Schinthia Islam

Co-authors:
Fredy Bridges
Madeline Laxa
Becky McKenna
Shawn Pugh

Abstract

Protein purification processes have become highly sophisticated in recent decades. Even the simplest separations require various steps to ensure the highest possible purity and maximum profit. The goal of this paper is to propose a configuration of unit operations that will purify a monoclonal antibody (Lucentis) grown and extracted from *e. coli* for the purpose of treating the "wet" form of macular degeneration.

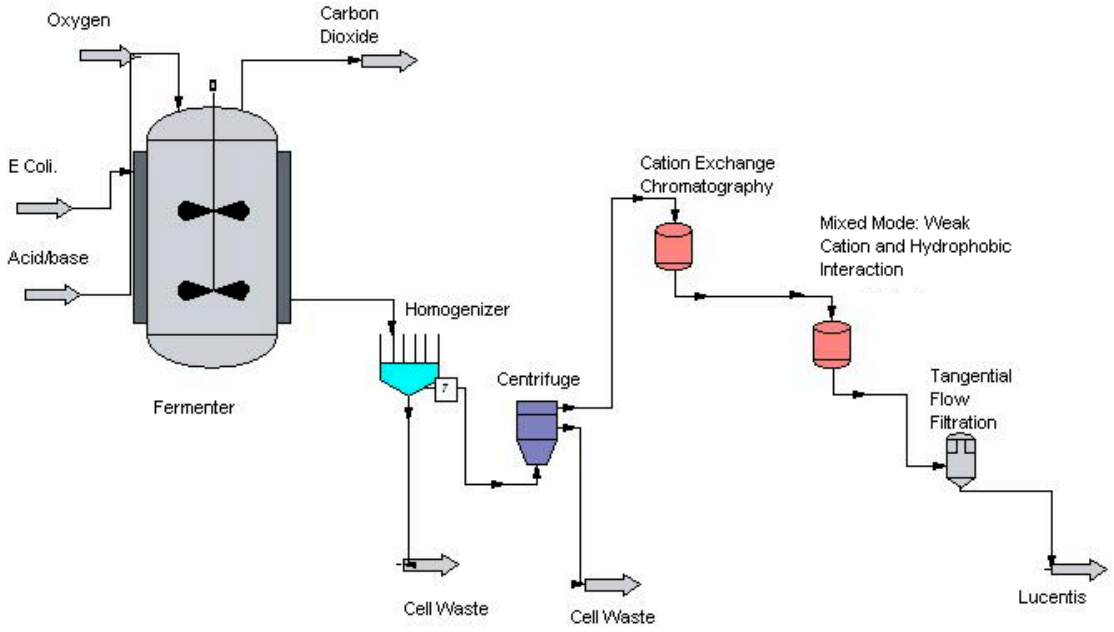
The following units will be explored, in this determined order: homogenizer, centrifuge, column chromatography, tangential flow filtration. Specifications for these units will be determined using data from either literature research or experimentation.

A sample of bacteria culture that has been processed through a homogenizer and centrifuge were obtained and used in our experimentations. A homogenizer is used to break apart the cell wall to release the proteins inside. A centrifuge is used to do a crude mass separation before more intrinsic steps to isolate the desired antibody are needed. Specifications for these two units such as reactor size and flow area will be theorized. Two types of column chromatography will be used to separate and purify the sample: cation exchange and mixed (hydrophobic interaction-anion exchange) mode. The resin height, column height, and column diameter will be determined for these units. Chromatography is commonly used to isolate and purify proteins. A tangential flow filtration is then used to remove any excess debris from the sample as the last purification procedure. The culmination of these units proposes a purification process that can ideally isolate a desired antibody and produce a pure product, ready for human use.

Introduction

Lucentis (Ranibizumab) is a monoclonal antibody derived from the murine parent antibody. It is smaller than the parent molecule, but provides a much stronger affinity for binding the VEGF-A (vascular endothelial growth factor). Lucentis prevents the formation of abnormal, new blood vessels in the eye and dries up the blood vessels which have already begun to leak blood and other fluid. It has been approved to treat the "wet" type of age-related macular degeneration, which causes vision loss. The Lucentis antibody is known to have a molecular weight of 50kDa and an isoelectric point (pI) of 9.

The following is an overall process flow diagram of the production and purification of Lucentis.



Theory

Fermentor

Synthesis of Lucentis by recombinant E. coli requires a fermentation process in which the bacteria can grow and multiply. The cultivation process can be done in either a batch or continuous fermentor. For small, lab-scale fermentations, a chemostat would be the ideal bioreactor. A chemostat, shown in Figure 1 below, is an example of a continuous bioreactor.

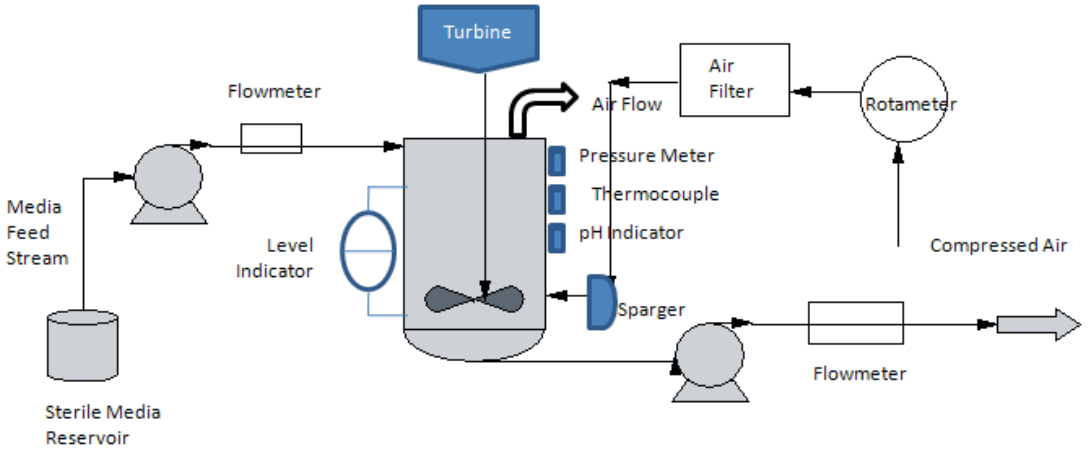


Figure 1: Chemostat Process Flow Diagram.

In the diagram above, fresh medium is supplied continuously into the reactor, while the culture volume is maintained at a constant level by continuously removing some of the culture fluid.

For a large, industry-scale fermentation, a fed-batch reactor, shown in Figure 2, is considered more practical. The reason behind this is the fact that fed-batch fermentations have the advantages of growing the maximal amount of biomass while avoiding substrate inhibition. Semi-continuous feeding also ensures that optimum feed conditions are maintained, allowing cultures of very high cell density while repressing the accumulation of by-products that can inhibit bacterial growth.

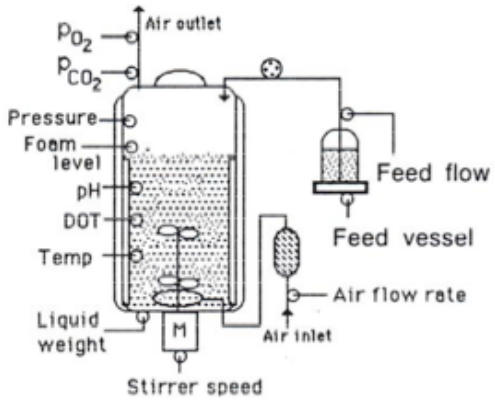


Figure 2: Fed-batch Reactor [1]

For both types of bioreactor, the following design parameters are to be critically considered[2]:

1. Optimal culture-medium temperature
2. Pressure within the reactor

3. Agitator shaft power (for maximum cell-substrate interaction)
4. Carbon source concentration and feed rate
5. Fermentation pH
6. Dissolved oxygen concentration and gas flowrate
7. Induction cell density (the cell density at which feeding initiates)

Controlling the fermentation process or the specific growth rate of the biomass entails the rigorous study of the specific microbe of interest, since the nature of the organism will ultimately dictate the optimum growth condition that must be provided by the reactor system.

Research and Design

The design of the bioreactor begins with understanding the characteristics and nature of the concerned microbe, specific kinetics of biomass growth and production of the desired product.

Microbial growth can be descried by the following reaction[2]:

$$\sum S + X \rightarrow \sum P + nX \qquad (1)$$

Where _S is the substrate, X is the cells, _P is the extracellular products and nX represents more cells. More specifically, a biological reaction can be written as[2]:

$$CH_mO_n + aO_2 + bNH_3 \rightarrow cCH_\alpha O_\beta N_\delta + dH_2O + eCO \qquad (2)$$

Where CH_mO_n represents a mole of carbohydrate and $cCH_\alpha O_\beta N_\delta$ represents c-moles of cellular material. The above reaction assumes that only water and CO are produced. An elemental composition of a bacteria was found to be typically $CH_{1.666}N_{0.20}O_{0.27}$ and a molecular weight of 20.7

The most simple growth kinetics model is given by the Monod Kinetics [3] below:

$$\mu_g = \frac{\mu_m S}{K_s + S} \qquad (3)$$

Where μ_g is the gross specific growth rate of the bacteria, μ_m is the max. growth rate, S is the rate limiting substrate concentration and Ks is the saturation constant or half-velocity constant. This type of kinetics assumes substrate-limited growth when the latter is slow and the population density is low.

In the production of Lucentis, however, it is assumed that the recombinant E. coli strain undergoes a much more complicated growth kinetics. The metabolic burden of the desired protein product on bacterial growth and the inhibition caused by production of unwanted by-products must be taken into consideration.

A study [4] by A.R. Cockshott, I.D.L. Bogle on growth kinetics modeling of recombinant E. coli fermentation suggests the following model:

$$\mu = \mu_{max} \cdot \left(\frac{1}{1 + K_{M1}/c_s}\right) \cdot \left(1 - \frac{c_a}{K_{ia1}}\right) \cdot \left(1 - \frac{r_p}{r_{p,l}}\right) \quad (4)$$

Where μ is the biomass specific growth rate [h^{-1}], μmax is the maximum specific growth rate [h^{-1}], KM1 [g/L] is the Michaelis-Menten coefficient, cs is the glucose concentration [g/L], ca is the acetate concentration [g/L], Kia1 is the acetate inhibition constant for growth [g/l], rp is the specific rate of product production [g product/g biomass/h], rpl is a term for inhibition of growth due to product production [g product/g biomass/hr]. This growth kinetics model clearly takes into account the effect of the protein specific production rate on the biomass growth rate; in other words, the energy cost of product production to the bacterial host is not taken to be negligible. Also, for large scale fermentations, production of growth-inhibiting by-products can be an issue, and is also considered in the above model If fermentation experiments are implemented on the production of Lucentis, the mention growth kinetics constants can be experimentally determined and used in the design of the bioreactor.

Given that growth kinetics are known, the pertinent equations that apply to a fed-batch reactor are given by Table 1 below[5]:

Table 1: Mass Balance Equations for Variable-volume Fed-Batch Reactor

Component	Mass Balance Equation
Overall	F = dV/dt
Biomass	dx/dt = x . (u . V -– K_d . V -– F) / V
Substrate	ds/dt = F . (s_o -– s)/V -– u. x/ $Y_{x/s}$
Product	dP/dt = q_p . x -–P . F / V

In the above equations, the notations used are defined as follows:

- V is the volume of the fermentor
- t is the time
- F is the feed rate [volume/time].
- x is the biomass concentration [mass biomass/volume]
- u is the specific growth rate [$time^{-1}$]
- K_d is the specific death rate [$time^{-1}$]
- s is the substrate concentration in the fermentor [mass substrate/volume]
- s_o is the substrate concentration in the feed [mass substrate/volume]
- $Y_{x/s}$ is the yield factor [mass biomass/mass substrate]
- P is the product concentration {mass product/volume] and
- q_p is the specific production rate of product [mass product/(mass biomass . time)

Other important factors in the fermentation process are the amount of inoculum and the composition of the growth media. Typical final culture volume to inoculum ratio is 150 to 1[5].
Also common typical culture medium composition is given by the following[5]:
(g/L)- Citric acid, 0.66; KH2PO4, 4; NH4Cl, 2.7; Na2HPO4, 2.1;

K2SO4, 13.2; CaCl2.2H2O, 0.66; MgSO4.7H2O, 1.3 and the following trace elements (mg/L): ZnSO4.7H2O, 6.4; Cu-SO4.5H2O, 0.66 and MnSO4.H2O, 1.14.Thiamine hydrochloride (0.0132 g/l), tetracycline hydrochloride (0.002 g/l) and glucose (12.5 g/l).

Homogenizer

Due to the fact that Lucentis is not excreted by E. coli and does not occur as a by-product during the fermentation process, a cell disruption mechanism is required to recover it from the bacterial cells. Both mechanical and chemical cell disruption techniques are available in industry today, but for the purpose of this study, the mechanical technique of cell homogenization will be focused on.

Homogenization involves passing a solution of cells through a restricted orifice under high pressure [7]; the cell is then subjected to high shear stress which results in cell lysis or disintegraton. This mechanism is shown in Figure 3 below[8]:

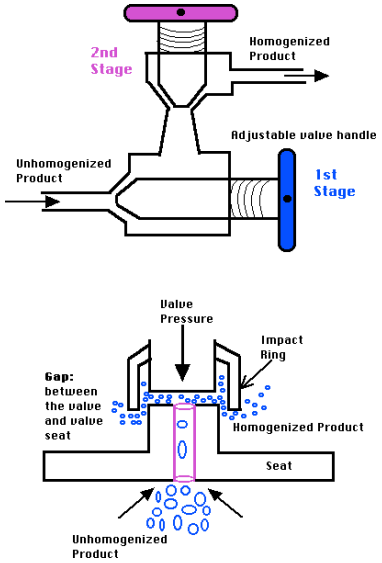


Figure 3: mechanism of homogenization.

Homogenizers typically use an air or motor-driven pump to provide the disruption pressure . A valve adjustment mechanism is also commonly provided to modify the amount of shear stress on the cells to be homogenized. For E. coli disruption, a pressure range of 10,000-20000 psi is usually required, depending on the specific strain and application.

There are three (3) cell processing techniques commonly employed with homogenizers; these are:

1. Single pass technique
2. Batch recycle
3. Continuous recycle and bleed.

These methods are shown in the following block diagram (Figure 4) with the top diagram corresponding to single pass technique, the middle diagram to batch recycle technique and the bottom diagram corresponding to continuous recycle and bleed technique:

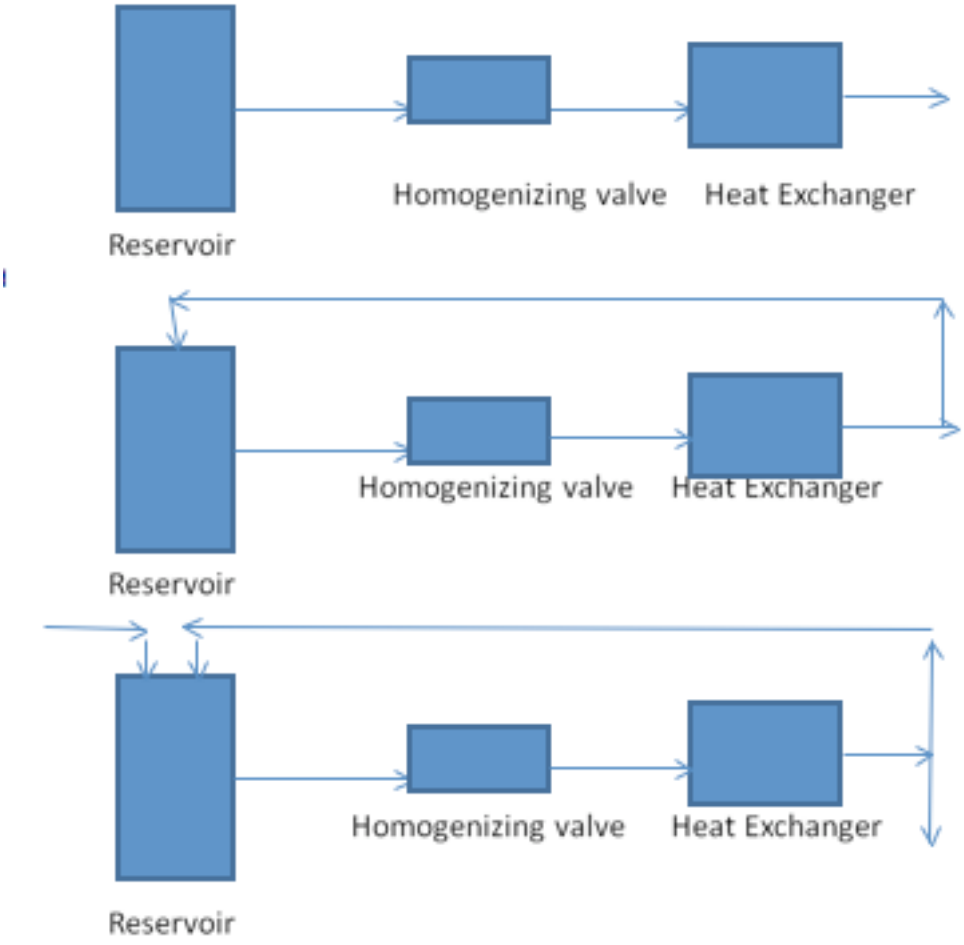


Figure 4: Homogenization techniques.

For the single pass technique, the following equations apply[7]:

$$\frac{C_{out}}{C_{in}} = K \qquad (6)$$

where K is the fraction of cells that survive each pass through the homogenizer and Cout and Cin are the cell concentrations before and after the pass.

If passes are done n times, then equation (6) becomes:

$$\frac{C_{out}}{C_{in}} = K^n \qquad (7)$$

The processing rate is then calculated by equation (8):

$$P = \frac{Flowratein(W)}{Numberofpasses(n)} \qquad (8).$$

For a batch recycle, the following equations apply[7]:

$$\frac{C_f}{C_0} = \exp(-\frac{Wt}{v(1-K)}) \qquad (9).$$

Where W is the flow rate of cell suspension and t is the processing time.

For a continuous recycle and bleed, the following equation (10) describes the process:

$$\frac{C_f}{C_0} = \frac{1}{\frac{W}{I}(\frac{1}{K}-1)+1} \qquad (10)$$

where I is the bleed rate.

Pending homogenization experiments, the optimum number of pass for effective homogenization will depend on the homogenizing capacity of the equipment, the maximum volume of solution it can process as well as its pressure range, and the viscosity of the solution to be processed. Currently, in industry, the most common homogenizer is the Gaulin-Manton homogenizer.

With experiments, the retention time in the homogenizer can be approximated, as well as the number of passes and total processing time, given the degree of cell disruption needed to recover the desired product protein.

Centrifuge

Centrifugation is a separation process that is used to remove cell debris from the homogenization step of this purification process. It uses the density difference between the solids and the surrounding liquid to separate them. When a mixture of solids and liquids is allowed to stand, sedimentation occurs. This is where the denser material settles to the bottom through gravity. The process is called centrifugation if the settling is accelerated with a centrifugal field. The velocity of the particles is affected by the buoyant force and the drag force. The particles are accelerated by the buoyant force which is based on the density differences and the drag force retards the particle from this movement caused by the buoyant force. Eventually, these two forces will become equal and allow the particle to move at constant velocity. This velocity is the terminal velocity and can be found from the following equation where v is the terminal velocity, d is the sphere's diameter, _ is the viscosity of the continuum, $_s$ is the density of the sphere, _ is

$$v = \frac{d^2}{18\mu}(\rho_s - \rho)\,a$$

the density f the fluid, and a is the acceleration due to some applied force. For centrifugation, the acceleration, a, can be found by substituting with $_^2$r where _ is the angular rotation in radians per second and r is the radial distance from the center of the centrifuge to the sphere. By

$$v = \frac{d^2}{18\mu}(\rho_s - \rho)\,\omega^2 r$$

integrating this equation, the separation time, t, can be found.

$$v_\omega = \frac{dr}{dt} = \frac{d^2}{18\mu}(\rho_s - \rho)\,\omega^2 r$$
$$ln\left(\frac{r}{r_o}\right) = \frac{d^2}{18\mu}(\rho_s - \rho)\,\omega^2 t$$

There are three basic types of centrifuges: tubular bowl, disc, and basket. A disc with nozzle has been chosen to replicate the centrifuge used at Genentech. It has also been chosen since it is commonly used for bioseparations and it has good efficiency and recovery time (Belter, Cussler, & Hu, 1988). Below is a diagram of a disc with nozzle centrifuge along with the descriptions for each part (Alfa Laval).

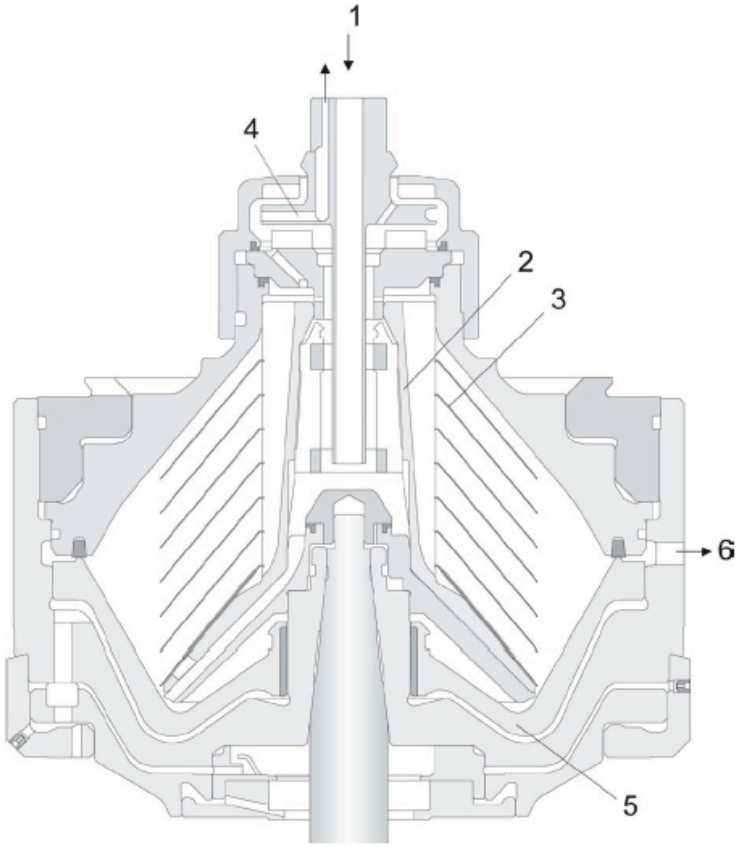


Figure 5: Disc with Nozzle Centrifuge.

1. Feed
2. Distributor – accelerates feed
3. Disc Stack – where separation occurs
4. Paring Disc – discharges liquid
5. Sliding Bowl Bottom – allows solids to be discharged, moved by hydraulic system
6. Solid Ports – where solids are discharged out

Designing large scale centrifugations involves using laboratory data. This is needed to predict the performance of commercially available centrifuges. The method of equivalent time is a qualitative way to obtain information. By calculating the product of the centrifugal force, G, and time, t, the difficulty of a separation can be found. In this equation R_o is a characteristic radius

$$Gt = \frac{\omega^2 R_o t}{g}$$

which is usually the maximum in the centrifuge. The following is a table listing typical Gt values. These values are used by commercial vendors.

Table 2: Generalized Centrifugation Conditions.

Solids	Centrifugation Conditions (Gt) (106 s)
Eukaryotic cells Chloroplasts	0.3
Eukaryotic cell debris Cell Nuclei	2
Protein precipitates	9
Bacteria Mitochondria	18
Bacterial cell debris Lysosomes	54
Ribosomes Polysomes	1100

The other type is the sigma factor. This is best used for determining which centrifuge on hand to use, while Gt is best for deciding which centrifuge to buy. In the following equation Q is the flow, v_g reflects the properties of the solids, and _ summarizes characteristics of the centrifuges.

$$Q = v_g \cdot \Sigma$$

The following is the sigma factor for a disc bowl centrifuge where n is number of discs and R is the radius in and out (Belter, 1988).

$$\Sigma = \frac{2n\pi\omega^2 (R_0^3 - R_1^3)\cot(\theta)}{3g}$$

Table 3: Centrifuge Parameters.

E. Coli	
Density (g/mL)	1.09
Protein	
Density (g/mL)	1.3
Sedimentation Radius (m)	5.00E-09
Sedimentation Velocity (cm/hr)	0.06
Reynold's Number	2.00E-09
Centrifuge	
Number of Discs	20
Angle of Discs (Degrees)	40
Flow Rate (L/hr)	250
Inner Radius (m)	0.05
Outer Radius (m)	0.45

Ion Exchange Chromatography

Ion exchange chromatography is a purification process which utilizes the charge of the molecules for separation. Within a chromatography column there is a stationary as well as a mobile phase. The stationary phase is a resin which possesses either positively or negatively charged functional groups supported on the surface of the resin beads. Once the proteins are loaded to the column, the mobile phase of the chromatography column is composed of buffers and a gradient of salt solutions for elutions. When designing an ion exchange column, it is important to understand how the isoelectric point of the desired molecule interacts in certain conditions. The isoelectric point (pI) of a molecule is defined as the pH at which that molecule has a net negative charge. When the pH of a solution is greater than the pI of the protein, the protein has a net negative charge. Similarly, when the pH of a solution is less than the pI of the protein, the protein has a net positive charge. During operation of the ion exchange column, when the buffer solution's pH reaches the pI of the protein, the protein's net charge will be zero. At this point the protein will no loner bind to the stationary phase and will be released from the column.

The two types of ion exchange chromatography include cation exchange chromatography and anion exchange chromatography. The principles of chromatography are similar; however, they differ in the resin selection as well as the buffer selection.

Cation Exchange Chromatography

While the resin involved with a cation exchanger are negatively charges, cation exchangers are named for their affinity to cations. To effectively bind proteins, the pH of the buffer in the system must be less than the pI of the protein of interest. The lower the pH of the system to the pI of the protein, the stronger the binding interaction will be between the protein of interest and the negatively charged resin

Anion Exchange Chromatography

The resin in an anion exchange chromatography column possesses positively charged functional groups to which negatively charged particles bind. In order to bind a target

protein to the column, the pH of the buffer must be greater than the pI of the protein. The higher the pH, the more negatively charged the protein will be and the stronger it will bind to the column.

Protein Binding Capacity

Whether a cation or anion exchange column is being designed, it is important when choosing a resin for the ion exchanger that the amount of protein that can bind per unit volume of the packed resin be within the required range. Generally, smaller proteins bind to the resin more easily than larger molecules. The binding capacity of proteins has a nearly linear relationship with its molecular weight. The semi-log plot below illustrates this linear relationship and shows that as the molecular weight of the molecule increases, the ability of the protein to bind to the resin linearly decreases.

Table 4: Binding capacity of various proteins on CM-Cellulose resin

MW	log (MW)	Capacity (mg/cm^3)
14300	4.155336037	130
21400	4.330413773	100
45000	4.653212514	70
60000	4.77815125	40
82000	4.913813852	35
88000	4.944482672	48
140000	5.146128036	21
145000	5.161368002	26
160000	5.204119983	22
228000	5.357934847	12.5

[Scopes 150-151]

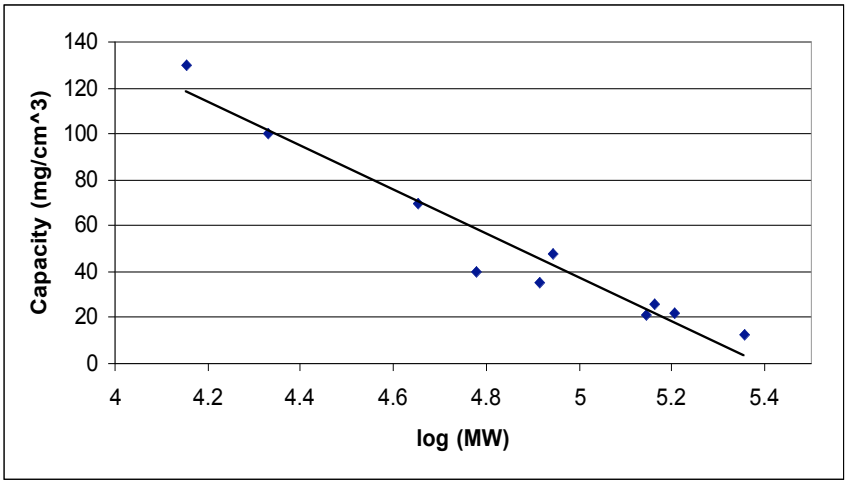


Figure 6: Semi-log plot of molecular weight and binding capacity

When designing an ion exchange column, it is important to account for the possibility of several proteins binding to the resin besides the one you are trying to isolate. However, the implications of this linear relationship illustrates that if the molecular weight of the other proteins (besides

Lucentis) are significantly higher than the molecular weight of Lucentis (50 kDa), they will not bind as readily to the resin. This will help in the purification of Lucentis since larger proteins will not bind in the column as readily as the smaller molecules

Properties which must be considered when determining best resin for chromatography

Operating pH Range

Whether the ion exchanger is cationic or anionic, the material of the resin is generally made from cellulose, agarose, dextran, silica, and synthetic polymers which have positively or negatively charged functional groups attached. Depending on the circumstances at which the resins will be used, some resins have the ability to sustain harsh conditions while other become uncharged. If a resin becomes uncharged, the experiment would essentially be ruined.

Cation Exchange: For basic cation exchange chromatography, carboxymethyl functional groups are generally used, but in the pH range from 1-6, the resin will become uncharged. If a experiment is designed to run at these conditions, it is suggested that a sulfopropyl group be used which stays charged at nearly the entire spectrum of pH.

Anion Exchange: The most common functional groups for anion exchange chromatography are diethylaminoethyl (DEAE) groups, but these groups become uncharged at a pH from 5-14 making them very difficult to work with. The primary alternative to DEAE functional groups are quaternary amino groups.

Resin Capacity

Each type of resin will have a limit to the amount of protein the resin can hold before it is fully saturated. Generally the capacity is on a basis of milli-equivalent of protein per milliliter. Based off the resin capacity, the necessary volume of the column may be determined based off the estimated protein loading required.

Resin Pore Size

When the resin reaches a point where the resin is completely saturated with proteins, the pore size of the resin will have a great effect on the resin loading of smaller molecules. If the larger proteins do not sterically block the resin pores, smaller molecules and proteins have the ability to bind.

The resin which was chosen for the cation exchange chromatography was POROS 50 HS. The sulfopropyl functional group on the resin beads will withstand the entire spectrum of pH making it a suitable resin for experimentation. Table 5 provides additional information on the cation exchange resin.

Table 5: Characteristics of Cation Exchange Resin (POROS 50 HS)

Characteristics of POROS 50 HS	
Matrix	Crosslinked poly(styrene-divinylbenzene)
Functional group	Sulfopropyl
Particle size	50 _m
Ionic range	0-5 M, all common salts
Flow velocity	maximum 1000 cm/h
Dynamic binding capacity	55 mg BSA/ml medium
Working temperatures	+4 to +30°C
Avoid	oxidizing agents, cationic detergents

Elution by Salt Gradient

After the resin has been loaded with the protein mixture, the protein of interest may be isolated by a salt gradient. Proteins which possess a pI near the pH of the solution will be weakly bound and will be eluted with a very low salt concentration (.05 M NaCl). Increasing the salt concentration will elute the more strongly bound protein. A salt concentration of 1 M NaCl should elute all proteins from the column. As the salt passes through the column in the mobile phase, the salt competes with the bound proteins to bind to the column thereby eluting the proteins. Figure 7 illustrates how bound proteins are eluted by NaCl.

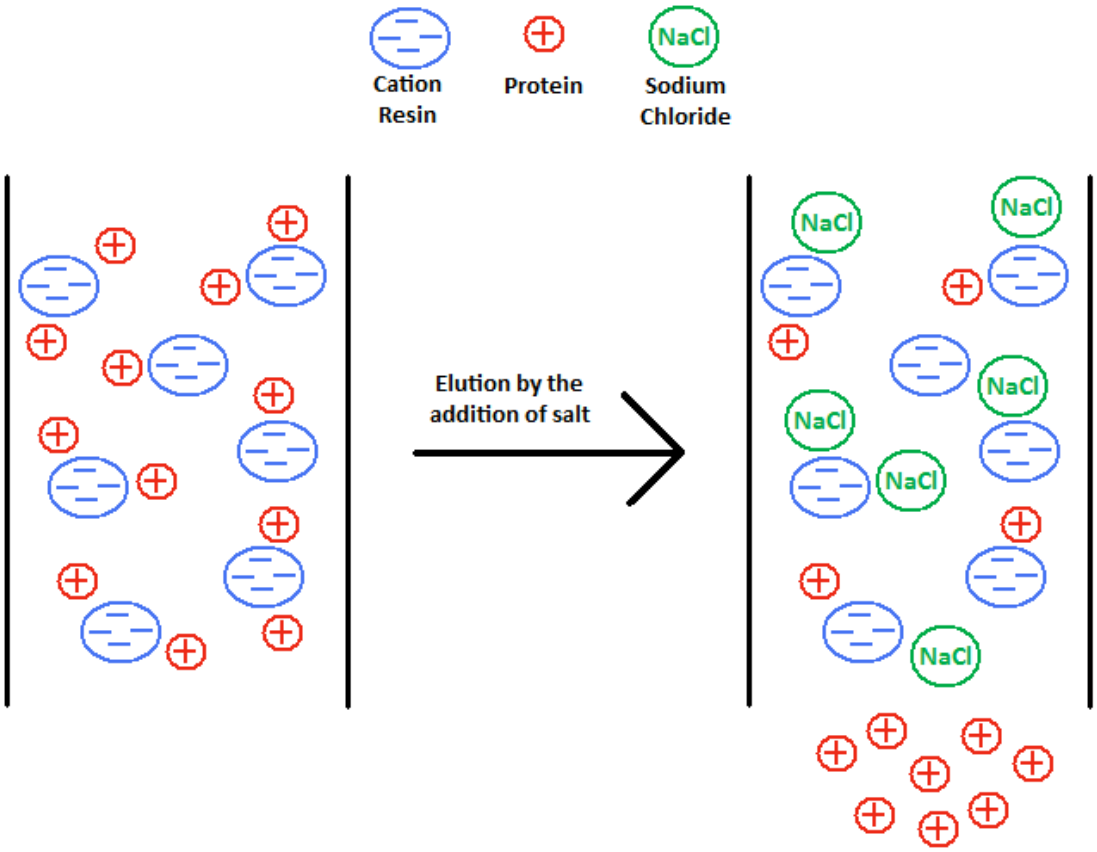


Figure 7: Effect of salt elutions on a cation exchange column

Mixed-Mode Chromatography

Capto MMC is a multimodal ligand with properties of a weak cation exchanger. As shown in Figure 8, the ligand also has properties which enable hydrogen bonding and hydrophobic interactions. The carboxyl group is mainly responsible for the cation exchanger properties. The resin matrix is salt tolerant so the proteins will bind at the feed conductivity. Since it allows binding at high conductivity, it may not be necessary to screen for optimal loading conductivity with respect to binding capacity. Since Capto MMC allows efficient capture of proteins at high conductivity, this limits the use of high salt concentrations when eluting proteins. The high concentrations of salts are bad for the environment and add extra costs to the process when they have to be separated out and treated separately. Since lower concentration of salts are needed for Capto MMC, the production costs will be reduced for purchasing the salts and treating them. The medium is made of a highly rigid agarose base matrix which allows for high flow rates and low back pressure at large scale. Properties of the resin itself may be found in Table 6.

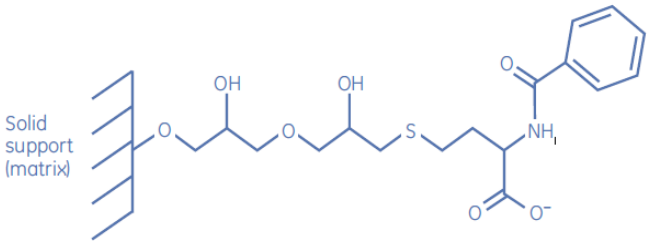


Fig. 8 Capto MMC Multimodal

Table 6: Characteristics of Capto MMC	
Matrix	Highly cross linked agarose
Functional group	multimodal weak cation exchanger
Particle size	75 _m
Total ionic capacity	0.07-0.09 mmol H+/ml medium
Flow velocity	at least 600 cm/h
Dynamic binding capacity	45 mg BSA/ml medium
Working temperatures	+4 to +30°C
Avoid	oxidizing agents, cationic detergents

Height Equivalent Theoretical Plates[1]

To obtain the number of theoretical plate and the height of the theoretical an elution curve in needed from the chromatography column. An ideal elution curve is shown in Figure 9.

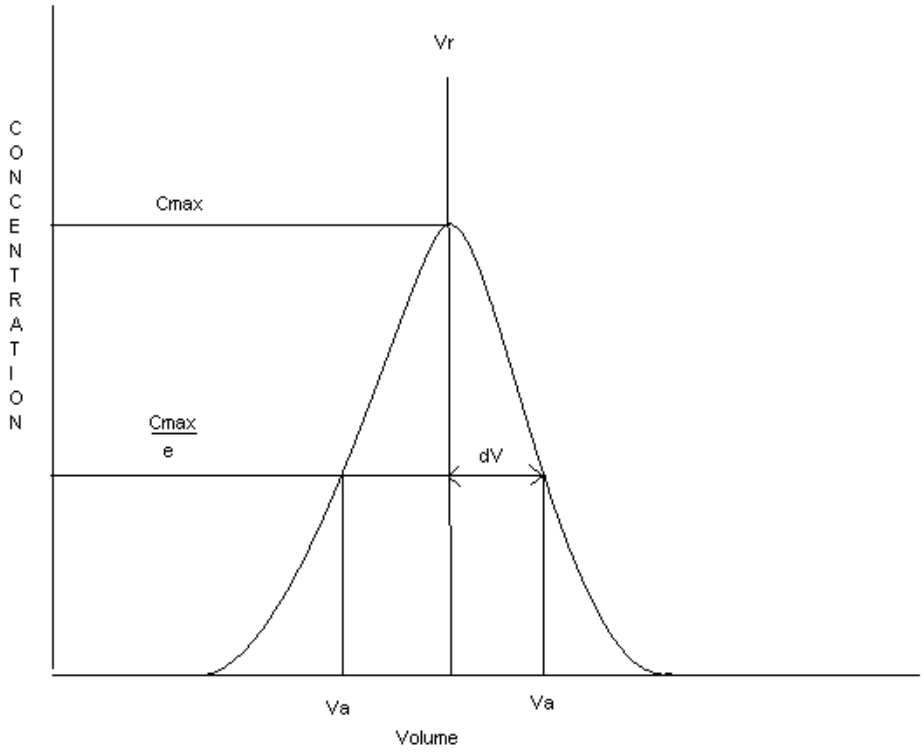


Figure 9: Ideal Elution Curve

[1] Reference: CRC Handbook of Chromatography

From the ideal elution curve the number of theoretical plates, N, can be found from the area under the curve with an infinitly small width. The width of the band dV is dependent of the number of theoretical plates and given by

$$dV = Vr\sqrt{\frac{2}{N}}$$

The maximum concentration is also related to N and found by the equation

$$C\max = \frac{m}{Vr}\sqrt{\frac{N}{2\Pi}}$$

The total plate number for each peak can be defined by

$$N = \left[\frac{2D}{D+1}\right]\left[\frac{Vr}{Va - Vr}\right]^2$$

where Va is the average of volumes at C=Cmax/e. The effect of column length can be shown by the equation

$$\sqrt{H} = \frac{3.29}{D_2 - D_1}\left(\frac{D_2 + 0.5}{\sqrt{P_2}} + \frac{D_1 + 0.5}{\sqrt{P_1}}\right)$$

where D is the amount of affinity, P is the number of plates per cm of column. The volume of peak with stepwise changes in eluant can be defined by the equation

$$Vr = \sum_1^n V_1 + V_M + D_{n+1}\left[V_m + \sum_1^n\left(\frac{V_n}{D_n}\right)\right]$$

For scale-up, some guidelines need to be followed which are listed in Table 7.[Sofer, Gail and Lars Hagel. *Handbook of Process Chromatography*. Academic Press. 1997]

Table 7: Guidelines for scale-up of chromatographic purifications[1]

Maintain	Bed Height Efluent velocity Sample Concentration Gradient slope/bed volume Sample residence time
Increase	Column diameter Sample volume in proportion to column cross sectional area Volumetric flow rate in proportion to column cross-sectional area Gradient volume in proportion to column cross-sectional area
Check	Increased pressure drop Sample distribution Piping and system dead volumes

Equipment design and scale-up

[Al-Jibbouri, Scale-up of chromatographic ion-exchange processes in biotechnology. Journal of Chromatography. 2006. 135-142.]

The efficiency to separate biological products depends on a variety of design and operation factors. Wilhelm and Riba presented a strategy and optimization of separation which requires

both a mathematical model of the chromatographic separation and an experimental evaluation of its parameters. The key parameters in the purification process are scalability, feasibility, and performance. A successful scale-up is defined by keeping the residence time of the fluid flowing through the column constant. The volumetric flow, gradient length, and load are scaled to the column volume.

The simplified Deemeter equation for liquid chromatography can be applied to the Lucentis case.

$$H = A_H + C_H \upsilon \text{ where } A_H = 5\lambda d_p \text{ and } C_H = 2\left(\frac{1+A}{p+1+A}\right)^2 \times \frac{\varepsilon}{6(1-\varepsilon)K_m}$$

The time scale for the flow t_o is given as,

$$t_o = \frac{\varepsilon_t V_{col}}{Q} = \frac{l_{col}}{\upsilon}.$$

An identical time scale can be achieved in all columns by scaling the volumetric flow rate to the total void volume $V_t = \varepsilon_t \times V_{col}$. The number of plates is also kept constant through the scale-up process by using the relation

$$N = \frac{l_{col}}{H} = \frac{l_{col}}{A_H + C_H \upsilon} = \frac{1}{A_H / l_{col} + C_H / t_o}.$$

When the column length is increased, the number of plates is then,

$$N_2 = \frac{N_1}{1 + A_H((l_{col,1} - l_{col,2})/(l_{col,1} l_{col,2}))N_1}.$$

The steric mass action relation was formalized by Brooks and Cramer to determine the concentration of the eluting salt ions. The distribution ratio, Ai, is given by

$$A_i = K_i \left(\frac{\Lambda}{c_{salt} z_{salt}}\right)^{\upsilon_i}$$

where Ki is the thermodynamic equilibrium constant given by

$$RT \ln(K_i) = -\Delta G^o_{protein,i} + \upsilon_i \Delta G^o_{counter-ion}.$$

The distribution ratio, A, is found from the retention volume

$$\frac{V_R}{V_{col}} = \varepsilon + (1-\varepsilon)\varepsilon_p k_d (1+A)$$

where _ is the void fraction. The eluting salt ion concentration is given by the equation

$$C_R = \left[GB\left(\frac{\Lambda}{z_{salt}}\right)^{\upsilon} (\upsilon + 1) + c^{\upsilon+1}_{salt,o} \right]^{1/\upsilon+1}.$$

From the eluting ion concentration, the corresponding retention volume can than be calculated from the equation

$$\frac{V_{R_g}}{V_{col}} = \frac{V_{R_{salt}}}{V_{col}} + \frac{1}{G'}\left[GB\left(\frac{\Lambda}{z_{salt}}\right)^{\upsilon} (\upsilon + 1) + c^{\upsilon+1}_{salt,o} \right]^{1/\upsilon+1} - \frac{c_{salt,o}}{G'}$$

where Vrsalt is the volume the salt ions travel,

$$G' = G \times V_{col}\,,\; G = (c_{salt,1} - c_{salt,o})/V_g$$
$$B = V_{col}(1-\varepsilon)\varepsilon_p k_d K$$

Tangential Flow Filtration

Filtration is separation process that is driven by pressure through membranes to separate based on size and charge differences. There are two types of filtration: Normal Flow Filtration (NFF) and Tangential Flow Filtration (TFF). The differences can be seen in the image below.

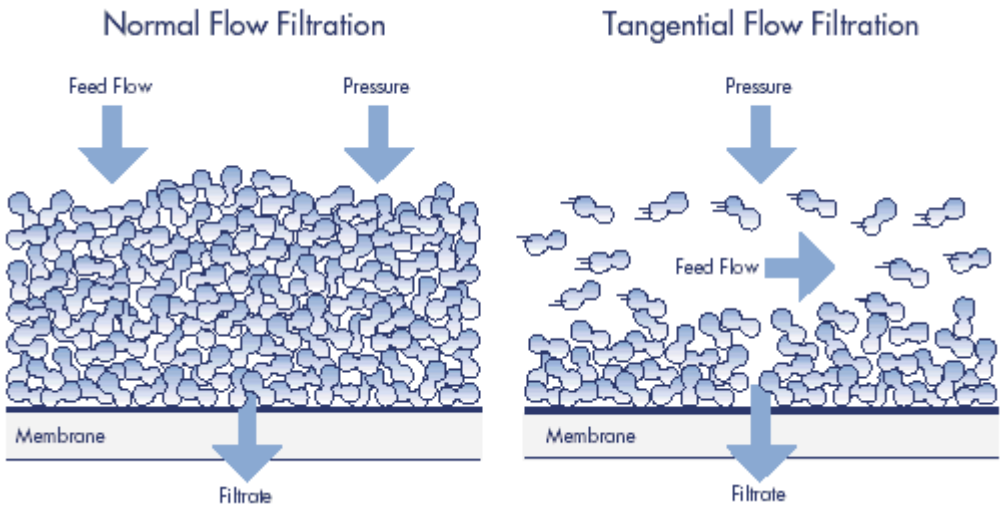


Figure 10: Normal Flow Filtration versus Tangential Flow Filtration.

Table 8: Types of Tangential Flow Filtration.

	Microfiltration	Virus Filtration	High-Performance Filtration	Ultrafiltration TFF	Nanofiltration/ Reverse Osmosis
Components retained by membrane	Intact cells Cell debris	Viruses	Proteins	Proteins	Antibiotics Sugars Salts
membrane membrane membrane membrane membrane membrane membrane membrane membrane membrane membrane membrane membrane membrane					
Components passed through membrane	Colloidal material Viruses Proteins Salts	Proteins Salts	Proteins Salts	Small Peptides Salts	(Salts) Water
Approximate membrane cutoff range	0.05 µm – 1 µm	100 kD – 0.05 µm	10 kD – 300 kD	1 kD – 1000 kD	<1 kD

The purpose of the TFF in this process is to increase the concentration factor, remove buffer solution, and exchange solution for a better environment Ultrafiltration will be used since the

protein that will be separated out is around 50 kDa and buffers will be separated through the membrane (Millipore). Ultrafiltration has three distinctive characteristics: use high cross flow, dominated by membrane, depend on membrane geometry in actual equipment. The cross flow in Ultrafiltration minimizes the development of a filter cake making it last longer. There are a few different types of membranes: flat sheets, Shell and tube, spiral wound, and hollow fiber. The flat sheets or plates were chosen for this experiment to replicate the Ultrafiltration that Genentech uses. It was also chosen because it can be easily disassembled for cleaning and replacement; it has the smallest area per volume; and has the lowest fluxes per volume of the types of membranes. The following is an image of flat sheets showing where the feed enters, the permeate stream (waste stream), and the retentate stream (recycle stream). Membranes can be made from polymers and other materials.

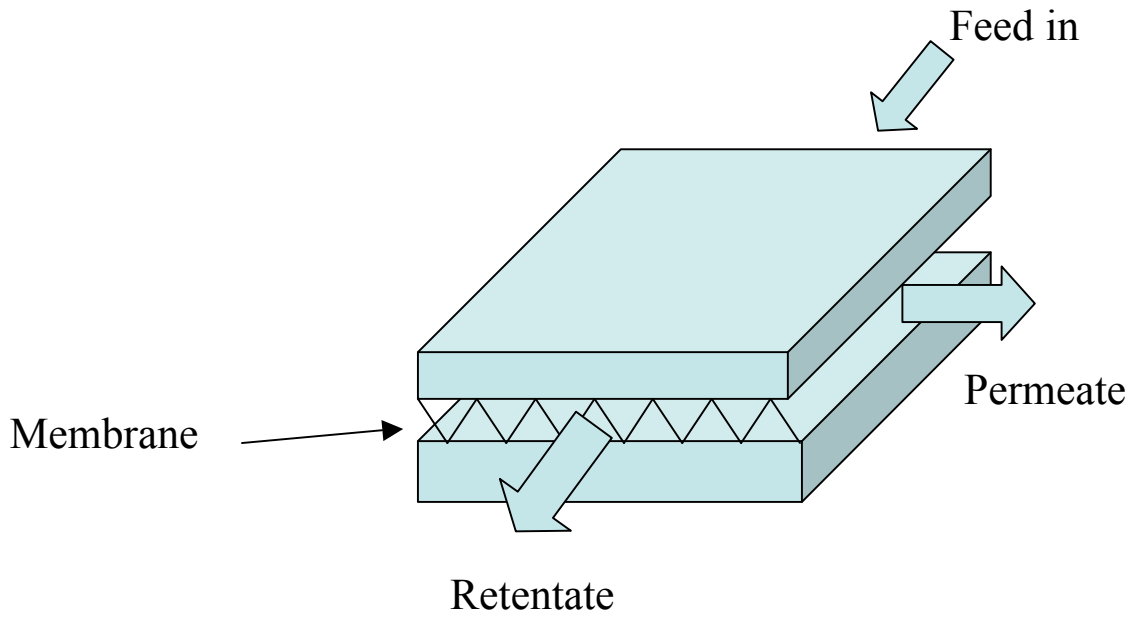


Figure 11: Flat Sheets Diagram.

The following is a Millipore image of a TFF set up.

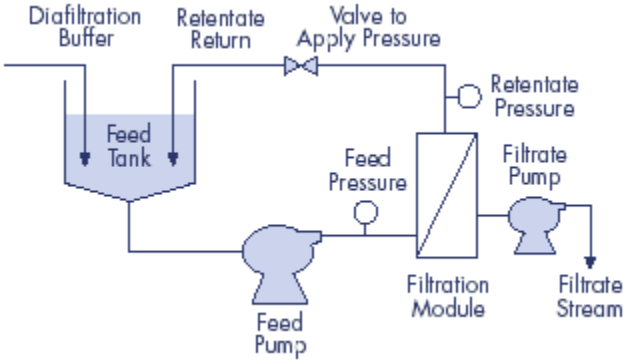


Figure 12: Tangential Flow Filtration Set Up.

The following is a typical graph for flux versus the transmembrane pressure (TMP). The optimum operation conditions are located at the circles in the graph. At this point the highest flux is achieved without exerting excessive amounts of pressure or reaching high protein wall

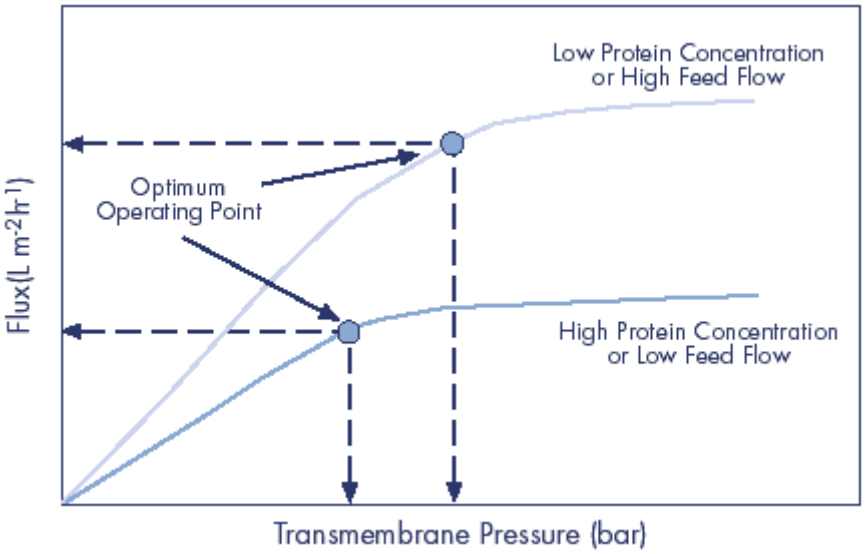


Figure 13: Flux versus Transmembrane Pressure.

concentrations. The following is an equation to find the transmembrane pressure which uses the

$$TMP = \left[\frac{P_{feed} + P_{ret}}{2}\right] - P_{perm}$$

feed, retentate, and permeate pressures (Millipore). The flux, J, is the amount of fluid passing through the membrane. It can be found using the following equation, which is derived from the Hagen-Poiseuille model for laminar flow where _ is the number of pores or porosity, d_p is the pore diameter, $\_P_T$ is the applied pressure, _x is the thickness of the membrane, and _ is the viscosity of the solvent (Belter).

$$J = \frac{\epsilon d_p^2 \Delta P_T}{32 \Delta x \eta}$$

The membrane area can be found using the following equation. Usually 20% extra membrane is used as a safety margin (Millipore). A linear scale up can be used.

$$Membrane\ area\ \left[m^2\right] = \frac{FIltrate\ volume\ \left[L\right]}{Flux\ \left[\frac{L}{m^2 h}\right]} \cdot \Pr ocess\ time\ \left[h\right]$$

To find the size of the pores, the Molecular Weight Cut-Off is used. This can be calculated by taking one-third and one-fifth of the protein size.

The concentration factor CF can be found by dividing the feed flow rate by the retentate flow rate. It needs to be concentrated to around 20 g/L.

The efficiency can be found by calculating the rejection of the system where _ is the rejection, C_P is the concentration of the permeate and C_R is the concentration of the permeate (Cheryan, 1986).

$$\sigma = 1 - P = 1 - \frac{C_P}{C_R}$$

Table 9: TFF Values.

Recovery	95%
Molecular Weight Cut-Off (kDa)	9.6-16
Transmembrane Pressure (psi)	20
Flux (L/m2h)	30
Membrane Area (m2)	5.5
Process Time (h)	5
Cost	$165,000

Preliminary Design

Chromatography Base Case Process Flow Diagram

The base case process flow diagram for last years chromatography steps is illustrated in Figure 14.

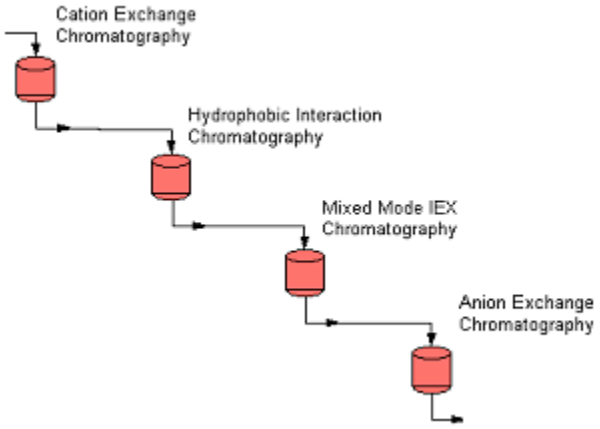


Figure 14: Last year's base case chromatography process

Chromatography Sizing and Costs

The preliminary design for the chromatography processes was based off the previous year's calculations (Barbara Coruna, Jason Hastings, Kevin Schmidt, and Trisha Wesner). Tables 10-16 provide the sizing and cost estimations for cation, anion, mixed-mode, and hydrophobic interaction chromatography columns.

Table 10: Sizing for cation-exchange column and anion-exchange column

Linear velocity	270.9 cm/hr
Column diameter	54 cm
Column length	46 cm
Resin height	40 cm
HETP	$3.086*10^{-5}$ cm
Number of Plates	12960
Pressure drop	1.033 bar
Number of columns	2

Table 11: Costs for cation-exchange column

2-(N-Morpholino)Ethanesulfonic acid	$238,120
NaCl	$109,960
Resin AB	$709,200
Column Extension	$4,854
Column Stand	$7,220
Column Millipore, ss	$23,110
TOTAL	**$1,092,464**

Table 12: Sizing for mixed-mode column

Linear velocity	270.9 cm/hr
Column diameter	54 cm
Column length	46 cm
Resin height	40 cm
HETP	$3.086*10^{-5}$ cm
Number of Plates	12960
Pressure drop	0.459 bar
Number of columns	2

Table 13: Costs for mixed-mode column

2-(N-Morpholino)Ethanesulfonic acid	$228,300
NaCl	$105,400
Resin AB	$1,723,000
Column Extension	$4,854
Column Stand	$7,220
Column Millipore, ss	$23,110
TOTAL	**$2,092,000**

Table 14: Costs for anion-exchange column

TRIS	$52,200
NaCl	$68,560
Resin AB	$487,600
Column Extension	$4,854
Column Stand	$7,220
Column Millipore, ss	$23,110
TOTAL	**$643,500**

Table 15: Sizing for HIC column

Linear velocity	175 cm/hr
Column diameter	45 cm
Column length	55 cm
Resin height (total)	269 cm
Theoretical number of plates	6.708×10^{6}
HETP[7]	8.199×10^{-6} cm
Pressure drop	1.078 bar
Cycle time (per column)	~3 hrs
Number of columns	5

Table 16: Cost for HIC column

Na_2SO_4 (per column run) Sigma-Aldrich	$42, 480
MES (per column run) ISC Bioexpress	$275, 300
NaOH (per column run) GFS Chemicals	$1,011
Resin GE	$1,107,000
Column Millipore, ss	$61, 530
TOTAL	**$1,487,321**

Chromatography Base and Alternative Case Process Flow Diagram

Chromatography Base Case Process Flow Diagram

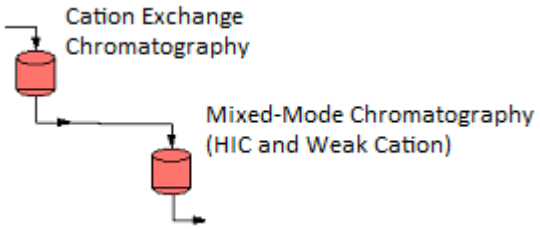


Figure 15: Base Case PFD

Justification for Order of Operations for Chromatography Process

The order of operations for the purification process was based off of a map of the *e. coli* proteins with molecular weight on the y-axis and isoelectric point on the x-axis, illustrated in Figure 16. Running a cation exchange column as the initial purification step would eliminate a majority of the undesirable proteins. A cation exchange chromatography experiment should eliminate all proteins to the left of the dividing line leaving only the proteins to the right of the line bound to the column. Since Lucentis is not naturally found in *e. coli* it's position on the protein map is not identified; however, a red dot and arrow illustrate it's theoretical position based off it's molecular weight (50 kDa) and it's isoelectric point (pI = 9).

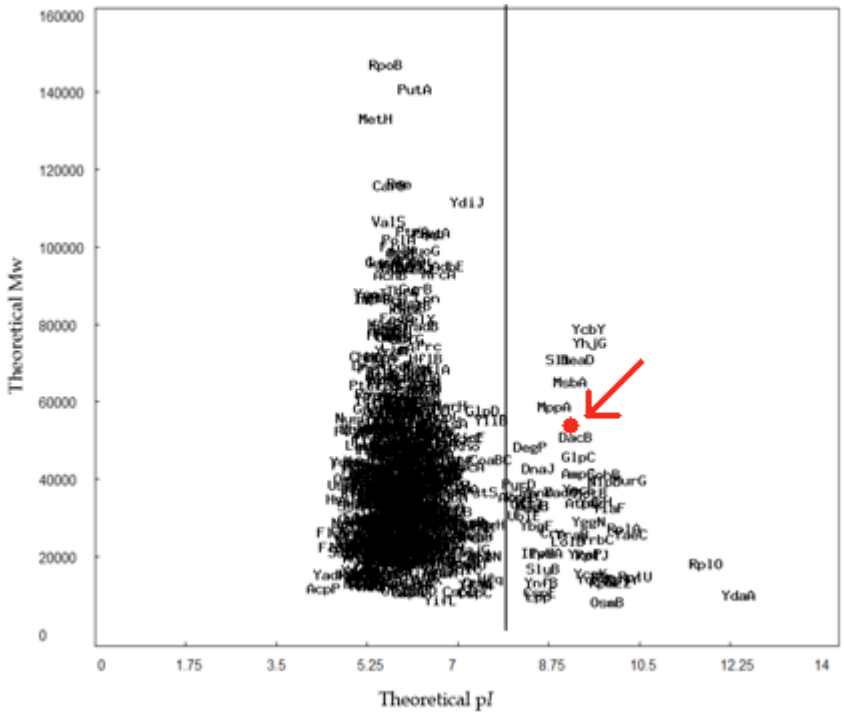


Figure 16: Theoretical Cation Chromatography Separation

Following cation exchange chromatography, mixed-mode chromatography, which is a combination of hydrophobic interaction chromatography and weak cation chromatography, is the next step of the purification process. The mixed-mode chromatography will separate the remaining proteins based off of their respective hydrophobicity.

Chromatography Alternative Case Process Flow Diagram

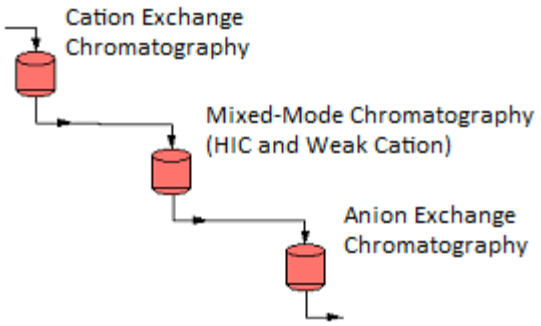


Figure 17: Alternative Case PFD

Based off of the experimental results, the need for the alternative case will become clear. If the cation and mixed-mode chromatography processes are insufficient for the purification of Lucentis, it may be necessary to include anion exchange chromatography. Anion exchange chromatography will have the ability to isolate Lucentis from all other proteins with a pI greater than 9. An additional alternative case is provided in Appendix 2 on monolithic chromatography.

Experiments

Experimental Procedure: Mixed Mode Chromatography

To perform the mixed mode chromatography using a raw, thawed sample of Lucentis, the following equipment was obtained:

- Omnifit 10 mm Empty Glass Column (10 cm in length, 10 mm in diameter)
- 40 um Omnifit frit
- Beakers: 1000 mL (1), 250 mL (3), 100 mL (1), 50 mL (1)
- Graduated cylinder: 10 mL (1)
- Plastic screw-cap bottles: 500 mL (1), 250 mL (8), 100 mL (2), 50 mL (1)
- Eppendorf tubes: 1 mL (500)
- Pipettes (assorted)
- Pipette bulb

Chemicals obtained for this experiment were the following:

- 0.5 M bicine
- Capto MMC
- Sodium hydroxide
- Sodium chloride

The resin chosen for this experiment was Capto MMC. This resin promotes hydrophobic and weak cationic interactions. A 40 um frit was placed at the bottom end of the column to be used as a membrane to ensure that the Lucentis molecule was not lost too quickly but also that too high of a back-pressure was not created. The column was prepared with enough Capto MMC resin to fill up to the 5 cm line. The resin was allowed to let settle for more than three hours and then introduced into the empty column in small portions, allowing each addition to settle before more was added.

The buffer used to introduce the Lucentis into the column was 0.5 M bicine mixed with enough sodium hydroxide (almost 50% by volume) to result in a pH of 7.90. This solution was poured into the packed column in small portions until about 20 mL of raw Lucentis in a variable amount of bicine-sodium hydroxide was introduced onto the resin. This buffer-Lucentis solution was run through the column three additional times to ensure that the Lucentis had been given a reasonable chance to bind to the resin.

Sodium chloride was used to elute the different proteins from the column. The concentration of the salt solution was gradually increased, to elute proteins at differing pIs. The following concentrations of sodium chloride solutions were made: 1.0 M, 0.5 M, 0.25 M, 0.15 M, 0.1 M, 0.05 M. To make these stock solutions, the following calculations were used.

$Molecular\ weight = 58.44 \frac{g}{mol}$

To create a 1.0 M solution, 58.844 g were placed in a 250 - mL bottle and enough water was added to make 100 mL.

To create a 0.5 M solution,

$$\frac{58.44\text{g}}{\text{mol}} \left| \frac{0.5 mol}{L} \right| \left| \frac{0.100 L}{} \right| = 2.922 g$$ were placed in a 250 - mL bottle and enough water was added to make 100 mL

This method was repeated for the remaining solutions.

Once the Lucentis sample and buffer solutions had been run through the column, the apparatus was then ready for running the various salt solutions, starting with the lowest concentration. Three-mL of each salt solutions in the following order were run: 0.05 M, 0.1 M, 0.15 M, 0.25 M, 0.5 M, 1.0 M (to finally elute any proteins left in the column). Using the eppendorf tubes, three 1-mL fractions of each concentration were collected. Pressurized air was used to speed up the flow of the salt solution through the column; however, the final pressure at the end of the column was atmospheric. Each fraction collected was labeled appropriately (Run 1, 2, 3) for each concentration, to be identifiable later during analysis.

To prepare the column for future trials, 1.0 M salt solution was run through the column to make sure the resin did not contain any more protein. The resin was removed for reuse.

Experimental Procedure: Cationic Exchange Chromatography

To perform the mixed mode chromatography using a raw, thawed sample of Lucentis, the following equipment was obtained:

- Omnifit 10 mm Empty Glass Column (10 cm in length, 10 mm in diameter)
- 40 um Omnifit frit
- Beakers: 1000 mL (1), 250 mL (3), 100 mL (1), 50 mL (1)
- Graduated cylinder: 10 mL (1)
- Plastic screw-cap bottles: 500 mL (1), 250 mL (8), 100 mL (2), 50 mL (1)
- Eppendorf tubes: 1 mL (500)
- Pipettes (assorted)
- Pipette bulb

Chemicals obtained for this experiment were the following:

- 0.5 M bicine
- POROS 50 HS
- Sodium hydroxide
- Sodium chloride

The resin chosen for this experiment was POROS 50 HS. This resin promotes strong cationic interactions. A 40 um frit was placed at the bottom end of the column to be used as a membrane to ensure that the Lucentis molecule was not lost too quickly but also that too high of a back-pressure was not created. The column was prepared with enough POROS 50 HS resin to fill up to the 5 cm line. The resin was allowed to let settle for more than three hours and then introduced into the empty column in small portions, allowing each addition to settle before more was added.

The buffer used to introduce the Lucentis into the column was 0.5 M bicine mixed with enough sodium hydroxide (almost 50% by volume) to result in a pH of 7.0. This solution was poured into the packed column in small portions until about 20 mL of raw Lucentis in a variable amount of bicine-sodium hydroxide was introduced onto the resin. This buffer-Lucentis solution was run through the column three additional times to ensure that the Lucentis had been given a reasonable chance to bind to the resin.

Sodium chloride was used to elute the different proteins from the column. The concentration of the salt solution was gradually increased, to elute proteins at differing pIs. The following concentrations of sodium chloride solutions were made: 1.0 M, 0.5 M, 0.25 M, 0.15 M, 0.1 M, 0.05 M. To make these solutions, the following calculations were used.

$Molecular\ weight = 58.44 \frac{g}{mol}$

To create a 1.0 M solution, 58.844 g were placed in a 250 - mL

bottle and enough water was added to make 100 mL.

To create a 0.5 M solution,

$$\frac{58.44\text{g}}{\text{mol}} \left| \frac{0.5 mol}{L} \right| \frac{0.100 L}{} \Bigg| = 2.922 g \text{ were placed in a } 250 - \text{mL}$$

bottle and enough water was added to make 100 mL

This method was repeated for the remaining solutions.

Once the Lucentis sample and buffer solutions had been run through the column, the apparatus was then ready for running the various salt solutions, starting with the lowest concentration. Three-mL of each salt solutions in the following order were run: 0.05 M, 0.1 M, 0.15 M, 0.25 M, 0.5 M, 1.0 M (to finally elute any proteins left in the column). Using the eppendorf tubes, three 1-mL fractions of each concentration were collected. Pressurized air was used to speed up the flow of the salt solution through the column; however, the final pressure at the end of the column was atmospheric. Each fraction collected was labeled appropriately (Run 1, 2, 3) for each concentration, to be identifiable later during analysis.

To prepare the column for future trials, 1.0 M salt solution was run through the column to make sure the resin did not contain any more protein. The resin was removed for reuse.

Quantification Techniques and Analysis

EZQ Protein Quantification

(http://wolfson.huji.ac.il/purification/PDF/Protein_Quantification/MOLECULAR_PROBES_EZQProtQuantKit.pdf)

Once samples have been run through a chromatography column, the quantification of the total protein content is important for further analysis. The total protein may be found using an EZQ Protein Quantification Kit. The fluorescence based quantification kit is not affected by detergents, reducing agents, urea, or tracking dyes making it an ideal quantification tool for total protein. The protein samples are spotted on an assay paper requiring only 1 _L of the sample. A

plot of the total fluorescence versus the protein concentration between .02-5 mg/ml is used to determine the total protein within a sample.

Acetone Precipitation

[(http://www.piercenet.com/files/TR0049dh4-Acetone-precipitation.pdf)]

Based off the results of the EZQ analysis, the proteins may need to be concentrated by acetone precipitation. Samples which are quantified to have .1 mg/ml or less of protein must be concentrated. The following protocol should be followed for acetone precipitation:

1. Cool the required volume of acetone to -20°C.
2. Place protein sample in acetone-compatible tube.
3. Add four times the sample volume of cold (-20°C) acetone to the tube.
4. Vortex tube and incubate for 60 minutes at -20°C.
5. Centrifuge 10 minutes at 13,000-15,000 x *g*.
6. Decant and properly dispose of the supernatant, being careful to not dislodge the protein pellet.
7. Allow the acetone to evaporate from the uncapped tube at room temperature for 30 minutes. Do not over-dry pellet, or it may not dissolve properly.
8. Add buffer appropriate for the downstream process and vortex thoroughly to dissolve protein pellet.

1-D Gel Electrophoresis

To perform gel electrophoresis, place about 5 ul of each vial obtained from the chromatography and pipet it in each hole in the gel. Place the gel on the apparatus and attach the electrodes so that the proteins will run from the negative to the positive (Figure 18). Turn the electricity on to about 100 volts. The proteins will begin to separate according to their molecular weight. Turn the electricity off when there appears to be a good separation. A good separation is defined by having the molecular weights spread out so that it is clear each band contains a different molecular weight protein. This may take one to two hours, and if there are too many proteins in one lane, a longer gel may be needed.

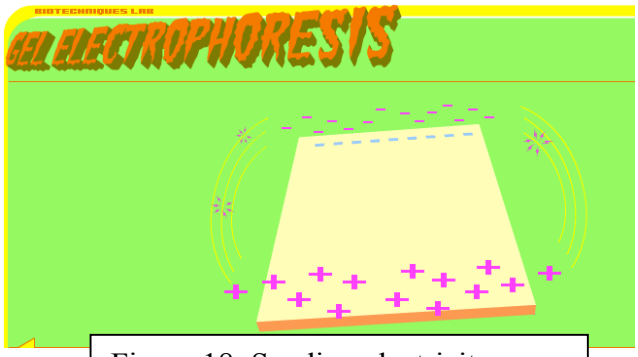


Figure 18: Sending electricity through the gel from negative to

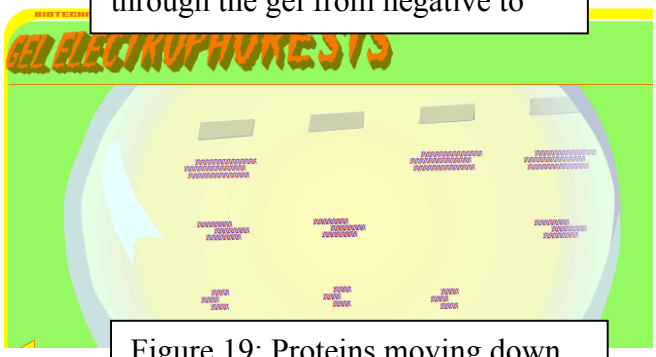


Figure 19: Proteins moving down the gel in order of size from largest to smallest.

Reference:
"Gel Electrophoresis." 2009. University of Utah. Jan 30 2009. <http://learn.genetics.utah.edu/units/biotech/gel/>.

2-D Gel Electrophoresis

An IEF gel (isoelectric focusing gel) can be used to separate proteins based on their isoelectric point. This is done by using a pH gradient in a tube or strip gel using a specially formulated

buffer system or an ampholyte mixture. Ampholytes are a mixture of amino acid polymers that have surface charges corresponding to different pH ranges. When the proteins are loaded on a gel electrophoresis with the anode at the acidic end and the cathode on the basic end, negatively charged proteins will move towards the anode. When the proteins reaches a zone where it has a net zero charge, it will stop moving.

After isoelectric focusing is performed, the proteins by than be separated by SDS-PAGE. This is known as a 2-D PAGE. This technique is used to resolve complex protein mixtures into the greatest number of individual protein "spots". The IEF gel is equilibrated and laid across the SDS-PAGE gel. Current is applied and the proteins migrate into the gel where separation occurs according to mass. Several thousand protein spots may be resolved on a single 2-D PAGE gel.

Reference: "Gel Electrophoresis." Pierce. 2007. Jan 30 2009. <https://www.piercenet.com/Proteomics/browse.cfm?fldID=21518847-2D72-475F-A5B9-B236EC5B641E#native%20page>.

Mass Spectrometry

Following gel electrophoresis, bands of interest may be isolated and cut from the gel for further analysis. Mass spectrometry (MS) is an analytical technique for the determination of a sample. Prior to mass spectrometry, the samples will be prepared with trypsin digestion. Trypsin is a serine protease that breaks down proteins. Trypsin cleaves peptide chains at the carboxyl side of lysine and arginine, Figure 20 shows the structures of lysine and arginine.

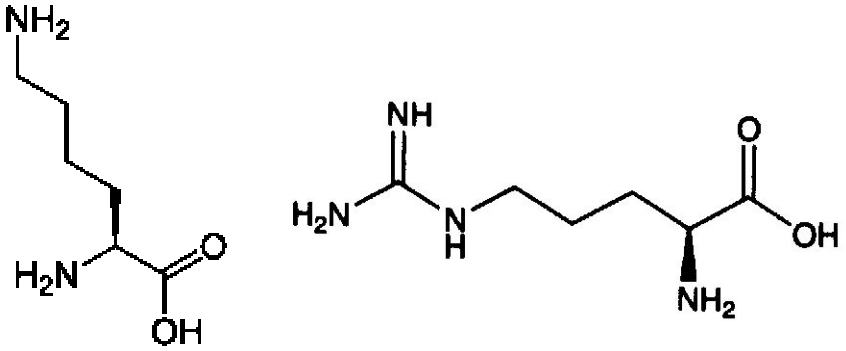


Figure 20: Structure of Lysine (left) and Arginine (right)

For analysis, after trypsin digestion, a sample is loaded onto the MS instrument and the components are ionized. The ionized sample is directed into an electric and/or magnetic field. Computations of the mass to charge ratio of the particles is based on their movement through the electromagnetic field. Detection of the ions provides information on the amino acid sequence. Using a protein database from Applied Biosystems, the proteins within each band are identified. The database provides the name of the protein, the molecular weight, and the isoelectric point.

Experimental Results

Cation Exchange Column

EZQ Protein Quantification

After running the cation exchange column, analysis of the samples will provide further information on what should be done next for studying this step of the purification process. The

first table describes the sample number with the sample description. The first samples were the original sample mixture. The remaining samples are analyzed in increasing concentration of salt.

Table 17: Sample number and description for EZQ analysis

Sample Number	Sample Description
1	Original
2	1st run through
3	2nd run through
4	.05 #1
5	.05 #2
6	.05 #3
7	.1 #1
8	.1 #2
9	.1 #3
10	1-0.15
11	2-0.15
12	3-0.15
13	0.25 #1
14	0.25 #2
15	0.25 #3
16	1-0.5
17	2-0.5
18	3-0.5
19	1M #1
20	1M #2
21	1M #3
22	1M #4
23	1M #5

In the EZQ analysis, some samples were analyzed twice and some were analyzed only once. The blue bar represents the first analysis, the red bar represents the second analysis, and the vanilla bar represents the average of the two analysis.

The EZQ analysis provided some knowledge into where the proteins were eluted. The following graph shows the mg/ml of protein in the sample.

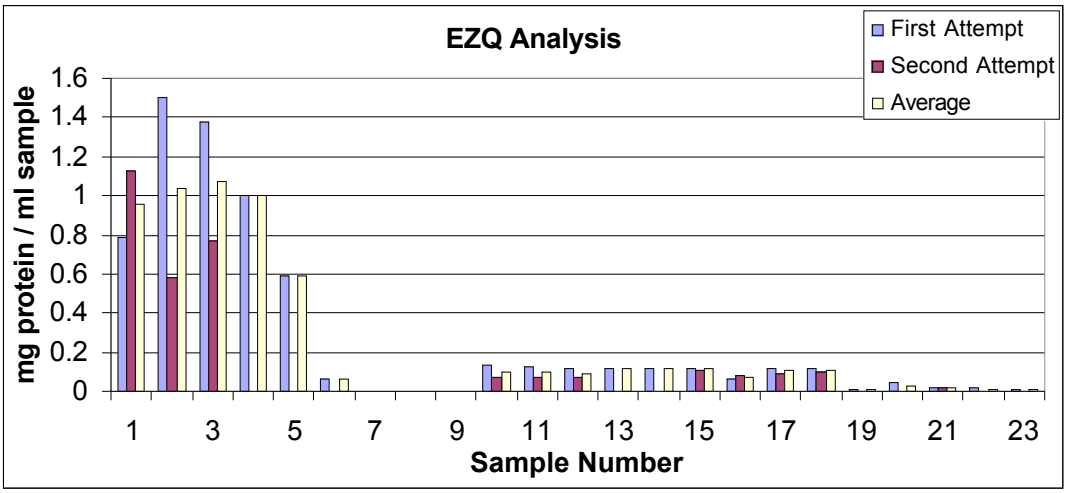


Figure 21: EZQ analysis all samples

If Lucentis bound to the column, Lucentis is most likely present in samples 10-18. The following graph highlights samples 10-18 from Figure 22.

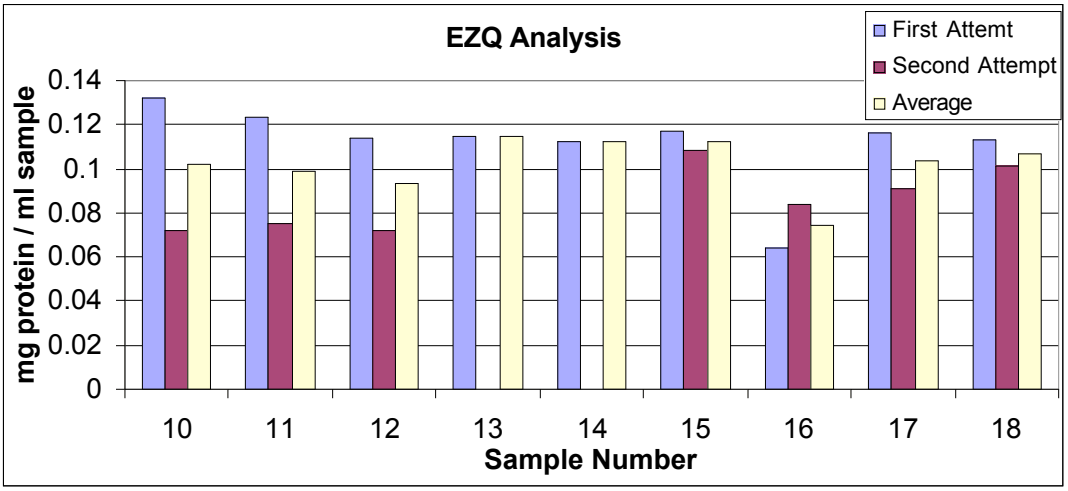


Figure 22: EZQ analysis samples 10-18

Acetone Precipitation

After EZQ protein quantification, several samples required acetone precipitation including samples: 8, 9, 10, 11, 12, 13, 14, 18, 19, 21, 22, 23. Theses samples had the lowest protein concentration and were precipitated for the gel electrophoresis analysis.

1-D Gel Electrophoresis

The samples, in order according to Table 18, were loaded onto the gel plate. Protein markers were used in wells 1, 12, and 26.

Table 18: 1-D cation gel well description

Well #	
1	Protein Markers
2	Original
3	1st run through
4	2nd run through
5	.05 #1
6	.05 #2
7	.05 #3
8	.1 #1
9	.1 #2
10	.1 #3
11	.15 #1
12	Protein Markers
13	.15 #2
14	.15 #3
15	.25 #1
16	.25 #2
17	.25 #3
18	.5 #1
19	.5 #2
20	.5 #3
21	1 M #1
22	2 M #1
23	3 M #1
24	4 M #1
25	5 M #1
26	Protein Markers

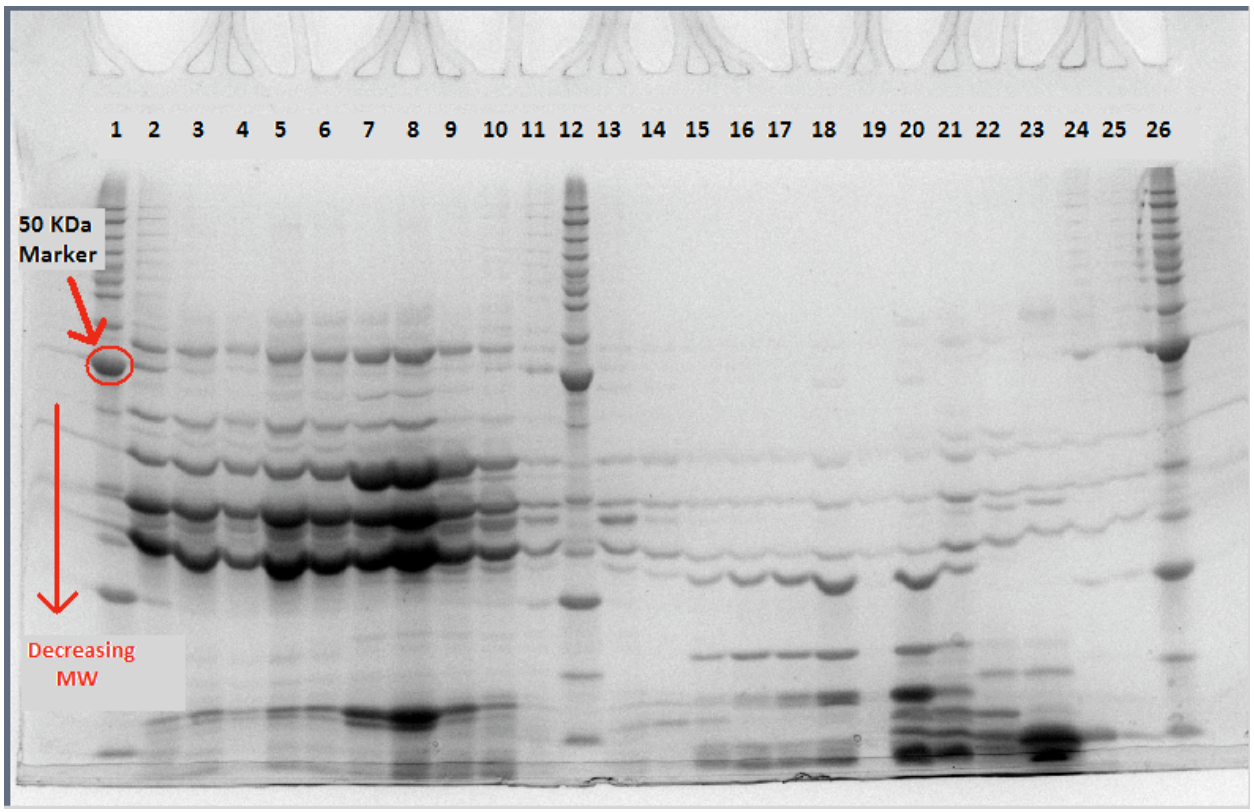


Figure 23: 1-D cation gel electrophoresis

The cation gel electrophoresis showed a clean separation from the column. There were several bands in the 50 kDa range which may be evidence of the presence of Lucentis. Mass spectrometry should provide clarification and further details of the location of Lucentis.

Mass Spectrometry

Based off the results of the cation gel electrophoresis, certain bands were of particular interest. The bands of interest are boxed in Figure 24. Several bands in well 8 were chosen since well 8 showed the most prominent bands with the highest concentrations. One band in well 11 was chosen since it seemed closest to the 50 kDa protein marker.

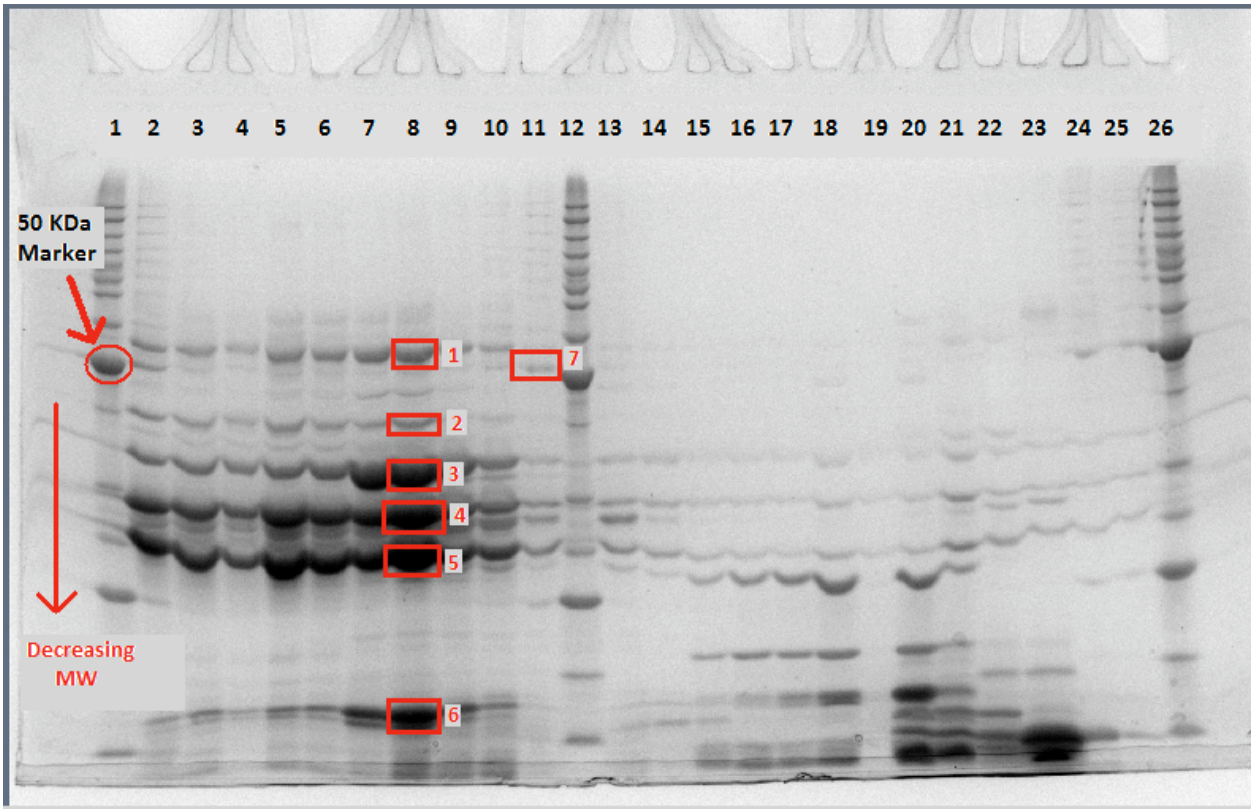


Figure 24: Selected bands from 1-D gel electrophoresis for mass spectrometry

Mixed-Mode Column

EZQ Quantification

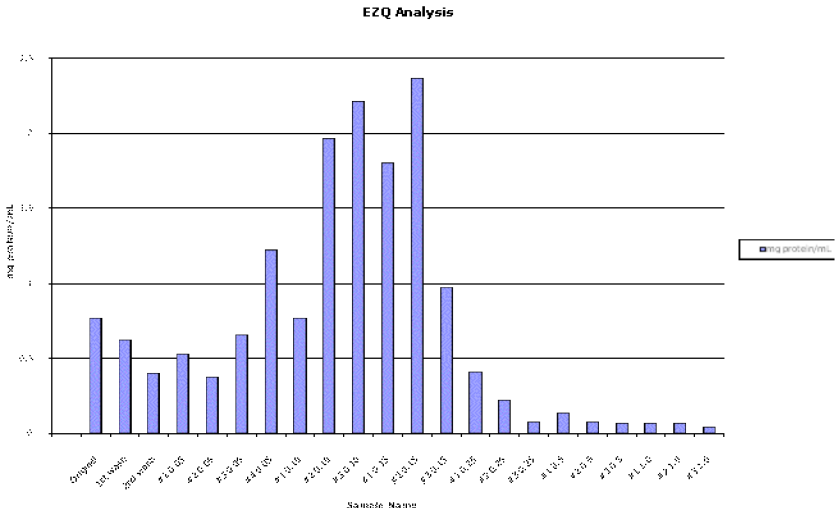


Figure 25: EZQ analysis graph showing elution trend of mixed mode chromatography experiment.

As given in Figure 25, the trend of elution can be noted, with the most protein being removed from the column at a concentration of 0.10 M and 0.15 M sodium chloride. Because this peak is still unidentifiable in terms of protein content, it cannot be determined whether or not Lucentis is present, so determining the height equivalent to theoretical plates would be futile. However, using these results, further testing using mass spectrophotometry will be conducted to determine the actual molecular weights of the proteins found in each band.

Table 19: Identification of wells in gel electrophoresis image for mixed mode chromatography.

Well number	Trial run	Concentration
1.	standard	N/A
2.	original	N/A
3.	1st run	N/A
4.	2nd run	N/A
5.	#1	0.05
6.	#1	0.1
7.	#1	0.15
8.	#1	0.25
9.	#1	0.5
10.	#1	1.0
11.	#2	0.05
12.	standard	
13.	#2	0.1
14.	#2	0.15
15.	#2	0.25
16.	#2	0.5
17.	#2	1.0
18.	#3	0.05
19.	#3	0.1
20.	#3	0.15
21.	#3	0.25
22.	#3	0.5
23.	#3	1.0
24.	#4	0.05
25.	original	N/A
26.	standard	N/A

The order of these trials did not show the trend of elution for each concentration. Rearranging the wells in order for each group of trials, one can mark the significant bands of elution. The following figure shows the concentration of protein in each well. Wells 7, 9, 10, 11, 12 were precipitated with acetone to increase the chances that the protein available would be analyzed thoroughly.

1-D Gel Quantification

The following shows a gel electrophoresis image differentiating the relative molecular weights of the proteins eluted in the fractions collected during the mixed mode chromatography experiment. The previous table shows the identities of each well.

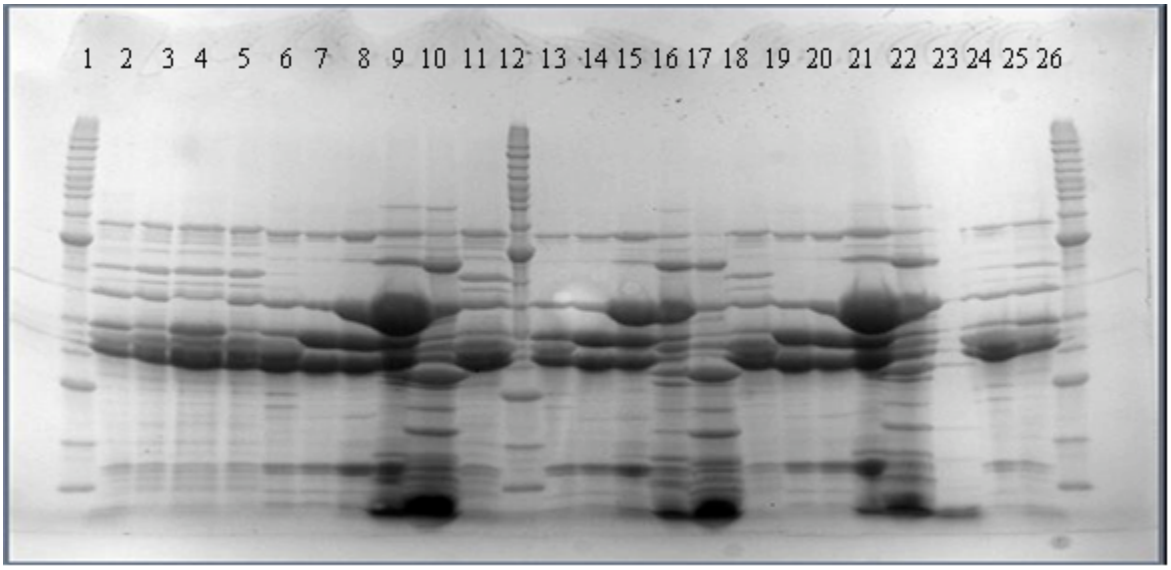


Figure 26: Gel electrophoresis image for mixed mode chromatography.

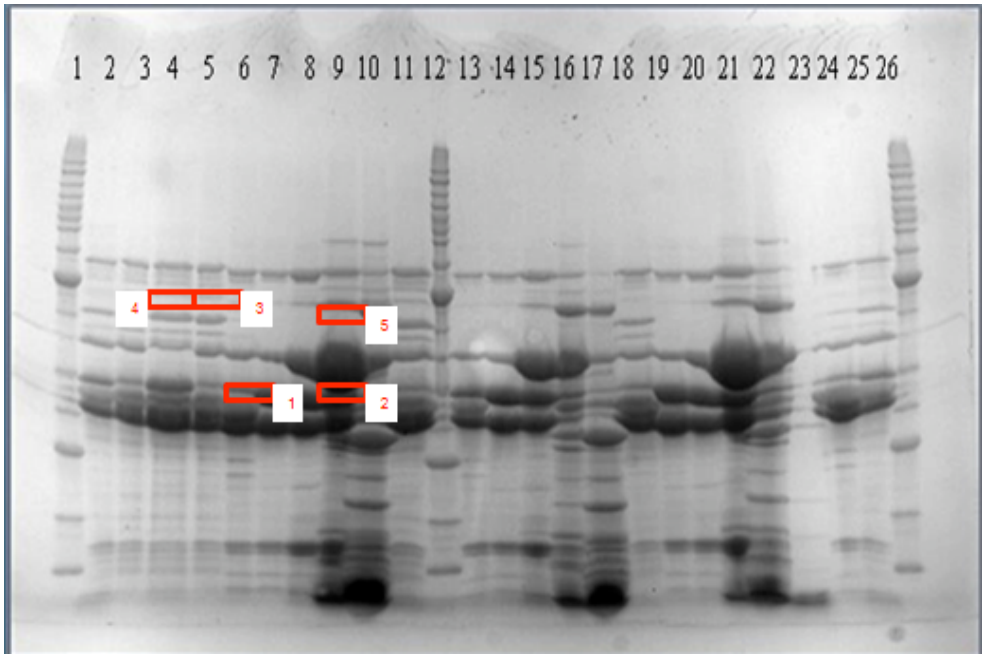


Figure 27: Bands selected for Trypsin Digest and Mass Spectrophotometry

The trypsin digest and mass spectrophotometry results are shown in Appendix 4. Each band gives results showing about ten proteins with their respective molecular weight and PI. There

was no protein found that was close to that of Lucentis, 50 kDa and PI of 9. The results should have shown a trend where the column had a very similar PI and as the column number increases, the PI also increases as it is eluted off the resin. This trend did not exist. The results of this protein analysis may have been improved though if all bands around 50 kDa were analyzed instead of picking random bands on the gel electrophoresis. This analysis did increase our knowledge of the proteins present in the E Coli centrifugate, but did not assist us in determining a productive method for isolating Lucentis from a Capto MMC column.

Scale-up and Economics

Fermentor

For a theoretical growth kinetics calculation, the Monod Kinetics was used along with the following assumptions:

-24 hours cycle time

- Biomass was calculated using E . Coli density of 1.089 g/ml.

Also, the targeted values given by Table 20 below were used:

Table 20: Targeted fermentation values.

Volume leaving fermentor	1000 L fermented culture
Solid content	20-25%
Concentration of antibody	5 g/L (5000 g of antibody total)

The product yield given by [6]:

$$P = P_0 \frac{V_0}{V} + q_p X_t (\frac{V_0}{V} + \frac{Dt}{2})t \quad (5)$$

resulted in the following values (Table 21)for specific product growth rate , dilution rate (D) and inoculum volume (V_0):

Table 21: Product yield calculations.

qp	D	V_0
(g product/g cell)	(h^{-1})	(L)
0.018	0.00353	0.015

Table 4 shows the calculated maximum specific growth rate and the final substrate concentration using a Michaelis-Menten constant derived from literature:

Table 22: Substrate concentration calculations.

um	Ks	Sf
(h^{-1})	(g glucose/L)	(g/L)
0.66	0.004	0.00002

The calculated reactor scale-up cost [9]for a fed-batch bioreactor (Table 23) was found to be:

Table 23: Reactor scale-up cost

Equipment	**Cost (2008 $)**
reservoir	4,286.00
tank	96,731.00
pump	36,080.00
Mass meter	5998.00
Rotameter	974.00
Panel mount pH Indicator	245.00
Digital Pressure /Temperature Indicator	675.00
Level Indicator	367.00
Total:	$145,356.00

For an alternative case of disposable bioreactor (Sartorius-Stedim Cultibag 200L) that includes the following components:

BIOSTAT® CultiBag RM 200 OPTICAL, 208 VAC
Rocker 200 with integrated Bag Holder
BIOSTAT® RM Twin Control Tower 200 optical
BioPAT® MFCS/DA
Package includes:
Rocker 200 with Bag Holder:
- Rocker platform with Bag Holder for 100L and 200L Bags
- Tinted Lid
- 2 Filter Heaters including power cords
- Rocking Angle: 5 - 10 degrees
- Rocking Rate: 8 - 42 [rocks/min]
- 2 x PT100 temperature probes (Yellow and Red)
- Power supply: 208 VAC / 50-60 Hz
- Power cord
Amplifiers for:
- temperature
- pH
- pO2
- Temperature control system for temperature range 20- 40 °C
- Glass Fibre cables
- Clamps for Glass Fibre cables
- Installed on fixed platform
- Aeration modules allow separate gassing strategies in two (2) Bags at the same time
- Each Aeration system consists of four (4) rotameters and two (2) mass flow controllers

(MFC)

The whole system was priced at $194,616.00 with a installation price of $1200/day.

[6]Fogler, H. Scott. Elements of Chemical Reaction Engineering 3rd Ed.New Jersey: Prentice Hall, 1999.
[2] Schuler, Michael and Fikret Kargi. Bioprocess Engineering, Basic Concepts 2nd Ed. New Jersey: Prentice Hall, 2002, pg 307-308.

[4] A.R. Cockshott, I.D.L. Bogle "Modelling the effects of glucose feeding on a recombinant E. coli fermentation". Bioprocess Engineering 20 (1999) 83-90

[5] Ferreira, Gisela. "Fed-Batch Fermentations". Department of Chemical and Biochemical Engineering, UMBC. May 1, 2009 <http://userpages.umbc.edu/~gferre1/fedbatch.html>.
[1] Ulla Saarela, Kauko Leiviskä and Esko Juuso . "MODELLING OF A FED-BATCH FERMENTATION PROCESS ". Control Engineering Laboratory,Department of Process and Environmental Engineering ,University of Oulu.June 21, 2003

Homogenizer

Using the following equipment in the diagram below (Figure 28), the following costs were calculated[9] in Table 24:

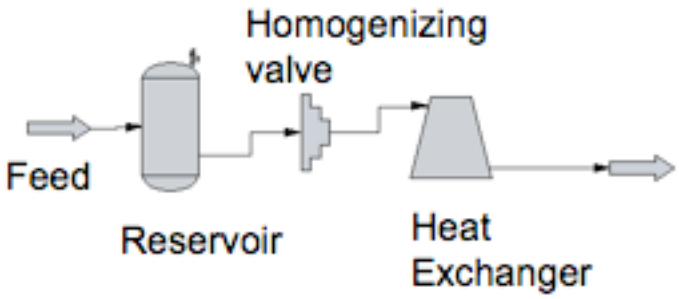


Figure 28: Homogenizer PFD

Table 24: Homogenizer Cost Calculation

Equipment	**Cost ($)**
Reservoir (1000L)	4286
Homogenizing Valve	204566
Heat Exchanger	15000
Total Cost	**$223,852**

[7] Garcia, Antonio et. Al. Bioseparation Process Science. Massachusetts: Blackwell Science, 1999. 341-345
[8] http://www.energymanagertraining.com/dairy/img/homogenizer.gif
[9] -R. Turton et al. **Analysis, Synthesis, and Design of Chemical Processes**, 2nd Ed.Prentice Hall PTR. New York 2007. Pg 915-920.

Cation Exchange Column

Table 25 : POROS 50 HS Column (100 L batch)	
Column Height	0.28 m
Column Diameter	0.33 m
Volume of resin	0.02 m^3
Linear Velocity	455 cm/hr
Pressure Drop	0.459 bar
Cost of Eluting Salts	$81,114
Cost of Resin	$307,500
Cost of Extension	$3,581
Cost of Stand	$5,326
Total Cost	$397,520

Table 26 : POROS 50 HS Column (500 L batch)	
Column Height	0.44 m
Column Diameter	0.52 m
Volume of resin	0.08 m^3
Linear Velocity	455 cm/hr
Pressure Drop	0.459 bar
Cost of Eluting Salts	$107,031
Cost of Resin	$1,537,500
Cost of Extension	$4,725
Cost of Stand	$7,028
Total Cost	$1,656,283

Mixed-Mode Column

The chromatography column was sized based on the binding capacity of the resin (shown in Table 27 to be 45 mg/ml). Only 20% of the binding capacity of the resin was used to obtain a

higher purification. Columns were scaled for processes with volumes of 100 and 500L. The height of the resin was set at 0.5 m and the diameter was found based off the total resin needed for a certain amount of Lucentis to bind. The linear velocity and pressure drop are found from experimental analysis. These parameters should be set from the bench scale to large size processes. Table 27 and Table 28 show the process scale up for 100 L and 500 L. The 500 L requires two passes. Sample calculations for the column parameters can be found in Appendix 1.

Table 27 : Capto MMC Column (100 L batch)	
Resin Height	0.5 m
Diameter	0.428 m
Volume of resin	0.072 m^3
Linear Velocity	606 cm/hr
Pressure Drop	0.459 bar
Cost of Eluting Salts	$30,000
Cost of Resin	$656,776
Cost of Extension	$4,854
Cost of Stand	$7,220
Total Cost	$698,850

Table 28 : Capto MMC Column (500 L batch)	
Resin Height	0.5 m
Diameter	0.678 m
Volume of resin	0.181 m^3
Linear Velocity	606 cm/hr
Pressure Drop	0.459 bar
Cost of Eluting Salts	$109,960
Cost of Resin	$709,200
Cost of Extension	$5,276
Cost of Stand	$7,848
Total Cost	$832,284

Future Work

Fermentor

In the future, fermentation experiments should be performed growing an *e. coli* culture in order to experimentally determine the reaction kinetics. Also, different types of media should be experimented with to see what provides the best environment for growth.

Chromatography

Additional experiments should be performed to improve the experimental design and isolate Lucentis. This includes improving the experimental design of the cation exchange chromatography, the mixed-mode chromatography, and the anion exchange chromatography. Based off the experimental results, it will most likely be necessary to include anion exchange chromatography in the base case. For each experiment, 1-D gel electrophoresis should be performed and bands of interest should be identified by mass spectrometry. Also, samples of interest should be analyzed by 2-D gel electrophoresis. Additional equipment should be purchased including new resins and buffers. Also, a pH probe should be purchased to experiment with elutions using a pH gradient rather than a salt gradient.

Appendix 1:

Sample Calculations

Linear Velocity of Experimental Column

The linear velocity of the experimental column was found by timing how long it took for 1 ml to leave to column filled with Capto MMC resin which can be seen in Table 29. The column height (height of resin) was divided by the time of elution. The average linear velocity was found to be about 606 cm/ hr. The characteristics of the resin specify that the resin can bare linear velocities of at least 600 cm/hr so this calculated linear velocity is within the characteristics of the resin.

Table 29: Linear Velocity Experimental Trials

Trial	Time, s	Time, hr	Column Height, cm	Linear Velocity, cm/hr
1	26	0.0072	5	692.308
2	28	0.0078	5	642.857
3	24	0.0067	5	750.000
4	42	0.0117	5	428.571
5	35	0.0097	5	514.286
AVERAGE	31	0.0086	5	605.604

Calculations for 100L and 500L batches

100 L batch

- 45 mg/ml binding capacity
- Only have 20% of resin bound for a high purification
- Assume 30% additional protein on top of the 5 g/l antibody present
  - 6.5 g/l total protein will stick to the resin
- 500 g of antibody present
- 650 g protein will stick to the resin
- 45 g/l binding capacity
  - Using only 20% of resin capacity- 9 g/l
- 72.2 l (650g/9(g/l)) of resin needed
  - 0.0722 m3 of resin
- Use a 0.5 m height and change the diameter
- Cost of resin- $2733/L
- Extension and stand scaled from last years' data using the six-tenths rule

For a 20% of resin allowed to be bound					
	For 100 L				
	Height (m)	Volume (m3)	Area m2	Diameter m	Ratio (height:diameter)
1 pass	0.5	0.072	0.144	0.428	1.167
2 pass	0.5	0.036	0.072	0.303	1.651
3 pass	0.5	0.024	0.048	0.247	2.022
4 pass	0.5	0.018	0.036	0.214	2.335
5 pass	0.5	0.0144	0.0288	0.192	2.610
6 pass	0.5	0.012	0.024	0.175	2.860

Economics:

Height	Diameter	Volume of Resin	Total Cost of Resin	Extension	Stand	Total Cost
m	m	m3	$	$	$	$
0.5	0.428	0.072	656776	4854	7220	698850

500 L batch

- 45 mg/ml binding capacity
- Only have 20% of resin bound for a high purification
- Assume 30% additional protein on top of the 5 g/l antibody present
  - 6.5 g/l total protein will stick to the resin
- 500 g of antibody present
- 650 g protein will stick to the resin
- 45 g/l binding capacity
  - Using only 20% of resin capacity- 9 g/l
- 361 l (650g/9(g/l)) of resin needed
  - 0.361 m3 of resin
- Use a 0.5 m height and change the diameter

	For 500 L				
	Height (m)	Volume (m3)	Area m2	Diameter m	Ratio (height:diameter)
1 pass	0.5	0.361	0.722	0.959	0.521
2 pass	0.5	0.181	0.361	0.678	0.737
3 pass	0.5	0.120	0.241	0.554	0.903
4 pass	0.5	0.090	0.181	0.480	1.043
5 pass	0.5	0.072	0.144	0.429	1.166
6 pass	0.5	0.060	0.120	0.392	1.277

Economics:

Height	Diameter	Volume of Resin	Total Cost of Resin	Extension	This stand	Total Cost
m	m	m3	$	$	$	$
0.5	0.678	0.181	709200	5276.08	7847.82	832284

Appendix 2:

Alternative Case Monolithic Column

Monolithic Chromatography[http://pubs.acs.org/email/cen/html/121106165845.html]
[http://images.google.com/imgres?imgurl=http://www.flowinjection.com/sichrom/onyx_cutaway.jpg&imgrefurl]

Monolithic columns are made of a new material used for column packing where the monolith is a one-piece porous solid. The sponge-like, which can be seen in Figure 27, in which the material is created from optimizes porosity and is shown to have a higher separation efficiency.

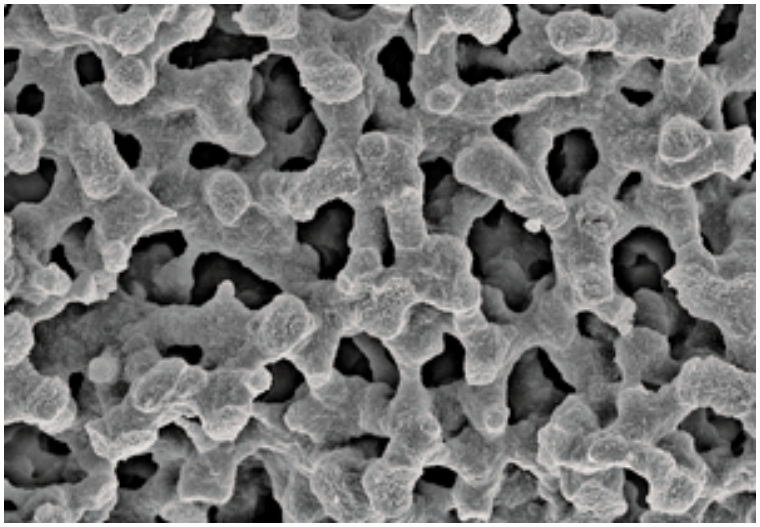


Figure 27: Monolithic Porous Material

Monoliths: A new breed of separation media for chromatography
by Frantisek Svec, *Lawrence Berkeley National Laboratory*
http://www.aesociety.org/areas/Monoliths.pdf

Monoliths can be considered one large particle that does not contain any interstitial voids where all the mobile phase travels through the stationary phase. The differences between monolithic material and packed bead material can be seen in Figure 28. Diffusion is the typical driving for a chromatography column, but in this case, the driving force is convection which greatly speeds up the process of separation. An up and coming application for monoliths is on the nano scale where the column can be used along with a nanoHPLC or a CEC to detect the peptides in microorganisms.

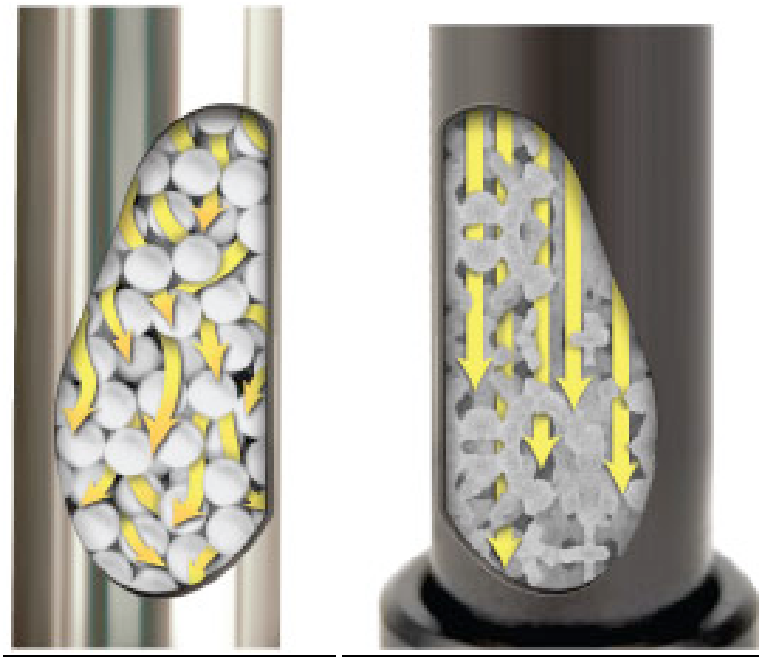


Figure 28: Packed Bead (left) and Monolithic (right) Column

Porous Monoliths: The Newest Generation of Stationary Phases for HPLC and Related Methods
by Frantisek Svec
http://chromatographyonline.findanalytichem.com/lcgc/data/articlestandard//lcgceurope/232003/59044/article.pdf

Buchmeiser has begun to use ring-opening metathesis polymerization of selected monomers for the preparation of monolithic columns. These new monoliths are polymerized in situ within a tube. The mould is filled with a mixture of monomers, a free-radical initiator and porogenic solvent, then sealed and the polymerization is performed under carefully controlled thermal conditions. Swift makes a variety of monolithic columns with a variety of functional groups which are shown to have an excellent stationary phase for a quick separation of proteins.

Large monolithic columns are desirable for production size processes, but the polymerization process which creates the columns is highly exothermic. Any significant change in the temperature within the mould would significantly reduce the performance of the monolith. This problem could be overcome by creating monoliths in an annular shape which allows for independent variation of both tube wall thickness and inner diameter.

Large-scale methacrylate monolithic columns: design and properties
[A. Podgornik, , J. Jan ar, M. Merhar, S. Kozamernik, D. Glover, K. u ek, M. Barut and A. trancar
BIA Separations. 2004. 60: 179-189.]

Large scale monolithic columns are difficult to prepare due to problems with the heat generated during polymerization of the monolith. The chromatographic method used was calculated from the small column and converted to the large column using the equation

$$t_{g.large} = t_{g.small}\left(\frac{V_{large}}{V_{small}}\right)\left(\frac{F_{small}}{F_{large}}\right)\left(\frac{T_{small}}{T_{large}}\right)$$

where tg is the gradient time, V is the volume of the column, F is the flow rate of the column, and T is the monolith radius. The average linear velocity of the large scale column was calculated by

$$v = \frac{F}{2\pi h}\frac{\ln\left(\frac{r_i}{r_o}\right)}{T}$$

where v is the average linear velocity, h is the monolith height, r_o is the outer radius, and r_i is the inner radius. The productivity or column yield can be calculated by

$$P = \frac{q}{\frac{q}{CF} + \frac{3V}{F}}$$

where P is for productivity, q is the total capacity of the column, c is the protein concentration in the loading solution, F is the flow rate, and V is the column volume. There was no difference in performance between axial and radial monolithic column in large column designs with fast separations. A problem with large monolithic columns is the changes in stationary phases to maintain a low pressure drop can affect the quality of separation. For monoliths, a single crack can cause a column to malfunction. Methacrylates are known to be mechanically stable. The mobile phase is applied in a direction so that the pressure squeezes the monolith and seals any crack that may appear. Table 30 shows experimental scale-up of monolithic columns and the parameters associated with scale-up.

Table 30: Scale up Parameters of Monolithic Columns

Column volume (mL)	Dimensions (cm)			Maximal flow rate (ml/min)	Average maximal linear velocity (cm/h)	BSA capacity per unit (g)	Productivity (g/h)
	h	r_i	T				
8	4.6	1.5	0.65	40	297	0.2	3.1
80	11	3.5	0.9	250	174	2	13.4
800	15	11	1.85	3000	383	20	107.1
8000	41.5	30	2.2	18,000	166	200	333.7

Since monolithic columns are difficult to scale up and there has been no evidence of scale-up greater than 8 liters. The design parameters used for HPLC sized monolithic columns are shown in Table 31.

Table 31: Monolithic HPLC Column Parameters

Sample Load	2 ug
Maximum loading capacity	10 pmol
Flow Rate	20 ul/min
Gradient	10- 50% MeCN over 20 min
Temperature	60 C
Diameter	500 um ID
Length	5 cm
Volume of Monolith	9.81×10^{-9} m

Dionex Design Parameters HPLC size column [http://www.dionex.com/en-us/webdocs/39001_AN529_V30_releasedJC060106.pdf]

The HPLC column was scaled up to a size of 10 L and the following design parameters were found (Table 32).

Table 32: 10 L Volume Monolithic Column Design Parameters

Sample Load	20 g
Maximum loading capacity	10 pmol
Flow Rate	20 ul/min
Gradient	10- 50% MeCN over 20 min
Temperature	60 C
Diameter	10.7 cm ID
Length	10.77 cm
Volume of Monolith	0.0981 m

The ratio of length to diameter of the column was kept the same due to the type of flow that is present in a monolithic column. The following calculations show the determination of the length and diameter of the column needed for 10 L. The last two equations were solved simultaneously to determine the length and diameter needed.

$$Sample\ Amount = Concentration \cdot Volume$$

$$Sample\ Amount = 2\frac{g}{L} \cdot 10L = 20g$$

$$\frac{Volume_{10L}}{Sample_{10L}} = \frac{Vol_{HPLC}}{Sample_{HPLCE}}$$

$$Volume_{10L} = \frac{9.81 \times 10^{-9} m^3}{2 \times 10^{-6} g} 20g$$

$$Volume_{10L} = 0.0981 m^3$$

$$Area = \frac{Volume}{Length}$$

$$Area = \pi\left(\frac{d}{2}\right)^2$$

$$\pi\left(\frac{d}{2}\right)^2 = \frac{Volume}{Length}$$

$$\frac{Length}{diameter} = 100$$

Overall the advantages to monolithic chromatography are having a better separation by molecular weight instead of isoelectric point and higher flow rates. The disadvantages to

monolithic chromatography were found to be that large scale processes are not common, it is a high pressure system, and since it is all one piece, a crack can ruin the entire system.

Appendix 3:

Cation Results of Mass Spectrometry

Band #	Protein Rank	Protein Name	MW	pI
1	1	Chain A, Dipeptide Binding Protein Complex With	57370.6	5.75
		Glycyl-L-Leucine		
	2	dipeptide ABC transporter, periplasmic	60225.3	6.36
		dipeptide-binding protein [Escherichia albertii TW07627]		
	3	periplasmic dipeptide transport protein precursor	60255.3	6.36
		[Escherichia coli CFT073]		
	4	Chain A, Dipeptide-Binding Protein	57398.6	5.66
	5	dipeptide transporter [Escherichia coli str. K-12 substr.	60255.2	6.21
		MG1655]		
	6	dipeptide ABC transporter, periplasmic	60239.2	6.21
		dipeptide-binding protein [Escherichia coli E110019]		
	7	dipeptide transport protein [Shigella sonnei Ss046]	60285.3	6.21
	8	dipeptide transporter [Escherichia coli O127:H6 str.	60281.3	6.31
		E2348/69]		
	9	dipeptide transporter ; periplasmic-binding component	60254.3	6.36
		of ABC superfamily [Escherichia fergusonii AT		
	10	dipeptide transport protein [Escherichia coli O157:H7	60285.1	6.21
		EDL933]		
2	1	Chain A, Crystal Structure Analysis Of Periplasmic	36749.8	5.28
		LeuILEVAL- Binding Protein In Superopen Form		
	2	high-affinity branched-chain amino acid ABC	39068	5.54
		transporter, periplasmic Leu/Ile/Val-binding protein Li		
	3	Leu/Ile/Val-binding protein precursor [Escherichia coli	41277.3	6.34
		CFT073]		
	4	leucine/isoleucine/valine transporter subunit ;	39054	5.54
		periplasmic-binding component of ABC superfamily [E		
	5	high-affinity amino acid transport protein, periplasmic	41314.2	6.25
		binding protein [Shigella flexneri 2a str.		
	6	leucine/isoleucine/valine transporter subunit	39066	5.54
		[Escherichia coli O127:H6 str. E2348/69]		

	7	high-affinity amino acid transport system periplasmic	41316.2	6.25
		binding protein [Shigella dysenteriae Sd197]		
	8	high-affinity branched-chain amino acid ABC	39082	5.54
		transporter, periplasmic Leu/Ile/Val-binding protein Li		
	9	hypothetical protein CKO_04883 [Citrobacter koseri	39619.3	5.91
		ATCC BAA-895]		
	10	RecName: Full=Leu/Ile/Val-binding protein;	39062.9	5.91
		Short=LIV-BP; Flags: Precursor		
3	1	Chain A, Phosphate-Binding Protein (Re-Refined)	34400.7	6.92
	2	Chain A, Phosphate-Binding Protein (Pbp) Complexed	34330.6	7.27
		With Phosphate		
	3	Chain A, Phosphate-Binding Protein Mutant With Asp	34399.8	7.94
		56 Replaced By Asn Complex With Monobasic Phosph		
	4	phosphate transporter subunit [Escherichia coli 536]	37014.1	8.39
	5	phosphate transporter subunit [Escherichia coli	37027.1	8.39
		O157:H7 EDL933]		
	6	Chain A, Phosphate-Binding Protein With Ala 197	34515.8	6.92
		Replaced With Trp		
	7	phosphate transporter subunit ; periplasmic-binding	37035.1	8.39
		component of ABC superfamily [Escherichia fergu		
	8	Chain A, Phosphate-Binding Protein Mutant With Asp	34399.8	7.94
		137 Replaced By Asn Complex With Phosphate		
	9	phosphate transporter subunit [Escherichia coli	36987.1	8.39
		CFT073]		
	10	Chain A, Phosphate-Binding Protein Mutant With Asp	34342.7	7.94
		137 Replaced By Gly Complex With Bromine And Pho		
4	1	Chain L, Crystal Structure Of Tissue Factor In Complex	23484.6	6.99
		With Humanized Fab D3h44		
	2	humanized antibody D3H44 [synthetic construct]	26016.9	8.18

	3	immunoglobulin light chain [Macaca mulatta]	9515.6	6.23
	4	immunoglobulin kappa light chain variable region [Homo	8611.3	10.29
		sapiens]		
	5	immunoglobulin kappa chain variable region [Homo	10774.2	6.92
		sapiens]		
	6	immunoglobulin light chain variable region [Homo	11682.7	9.04
		sapiens]		
	7	immunoglobulin light chain V-J region [Homo sapiens]	11612.7	7.96
	8	immunoglobulin kappa light chain [Homo sapiens]	11477.7	8.64
	9	anti-tetanus toxoid immunoglobulin light chain variable	11585.8	7.96
		region [Homo sapiens]		
	10	immunoglobulin light chain variable region [Homo	11820.9	9.2
		sapiens]		
5	1	Chain A, Phosphate-Binding Protein (Re-Refined)	34400.7	6.92
	2	Chain A, Phosphate-Binding Protein Mutant With Asp	34399.8	7.94
		56 Replaced By Asn Complex With Monobasic Phosph		
	3	Chain A, Phosphate-Binding Protein (Pbp) Complexed	34330.6	7.27
		With Phosphate		
	4	Chain A, Phosphate-Binding Protein Mutant With Asp	34342.7	7.94
		137 Replaced By Gly Complex With Bromine And Pho		
	5	Chain A, Phosphate-Binding Protein With Ala 197	34515.8	6.92
		Replaced With Trp		
	6	phosphate transporter subunit [Escherichia coli 536]	37014.1	8.39
	7	Chain A, Phosphate-Binding Protein Mutant With Thr	34344.6	6.06
		141 Replaced By Asp (T141d), Complexed With Phos		
	8	phosphate transporter subunit [Shigella sonnei Ss046]	37073.1	8.39
	9	phosphate transporter subunit [Escherichia coli	36987.1	8.39
		CFT073]		
	10	phosphate transporter subunit [Escherichia coli	37027.1	8.39
		O157:H7 EDL933]		

6	1	serine endoprotease [Shigella boydii Sb227]	47222.1	5.76
	2	serine endoprotease [Escherichia coli APEC O1]	47217.2	5.76
	3	serine endoprotease [Escherichia coli CFT073]	48751.9	6.26
	4	serine peptidase DegQ [Shigella dysenteriae 1012]	47163.2	5.76
	5	serine endoprotease [Shigella sonnei Ss046]	47146.1	5.77
	6	serine peptidase DegQ [Escherichia coli E22]	47166.1	5.76
	7	serine endoprotease, periplasmic [Escherichia coli	47231.2	5.76
		ED1a]		
	8	serine endoprotease [Escherichia coli O157:H7	47188.1	6.08
		EDL933]		
	9	serine peptidase DegQ [Escherichia coli O157:H7 str.	48740.9	6.26
		EC4113]		
	10	serine endoprotease [Escherichia coli 536]	47231.2	6.1
7	1	glutathione oxidoreductase [Escherichia coli str. K-12	48741.6	5.64
		substr. MG1655]		
	2	glutathione reductase [Escherichia coli HS]	48697.6	5.64
	3	glutathione oxidoreductase [Escherichia coli ED1a]	48649.6	5.72
	4	Chain L, Crystal Structure Of Tissue Factor In Complex	23484.6	6.99
		With Humanized Fab D3h44		
	5	Chain A, Anatomy Of An Engineered Nad-Binding Site	48656.4	5.23
	6	Chain L, Murine 6a6 Fab In Complex With Humanized	23381.5	7
		Anti-Tissue Factor D3h44 Fab		
	7	glutathione reductase [Salmonella typhimurium LT2]	48661.7	5.77
	8	humanized antibody D3H44 [synthetic construct]	26016.9	8.18
	9	glutathione reductase [Salmonella enterica subsp.	27140.9	7.78
		enterica serovar Typhi str. AG3]		
	10	glutathione reductase [Shigella dysenteriae Sd197]	48751.7	5.64

Appendix 4:

Mixed-Mode Results of Mass Spectrometry

Band #	Protein Rank	Protein Name	MW	pI
1	1	Chain H, Murine 6a6 Fab In Complex With Humanized	23106.4	6.34
		Anti-Tissue Factor D3h44 Fab		
	2	Chain H, Crystal Structure Of Tissue Factor In Complex	24006.8	7.11
		With Humanized Fab D3h44		
	3	humanized antibody D3H44 [synthetic construct]	26539.2	8.19
	4	Chain B, Crystal Structure Of Phage Derived Fab Bdf1	24099.1	9.07
		With Human Death Receptor 5 (Dr5)		
	5	Chain F, C3b In Complex With A C3b Specific Fab	23760.7	8.9
	6	Chain A, Crystal Structure Of An Autonomous Vh	13035.3	6.51
		Domain		
	7	Chain H, Crystal Structure Of Hbr3 Bound To Cb3s-Fab	24376.1	8.82
	8	immunoglobulin VH region=humanized bispecific	13474.5	8
		antibody [human, Peptide Recombinant, 122 aa]		
	9	Chain B, Crystal Structure Of The Fab Yads2	23342.5	7.86
		Complexed With H-Vegf		
	10	Chain H, Short Form Hgfa With Inhibitory Fab75	23267.5	8.79
2	1	Chain L, Crystal Structure Of Tissue Factor In Complex	23484.6	6.99
		With Humanized Fab D3h44		
	2	humanized antibody D3H44 [synthetic construct]	26016.9	8.18
	3	Chain A, X-Ray Structures Of The Antigen-Binding	23394.5	6.99
		Domains From Three Variants Of Humanized Anti-P185		
	4	Chain A, The Crystal Structure Of The Ysd1 Fab Bound	23113.3	6.9
		To Dr5		
	5	Chain L, Antibody To Campath-1h Humanized Fab	23556.7	8.54
	6	Chain A, Bence Jones Protein Del: An Entire	22972.3	5.93
		Immunoglobulin Kappa Light-Chain Dimer		
	7	Chain L, Vascular Endothelial Growth Factor In	23436.4	6.34
		Complex With A Neutralizing Antibody		
	8	immunoglobulin kappa 1 light chain [Homo sapiens]	23243.4	6.17
	9	anti-Entamoeba histolytica immunoglobulin kappa light	23290.6	8.26
		chain [Homo sapiens]		
	10	Chain L, Crystal Structure Of A Humanized Fab	24027.8	8.27
		(Hu3s193) In Complex With The Lewis Y		
		Tetrasaccharide		
3	1	glycerol-3-phosphate-binding periplasmic protein	48417	6.57
		precursor [Escherichia coli E22]		
	2	glycerol-3-phosphate transporter periplasmic binding	48388	6.29
		protein [Shigella dysenteriae Sd197]		
	3	glycerol-3-phosphate transporter periplasmic binding	48434	6.29
		protein [Escherichia coli E24377A]		
	4	unnamed protein product [Escherichia coli]	48422	6.29
	5	glycerol-3-phosphate-binding periplasmic protein	48435.1	6.49
		precursor [Escherichia coli SMS-3-5]		

	6	glycerol-3-phosphate transporter periplasmic binding	48462.2	7.6
		protein [Escherichia coli APEC O1]		
	7	glycerol-3-phosphate-binding periplasmic protein	48462.1	6.2
		precursor [Escherichia albertii TW07627]		
	8	glycerol-3-phosphate transporter periplasmic binding	48434.1	6.29
		protein [Shigella flexneri 2a str. 301]		
	9	glycerol-3-phosphate transporter periplasmic binding	48424.3	7.63
		protein [Salmonella enterica subsp. enterica s		
	10	sn-glycerol-3-phosphate-binding periplasmic protein	48366.2	6.98
		UgpB [Salmonella enterica subsp. enterica serov		
4	1	glycerol-3-phosphate-binding periplasmic protein	48417	6.57
		precursor [Escherichia coli E22]		
	2	glycerol-3-phosphate transporter periplasmic binding	48388	6.29
		protein [Shigella dysenteriae Sd197]		
	3	glycerol-3-phosphate transporter periplasmic binding	48434	6.29
		protein [Escherichia coli E24377A]		
	4	unnamed protein product [Escherichia coli]	48422	6.29
	5	glycerol-3-phosphate-binding periplasmic protein	48435.1	6.49
		precursor [Escherichia coli SMS-3-5]		
	6	glycerol-3-phosphate transporter periplasmic binding	48462.2	7.6
		protein [Escherichia coli APEC O1]		
	7	glycerol-3-phosphate-binding periplasmic protein	48462.1	6.2
		precursor [Escherichia albertii TW07627]		
	8	glycerol-3-phosphate transporter periplasmic binding	48434.1	6.29
		protein [Shigella flexneri 2a str. 301]		
	9	glycerol-3-phosphate transporter periplasmic binding	48424.3	7.63
		protein [Salmonella enterica subsp. enterica s		
	10	sn-glycerol-3-phosphate-binding periplasmic protein	48366.2	6.98
		UgpB [Salmonella enterica subsp. enterica serov		
5	1	Chain A, Phosphate-Binding Protein (Re-Refined)	34400.7	6.92
	2	Chain A, Phosphate-Binding Protein (Pbp) Complexed	34330.6	7.27
		With Phosphate		
	3	Chain A, Phosphate-Binding Protein Mutant With Asp	34399.8	7.94
		56 Replaced By Asn Complex With Monobasic Phosph		
	4	phosphate transporter subunit [Escherichia coli 536]	37014.1	8.39
	5	phosphate transporter subunit [Escherichia coli	37027.1	8.39
		O157:H7 EDL933]		
	6	Chain A, Phosphate-Binding Protein With Ala 197	34515.8	6.92
		Replaced With Trp		
	7	phosphate transporter subunit ; periplasmic-binding	37035.1	8.39
		component of ABC superfamily [Escherichia fergu		
	8	Chain A, Phosphate-Binding Protein Mutant With Asp	34342.7	7.94
		137 Replaced By Gly Complex With Bromine And Pho		
	9	Chain A, Phosphate-Binding Protein Mutant With Thr	34344.6	6.06
		141 Replaced By Asp (T141d), Complexed With Phos		
	10	phosphate ABC transporter, periplasmic	37069.2	7.68
		phosphate-binding protein PstS [Escherichia coli 53638]		

References:

Belter, P. A., Cussler, E. L., & Hu, W. (1988). *Bioseparations: Downstream Processing for Biotechnology*. New York, NY: Wiley and Sons, Incorporated.

BTPX 305. Retrieved May 6, 2009, from Alfa Laval Web site: http://www.alfalaval.com/solution-finder/products/btpx-separator/Documents/BTPX%20Series-BTPX%20305%20S.pdf.

Cheryan, Munir (1986). *Ultrafiltration Handbook*. Lancaster, PA: Technomin Publishing Company, Incorporated..

Millipore. (2003). Protein Concentration and Diafiltration by Tangential Flow Filtration -- An Overview. Retrieved May 6, 2009, from Millipore Web site: http://www.millipore.com/techpublications/tech1/tb032.