; thus providing, in theory, a better product.

It is better to change the protein structure on the genetic level rather than on the chemical level since methods are already in place to do so. Chemically attaching the His-tag will be difficult due to the unfavorable kinetics of the reaction as well as loss of specificity with regards to where the marker will be placed. By inserting the gene for the His-tag in the appropriate place of the plasmid, the marker is ensured to be in the correct place for all expressed desired proteins.

In order for this alternate case to be valid, assumptions have been made. First it is assumed that adding a His-tag is possible. If this assumption is not met then the process design is not feasible since it is made specifically for the His-tag. The second assumption made is that the C-terminus of the antibody is not folded within the protein. That is, the C-terminus is located on the outside of the protein. Literature suggests that the C-terminus region is located on the constant (non-interacting) region of the monoclonal antibody whereas the N-terminus is located on the variable (interacting) region<sup>32</sup>. In some instances, the protein “begins” in the middle of a globular region. Thus, the assumption that the C-terminus is on the outer end of the protein is valid since adding a His-tag there would allow for some interaction with the surrounding environment. Third,

it is assumed that the addition of the His-tag does not affect antibody performance. This is an important assumption to make since if it were found that the His-tag decreases performance, then there would be no reason to alter the protein in the first place. If scientists can demonstrate that there is no difference in performance from the marked and unmarked antibodies, then the process design can proceed. Lastly, it is assumed that since protein performance is unaffected by the addition of the His-tag, it does not need to be removed from the antibody. Figure 16 illustrates a block flow diagram in which the His-tag does not need to be cleaved off of the antibody.

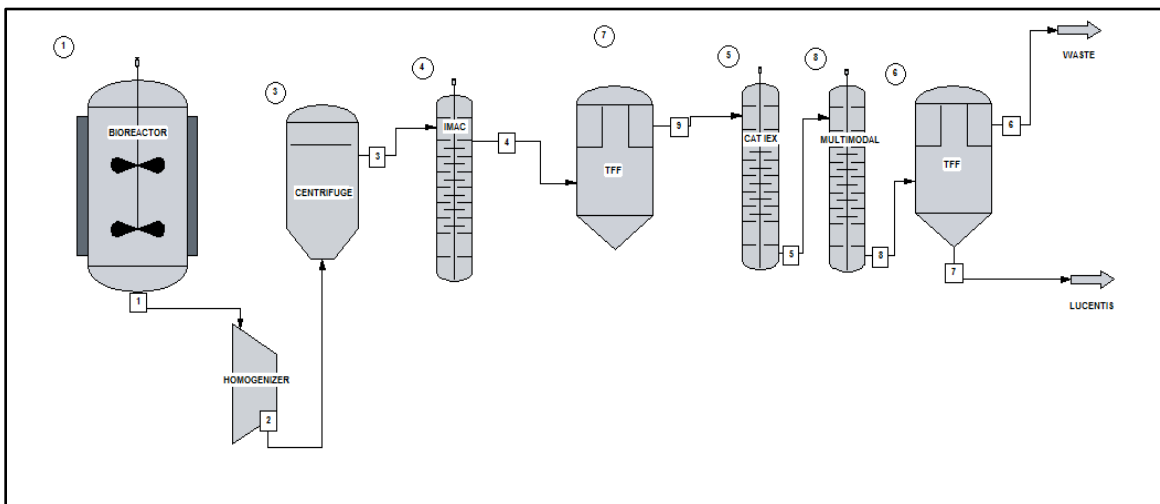


Figure 16: Block flow diagram of Alternate case 2a.

From figure 16, it is seen that the first three unit operations are the same as the base case. The next unit operation is an immobilized metal affinity chromatography (IMAC) column. IMAC is an excellent capturing step since proteins with His-tags bind onto it with a high degree of efficiency<sup>33</sup>. This is why this type of chromatography is referred to as an affinity chromatography since it is so specific with respect to what binds

to it. One potential drawback of this type of chromatography is that if an undesired protein has a series of histidine residues it will bind to the resin as well. Consequently, tests ought to be conducted when using this type of chromatography to determine if other proteins other than what is desired are binding.

The Nuvia™ IMAC resin manufactured by BIO RAD will be used in the first chromatography column. This resin has a charged nickel ligand that attracts the His-tag during operation. It has a binding capacity of 40 mg/mL<sup>34</sup>. It was determined that 122.5L of the resin is needed to achieve a successful capturing separation. As was the case with multimodal chromatography, IMAC relies upon a concentration gradient. Table 6 displays the dimensions needed for the IMAC resin.

Table 6: Column conditions for IMAC.

Height of Resin (cm)	29.3
Volume of Resin in each column (L)	122.5
Column Diameter (cm)	60
Number of Required columns	1
Starting pH	4.5
Finishing pH	4.5
Buffer recommended	20 mM NaH <sub>2</sub> PO <sub>4</sub> , 500mM NaCl, 5mM Imidazole to 20 mM NaH <sub>2</sub> PO <sub>4</sub> , 500mM NaCl, 200mM Imidazole

The multimodal chromatography resin that will be used is Capto Adhere ImpRes which is manufactured by GE Healthcare Life Sciences. Capto Adhere ImpRes is a strong anion multimodal resin that utilizes a phenyl group, OH group, and quaternary amine to bind to various regions of a monoclonal antibody<sup>35</sup>. It has a binding capacity of 65

mg/mL. From calculations, it was determined that 77.4L of the resin is needed to achieve a successful polishing separation. The low resin requirement is due to the efficient binding capacity. Table 7 displays the dimensions needed for this multimodal chromatography.

Table 7: Column conditions for multimodal chromatography.

Height of Resin (cm)	21.0
Volume of Resin in each column (L)	77.4
Column Diameter (cm)	60
Number of Required columns	1
Starting pH	6.5
Finishing pH	6.5
Buffer recommended	40 mM NaH <sub>2</sub> PO <sub>4</sub> , 100mM NaCl to 40 mM NaH <sub>2</sub> PO <sub>4</sub> , 100mM NaCl

To further explore the feasibility of marked protein, an additional assumption was made that the His-tag needed to be removed from the antibody. Consequently an additional reactor is necessary in order to cleave the His-tag. This is shown in figure 17 below.

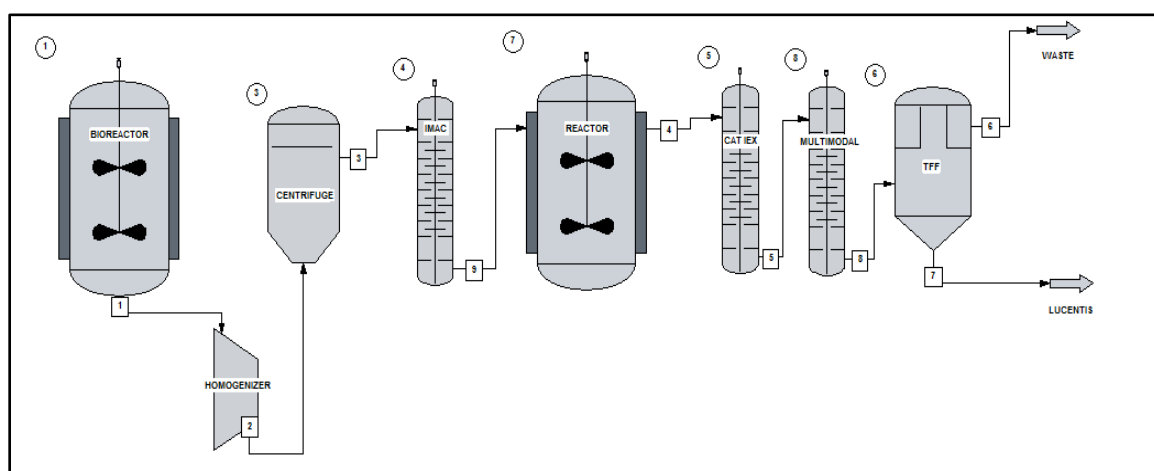


Figure 17: Block flow diagram of alternate case 2b.

This block flow diagram is the same as figure 16 with only exception being that the TFF is replaced with a reactor to cleave the His-tag. For this reaction, BoCPA, or bovine carboxypeptidase would be needed to remove the His-tag from the antibody. An assumption was made that the reaction to remove the His-tag would be 90% efficient for the sake of process design, literature was not able to show if 90% efficiency for this reaction was possible.

If it is found that the His-tag needs to be removed from the antibody, then it is better from a manufacturing standpoint to never alter the genetics behind the protein. Although a process design is presented that allows for the removal of the His-tag, it is not recommended to be further developed. There are many reasons for this; first, it is very costly. As the economic analysis will show, adding a His-tag for one the sake of one unit operation creates costs instead of shed cost. Secondly, the kinetics of this reaction makes it highly unlikely that even a 90% His-tag removal is possible. Lastly, patients may not be comfortable with the fact that an enzyme derived from a cow was used in the making of Lucentis.

#### Alternate Case 3

As seen in figure 18, alternate case 3 is very similar to the base case and in fact only one unit operation in particular has been replaced. The HIC unit operation has been replaced with a monolithic chromatography column. Through experimentation and literature, the ion exchange chromatography used upstream provides good separation of the monoclonal antibody and the hydrophobic chromatography does a sufficient job to purify the protein as well. The ultimate goal of this alternate case, and the reason for its proposal, is to increase the purification efficiency of the process.

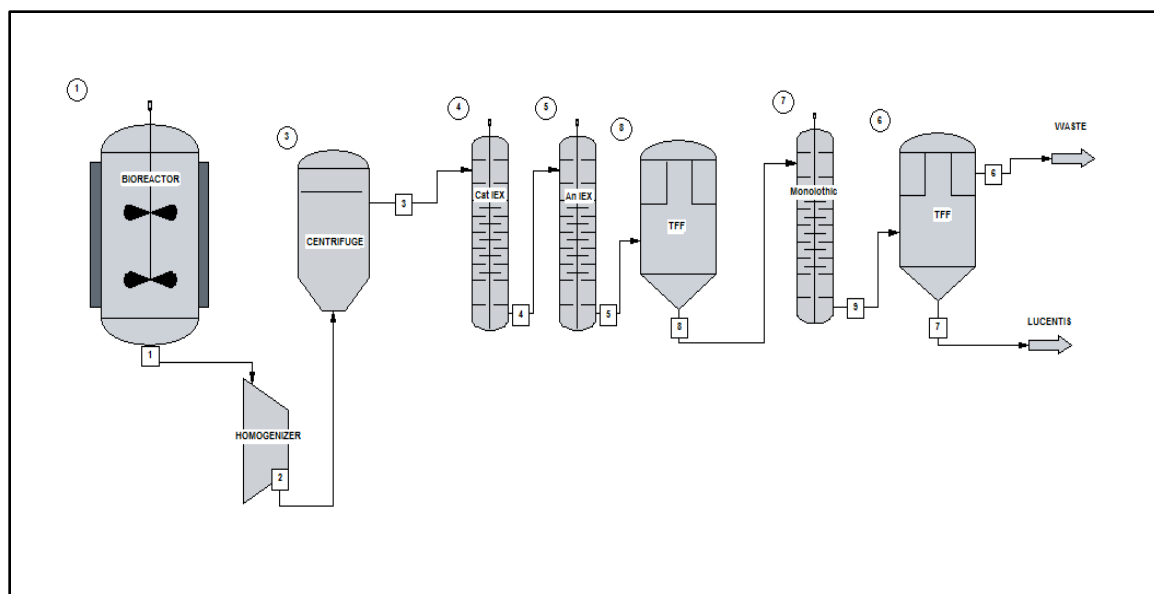


Figure 18: Block flow diagram of alternate case 3.

Monolithic chromatography has porous channels rather than the typical packed beads in packed bed chromatography. This porous resin and channels allow for high permeability, more channels, and increase in surface area for more mass transfer to occur hence making the separation and purification process more efficient. The high efficiency seen in the monolithic columns decreases at a very slow rate with an increasing flow velocity which in turn allows for a faster process <sup>36</sup>. With this information, a high-performance liquid chromatography monolithic column was explored for implementation into the base case as a purifying step.

Research has indicated successful and efficient removal of aggregates in monoclonal antibody production using monolithic columns. Literature has suggested that monoliths were indeed able to purify more protein per milliliter. The monoclonal antibody collected in the column was yielding greater than 90% whereas the traditional cation packed bed chromatography was only able to yield 70% <sup>37</sup>. This was tested among

different flow rates as well as different monolith media, some with much higher binding capacity, but the overall conclusion of this research was that the monolithic chromatography was successfully able to purify the antibody of interest at a higher yield than the traditional packed beads. It is because of the higher binding capacity compared to convective matrices, the SO<sub>3</sub> monoliths have a higher capacity to retain the antibody of interest and remove other proteins, hence increasing the overall purity.

The monolithic column chosen to replace the hydrophobic column is BIA Separations CIMmultus<sup>TM</sup> SO<sub>3</sub> which is a strong cation exchanger and is specifically used as a polishing step in monoclonal antibody production to remove aggregates and increase purity. The sulfate media in this column contains a 2 $\mu$ m pore size which is optimal for small molecules to flow through the channels and bind to the binding sites. The monolith media, SO<sub>3</sub>, selectively binds to molecules with positive charges over a large pH range of 2-13. The monoclonal antibody solution will be in a pH range close to that of physiological pH of around 7 to prevent denaturing. As discussed above, the flow-through mode operation allows for this column to be run at higher flow rates because of the stable efficiency. The SO<sub>3</sub> monolith has a binding capacity of approximately 21 mg/mL which coupled with the high surface area available through the porous resin increases the overall efficiency of the column <sup>37</sup>.

In order to implement this column into the process design for this project, large and industrial size columns were researched. A total of 6 pre-packed CIMmultus<sup>TM</sup> SO<sub>3</sub> 40L columns will be connected in parallel as a polishing step. This calculation was conducted using the parameters provided by BIA separations and the larger columns will allow for lower pressure drop preventing any hindering of the resin <sup>38</sup>. The design

parameters and operating conditions for such columns is displayed below. BIA separations mention the ability for linear scale up in terms of operating conditions and the numbers displayed below in Table X were tabulated with linearity.

Table 8: Operating parameters for monolithic column.

Flowrate (L/min)	40
Pressure	1.4 MPa
pH range	2-13
Temperature Range (°C)	4 – 40

Implementation of the SO<sub>3</sub> monolithic columns as purification step provides many benefits as discussed above. Between having a high binding capacity, long shelf life, and the increased efficiency because of more surface area in the monoliths the SO<sub>3</sub> column could be a successful replacement to the hydrophobic columns to increase purity of the monoclonal antibody of interest.

## **ECONOMIC ANALYSIS**

An economic analysis was performed on base and alternate cases in order to determine which process would be the most profitable and overall viable. Members from the team contacted vendors in order to obtain prices corresponding to the design parameters design specifications. The amount of resin required for each case was determined as described in above and divided among parallel columns of each type of chromatography. In addition to this, cash flow diagrams were made to graphically illustrate the more economically feasible design processes. The payback period (PBP) and the rate of return on investment (ROROI) were determined for the base case as well

as all alternate cases. Table 8 displays the capital cost calculations for the base case. Similar calculations for each case can be found in Appendix B.

Table 8: Capital Cost Analysis for the Base Case

Equipment	Size	Manufacturer	Quantity	Unit Price (\$/equipment)	Cost
Bioreactor	1300 L	Sartorius	1	\$1,200,000.00	\$1,200,000.00
High- Pressure Homogenizer	20,000 PSI (max) 6 L/min	BEE International	1	\$250,000.00	\$250,000.00
Continuous Centrifuge	2500 L/hr 9650 rpm	Alfa Laval	1	\$650,000.00	\$650,000.00
Tangential Flow Filtration	10 L/min	EMD Milipore	1	\$26,430.00	\$26,430.00
Chromatography Columns	Width: 296 mm Height: 900 mm	Verdot Ips	8	\$29,731.00	\$226,270.95
<b>Resin</b>					
<b>Resin</b>	<b>Amount Needed (L)</b>		<b>Unit Price (\$/L)</b>	<b>Price (\$)</b>	
Capto S ImpAct	217.8840918		3650	\$795,276.93	
Capto Q ImpRes	216.46926		3063	\$663,045.34	
Capto Phenyl ImpRes	263.1810477		3130	\$823,756.68	
<b>Total</b>				<b>\$4,634,779.90</b>	

A large portion of each case's capital cost is associated with the price of the resin, which has to be exchanged on average every five years. Each resin amount listed in Table 8 represents a 5 percent excess in order to account for error in chromatography calculations. The next step in the analysis involved estimating expenses associated with each case's operation. For each case the amount of labor, yearly maintenance, buffer, TFF/Depth Filter, administration, and laboratory costs were computed. Labor expenses were obtained from equation 16 as shown below<sup>39</sup>.

$$C_{OL} = \left( (6.29 + 0.23 * \sum_{i=1}^8 N_p)^5 \right) * 4.5 * \frac{\$59,580}{yr} \quad \text{Eqn. 16}$$

Where  $C_{OL}$  is the cost of operating labor and the summation is the total number of unit operations used in each process, which is constant at 8 for each case. Notice that because all cases utilize 8 unit operations, they all have the same labor cost. The cost associated with supervision is approximated using equation 17. The yearly maintenance costs are given by equation 18.

$$C_{SOL} = 0.18 * C_{OL} \quad \text{Eqn. 17}$$

$$C_{maint} = 0.06 * C_{cap} \quad \text{Eqn. 18}$$

Where  $C_{cap}$  is the capital cost associated with each case. Administration and laboratory costs for each case can be approximated using equation 19 and 20, respectively.

$$C_{adm} = 0.177 * C_{OL} + 0.009 * C_{cap} \quad \text{Eqn. 19}$$

$$C_{adm} = 0.15 * C_{OL} \quad \text{Eqn. 20}$$

Buffer expenses were computed by assuming that each chromatography column requires five column volumes of each of the five types of elution buffers. Table 9 and Table 10 illustrates the buffer cost calculation for the base case. Similar tables, pertaining to each of the alternative cases, can be found in Appendix B.

Table 9: Buffer requirements for the base case.

Column Volume (L)	Resin Type	Buffer runs per column	Number of columns in parallel	buffer per chromatography (L)
73.70	Cation	25	3	5187.72
73.36	Anion	25	3	5154.03
83.97	HIC	25	3	6266.22

Table 10: Buffer cost calculations for the base case.

	Cation and Anion		HIC
Buffer Components	Na <sub>2</sub> PO	NaCl	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>
Price (\$/kg)	\$150	\$71.50	\$89.90
MW (g/mol)	119.98	58.44	132.14
Concentrations Needed (mol/L)	0.05	0.3	1.2
Moles Needed (mol)	5170.873235	3102.523941	7519.458505
Kilograms Needed (kg)	620.4013708	181.3114991	993.6212469
Price (\$)	\$93,060.21	\$12,963.77	\$89,326.55
<b>Total</b>	<b>\$195,350.53</b>		

The TFF requirements and costs were obtained through a vendor. The calculations for the base case are shown below in Table 11.

Table 11: TFF filter operating expenses for the base case.

Number of Filters per batch per TFF	Number of TFFs	Total Number of Filters per batch	Price (\$/filter)	Total cost per batch (\$)	Stream Factor (SF)	Operating Time (day/batch)	Days of Operation (day/yr)
2	2	4	\$648.00	\$2,592	0.9	8	328.5
<b>Total Cost (\$/yr)</b>				<b>\$106,434</b>			

Note that a conservative operating time is used. It is assumed that the plant operates for 90 percent of the year and that the batch time is equivalent to 8 days. This maximum

batch time allows for a 24 hour operation time for each one of the unit operations performed in each case. Revenue, estimated to be the same for all cases, was calculated using the same assumptions mentioned above. The results are displayed in Table 12.

Table 13 summarizes the cost and revenue associated with each case.

Table 12: Revenue calculation for all cases.

Fab produced (g/batch)	overall yield (%)	overall Fab produced (g/batch)	Price of dose (\$/dose)	Dose (mg)	Price of product (\$/g)	Operating Time (day/batch)	Days of Operation (day/yr)
5000	0.75	3750	\$2,000	0.5	\$4,000,000	8	328.5
<b>Minimum Revenue (\$/year)</b>					<b>\$5,235,468.75</b>		

Table 13: Expenses summary for all cases.

	Base Case	Alternate Case 1	Alternate Case 2a	Alternate Case 2b	Alternate Case 3
Revenue (\$/yr)	\$5,235,468.75	\$5,235,468.75	\$5,235,468.75	\$5,235,468.75	\$5,235,468.75
Expenses					
Labor (\$/yr)	-\$902,070.11	-\$902,070.11	-\$902,070.11	-\$902,070.11	-\$902,070.11
Maintenance and Repairs (\$/yr)	-\$278,086.79	-\$254,437.73	-\$269,293.52	-\$289,273.73	-\$280,655.47
Laboratory Charges (\$/yr)	-\$135,310.52	-\$135,310.52	-\$135,310.52	-\$135,310.52	-\$135,310.52
TFF/ Depth Filters (\$/yr)	-\$106,434.00	-\$106,434.00	-\$106,434.00	-\$53,217.00	-\$106,434.00
Administration Costs (\$/yr)	-\$201,379.43	-\$197,832.07	-\$200,060.44	-\$203,057.47	-\$201,764.73
Buffer (\$/yr)	-\$195,350.53	-\$120,895.98	-\$97,841.26	-\$91,346.26	-\$142,744.19
Capital Cost (\$)	-\$4,634,779.90	-\$4,240,628.78	-\$4,488,225.39	-\$4,821,228.76	-\$4,677,591.24
Tax rate			35%		

All of the manufacturing expenses shown in Table 13 can be combined using equation 21 to generate an overall operating cost for each of the five cases shown in Table 14. Where  $C_{buffer}$  and  $C_{TFF\ filters}$  are the buffer TFF/Depth filter costs per year.

$$C_{COMd} = 0.18 * C_{cap} + 2.73 * C_{OL} + 1.23 * (C_{buffer} + C_{TFF\ filters}) \quad \text{Eqn. 21}$$

Table 14: Overall operating costs for all cases.

	Base Case	Alternate Case 1	Alternate Case 2a	Alternate Case 2b	Alternate Case 3
Operating Costs (\$/yr)	-\$4,131,584.74	-\$3,929,643.33	-\$3,970,613.08	-\$3,990,408.27	-\$4,078,866.12

The overall after-tax Cash Flow (ATCF) associated with each case can be computed using equation 22.

$$ATCF = (R - C_{COMd} - d) * (1 - t) + d \quad \text{Eqn. 22}$$

Where R is revenue, t is tax rate (35%), and d is depreciation which was estimated using a five year recovery period MACRS method starting at year 1 since it is assumed that all equipment is purchased at year 0 and the process becomes operational at year 1. Figure 17 displays the discrete cash flow diagram for all of the production cases while figure 18 shows the non-discounted cumulative cash flow diagram.

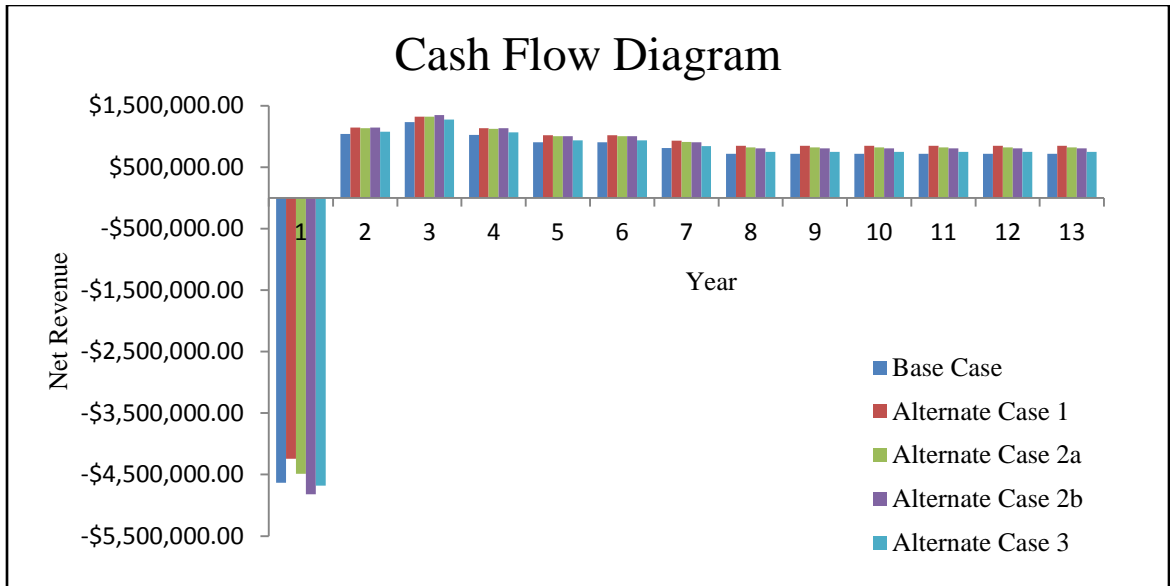


Figure 19: Discrete cash flow diagram for all cases.

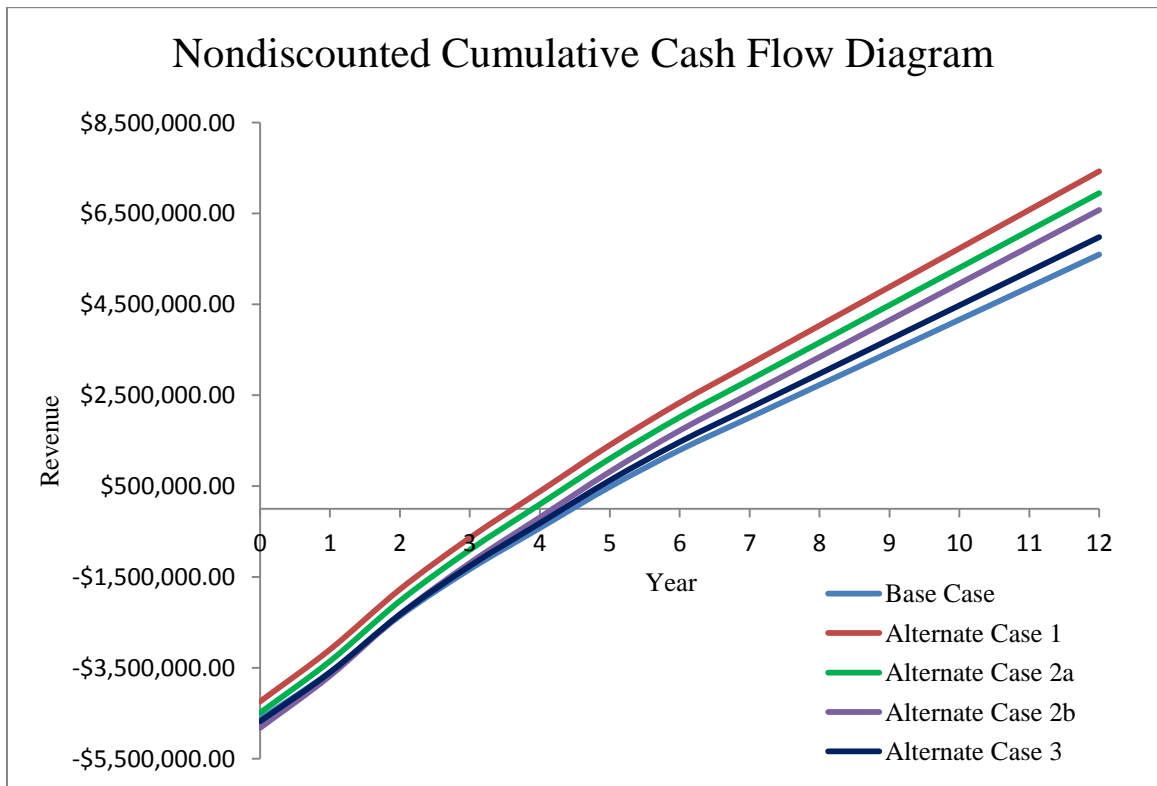


Figure 20: Non-discounted cumulative cash flow diagram for all cases.

Note that even though the base case has a lower capital cost than all the other cases, except alternative case 1, it is the least profitable option. It is also evident from figure 18 that alternate case 1 has the shortest payback period with a value of 3.63 years and an ROROI value of 14.6%. Payback periods and ROROI values for all cases are shown in Table 15.

Table 15: Payback periods and ROROI values for all cases.

	Pay Back Period (yr)	ROROI
Base Case	4.47	10.06%
Alternate Case 1	3.63	14.60%
Alternate Case 2a	3.90	12.90%
Alternate Case 2b	4.19	11.37%
Alternate Case 3	4.34	10.66%

## CONCLUSION

The goal of this project was to analyze and design a process in which a monoclonal antibody fragment is separated and purified for use in medicine. A base case was made to serve as a reference point, this would allow for deviations in the form of three different alternate cases. To better understand the process, the senior design team worked in conjunction with the pharmaceutical company, Genentech, to design this purification process. Genentech provided various parameters to better guide the design process. First, the design was made to accommodate 1000L of fermented culture in which the original Fab concentration is 5 g/L. Within the 1000L fermented culture, 20% to 25% was assumed to be solid cell content which includes cells and cellular debris. A maximum of eight unit operations were used from start to finish. To simplify the economic analysis, it was assumed that each unit operation was at least 90% efficient

with respect to its operation and performance. An overall industrial yield of 65% was obtained as seen in the economic analysis.

To get an in depth understanding of the overall process, various experiments were conducted. These experiments included operating a bioreactor for the first time in the unit operations laboratory, cation exchange chromatography experiment, and a tangential flow filter experiment. From the bioreactor, it was determined that the E. Coli growth cycle can be divided into a lag phase, a growth phase, and a stationary phase before the death phase. The cation exchange experiment demonstrated a very effective isolation and purification of the antibody of interest as seen with the spectrophotometric measurements and gel electrophoresis. The tangential flow filter demonstrated the filtration of smaller proteins while the larger antibody was retained in the original feed solution.

Changes to the base case were made in order to streamline the process or to make the purification easier. Alternate case 1 introduced the concept of depth filtration which was used to filter out small particulates that escaped centrifugation. Alternate case 2 altered the antibody on the genetic level in order to allow easier and more efficient purification. Alternate case 3 introduced monolithic chromatography as a means of polishing off the chromatography process.

The alternate cases were followed by economic analysis which demonstrated which process designs are recommended for further research and development. Altogether the economic analysis has shown that alternate case 1 is the most profitable as seen with the lowest overall capital cost, largest ROROI as well as the most net return of revenue. It is recommended that future senior design teams build off of the information provided so that the process design can go more in depth.

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## APPENDIX A: UNIT OPERATION CHECKLIST FOR FUTURE SENIOR DESIGN TEAMS

<b>Table A.1: Bioreactor Design Checklist</b>	
Diameter	The diameter should follow a 2-3:1 diameter to height ratio.
Liquid level	The liquid level within the reactor is optimum when equal to the tank diameter.
Reactor Temperature	Optimal Temperature for E. Coli growth is around 37°C.
Temperature Effect on Product Production	For a typical reaction, the reaction rate about doubles for every 10°C. For cell growth, operating temperatures above the optimal temperature decreases bioproduct yield.
Reactor pH	Generally the acceptable pH range varies about the optimum by $\pm 1$ to 2 pH units. Optimum pH is around 7.
Oxygen Requirements	Dissolved oxygen is an important substrate and may be a limiting component in the design. A method for checking where the system is obtaining enough oxygen is to run the reactor in a fed batch manner in which growth media is continuously fed into the reactor in excess. The reactor pH should be monitored during growth. If oxygen is limiting, pH will drop due to the generation of lactic acid brought on by the anaerobic metabolism occurring by some of the E. coli. Typical oxygen requirements range from 40 to 60 mmol/L hr.
Impeller	Hydrofoil over Rushton impellers since they require less energy input. Impeller diameter should be between 30 to 40 percent of the reactor's diameter.
MOC	Preference of steel over any other material due to its stability with large ranges of operating pressures and easy sterility.
Reactor Pressure	Always preferred to operate at higher than atmospheric pressure to prevent air and contaminants from leaking into the reactor.

Revolutions Per Minute for Separation	About 3000-12,000 RPM is a good range for accurate cell separation
Time	Time can range from anywhere to 5 minutes (lab scale) to hours (industry) depending on amount of cell culture. Most likely just a few minutes.
Angle of Application	About 4 degree angle seems to be sufficient for proper separation. Fixed angle important.
Flow rate	For industrial use around 350L for continuous flow
Solids Removal	Should be around 75% of the inlet flow rate

Operating Pressure	Below 20,000 psi. While the proposed high-pressure homogenizer can operate at 45,000psi, this would exceed the safety factor, cost more to cool, and lead to a significant E-coli inactivation <sup>i</sup>
Percent Yield	98% (90% is the bare minimum in case study but this is highly discouraged since later stages need to have as much protein from early steps as possible)
Reverse or Parallel Flow? (Hint, it's not parallel)	Recommended: Reverse Flow Pattern Run the homogenizer in a parallel flow pattern to maximize laminar flow, minimize shear, shorten the operating time and create less impact; run the homogenizer in a reverse flow pattern to <u>maximize shear</u> , <u>maximize product on product shear</u> vs. <u>product on equipment</u> and create more impact
Pre-Treatment	Keep E-coli cool, understand what inlet viscosity, particle size should be below 200 micrometers
Post-Treatment	Heat exchanger must be able to compensate for a temperature increase of two degrees Celsius for every 10 MPa increase in operating pressure; particle size after process is one micrometer
Power	Every 100 MPa increase in operating pressure requires the consumption of 3.5 kW
Process duration	Must not exceed 24 hours in addition to a safety factor of at least 10% of this (22.5 hours)
Inlet Flow Rate	Lower flow rates for higher operating pressures
Continuous process	For future groups it is recommended to find the following design parameters: recycle ratio, bleed rate, fraction of cells after each pass, flow rate through homogenizer valve, initial and final concentration of unbroken cells

<b>Table A.4: Tangential Flow Filter Design Checklist</b>	
Filter Surface Area	The larger the filter surface area the better the mass transfer. Around 2.5m <sup>2</sup> is a good membrane to look at.
Filter Pore Size	It is recommended to have filter pore sizes that are at least three times as small as the mAb fragment to increase protein retention.
Operating Pressure	A decent degree of pressure is required. Around 20 psi with respect to atmospheric pressure is a good amount to ensure efficient mass transfer. Research durable TFF's with high pressure capacities.
Buffer Flow	In order for proper TFF function, buffer should be allowed to flow through readily. Flow rates on industrial scale should be around 3500 L.
Feed/Protein Viscosity and Concentration	Too viscous and concentrated feed solution can cause clogging of the pores in the membrane and lead to poor results. Be sure to dilute and maybe run through chromatography columns first before feeding into the TFF.
Feed Flow Rate	After accounting for concentration and viscosity, a large diluted amount should be flowed through. On an industrial level around 4-10 L/min

<b>Table A.5: Chromatography Design Checklist</b>	
Resin Height	Typical resin height is approximately 15-20'' (Do not exceed 30'' in height).
Diameter	Increase diameter of columns with resin volume. This is an important parameter that Genentech looks for, the wider the column the better. Build outwards not upwards.
Number of Theoretical Plates	Approximately 1,400 to 10,000 theoretical plates expected because the number of theoretical plates depends on the resin (many small beads).

HETP	Should be same as the diameter of the individual resin bead approximately 50 $\mu$ m. This is common for almost all columns and should be understood.
Pressure Drop	Decrease column height and increase diameter to prevent increase pressure drop and destroying the resin. The greater the pressure drop, the more damage to the resin.
Number of Columns	Should be minimized to decrease maintenance costs. Running in parallel allowed for multiple smaller columns but look into designing fewer larger columns. Again, build wider columns.
Column Material	Preference of stainless steel. Acrylic is cheaper and can handle the same amount of pressure and design specs. However, acrylic is harder to sterilize and should be taken into consideration.

## APPENDIX B: ECONOMIC ANALYSIS

### Base Case

Table B.1: Capital costs for base case

Equipment	Size	Manufacturer	Quantity	Unit Price (\$/equipment)	Cost
<b>Bioreactor</b>	1300 L	Sartorius	1	\$1,200,000.00	\$1,200,000.00
<b>High- Pressure Homogenizer</b>	20,000 psi 6 L/min	BEE International	1	\$250,000.00	\$250,000.00
<b>Continuous Centrifuge</b>	2500 L/hr 9650 rpm	Alfa Laval	1	\$650,000.00	\$650,000.00
<b>Tangential Flow Filtration</b>	10 L/min	EMD Milipore	1	\$26,430.00	\$26,430.00
<b>Chromatography Columns</b>	Width: 296 mm Height: 900 mm	Verdot Ips	8	\$29,731.00	\$226,270.95
Resin	Amount Needed (L)	Unit Price (\$/L)	Price (\$)		
<b>Capto S ImpAct</b>	217.8840918	3650	\$795,276.93		
<b>Capto Q ImpRes</b>	216.46926	3063	\$663,045.34		
<b>Capto Phenyl ImpRes</b>	263.1810477	3130	\$823,756.68		
<b>Total</b>				<b>\$4,634,779.90</b>	

Table B.2: Amount of resin needed for base case

Column Volume (L)		Buffer runs per column	Number of columns in parallel	buffer per chromatography (L)
73.70335328	Cation	25	3	5187.716471
73.36273722	Anion	25	3	5154.03
83.96975654	HIC	25	3	6266.215421

Table B.3: Buffer costs for base case.

	<b>cat/An</b>		<b>Hy</b>
<b>buffer components</b>	Na <sub>2</sub> PO <sub>4</sub>	NaCl	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>
<b>Price (\$/kg)</b>	\$150	\$71.50	\$89.90
<b>MW (g/mol)</b>	119.98	58.44	132.14
<b>Concentrations need (mol/L)</b>	0.5	0.3	1.2
<b>Moles Needed (mol)</b>	5170.873235	3102.523941	7519.458505
<b>Kilograms Needed (kg)</b>	620.4013708	181.3114991	993.6212469
<b>Price (\$)</b>	\$93,060.21	\$12,963.77	\$89,326.55
<b>Total</b>	\$195,350.53		

Table B.4: Filter costs for base case.

Filters per batch per TFF	Number of TFFs	Total Filters per batch	Price (\$/filter)	Total cost per batch (\$)	Stream Factor (SF)	Operating Time (day/batch)	Days of Operation (day/yr)
2	2	4	\$648.00	\$2,592.00	0.9	8	328.5
Total Cost (\$/yr)				\$106,434.00			

## Alternate Case 1

Table B.5: Capital costs for alternate case 1.

Equipment	Size	Manufacturer	Quantity	Unit Price (\$/equipment)	Cost
<b>Bioreactor</b>	1300 L	Sartorius	1	\$1,200,000.00	\$1,200,000.00
<b>High- Pressure Homogenizer</b>	20,000 psi 6 L/min	BEE International	1	\$250,000.00	\$250,000.00
<b>Continuous Centrifuge</b>	2500 L/hr 9650 rpm	Alfa Laval	1	\$650,000.00	\$650,000.00
<b>Depth Filtration</b>	n/a	EMD Milipore	1	\$12,950.00	\$12,950.00
<b>Tangential Flow Filtration</b>	10 L/min	EMD Milipore	1	\$26,430.00	\$26,430.00
<b>Chromatography Columns</b>	Width: 296 mm Height: 900 mm	Verdot Ips	7	\$29,731.00	\$209,236.01
<b>Resin</b>	Amount Needed (L)		Unit Price (\$/L)	Price (\$)	
<b>Capto S ImpAct</b>	266.9347059		3650	\$974,311.68	
<b>Capto Q ImpRes</b>	212.1610909		3063	\$649,849.42	
<b>Capto MMC ImpRes</b>	65.345616		4099	\$267,851.68	
<b>Total</b>				<b>\$4,240,628.78</b>	

Table B.6: Resin requirements for alternate case 1.

Column Volume (L)		buffer runs per column	Number of columns in parallel	buffer per chromatography (L)
84.76843398	Cation	25	3	6355.588235
72.31760883	Anion	25	3	5051.454545
50.00460194	Multimodal	25	1	1555.848

Table B.7: Buffer cost for alternate case 1.

	cat/An		Multimodal	
buffer components	Na <sub>2</sub> PO <sub>4</sub>	NaCl	Na <sub>2</sub> PO <sub>4</sub>	NaCl
Price (\$/kg)	\$150	\$71.50	\$150	\$71.50
MW (g/mol)	119.98	58.44	119.98	58.44
Concentrations need (mol/L)	0.5	0.3	0.025	0.5
Moles Needed (mol)	5703.52139	3422.11283	38.8962	777.924
Kilograms Needed (kg)	684.3084964	199.988274	4.6667661	45.46187856
Price (\$)	\$102,646.27	\$14,299.16	\$700.01	\$3,250.52
<b>Total</b>	<b>\$120,895.98</b>			

Table B.8: Filter costs for alternate case 1.

Filters per batch per TFF	Number of Depth Filters	Total Number of Filters	Price (\$/filter)	Total cost per batch (\$)	Stream Factor (SF)	Operating Time (day/batch)	Days of Operation (day/yr)
2	2	4	\$648.00	\$2,592.00	0.9	8	328.5
<b>Total Cost (\$/yr)</b>				<b>\$106,434.00</b>			

## Alternate Case 2a

Table B.9: Capital costs for alternate case 2a.

Equipment	Size	Manufacturer	Quantity	Unit Price (\$/equipment)	Cost
<b>Bioreactor</b>	1300 L	Sartorius	1	\$1,200,000.00	\$1,200,000.00
<b>High- Pressure Homogenizer</b>	20,000 psi 6 L/min	BEE International	1	\$250,000.00	\$250,000.00
<b>Continuous Centrifuge</b>	2500 L/hr 9650 rpm	Alfa Laval	1	\$650,000.00	\$650,000.00
<b>Tangential Flow Filtration</b>	10 L/min	EMD Milipore	2	\$26,430.00	\$52,860.00
<b>Chromatography Columns</b>	Width: 296 mm Height: 900 mm	Verdot Ips	6	\$29,731.00	171062.2171
<b>Resin</b>					
<b>Resin</b>	<b>Amount Needed (L)</b>		<b>Unit Price (\$/L)</b>	<b>Price (\$)</b>	
<b>Nuvia™</b>	128.625		6636.36	\$853,601.81	
<b>Capto S ImpAct</b>	258.737359		3650	\$944,391.36	
<b>Capto Adhere ImpRes</b>	81.20372497		4511	\$366,310.00	
<b>Total</b>				<b>\$4,488,225.39</b>	

Table B.10: Resin requirement for alternate case 2a.

Column Volume (L)		buffer runs per column	Number of columns in parallel	buffer per chromatography (L)
82.69235056	IMAC	25	1	3062.5
83.01448695	Cationic	25	3	6160.41331
59.3113021	Multimodal	25	1	1933.42202

Table B.11: Buffer costs for alternate case 2a.

	cat		IMAC			Multimodal	
buffer components	Na <sub>2</sub> PO <sub>4</sub>	NaCl	Na <sub>2</sub> PO <sub>4</sub>	NaCl	Imidazole	Na <sub>2</sub> PO <sub>4</sub>	NaCl
Price (\$/kg)	\$150	\$71.50	\$150	\$71.50	\$243.00	\$150	\$71.50
MW (g/mol)	119.98	58.44	119.98	58.44	68.08	119.98	58.44
Concentration need (mol/L)	0.5	0.3	0.02	0.5	0.5	0.03	0.1
Moles Needed (mol)	3080.2066	1848.123	61.25	1531.25	1531.25	58.002660	193.3422
Kilograms Needed (kg)	369.56319	108.0043	7.348775	89.4862	104.25	6.9591592	11.2989
Price (\$)	\$55,434.48	\$7,722.31	\$1,102.32	\$6,398.27	\$25,332.14	\$1,043.87	\$807.87
<b>Total</b>	<b>\$97,841.26</b>						

Table B.12: Filter costs for alternate case 2a.

Filters per batch per TFF	Number of TFFs	Total Number of Filters	Price (\$/filter)	Total cost per batch (\$)	Stream Factor (SF)	Operating Time (day/batch)	Days of Operation (day/yr)
2	2	4	\$648.00	\$2,592.00	0.9	8	328.5
<b>Total Cost (\$/yr)</b>				<b>\$106,434.00</b>			

## Alternate Case 2b

Table B.13: Capital costs for alternate case 2b.

Equipment	Size	Manufacturer	Quantity	Unit Price (\$/equipment)	Cost
<b>Bioreactor</b>	1300 L	Sartorius	1	\$1,200,000.00	\$1,200,000.00
<b>High- Pressure Homogenizer</b>	20,000 psi 6 L/min	BEE International	1	\$250,000.00	\$250,000.00
<b>Continuous Centrifuge</b>	2500 L/hr 9650 rpm	Alfa Laval	1	\$650,000.00	\$650,000.00
<b>Histidine BioRxR</b>	600 L	Sartorius	1	\$600,000.00	\$600,000.00
<b>Tangential Flow Filtration</b>	10 L/min	EMD Milipore	1	\$26,430.00	\$26,430.00
<b>Chromatography Columns</b>	Width: 296 mm Height: 900 mm	Verdot Ips	6	\$29,731.00	167283.0343
<b>Resin</b>					
<b>Resin</b>	Amount Needed (L)		Unit Price (\$/L)	Price (\$)	
<b>Nuvia™</b>	128.625		6636.36	\$853,601.81	
<b>Capto S ImpAct</b>	232.8869118		3650	\$850,037.23	
<b>Capto Adhere ImpRes</b>	73.09066154		3063	\$223,876.70	
<b>Total</b>				<b>\$4,821,228.76</b>	

Table B.14: Resin requirements for alternate case 2b.

Column Volume (L)		Buffer runs per column	Number of columns in parallel	buffer per chromatography (L)
82.69235056	IMAC	25	1	3062.5
77.23794748	Cationic	25	3	5544.92647
54.65833301	Multi anion	25	1	1740.25385

Table B.15: Buffer costs for alternate case 2b.

buffer components	Cation		IMAC			Multimodal	
	Na <sub>2</sub> PO <sub>4</sub>	NaCl	Na <sub>2</sub> PO <sub>4</sub>	NaCl	Imidazole	Na <sub>2</sub> PO <sub>4</sub>	NaCl
Price (\$/kg)	\$150	\$71.50	\$150	\$71.50	\$243.00	\$150	\$71.50
MW (g/mol)	119.98	58.44	119.98	58.44	68.08	119.98	58.44
Concentrations need (mol/L)	0.5	0.3	0.02	0.5	0.5	0.03	0.1
Moles Needed (mol)	2772.46324	1663.4779	61.25	1531.25	1531.25	52.20762	174.0254
Kilograms Needed (kg)	332.640139	97.213651	7.348775	89.48625	104.25	6.26387	10.17004
Price (\$)	\$49,896.02	\$6,950.78	\$1,102.32	\$6,398.27	\$25,332.14	\$939.58	\$727.16
<b>Total</b>	<b>\$91,346.26</b>						

Table B.16: Filter costs for alternate case 1.

Filters per batch per TFF	Number of TFFs	Total Number of Filters	Price (\$/filter)	Total cost per batch (\$)	Stream Factor (SF)	Operating Time (day/batch)	Days of Operation (day/yr)
2	1	2	\$648.00	\$1,296.00	0.9	8	328.5
<b>Total Cost (\$/yr)</b>				<b>\$53,217.00</b>			

## Alternate Case 3

Table B.17: Capital costs for alternate case 3.

Equipment	Size	Manufacturer	Quantity	Unit Price (\$/equipment)	Cost
<b>Bioreactor</b>	1300 L	Sartorius	1	\$1,200,000.00	\$1,200,000.00
<b>High- Pressure Homogenizer</b>	20,000 psi 6 L/min	BEE International	1	\$250,000.00	\$250,000.00
<b>Continuous Centrifuge</b>	2500 L/hr 9650 rpm	Alfa Laval	1	\$650,000.00	\$650,000.00
<b>Tangential Flow Filtration</b>	10 L/min	EMD Milipore	2	\$26,430.00	\$52,860.00
<b>Monolithic Column</b>	8 mL	BIA	6	\$127,660.17	\$723,833.16
<b>Chromatography Columns</b>	Width: 296 mm Height: 900 mm	Verdot Ips	5	\$29,731.00	\$143,590.83
<b>Resin</b>					
<b>Resin</b>	<b>Amount Needed (L)</b>		<b>Unit Price (\$/L)</b>	<b>Price (\$)</b>	
<b>Capto S ImpAct</b>	272.3823529		3650	\$994,195.59	
<b>Capto Q ImpRes</b>	216.4909091		3063	\$663,111.65	
<b>Total</b>				<b>\$4,677,591.24</b>	

Table B.18: Resin requirements for alternate case 3.

Column Volume (L)		Buffer runs per column	Number of columns in parallel	buffer per chromatography (L)
85.91432681	Cationic	25	3	6485.294118
73.36795884	Anionic	25	3	5154.545455
40	Monolithic	25	6	5670

Table B.19: Buffer costs for alternate case 3.

	<b>cat/An</b>		<b>Mono</b>
<b>buffer components</b>	Na <sub>2</sub> PO <sub>4</sub>	NaCl	Tris HCl
<b>Price (\$/kg)</b>	\$150	\$71.50	\$1,310.00
<b>MW (g/mol)</b>	119.98	58.44	157.6
<b>Concentrations need (mol/L)</b>	0.5	0.3	0.02
<b>Moles Needed (mol)</b>	5819.919786	3491.95187	113.4
<b>Kilograms Needed (kg)</b>	698.2739759	204.069667	17.87184
<b>Price (\$)</b>	\$104,741.10	\$14,590.98	\$23,412.11
<b>Total</b>	<b>\$142,744.19</b>		

Table B.20: Capital costs for alternate case 3.

Filters per batch per TFF	Number of TFFs	Total Number of Filters	Price (\$/filter)	Total cost per batch (\$)	Stream Factor (SF)	Operating Time (day/batch)	Days of Operation (day/yr)
2	2	4	\$648.00	\$2,592.00	0.9	8	328.5
<b>Total Cost (\$/yr)</b>				<b>\$106,434.00</b>			

Table B.21: Labor costs for all cases.

Number of Unit Operations	Required Operating Labor per shift	Operating Labor	Cost of Labor per Operator (\$)	Total Cost of Operating Labor (\$)	Supervisory Cost (\$)	Total Labor Cost (\$)
8	2.851	12.83091	\$59,580.00	\$764,466.19	\$137,603.92	\$902,070.11

Table B.22: Depreciation Values

Depreciation Schedule for MACRS Method (5 year recovery period)						
Year	Depreciation Allowance (% of Capital Costs)	$d_{kbase}$	$d_{kalt1}$	$d_{kalt2a}$	$d_{kalt2b}$	$d_{kalt3}$
1	20.00%	\$926,955.98	\$848,125.76	\$897,645.08	\$964,245.75	\$935,518.25
2	32.00%	\$1,483,129.57	\$1,357,001.21	\$1,436,232.12	\$1,542,793.20	\$1,496,829.20
3	19.20%	\$889,877.74	\$814,200.73	\$861,739.27	\$925,675.92	\$898,097.52
4	11.52%	\$533,926.64	\$488,520.44	\$517,043.56	\$555,405.55	\$538,858.51
5	11.52%	\$533,926.64	\$488,520.44	\$517,043.56	\$555,405.55	\$538,858.51
6	5.76%	\$266,963.32	\$244,260.22	\$258,521.78	\$277,702.78	\$269,429.26

Table B.23: Cash flows for all cases.

Cash Flow Diagram					
Year	Base Case	Alternate Case 1	Alternate Case 2a	Alternate Case 2b	Alternative Case 3
0	-\$4,634,779.90	-\$4,240,628.78	-\$4,488,225.39	-\$4,821,228.76	-\$4,677,591.24
1	\$1,041,959.20	\$1,145,630.54	\$1,136,331.96	\$1,146,775.33	\$1,079,223.10
2	\$1,236,619.95	\$1,323,736.95	\$1,324,837.43	\$1,349,266.94	\$1,275,681.93
3	\$1,028,981.81	\$1,133,756.78	\$1,123,764.93	\$1,133,275.89	\$1,066,125.84
4	\$904,398.93	\$1,019,768.68	\$1,003,121.43	\$1,003,681.26	\$940,392.19
5	\$904,398.93	\$1,019,768.68	\$1,003,121.43	\$1,003,681.26	\$940,392.19
6	\$810,961.77	\$934,277.60	\$912,638.81	\$906,485.29	\$846,091.95
7	\$717,524.60	\$848,786.52	\$822,156.18	\$809,289.32	\$751,791.71
8	\$717,524.60	\$848,786.52	\$822,156.18	\$809,289.32	\$751,791.71
9	\$717,524.60	\$848,786.52	\$822,156.18	\$809,289.32	\$751,791.71
10	\$717,524.60	\$848,786.52	\$822,156.18	\$809,289.32	\$751,791.71
11	\$717,524.60	\$848,786.52	\$822,156.18	\$809,289.32	\$751,791.71
12	\$717,524.60	\$848,786.52	\$822,156.18	\$809,289.32	\$751,791.71

Table B. 24: Cumulative cash flows for all cases.

Cumulative Cash Flow					
Year	Base Case	Alternate Case 1	Alternate Case 2a	Alternate Case 2b	Alternative Case 3
<b>0</b>	-\$4,634,779.90	-\$4,240,628.78	-\$4,488,225.39	-\$4,821,228.76	-\$4,677,591.24
<b>1</b>	-\$3,592,820.71	-\$3,094,998.25	-\$3,351,893.42	-\$3,674,453.43	-\$3,598,368.14
<b>2</b>	-\$2,356,200.75	-\$1,771,261.30	-\$2,027,056.00	-\$2,325,186.50	-\$2,322,686.22
<b>3</b>	-\$1,327,218.94	-\$637,504.52	-\$903,291.07	-\$1,191,910.61	-\$1,256,560.37
<b>4</b>	-\$422,820.01	\$382,264.16	\$99,830.36	-\$188,229.35	-\$316,168.18
<b>5</b>	\$481,578.92	\$1,402,032.83	\$1,102,951.80	\$815,451.91	\$624,224.00
<b>6</b>	\$1,292,540.69	\$2,336,310.43	\$2,015,590.60	\$1,721,937.19	\$1,470,315.95
<b>7</b>	\$2,010,065.30	\$3,185,096.95	\$2,837,746.79	\$2,531,226.51	\$2,222,107.66
<b>8</b>	\$2,727,589.90	\$4,033,883.48	\$3,659,902.97	\$3,340,515.82	\$2,973,899.37
<b>9</b>	\$3,445,114.51	\$4,882,670.00	\$4,482,059.15	\$4,149,805.14	\$3,725,691.08
<b>10</b>	\$4,162,639.11	\$5,731,456.53	\$5,304,215.34	\$4,959,094.45	\$4,477,482.79
<b>11</b>	\$4,880,163.71	\$6,580,243.05	\$6,126,371.52	\$5,768,383.77	\$5,229,274.50
<b>12</b>	\$5,597,688.32	\$7,429,029.57	\$6,948,527.71	\$6,577,673.08	\$5,981,066.21