Production of a Recombinant Spike Protein-Based SARS-CoV-2 Vaccine Using the Baculovirus Expression Vector System

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On my honor as a University Student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments

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1. Executive Summary

The purpose of this capstone design project is to design a manufacturing facility based in South Africa capable of producing 400 million doses of COVID-19 vaccines each year. This vaccine will be a recombinant subunit protein vaccine made using the baculovirus expression vector system. The facility will use single-use technologies to reduce the risk of contamination and to provide more flexibility in the manufacturing process. The upstream process consists of an Sf9 insect cell seed train and a baculovirus amplification train that culminates in protein production in five 2000 L production bioreactors. The upstream process will produce 300 g of spike protein per batch. The downstream process consists of centrifugation, homogenization, depth filtration, an ultrafiltration and diafiltration step, anion exchange chromatography, viral inactivation, cation exchange chromatography, ceramic hydroxyapatite chromatography, viral filtration, and a second ultrafiltration and diafiltration step. The downstream process will yield 133.5 g of antigen per batch. The downstream antigen solution will be formulated with preservatives, a surfactant, and phosphate buffered saline, and then will be filled into 10 mL vials. AS03 adjuvant will also be formulated separately and filled into 10 mL vials. The filled vials will be stored as a liquid at a temperature of 2 - 8°C. This plant aims to produce 400 million vaccine doses per year to satisfy global demand.

The manufacturing facility will have a five-year lifespan. Financial analysis over this timeframe was used to determine the economic feasibility of this project. The cost of operating this facility includes fixed capital cost and operating costs which include raw materials, utilities, labor, and taxes. The first year of operation yields $4.6 million in profits. Additionally, over a five year timeframe, the net present value of this plant is $15.6 billion using a 15% interest rate. Therefore, this project is economically feasible.

2. Introduction

Coronavirus disease 2019 (COVID-19) remains a major global health concern. To date, there have been over 465 million confirmed cases and 6.1 million deaths worldwide (World Health Organization, 2021a). Nevertheless, 6 billion doses of vaccines have been administered, with many well-developed nations exceeding 50% vaccination rate (World Health Organization, 2021b; Mwai, 2021). However, only 2.5% of people in low-income countries have received at least one vaccine dose (Ritchie et al., 2020). To reach a target of 70% vaccination worldwide, an estimated additional 11 billion doses are required. COVAX, an organization co-led by CEPI, Gavi, and WHO, aims to donate enough vaccine doses to vaccinate 20% of low-middle income countries (World Health Organization, 2021b). However, a low supply of vaccines has prevented COVAX from reaching their initial goal (Paton & Bloomberg, 2021). More vaccine doses are sorely needed.

Pfizer-BioNTech's and Moderna's mRNA-based vaccines have efficacies over 90% against the Wuhan strain but require extremely cold storage: between -50 °C and -15 °C for Moderna and between -90 °C and -60 °C for Pfizer (Centers for Disease control and Prevention, 2021). This cold storage supply chain is an issue for the 3 billion people in locations where cold chain storage is not easily accessible (Hinnant, 2020). Currently, Sanofi and GlaxoSmithKline (GSK) are developing a recombinant protein vaccine that is in phase 3 clinical trials with 95% efficacy against the Wuhan strain after the 2nd dose (Sanofi, 2021). This vaccine is manufactured using the baculovirus expression vector system and can be stored at normal refrigeration temperatures (2 to 8°C), providing considerable potential for low-income nations (Sagonowsky, 2020).

Baculoviruses are a family of viruses that are known to infect insects. The baculovirus expression vector system (BEVS) is an important biotechnology tool because it can be used to insert protein-coding DNA into insect cells (Felberbaum, 2015). Once infected, the insect cells are instructed to reliably produce the antigen protein which, when inserted in the human body, initiates an immune response, producing antibodies that protect against future infection. Baculoviruses are very selective in their choice of hosts to infect; they cannot infect mammals, plants, fish, or non-target insects (Hu, 2005). Unlike many other vaccine production processes, BEVS does not require handling of live, potentially-dangerous pathogens, reducing the biocontainment requirements (Felberbaum, 2015). Compared to other biopharmaceutical manufacturing platforms, such as mRNA- and viral vector-based vaccines, BEVS has lower manufacturing costs and easier scalability. Therefore, utilizing the existing global bioreactor capacity can reduce initial investment costs for BEVS facilities (Felberbaum, 2015). Furthermore, genetic and fermentation-based approaches exist that are known to improve product yield (Cox, 2012). These facilities can manufacture multiple types of vaccines using the same cell line and equipment (Josefsberg, 2012). There are currently four BEVS-derived products approved for human use including the Flublok® vaccine for seasonal influenza and the Cervarix® vaccine to prevent certain types of cancer-causing human papillomavirus (HPV). For all of these reasons, BEVS is ideal to manufacture another high-efficacy COVID-19 vaccine.

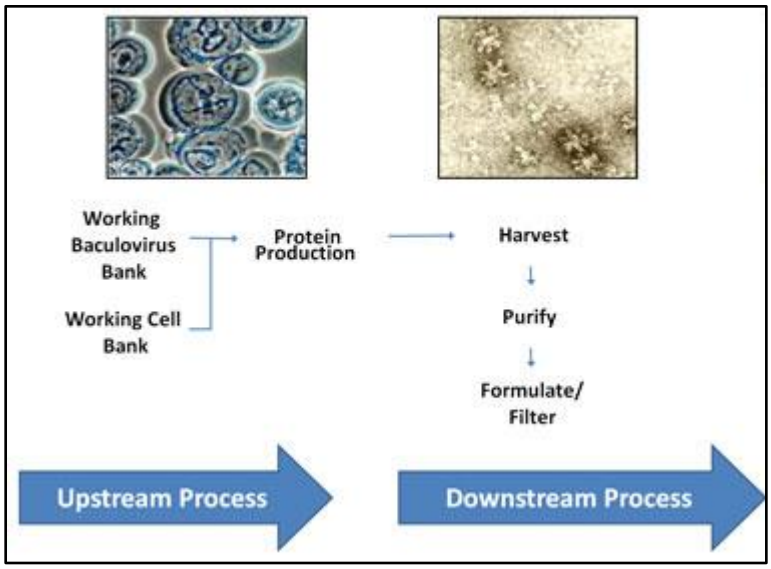


Figure 2.1. Simplified flow chart of upstream and downstream BEVS process (Genovesi, 2018)

2.1. Scale of Project

As of November 2021, 41% of the global population is fully vaccinated against COVID-19, and 53% of the global population is partially vaccinated (Our World in Data, 2021). Assuming that most of the vaccines require two doses, in order to vaccinate the entire global population of 7.9 billion people, 8.374 billion vaccine doses need to be produced. There are currently 22 authorized vaccines that are either approved or are in emergency use around the world (Zimmer et al., 2021). Therefore, each manufacturer is responsible for producing 380.6 million vaccine doses as soon as possible. Many of the current vaccines in use are beginning to recommend a third vaccine dose. Therefore, when considering yearly COVID-19 booster shots and global population growth, each vaccine manufacturer needs to produce approximately 367 million doses per year. The process outlined in this paper will be capable of manufacturing 400 million vaccine doses. Excess doses are needed in order to account for any losses during production and/or transit. The vaccine dose is 0.5 mL and contains 10 µg of antigen (Sanofi, 2021). Thus, 4 kg of antigen will be produced per year in order to produce 400 million vaccine

doses each year. The manufacturing facility will be cited in South Africa as an entrypoint into the African continent, which is in dire need of COVID-19 vaccines.

3. Discussion

3.1. Upstream

3.1.1. Recombinant Protein Subunit SARS-CoV-2 Vaccine

Overview

The baculovirus expression vector system (BEVS) is widely used for recombinant protein production. This process works by first transferring the SARS-COV-2 spike gene via a plasmid into a baculovirus. Baculovirus is a virus that can only infect insect cells. Next, the recombinant baculovirus infects the insect cells and uses the host cell's machinery to produce spike protein.

The advantages of this subunit vaccine is that it can be stored at refrigeration temperatures. Additionally, BEVS has more scalability, is safer, and is more cost effective. This process is inspired by the Sanofi-GSK and the Novavax vaccines being developed for COVID-19.

Insect Cell Line

The most common lepidopteran insect cell lines used for BEVS applications are clonal isolates from *Spodoptera frugiperda* (Sf9 and Sf21) and *Trichoplusia ni* (High Five™ BTI-Tn-5B1-4). These host cell lines are ideal for large-scale protein production due to their ability to grow freely in suspension while maintaining a high expression level of biologically active product. Although High Five™ cells have been demonstrated to express more recombinant proteins than the Sf cell line, High Five™ cells are less efficient in virus amplification (Scholz & Suppmann, 2021) and more rapidly consume growth-limiting nutrients (Rhiel et al., 1997). The Sf21 cell line was the first to be used in research and industrial applications, but the use of Sf21 cells has diminished to the benefit of Sf9 cells, owing to their higher growth rates and superior resistance to pH and shear stress (Drugmand, 2012) The Sf9 cell line is well-characterized by

publicly available research on its carbohydrate and amino acid metabolism, growth kinetics in suspension, and dissolved oxygen requirements. For these reasons, the Sf9 cell line will be used for this vaccine production process, and these cells will be purchased from Thermo Scientific's Gibco™ cell culture catalog.

Growth Medium

To grow the Sf-9 cells, a growth medium, that is specifically adapted for large-scale protein production using the Sf-9 cell line, is required. Sf-9 cells can be grown in serum-supplemented or serum-free media. A majority of insect cell culture media contains varying concentrations of animal serum (e.g. fetal bovine serum-FBS) as a growth supplement and protectant against shear force. In our design, however, we decided to use serum-free formulations due to several advantages that they offer over serum. Serum-free media is less likely to introduce adventitious agents (prions, viruses, bacteria) that could complicate downstream purification (Babcock et al., 2007). Serum-free media also has more consistent cost, quality, and availability. Two serum-free media, Sf-900 II SFM and EXPRESS-FIVE SFM, were considered since they both support faster population doubling times and higher saturation cell densities than other traditional media (Thermo Fisher, 2002). Gibco™ Sf-900™ II SFM was selected because it contains optimized concentrations of amino acids, carbohydrates, vitamins, and lipids that reduce or eliminate the effect of rate-limiting nutritional restrictions or deficiencies (Thermo Fisher, 2014). This media is ready-to-use, and does not require the addition of glutamine (nitrogen source) or shear-protecting surfactants (Thermo Fisher, 2014). The rheological characteristics of the medium, even in the presence of high cell concentrations and the release of host cell material, do not change significantly with time, remaining essentially

Newtonian and very similar to water (Godoy-Silva et al., 2010). Therefore, the medium will be approximated as water in all pertinent calculations.

Baculovirus Line

The specific baculovirus that will be used is *Autographa californica nuclear* polyhedrosis virus (AcNPV) expressing the SARS-CoV-2 spike protein. AcNPV is one of the most extensively studied baculoviruses and can infect a wide range of insect cell lines.

Growth Kinetics and Culture Conditions

Invertebrate cell cultures are extremely vulnerable to environmental conditions, and the low-protein nature of serum-free formulations increases this sensitivity. Therefore, it is critical that the physical conditions of the culture be closely monitored and regulated to ensure optimal cell proliferation and expression of recombinant protein. The Sf9 cell density (X) in the medium at t hrs after inoculation was determined using the following exponential growth model:

$$X(t) = X_0 e^{\mu t} \tag{3.1.1a}$$

$X(t) = X_0 e^{\mu t}$; μ = maximum specific growth rate (0.033 hr^{-1})

Sf9 cells have a maximum specific growth rate (μ) of 0.033 hr^{-1} , a maximum viable cell density of $8.1x10^6$ cells/mL, and grow optimally at 27 °C and a pH of 6.3 (Rhiel et al., 1997). During fermentation, carbon dioxide is produced as a waste by-product from the metabolism, cell growth, and protein production reactions. High levels of dissolved CO_2 are inhibitory to cell growth and protein production and can acidify the medium (Godoy-Silva et al., 2010). A probe will monitor the pH of the medium, and any reductions in pH will be neutralized by adding 1 M of NaOH. Additionally, in order to suppress foam formation, drops of antifoam emulsion will be added to the bioreactors as needed.

The cells must receive nutrients for metabolism. In addition to the Sf-900™ II SFM, glucose and pure oxygen will be added. Glucose provides a source of carbon for the insect cells, and glucose depletion triggers cell death. Rhiel et al. (1997) measured the specific glucose uptake rate of infected and uninfected Sf9 cells in Sf-900™ II SFM to be $2.4x10^{-17}$ mol glucose/cell/s and $1.7x10^{-17}$ mol glucose/cell/s, respectively. Insect cells must grow under aerobic conditions. Oxygen supply is a key limiting factor for insect cell growth and product formation due to the low solubility of oxygen in the culture medium (Karimi et al., 2013). Therefore, pure oxygen will be continuously sparged through the culture medium during bioreactor operation. This oxygen sparging will also help to strip dissolved CO_2 from the medium.

Bioreactors

The Thermo Scientific™ HyPerforma™ 5:1 Single-Use Bioreactor (S.U.B.) System - consisting of an outer support vessel, a BioProcess Container (BPC), and a PID control system for agitation and temperature will be used for all stirred, aerated fermentation operations. Although single-use bioreactors can have high life-cycle costs (polymeric bioprocess films can be expensive) and require coordination around the lead times of disposable equipment in the supply chain, single-use systems offer many advantages over conventional stainless steel bioreactors, including flexible production capacity, rapid campaign turnarounds due to the elimination of unproductive downtimes for clean-in-place (CIP) and steam-in-place (SIP) validation, and lower risk of cross-contamination (Rogge et al., 2015). Although single-use systems are not appropriate for the high oxygen demands of microbial fermentations, the oxygen mass transfer in disposable bags is sufficient to support the cultivation of animal cells, including Sf9 cells (Card et al., 2011).

Scale was a major factor in deciding which commercial single-use bioreactors to implement in our design. Since protein production will be performed at the 10,000 L scale, selecting as few reactors as possible to minimize costs while meeting this target capacity was a main priority. Furthermore, we wanted all of our bioreactors to be from the same manufacturer to reduce variability in reactor performance and in the quality of disposable material. These criteria narrowed our selection to two bioreactor series from Thermo Scientific™: The HyPerforma™ S.U.B and the HyPerforma™ DynaDrive™ S.U.B. The DynaDrive™ model offers several improvements over the standard HyPerforma™ bioreactor including a multi-blade driveshaft and a cubic design for maximum mixing efficiency as well as modern sensor technology. The DynaDrive™ portfolio includes a 5,000 L S.U.B. - the only one of that size on the market. Although the DynaDrive™ series seems ideal for our high-density cell culture operations, the HyPerforma™ line will be used in this upstream process since it is a more established technology (the 5,000L DynaDrive™ S.U.B. was released one year ago in March 2021) with well-documented performance characteristics. The HyPerforma S.U.B. vessel is available in sizes ranging from 50 to 2000 L. For Sf9 cell propagation, baculovirus amplification, and protein production, we will employ 1x100 L, 1x1000 L, and 6x2000 L single-use stirred tank bioreactors. The HyPerforma™ bioreactors are available with either electrical or jacketed cooling systems for culture temperature control. Leveling casters are affixed to the bottom of the bioreactors for increased portability around the production floor. The bioreactors feature sealed ports to introduce autoclavable probes for real-time monitoring of pH and dissolved oxygen.

BioProcess Container

The HyPerforma S.U.B. BioProcess Container (BPC) is the single-use, pre-sterilized consumable installed within the outer support bioreactor vessel. The BPC is constructed from

Thermo Scientific's proprietary CX5-14 polymeric film. This five layer, 14 mil cast film is manufactured in a cGMP facility using no animal-derived components. The outer layer is a polyester elastomer coextruded with an ethyl vinyl alcohol (EVOH) barrier layer and a low-density polyethylene product contact layer (Thermo Scientific, 2016). The BPC features a dual-sparger design with a drilled-hole sparger for cultures at maximum working volume, and a cross-flow sparger for when the reactor is operated with seed cultures at a 5:1 turndown ratio. The BPC comes with vent filters, sensor ports, and line sets for harvest and for addition of media and supplemental nutrients. The BPC arrives gamma-irradiated and ready to use.

Impellers

Bioreactor impellers are designed to evenly distribute oxygen and nutrients to cells for healthy growth, to prevent cells from settling to the bottom of the vessel, and to maintain a uniform culture temperature and pH (Mirro & Voll, 2009). It is critical to choose the impeller type that is best suited to the specific process conditions. Like all animal cells, insect cells lack a cell wall and are thus susceptible to damage by the hydrodynamic forces of excessive agitation (Chalmers, 1996). Therefore, impeller designs generating low shear were investigated. Two types of shear-sensitive impellers were identified: pitched-blade and marine. Pitched-blade impellers feature blades oriented at 45° angles, which generate simultaneous axial (fluid pushed parallel to the impeller shaft) and radial (fluid pushed perpendicular to the impeller shaft) flow. The leading face of the blades on marine impellers can be flat or concave, while their backsides are convex. This produces an axial flow. Due to this unidirectional flow, the oxygen mass transfer rate ($k_L a$) provided by marine impellers is generally lower than that of pitched-blade impellers (Mirro & Voll, 2009). Therefore, pitched-blade impellers will be used for all bioreactors in the upstream process. The HyPerforma™ S.U.B. BPC comes equipped with a

single, magnetically-coupled, 45°-pitch, three-blade impeller. This impeller has a calculated power number (N_p) of 2.1. The aspect ratios of the bioreactors that will be implemented in this upstream process are not high (~1.5), so one impeller should be adequate to guarantee efficient aeration and agitation.

Oxygen Delivery

The performance of a stirred-tank bioreactor is often assessed by the volumetric oxygen mass transfer coefficient ($k_L a$), a measure of how efficiently oxygen is transferred from gas bubbles into the liquid culture medium and therefore a critical scale-up criterion. The $k_L a$ needed to sustain cell growth is determined by the following oxygen mass balance:

$$q_{O_2} X_{max} = k_L a (C^*_{O_2} - C_{O_2}) \quad (3.1.1b)$$

q_{O_2}= specific oxygen utilization rate
X_{max}= maximum cell density
$C^*_{O_2}$= solubility of oxygen in medium at 27 ℃
C_{O_2}= dissolved oxygen concentration (set to 50% air sat.)
where $q_{O_2}$is the specific oxygen uptake rate (SOUR), X_{max} is the maximum cell density achieved in the reactor, $C^*_{O_2}$is the saturated dissolved oxygen concentration in the culture medium (the solubility of oxygen in water is 7.97 mg/L at 27 ℃), and C_{O_2} is the actual dissolved oxygen concentration in the medium. Equation 3.1.1b assumes a steady state at which the rate of oxygen consumption (left-hand side) equals the rate of oxygen transfer (right-hand side). Rhiel et al. (1997) measured the SOUR of uninfected and infected Sf9 cells in Sf-900 ™ II SFM to be $2x10^{-17}$ mol O_2/cell/s and $2.5x10^{-17}$ mol O_2/cell/s, respectively. Below critical oxygen concentration for animal cells (1-10% of air saturation), there is a reduction in product yield as well as an increase in glucose consumption to compensate for impaired mitochondrial activity (Godoy-Silva et al., 2010). Conversely, elevated oxygen concentration (~100% of air saturation) may stimulate the formation of cytotoxic reactive oxygen species (ROS) that can impair cell growth

or even cause death (Godoy-Silva et al., 2010). To avoid these adverse conditions, pure oxygen will be sparged through all stirred-tank bioreactors to maintain dissolved oxygen in the cell culture at 50% of air saturation.

The process of determining the gas flow rate (Q_g) needed to provide a desired $k_L a$ for a given impeller type and speed (N) first starts with guessing a Q_g and then calculating the impeller ungassed power (P) consumption and the aeration number (N_a) using the following two equations, respectively:

$$P = N_P \rho N^3 D_I^5 \tag{3.1.1c}$$

$$N_a = \frac{Q_g}{N D_I^3} \tag{3.1.1d}$$

where ρ is the medium density (the density of water at 27 ℃ is 990.305 kg/m^3) and D_I is the impeller diameter.

The aeration number correlates to a specific gassing factor according to Figure 3.1.1. This ratio can then be multiplied by the calculated ungassed power consumption to determine the power (P_g) drawn by the impeller in the aerated system.

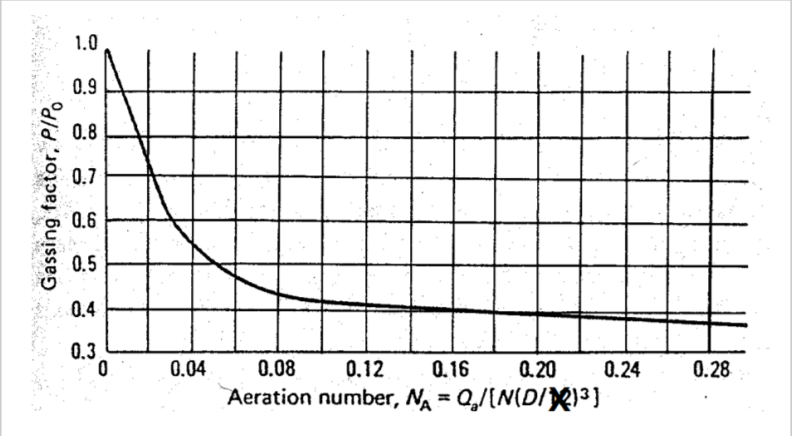


Figure 3.1.1. Power requirements for agitation in a gassed system (Blanch & Clark, 1997)

The empirical van't Riet (1979) correlation can then be applied to predict the oxygen mass transfer for the reactor conditions:

$$k_L a\ [s^{-1}] = 0.026(\frac{P_g}{V})^{0.4}(v_s)^{0.5} \quad (3.1.1e)$$

$$v_s = \frac{4Q_g}{\pi D_T^2} \quad (3.1.1f)$$

$$k_L a\ = 0.026(\frac{P_g}{V_W})^{0.4}(\frac{Q_g}{A_T})^{0.5}$$

P_g = impeller power input in presence of gas
V_W = bioreactor working volume
Q_g= volumetric gas flow rate
A_T = tank cross-sectional area

where V is the reactor working volume, v_s is the superficial flow velocity of gas through the reactor, and D_T is the reactor diameter. Q_g is adjusted until the predicted $k_L a$ matches the required $k_L a$.

The oxygen sparged into the bioreactors must be purified to reduce the possibility of contaminating the final product. The Thermo Scientific HyPerforma™ S.U.B.s have Meissner 0.2 μm hydrophobic Steridyne™ PVDF membrane filters and Pall Emflon™ II membrane filters in Kleenpak™ capsules built into the drilled-hole and overlay spargers, respectively, so no additional oxygen filtration will need to be implemented.

3.1.2. Insect Cell Growth Seed Train

Overview of Seed train

The purpose of the seed train is to generate a sufficient amount of insect cells to produce the spike protein of interest. Cells are grown in batches of increasing volume, allowing the engineers to control many growth factors and improve reproducibility (Hernándes Rodríguez et al., 2013). As cell numbers increase, larger containers will be used to house and provide oxygen to the cells. By conducting a seed train, we can also control the cell density, clear out cell debris,

and replenish the cells with fresh medium between stages, therefore optimizing cell growth (Hernándes Rodríguez et al., 2013. Our seed train is modeled after Flublok, a BEVS-based vaccine for influenza. Using that process as a model, we will scale up volume in roughly 10x increments: 40 mL, 500 mL, 22.4 L, 100 L, 2,000 L, 10,000 L (Buckland, 2014). Cells from the 22.4 L and 2,000 L will be split to the baculovirus amplification train where viruses will be grown in increments, similar to the insect cells.

Master Cell Stock

To start the seed train, we will defrost ten 4 mL vials of cells in a water bath set at 37 ℃. The master stock solution is prepared according to Protocol 5 of the Thermofisher BEVS Guide (Thermo Fisher, 2002). Methods for preparing the master stock are outside the scope of this project and we will assume that it is prepared in sufficient supply to run our upstream process for the entire year.

Table 3.1.2a. Specifications for master stock stage in seed train

Specifications	**Stage G0: Stock (10, 4 mL Flasks)**
Cell Density (cells/mL):	4.00E+06
Vessel Type:	4 mL Flasks
Vessel Size (L):	0.004
Vessel Quantity:	10
Total Volume (L):	0.04
Amount of Cells:	1.60E+08

The cell density of the master stock was based on the assumption that cryopreservation took place during the mid-exponential growth period, which yields a cell density in that range (Reuveny et al., 1993).

Shake Flasks

The first shake flask will be 500 mL in volume and the second will be 22.4 L in total volume. The first shake flask volume was chosen because it would be a roughly ten times increase in volume from the master stock solution. The 22.4 L in total volume will be split between five 3L flasks and one 10 L flask. This volume increase is higher than ten times because of the need to produce cells for baculovirus amplification.

Table 3.1.2b. Specifications for shake flasks in seed train

Specifications	**Stage G1: 500 mL Flask**	**Stage G2: 5 x 3 L Flasks + 7.4L for BV**
Starting Cell Density (cells/mL):	3.20E+05	1.81E+05
Target Cell Density for Next Stage (cells/mL):	8.10E+06	8.10E+06
Total Volume (L):	0.5	22.4
Initial Amount of Cells:	1.60E+08	4.05E+09
Final Amount of Cells:	4.05E+09	1.81E+11
Media Required (L):	0.46	21.91
Total Growth Time (days):	4.08	4.80
Volume to Seed Train (L):	0.5	15
Volume to BV Train (L):	-	7.4

The final cell density for the shake flasks was chosen because it was the maximum viable cell density recorded in a study of Sf9 cells with recombinant protein production in mind (Rhiel et al., 1997). The amount of cells allocated to the Baculovirus train depended on the total number of viruses needed in the production bioreactors and the factor that the baculovirus scales for each stage. More information can be found in the Baculovirus train section.

100 L Bioreactor

The 100 L Bioreactor is a scale up from the 15 L of cell solution that came before it. Similar to the previous stages, the targeted cell density is the maximum cell density recorded in

literature. The volume was chosen because it was roughly ten times the previous volume. Glucose will be added to the bioreactor in order to improve growth. All of the contents of the 100 L Bioreactor will move forward in the seed train.

Table 3.1.2c. Specifications for 100 L Bioreactor in seed train

Specifications	**Stage G3: 100 L Bioreactor**
Starting Cell Density (cells/mL):	1.22E+06
Target Cell Density for Next Stage (cells/mL):	8.10E+06
Total Volume (L):	100
Initial Amount of Cells:	1.22E+11
Final Amount of Cells:	8.10E+11
Media Required (L):	85.00
Glucose Required (kg):	0.11
Aeration (L/min):	2
Total Growth Time (days):	2.40

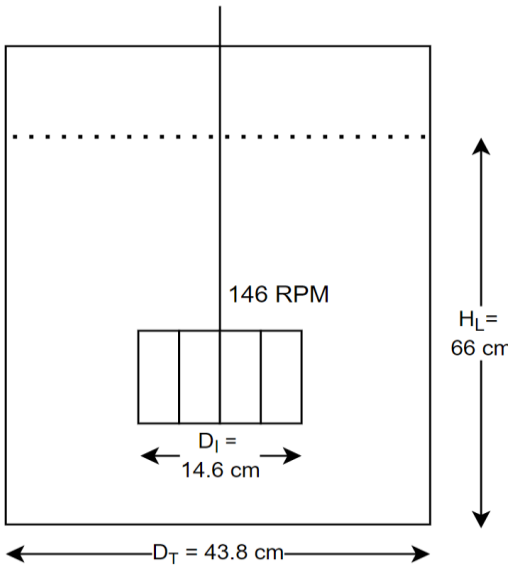


Figure 3.1.2a. Dimensions of 100 L Bioreactor in Seed Train

Table 3.1.2d. Aeration for 100 L Bioreactor in seed train

Impeller Characteristics:	
Impeller type	45° Pitched Blade (Quantity: 1; No. Blades: 3)
Power requirement (W)	2
Time to reach 95% homogeneity (s)	15 (<60 is desirable)
Aeration: To maintain DO at 50% of air saturation	
Aeration rate (L/min)	2
k_La (hr^{-1})	4.68
Glucose Consumption:	
Glucose added (kg)	0.11

2000 L Bioreactor

After the target cell density is reached, 100 L of material containing 8.10E+11 cells will be transferred to the reactor bag in the 2000 L Thermo Scientific Hyperforma single use bioreactor. In this stage, cells will be grown to provide for the next step in the seed train (1000 L) and for the baculovirus amplification train (740.7 L). Therefore, 1640.7 L of media will be added to meet this requirement, which yields a starting cell density of 4.65E+05 cells/mL. 1.09 kg of glucose and an aeration of 4 L/min is required. The reactor, having a working volume of 1740.7 L will operate at a 72.5 RPM impeller agitation speed which will require 36.25 W of power. It will take 3.61 days to reach a target cell density of 8.1E+06 cells/mL. After, 740.7 L containing 6.0E+12 cells will be transferred to the baculovirus amplification reactor. The rest of the material (1000 L) containing 8.1E+12 cells will be transferred to the five 2000 L protein production bioreactors.

Table 3.1.2e. Details for 2000 L Bioreactor in seed train

Specifications	**Stage G4: 2000 L Bioreactor (1000L Seed+ 740.7L for BV)**
Starting Cell Density (cells/mL):	4.65E+05
Target Cell Density for Next Stage (cells/mL):	8.10E+06
Total Volume (L):	1740.7
Initial Amount of Cells:	8.10E+11
Final Amount of Cells:	1.41E+13
Media Required (L):	1640.7
Glucose Required (kg):	1.09
Aeration Rate (L/min):	4
Total Growth Time (days):	3.61
Volume to Seed Train (L):	1000
Volume to BV Train (L):	740.7

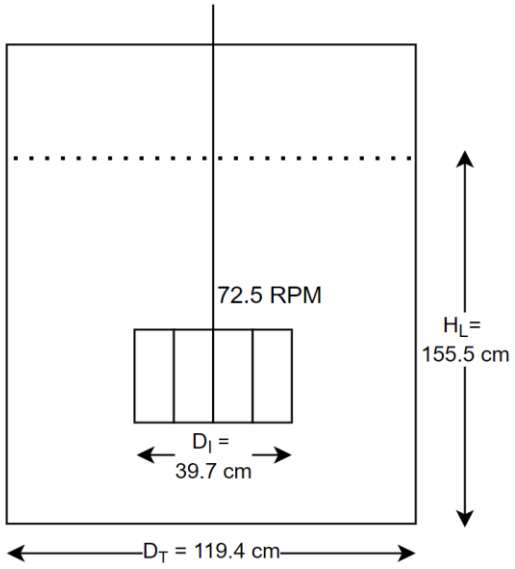


Figure 3.1.2b. Dimensions of 2000 L Bioreactor in Seed Train

Table 3.1.2f. Operating Conditions for 2000 L Bioreactor in seed train

Impeller Characteristics:	
Impeller type	45° Pitched Blade (Quantity: 1; No. Blades: 3)
Power requirement (W)	36
Time to reach 95% homogeneity (s)	31 (<60 is desirable)
Aeration: To maintain DO at 50% of air saturation	
Aeration rate (L/min)	4
k_La (hr^{-1})	4.68
Glucose Consumption:	
Glucose added (kg)	1.09

3.1.3. Baculovirus Amplification Train

In the first stage, 74 ml of baculovirus stock will be transferred into a 10 L shake flask. 7.4 L of cell culture from the cell seed train will be added to the shake flask at the maximum optimal cell density of 8.1E+6 cells/ml. The 7.4 L of cell culture will be infected at a multiplicity of infection (MOI) of 0.1 as this is the most optimal for virus amplification (Thermo Fisher, 2002). MOI describes the ratio between the number of virions and cells. An MOI of 0.1 means that for every 10 cells, there is 1 virion. The virus is then allowed to amplify for a period of 2 days. After 2 days, there is a 2 log increase in the amount of virions (Thermo Fisher, 2002). The initial amount of baculovirus stock needed was calculated using the following equation:

$$Inoculum\ required\ (mL) = \frac{Desired\ MOI\ (pfu/cell) \times (total\ number\ of\ cells)}{Titer\ of\ viral\ inoculum\ (pfu/mL)} \qquad (3.1.3a)$$

where the desired MOI is 0.1. A titer of the viral inoculum was suggested between 1E+7 and 1E+8 (Thermo Fisher, 2002). At the beginning of this stage, there will be 6.0E+9 virions. After

the 2 day amplification period, there will be 6.0E+11 virions. To have a virus stock of 6.0E+9, a titer of 8.1E+07 pfu/ml will be used.

Table 3.1.3a. Details for Baculovirus amplification shake flask

Specifications	**BV Stage 1: 10 L Shake Flask**
Starting Cell Density (cells/mL):	8.10E+06
MOI	0.1
Virus Titer (pfu/mL)	8.10E+07
Total Volume (L):	7.41
Inoculum Required (L):	7.41E-02
Initial Virion Number:	6.00E+9
Final Virion Number:	6.00E+11
Total Growth Time (days):	2

After the 2 day amplification period, the contents of the 10 L shake flask are transferred to the 1000 L Baculovirus amplification bioreactor. Additionally, 740.7 L from the seed train containing 6.0E+12 cells will be transferred to this 1000 L Thermo Scientific Hyperforma single-use bioreactor. The cell density will be at the maximum optimal cell density of 8.1E+6 cells/ml. An MOI of 0.1 will once again be used to infect the cells as this was suggested from literature as an optimal MOI for virus amplification. The virus will then amplify for a period of 2 days. After this amplification period, the amount of virions present will be 6.0E+13, which is enough to infect our production bioreactors at an MOI of 2. The reactor requires an aeration rate of 3 L/min. This aeration rate is necessary to meet the oxygen requirements of the cells when they are amplifying the virus. A kLa of 1.73 is optimal to maintain 50% dissolved oxygen in the bioreactor. It will operate at a 78.5 RPM impeller agitation speed which will require 15.9 W of power. After the 2 day amplification period, the contents of the 1000L Baculovirus bioreactor

will remain in the bioreactor for a holdtime of 4 days until the cells in the production bioreactors are ready for infection. The virions can remain stable and infectious for days at elevated temperatures, weeks at room temperature, and months at 4°C (Thermo Fisher, 2002). For this process, the bioreactor will be kept at room temperature because it requires no additional energy for cooling, yet remains stable and infectious.

Table 3.1.3b. Details for Baculovirus amplification bioreactors

Specifications	**BV Stage 2: 1000 L Bioreactor**
Starting Cell Density (cells/mL):	8.10E+06
MOI	0.1
Virus Titer (pfu/mL)	8.10E+07
Total Volume (L):	740.74
Inoculum Required (L):	7.41
Initial Virion Number:	6.00E+11
Final Virion Number:	6.00E+13
Aeration Rate (L/min):	3
Total Growth Time (days):	2

3.1.4. Antigen Production

Five 2000 L production single-use bioreactors are used to collectively express 300 g of spike protein over 72 hours by infecting $3x10^{13}$ Sf9 cells at an MOI of 2.0 (i.e., $6x10^{13}$ baculovirus). The geometric properties and operating conditions of the 2000 L bioreactors were obtained in the same fashion as the smaller bioreactors, and these characteristics are summarized in Figure 3.1.4a and Table 3.1.4a, respectively. The dissolved oxygen in the culture is controlled at 50% air saturation by supplying pure oxygen at 4 LPM through a drilled-hole sparger. This flow rate is sufficient to meet oxygen transfer requirements and to strip carbon dioxide for pH control and decreased foaming.

Table 3.1.4a. 2000 L bioreactor performance

Impeller Characteristics:	
Impeller type	45° Pitched Blade (Quantity: 1; No. Blades: 3)
Power requirement (W)	40
Time to reach 95% homogeneity (s)	30 (<60 is desirable)
Aeration: To maintain DO at 50% of air saturation	
Aeration rate (L/min)	4
k_La (hr^{-1})	2.2
Limiting Substrate (Glucose) Consumption:	
Glucose added (kg)	3.5 (growth) + 4.8 (infection)
Recombinant Spike Protein Production:	
Spike protein produced by one 2000 L S.U.B.s (g)	60

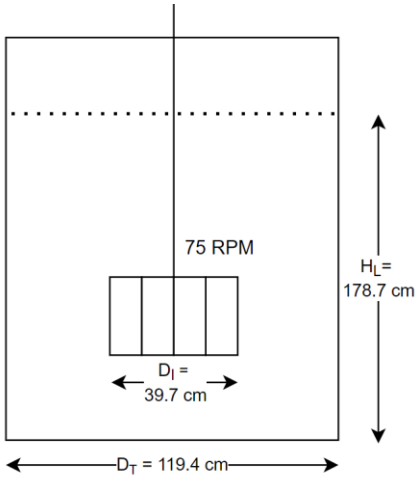


Figure 3.1.4a. Thermo Scientific™ HyPerforma™ 5:1 2000 L S.U.B. Dimensions

Only $8.1x10^{12}$ Sf9 cells are grown in stage 4, so an initial growth phase is necessary to reach the cell density appropriate for infection. While Sf9 cells are cultivated to maximum cell densities in the previous growth stages, studies indicate that infecting at maximal cell density

leads to low product yields. Maiorella et al. (1988) observed a 60% reduction in recombinant protein production when viral infection of Sf9 cells occurred during the stationary phase as compared to infection in the middle exponential phase. Reuveny et al. (1993) specifically recommend infecting during the early to middle exponential growth phase, when the cell density ranges between 1 to $3x10^6$ cells/mL, which agrees with the protocol outlined in Thermo Scientific's (2002) insect cell culture manual. We will infect at $3x10^6$ cells/mL to maximize the number of Sf9 cells available for infection, and thus recombinant spike protein expression, before the medium nutrients are exhausted and begin limiting further cell growth. To reach this target cell density, each 2000 L production bioreactor will be loaded with 200 L of cell culture from stage 4 of the seed train along with 1652 L of Sf-900™ II SFM. Based on the specific glucose uptake rate reported by Rhiel et al. (1997), the culture will also be supplemented with 3.5 kg of glucose. The resulting 1852 L cell culture will be grown in batch mode for 1.56 days to achieve a final cell density of $3x10^6$ cells/mL prior to viral infection. The conditions during the Sf9 growth phase are highlighted in Table 3.1.4b.

Table 3.1.4b. Sf9 growth conditions in 2000 L bioreactors

Specifications	2000 L Production Single-Use Bioreactors
Mode	Batch
Vessel Type:	2000 L S.U.B.
Vessel Quantity:	5
Starting Cell Density (cells/mL):	8.75E+05
Target Cell Density for Infection (cells/mL):	3.00E+06
Single STR Working Volume During Cell Growth (L):	1852
Initial Amount of Cells:	8.10E+12
Final Amount of Cells:	3.00E+13
Total Media Added (L)	8260

The fraction of insect cells infected by a specific number (n) of baculovirus particles at a given MOI can be predicted by the Poisson distribution:

$$F(n, MOI) = \frac{(MOI)^n e^{-MOI}}{n!} \quad (3.1.4a)$$

Therefore, the fraction of baculovirus-infected cells can be determined by subtracting the fraction of uninfected cells (i.e., $n = 0$) from 1. As MOI decreases, post-infection cell growth increases along with the dispersion of virally induced cell lysis. Increased levels of cell lysis at low MOIs (< 1 pfu/cell) have been shown to prolong the duration of product exposure to cell debris and host cell and virus-induced intracellular proteins, including proteases (Radford et al., 1997). Contaminating proteins can interfere with target product stability and downstream

purification, even if the product is not secreted like the spike protein; therefore, an MOI above unity is ideal for recombinant protein production (Radford et al., 1997). A high MOI will also allow protein expression to occur sooner, reducing production time.

Table 3.1.4c. Sf9 infection and protein production conditions in 2000 L bioreactors

Specifications	2000 L Production Single-Use Bioreactors
Cell Density at TOI (cells/mL):	3.00E+06
Total Viral Inoculum Added (L):	740
MOI:	2
Virus Titer (pfu/mL):	8.10E+07
Single STR Working Volume During Protein Production (L):	2000

In this process, viral infection at an MOI of 2 will result in approximately 86.5% of the cells being successfully infected during the initial round of virus replication. Subsequent cell growth in the culture will proceed until secondary absorption and infection of the previously non-infected cell population. To support this additional cell growth following infection and to provide a nutrient environment conducive to protein synthesis, Sf9 cells will be harvested from each bioreactor prior to infection during the middle exponential growth phase at a cell concentration of $3x10^6$ cells/mL. After 36 hours of viral infection and protein expression, 2.4 kg of glucose will be added to each 2000 L culture to replenish the carbon source that is depleted during the first half of production. Rhiel et al. (1997) observed maximal product concentration between 50 and 100 hours post-infection. We will operate intermediate of these extremes at 3 days (72 hours) of infection in order to maximize product yield while avoiding overlap with the expression of late viral apoptotic proteins that lead to cell lysis (Yee et al., 2018). At the

completion of the production process, each reactor will contain 30 mg of intracellular spike protein per L of culture, representing a cumulative batch yield of 300 g of spike protein (Oosten et al., 2021). The 10,000 L cell culture will be transferred to a centrifuge to commence the downstream recovery and purification processes. The conditions during Sf9 infection and production are summarized in Table 3.1.4c.

3.2. Downstream

3.2.1. Centrifugation

Centrifugation is a necessary process as our spike protein antigen product is intracellular. Therefore, after spike protein production, our cells will be disrupted to collect the spike protein. Centrifugation is the first step in that process. Centrifugation is based on sedimentation (Carta, 2021b). By applying a gravitational force to our cell broth, the cells will separate from the supernatant based on density at a certain velocity. This can be modeled by the following equation:

$$v_g = \frac{4r_p^2(\rho_p - \rho_f)g}{18\eta} \quad (3.2.1a)$$

where v_g is the sedimentation velocity of our cells, r_p is the radius of our cells, ρ_p is the density of our cells, ρ_f is the density of the supernatant, g is the gravitational constant, and η is the viscosity of the supernatant (Carta, 2021b). As demonstrated by the equation, a larger difference between the densities of the particles and the fluid will result in more quick and effective centrifugation. There are several different types of centrifuges including tubular bowls, disk stacks, scrolls, and basket centrifugal filters. Tubular bowl centrifuges are incapable of discharging solids and have a limited capacity, which is insufficient for our 10,000 L batches (Carta, 2021b). While scroll centrifuges are capable of continuously discharging centrifuges, they result in high shear forces and turbulence, which is problematic for our shear sensitive cells. Basket centrifugal filters are able to handle a large capacity, however, it is difficult to recover the solid cell pellet from them (Carta, 2021b). Ultimately, we chose a disk stack centrifuge for our process. Disk stack centrifuges are capable of continuously discharging solids once a certain solid depth or density has been reached. Disk stack centrifuges are also capable of handling large volumes. A disk stack centrifuge contains a stack with a number of disks as demonstrated in

Figure 3.2.1. These disks increase the amount of surface area available allowing for quick sedimentation (Alfa Laval, 2021a). The centrifugal force applied pushes the settled particles on the disks outwards where they can then be collected or ejected (Alfa Laval, 2021a). Disk stack centrifuges can be modeled by the following equation:

$$Q = \frac{v_g 2\pi(n-1)\omega^2}{3g} cot(\theta)(R_o^3 - R_i^3) \quad (3.2.1b)$$

where Q is the feed flow rate entering the disk stack centrifuge, n is the number of disks, θ is the disk angle, R_o is the distance of the outer disk from the center of rotation, and R_i is the distance of the inner disk from the center of rotation (Carta, 2021b). For purchase, our site will be using the BTPX 305 disk stack centrifuge from Alfa Laval. This centrifuge is capable of processing volumes of 2000 L per hour and can automatically discharge the cell pellet to a mixing bag (Alfa Laval, 2021b). With this disk stack centrifuge, the centrifugation process will take 5 hours. This process will be assumed to be 100% effective. 18.3 kg of cell pellet will be discharged for cell lysis (Chiou et al., 2000).

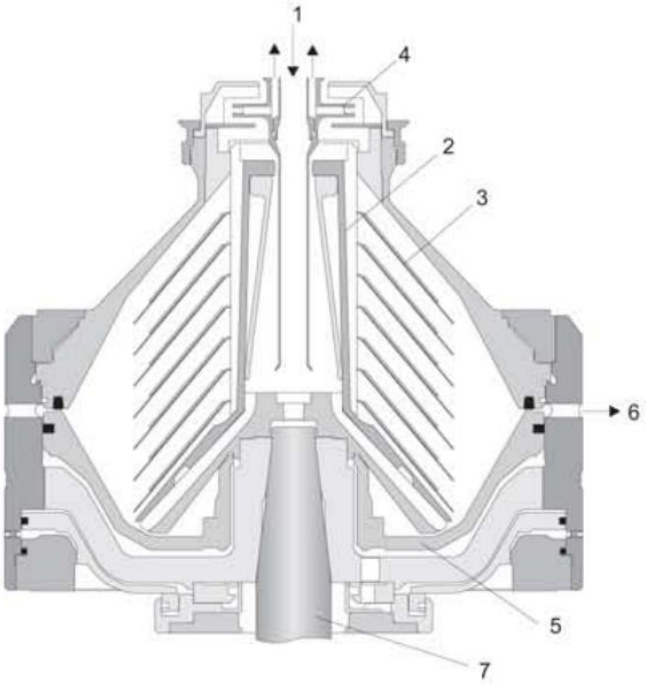


Figure 3.2.1 Disk Stack Centrifuge (Alfa Laval, 2021b)

3.2.2. Cell Lysis

Cell lysis is necessary because the spike protein is produced on the surface of the cells and stays within the cell pellet after centrifugation (Hahn TJ et al., 2015; Buckland B et al., 2014). Therefore, we need to break apart the cell membranes to extract the desired spike protein. We will use high pressure homogenization because it is highly effective for lysing cells at the production scale and requires no additional detergents (Shehadul Islam Mohammed et al., 2017).

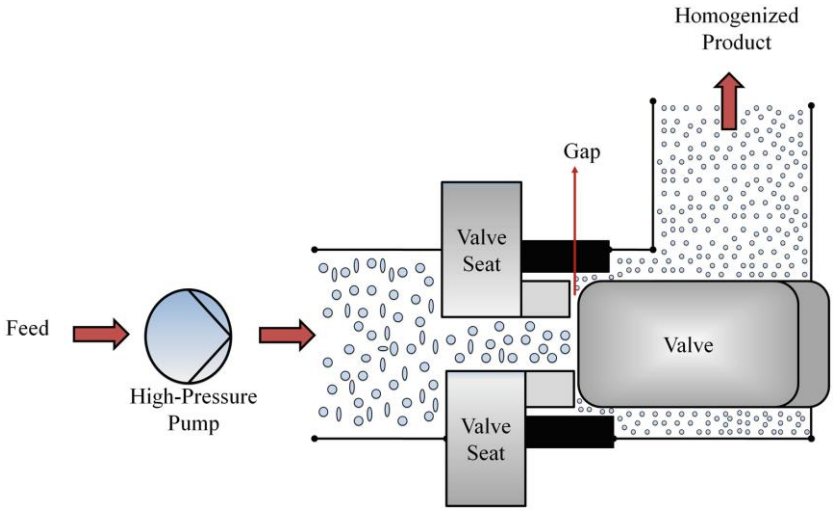


Figure 3.2.2a. Schematic of high pressure homogenizer

High pressure homogenization is an effective tool for cell lysis in the pharmaceutical industry. The process works by subjecting the cell solution to high pressure, followed by flowing through a narrow gap where pressure drops quickly. This sudden pressure change creates shear force that lyses the cells' membranes. Factors that affect homogenization performance include pressure, temperature, and number of passes. Finding the optimal pressure to operate the homogenization is important because higher pressure does not equate to better cell lysis (Biopharma group, 2018). Instead, high pressure increases the temperature of the cell solution, which may denature proteins. Therefore, it is important to operate homogenization at colder temperatures using heat exchangers. If the target protein is sensitive to temperature and pressure, then increasing the number of passes through the homogenizer can improve cell lysis while operating at lower pressures. Homogenization can be manipulated by changing its operating variables to meet the user's needs.

Before homogenization, we will resuspend the cell pellet in 1830 liters of WFI and various protease inhibitors using the Thermo Scientific Hyperforma Single-Use 2000 L Jacketed Mixer. The resuspension volume was calculated by using the optimal biomass density of 10g/L specified by a study and equation by the following equation (Tam et al., 2012):

$$Resuspension\ volume = \frac{mass\ of\ cell\ pellet}{biomass\ density} \quad (3.2.2a)$$

Our homogenization process will operate at conditions specified by a purification study on Sf9 cells (Date SS et al., 2017). We will operate at 4,000 psi and 4℃, and use a one-pass configuration. We assume that this pass of homogenization will result in a 100% lysis rate and 100% protein recovery. Before homogenization, we will put in protease inhibitors to prevent the spike protein from being degraded by intracellular enzymes. Concentrations of these protease inhibitors include: 10 μM chymostatin, 10 μM leupeptin, 1 μM pepstatin A and 0.2 mM phenylmethylsulfonyl fluoride.

We will use the EmulsiFlex-C1000 homogenizer manufactured by Avestin due to its high flow rate capacity of 1000 L/hr. At this flow rate, it will take 1.83 hours to process 1830 liters of resuspended cell solution.

3.2.3. Depth Filtration

Depth filtration is a common method to remove large cell debris from vaccines and cell culture. These filters predominately capture solids through size-exclusion but can also retain particles through surface adsorption depending on the properties of the selected filter material. An advantage of depth filters is that, like a sponge, they can trap large amounts of contaminants within themselves due to their high surface area and porous inner structure. In the downstream process, an initial clarification step is necessary because large particles can foul ultrafiltration and diafiltration membranes and block chromatography resins used later in the process. The spike protein released in the preceding lysis step will be smaller than the pores of the depth filter, so it will flow through the filter media at high yields while being separated from large cell debris as well as some large bacteria and viruses, as illustrated in Figure 3.2.3a. Before depth filtration, the inlet stream consists of an estimated 300g of spike protein. At the end of depth filtration, the

outlet stream consists of the desired spike protein along with host cell components (e.g., proteins, lipids, nucleic acids), target protein variants (e.g., truncated, oxidized, misfolded, glycovariants, aggregates), and adventitious agents that have been unintentionally introduced into the manufacturing process (e.g., bacteria, fungi, viruses, mycoplasma).

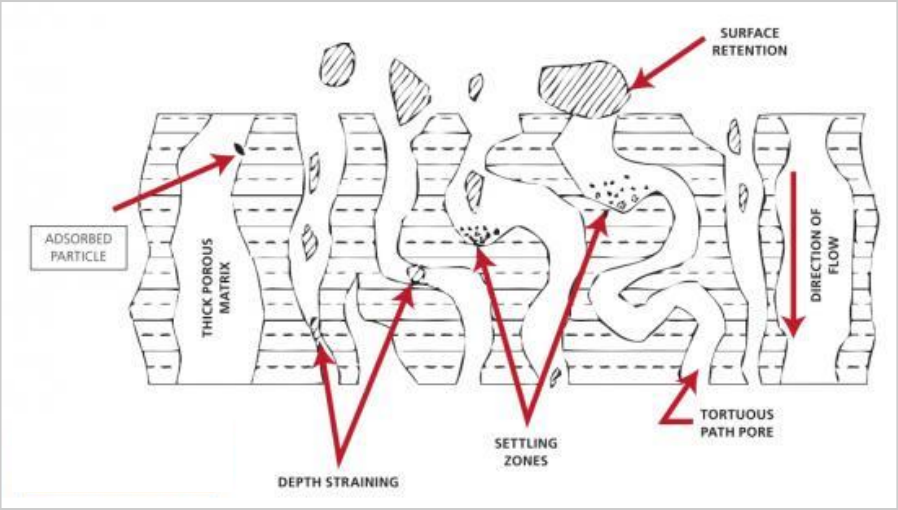


Figure 3.2.3a. Visualization of depth filter inner membrane

For our process, we will use Millipore Millistak+® CE50 Pod Depth Filters. These filters consist of a fibrous bed of cellulose. The CE50 series has pore sizes between 0.6-1 microns wide, which are large enough for the spike protein to pass through, but small enough to exclude most cell debris. For our filter setup, we will use Millipore's 3-rack which can hold up to thirty depth filter pods at once, expanding the surface area for filtration.

The steps of depth filtration include washing, filtering, and flushing. During washing, water for injection (WFI) is pumped through the filter to remove loose particles that come from the filter manufacturing process. According to the Millipore buying guide, washing is run at a flux of 600 LMH and at a pressure drop of 30 psi (Millipore Sigma, 2021). Also, Millipore recommends using 50L of WFI per m^2 of filter surface area (Millipore Sigma, 2022). To maximize surface area and reduce filtration time, a full capacity consisting of thirty filters will be

used. Since we will be using thirty filters each with 1.4 m^2 of surface area, we will need 2100 L of WFI to pump through the depth filter. These operating conditions will be the same for the flushing stage. The purpose of the flushing stage is to recover any spike protein remaining in the filter after loading the cell solution through the filtration stage.

The depth filtration process will take 23.81 minutes and will require a flow rate of 420 L/min (25,200 L/h). The flow rate is determined by multiplying the flux by the filter surface area. The spike protein recovery is expected to be 90%, resulting in 270g of spike protein leaving the process. (BioProcess International, 2014). The outlet stream is believed to contain many small protein impurities because the main mechanism behind depth filtration is size-exclusion, which fails to filter out compounds smaller than .6 microns wide based on the filter we chose.

3.2.4. Ultrafiltration and Diafiltration for Anion Exchange Chromatography

Ultrafiltration and diafiltration are two types of tangential flow filtration (TFF). In TFF, pressure is applied tangentially to a feed stream against a membrane. Each membrane is defined by its molecular weight cutoff limit (MWCO). If a particle's molecular weight is less than the MWCO, it will pass through the membrane pores into the permeate stream. If a component's weight is greater than the MWCO, then it will remain in the retentate stream as it cannot penetrate through the membrane. Benefits of TFF include the ability to recirculate the feed contents (unlike in dead-end filtration) and the prevention of material accumulation on the membrane surface. The purpose of ultrafiltration is to concentrate a target molecule in solution while the objective of diafiltration is to exchange a buffer.

The first step in choosing a membrane for ultrafiltration and diafiltration is to determine the molecular weight of the target protein. In this case, the spike protein has an estimated molecular weight of 141 kDa (Scheller et al., 2020). To retain as much protein as possible, a

MWCO between 3-6 times smaller than the MW of the target protein is recommended (Pall, 2022). Thus, a MWCO of 30 kDa was chosen for our membrane. A hollow fiber membrane was selected over other designs because of its high membrane area to volume ratio and its resistance to shear stress (Carta, 2021; Besnard et al., 2016). In this ultrafiltration step, a protein solution volume of 3,930 L is being fed from depth filtration at a spike protein concentration of 0.0687 mg/mL. It is necessary to reduce the volume for the following anion exchange chromatography step because ion exchange chromatography performs optimally between 0.5- 5 mg/mL (O'Shaughnessy et al., 2010). A concentration factor of 20 was chosen and Cytiva's Uniflux 400 cross flow filtration system was chosen because of its capability to process large feeds (about 10,000 L) (Cytiva, 2022). Membrane area and flux determine the time needed to process a feed volume. The relationship is displayed by the following equation:

$$t = \frac{V_0 - V}{A * u_{p,avg}} \tag{3.2.4a}$$

where t is time, V_0 is the initial feed volume, V is the final volume, A is the membrane area, and $u_{p,avg}$ is the average flux in units of LMH (liters/hour/m^2). An average flux of 30 LMH was recommended from cytiva for their membranes when used for protein concentration and buffer exchange (Cytiva, 2020). Since a concentration factor of 20 was chosen, the final volume was 196.5 L. The Uniflux 400 system is capable of having a total membrane area of 52 m^2 if equipped with 4 UFP-30-C-85 hollow fiber cartridges, each with an area of 13 m^2. There will be a cross-flow pressure of 8 psi along the membrane (Cytiva, 2020). Using eq. (3.2.4a), a process time of 2.39 hours was calculated. The protein solution was concentrated from 0.0687 mg/mL to 1.37 mg/mL. The protein yield was calculated using the following equation:

$$Yield = (CF)^{\sigma-1} \tag{3.2.4b}$$

where *CF* is the concentration factor (20) and σ is the protein rejection coefficient. The rejection coefficient can be approximated as 1 (0.999) since the molecular weight of our protein is significantly greater than the MWCO of our chosen membrane. The protein recovery yield was calculated to be 99.7%.

The purpose of diafiltration is to exchange the buffer to prepare for anion exchange chromatography in which the pH must be above the isoelectric point of our spike protein. This can be accomplished with continuous diafiltration, in which the diafiltration buffer is added to the feed reservoir at the same rate as the permeate is exiting the membrane. This method maintains a constant volume. Continuous diafiltration was chosen because it is a more efficient process than other diafiltration methods in that less diafiltration volume is needed to replace the previous buffer (Pall, 2022). Diafiltration was modeled using the following equations:

$$\frac{C}{C_0} = e^{-\frac{V_D}{V_0}(1-\sigma)} \tag{3.2.4c}$$

$$t = \frac{V_D}{A*u_{p,0}} \tag{3.2.4d}$$

$$Q_D = \frac{V_D}{t} \tag{3.2.4e}$$

where *C* is the final protein concentration, C_0 is the initial protein concentration, V_D is the diafiltration volume, V_0 is the initial volume, σ is the protein rejection coefficient, *A* is the membrane area, $u_{p,0}$ is the constant permeate flux, and Q_D is the diafiltration buffer feed volumetric flow rate. Equation (3.2.4c) is used to calculate the ending spike protein concentration, (3.2.4d) the process time, and (3.2.4e) the diafiltration buffer flow rate. The following equation is used to calculate the diafiltration volume.

$$(\frac{C}{C_0})_{salt} = 0.001 = e^{-\frac{V_D}{V_0}} \tag{3.2.4f}$$

where $(C/C_0)_{salt}$ is the percentage of low-molecular weight solutes (e.g., salt) we want remaining in the buffer at the end of the diafiltration process. Equation (3.2.4f) assumes all of the salt passes through the membrane (i.e., $\sigma_{salt} = 0$). For our design, we want to remove 99.9% of the previous buffer and replace it with Tris-HCl. Applying eq. (3.2.4f), the diafiltration volume is determined to be 1357.37 L. The time for diafiltration is calculated to be 0.87 hours with a protein recovery of 99.3%. 20 mM of Tris-HCl was added to the 1357.37 liters of WFI for a mass of 4.278 kgs. The same Uniflux 400 system used for ultrafiltration will be used for this diafiltration step. After diafiltration, 196.5 L of protein solution at a concentration of 1.36 mg/mL will be transferred to anion exchange chromatography. For both diafiltration and ultrafiltration, a transmembrane pressure of 16 psid will be used as recommended by Cytiva.

3.2.5. Anion Exchange Chromatography

Following buffer exchange in diafiltration, the retenate is sent to a capture chromatography process in order to concentrate and isolate the spike protein. The majority of published processes for the purification of recombinant spike proteins employ selective affinity resins, predominantly in the form of immobilized metal affinity chromatography (IMAC). Recombinant proteins can be tagged with a peptide sequence that selectively binds to specific chemical or biological ligands. For example, poly-histidine (His) is a common protein tag that forms complexes with metal ions such as nickel (Ni) or zinc (Zi). By immobilizing these metal ions onto a chromatographic medium by chelation, His-tagged proteins can bind to the medium and be eluted with a pH change or addition of a competitive molecule. Although these methods yield a highly pure product (purity up to 95% with a recovery of 90% can be achieved in a single IMAC step [Bornhorst & Falke, 2000]) and require minimal optimization, they are difficult to scale to large manufacturing processes. Since heavy metals can leach from the column during

purification, testing for these metals would increase the costs of validation. Additionally, the removal of the tag from the target protein requires expensive proteases and additional purification steps, which would further increase downstream processing costs.

To address the limitations of affinity-based separation methods, ion exchange (IEX) chromatography is being implemented as an initial step for capturing the spike protein. This mode of chromatography binds the spike protein via electrostatic interactions with an oppositely charged stationary phase. Due to their unique content of acidic and basic amino acids, proteins have different net surface charges and these charges vary with pH (Carta and Jungbauer, 2010). At a pH equal to a protein's isoelectric point (pI), the protein will carry no net charge. If the pH is above the pI, then the protein will have a net negative charge; if the pH is below the protein's pI, then the protein will acquire a net positive charge. The pI of the spike protein is estimated to be 6.24 on the basis of its polypeptide sequence (Scheller et al., 2020). In this chromatography process, the column will be loaded with 267 g of spike protein in 196.5 L of 20 mM Tris-HCl buffer at pH 7.4. Since this pH is above the pI of the spike protein (6.24), the protein will bear a negative charge and a positively charged anion exchange (AEX) resin will be packed into the column to adsorb the target protein.

The Thermo Scientific™ POROS™ XQ Strong AEX Resin - a 50-µm, rigid, polymeric resin - was selected for this chromatography step due to its high dynamic binding capacity (140 mg/mL at 5% breakthrough) and its robust pH and salt tolerance (Thermo Scientific, n.d.-b). The resin is assumed to bind all species that are negatively charged at the pH of the feed solution; therefore, some impurities - namely, host cell proteins (HCPs), lipopolysaccharides (e.g., endotoxin) from Gram-negative bacterial contaminants, and nucleic acids (e.g., host cell DNA) - will be retained along with the target product. Some of the neutrally and positively charged

impurities will flow through the column without binding during the loading stage, but any remaining small molecules and unbound proteins trapped in the extra-particle space will be removed in a pre-elution washing step. Although elution of reversibly bound molecules is commonly achieved by gradually increasing the ionic strength of the buffer via a salt concentration gradient (salt ions compete with protein molecules for binding sites on the charged ligands), a low pH (3.5) elution buffer will be used instead to modify the net charge of the bound spike protein and to ensure the eluate can be directly fed to the subsequent viral inactivation step without an intermediate buffer exchange. Although many adsorbed contaminants will be released along with the spike protein in this low pH elution condition, a downstream cation exchange chromatography process will be implemented to separate the spike protein from most of these impurities.

Column geometry and mobile phase flow properties

Based on the selected resin's dynamic binding capacity (*DBC*), the volume of resin (V_c) required to capture 267 g of spike protein is 1.91 L. By assuming the column can be approximated as a randomly packed bed of spherical particles, the extra-particle porosity (ε) is taken to be a typical value of 0.35 (Carta and Jungbauer, 2010). The resin manufacturer recommends a residence time (L/u) of at least 3 minutes. No studies could be found that investigated the effect of residence time on the productivity of AEX capture of the spike protein, so the residence time was set to 3.5 minutes to ensure adequate time for protein binding without a significant drop in productivity. According to the resin manufacturer, the pressure drop (ΔP) across the column should not exceed 3 bar, so the column was designed to operate below this threshold at a pressure drop of 2.5 bar (Thermo Scientific, POROS AEX). This low pressure drop will allow a shorter column to be used with a high flow rate, which is common for

industrial applications of capture chromatography processes (Carta & Jungbauer, 2010). The pressure generated when liquid flows through a packed bed of incompressible particles is influenced by factors such as linear flow velocity (u), liquid viscosity (η), length of the packed bed (L), and particle size (d_p) and extra-particle porosity (ε) of the chromatography resin. This relationship is described by the Carman-Kozeny equation as follows:

$$\Delta P = \frac{150(1-\varepsilon)^2}{d_p^2 \varepsilon^3} \times \eta L u \tag{3.2.5a}$$

Therefore, by constraining the pressure drop across the column, eq. (3.2.5a) can be rearranged to solve for the product Lu. In solving for this product, the viscosity of the Tris-HCl binding buffer was assumed to be 1.003 cP based on prior research (Chairatana et al., 2016). Using the product from eq. (3.2.5a) and the desired residence time, the column length and linear velocity were determined to be 29.8 cm and 510.1 cm/hr, respectively. The column diameter (d_c) was then calculated by rearranging the equation for the volume of a cylinder.

$$d_c = \sqrt{\frac{4V_c}{\pi L}} \tag{3.2.5b}$$

The resulting diameter is 9 cm. Multiplying the mobile phase linear velocity and the column cross-sectional area (S) yields a flow rate (Q) of 32.7 L/hr.

$$Q = u \times S \tag{3.2.5c}$$

Key geometric features and operating conditions of the AEX column are summarized in Table 3.2.5a.

Table 3.2.5a. AEX Column Specifications

Column Length, L (cm)	29.8
Column Diameter, d_c (cm)	9
Column Volume, V_c (L)	1.91
Flow Rate, Q (L/hr)	32.7
Linear Velocity, u (cm/hr)	510.1
Maximum Pressure Drop, ΔP_{max} (bar)	2.5
Operating Temperature, T (°C)	25
Cycle Time, t_{cycle} (hr)	7.2
Spike Protein Yield (%)	80

Cycle time and protein recovery

The total AEX process consists of five steps: equilibration, loading, washing, elution, and clean-in-place (CIP). All of these steps are assumed to operate at the same linear flow velocity of 510.1 cm/hr. With the exception of the loading stage, the time required to perform the i^{th} step of the chromatography process is described by the product of the buffer volume - expressed as a number of column volumes (CV) - and the residence time.

$$t_i = (CV)_i \times \frac{L}{u} \tag{3.2.5d}$$

Prior to loading the feed solution, the column will be equilibrated over 17.5 minutes with 5 CV of binding buffer (20 mM Tris-HCl, pH 7.4) to ensure the spike protein effectively interacts with the charged resin. The protein solution will be applied to the column in the binding buffer with contaminants flowing through. The load time (t_{load}) is calculated as follows:

$$t_{load} = \frac{DBC_{10\%}}{C_F}\frac{L}{u} \quad (3.2.5e)$$

where C_F is the concentration of the spike protein in the feed solution. Assuming the DBC at 10% breakthrough can be approximated by the resin manufacturer's published DBC of Bovine Serum Albumin at 5% breakthrough, the loading process will take 6 hours. Over 17.5 minutes, unbound and weakly retained contaminants will be washed from the column with 5 CV of the binding buffer. The elution process will take 17.5 minutes, over which 5 CV of 20 mM sodium acetate (NaOAc) at a pH of 3.5 will flow through the column. The 9.55 L eluate containing the spike protein and remaining contaminants will be sent directly to viral inactivation. Based on a study of recombinant spike protein purification using POROS™ AEX resins, the yield and purity of the spike protein were assumed to be 80% and 90%, respectively (Cibelli et al., 2021). Finally, the column will undergo a 21-minute CIP procedure using 3 CV of 1 mM NaCl followed by 3 CV of 1 mM NaOH. The total cycle time is 7 hours and 14 minutes. The sequence and duration of the steps performed in this AEX capture process are summarized in Table 2 along with the contents and quantities of the buffers that will be used.

Table 3.2.5b. AEX Chromatography Process

Phase	Buffer	Column Volume (CV)	Time (min)
1: Equilibrate	20 mM Tris-HCl pH 7.4	5	17.5
2: Load	20 mM Tris-HCl pH 7.4	196.5 L	360.16
3: Wash	20 mM Tris-HCl pH 7.4	5	17.5
4: Elute	20 mM NaOAc pH 3.5	5	17.5
5: CIP	1 mM NaCl → 1 mM NaOH	3 NaCl → 3 NaOH	21.0

3.2.6. Viral Inactivation

Viral inactivation is performed to denature the baculovirus and any other viral contaminants while keeping the spike protein intact. This step is crucial to produce a safe product. Additionally, this step allows for a lower containment level, from biosafety level 3 down to level 2, to be used in further downstream processes (Patterson et al., 2020). There are several methods to inactivate the virus: heat, ultraviolet radiation, detergents, reagents, and pH change.

For this process, a low pH will be used for viral inactivation. The target spike protein is more resistant to low pHs and high temperatures than viral impurities (Olia et al., 2021). For enveloped viruses, a target low pH range is 3.5-4 (Mettler-Toledo International, 2021). This is commonly done by adding an acid to lower the pH, starting a time based hold, and then adding a base to raise the pH to a more stable neutral pH range suitable for further processing. A short low pH hold time could be insufficient for complete viral inactivation, and overexposure to the low pH may denature the target protein (Mettler-Toledo International, 2021). A 60 minute hold time is expected to sufficiently inactivate the virus without denaturing the spike protein (Liang et al., 2021). Additionally, heat can denature the structure of the protein, so inactivation will occur at a temperature of 25℃.

Viral inactivation takes place directly after AEX chromatography, which will elute at a pH of 3.5. This is a suitable pH for inactivation, so further acid will not need to be added to adjust the pH. Viral inactivation will occur at a pH of 3.5 in the Thermo Scientific HyPerforma Single-Use 50L Jacketed Mixer. To effectively inactivate the virus, the mixer will be maintained at 25℃ for 60 minutes. The mixer will operate at a 141 RPM impeller agitation speed which will

require 1.80 W of power. (Thermo Scientific, n.d.-a). After inactivation is complete, the material exits at a pH of 3.5. It is then fed to cation exchange chromatography, where the material will be adjusted to a neutral pH range when it elutes out of the column.

3.2.7. Cation Exchange Chromatography

Following viral inactivation, an intermediate chromatography step will be performed to remove the negatively charged impurities that were eluted along with the spike protein in the upstream AEX process. Since the protein solution is at an acidic pH condition below the spike protein's pI, the spike protein has a net positive charge and will bind to a negatively charged cation exchange (CEX) resin. Thermo Scientific™ offers a 50-µm POROS™ XS CEX resin with a high DBC (100 mg/mL at 5% breakthrough) over a wide range of pH, conductivity, and flow conditions (Thermo Scientific, n.d.-c). The resin manufacturer recommends identical pressure drop (2.5 bar) and residence time (3.5 minutes) conditions as used for the AEX process, so the CEX column has similar geometry and operating conditions as the column designed for AEX capture. The same equations could be used to design the CEX column. The CEX column specifications are outlined in Table 3.2.7a.

Table 3.2.7a. CEX Column Specifications

Column Length, L (cm)	31.3
Column Diameter, d_c (cm)	9.3
Column Volume, V_c (L)	2.14
Flow Rate, Q (L/hr)	36.6
Linear Velocity, u (cm/hr)	535.8
Maximum Pressure Drop, ΔP_{max} (bar)	2.5
Operating Temperature, T (°C)	25
Cycle Time, t_{cycle} (minutes)	89
Spike Protein Yield (%)	80

Most of the negatively charged HCPs, DNA, and other impurities will flow through the column during loading or be washed out before elution as waste. Similar to the AEX process, the elution step in this CEX operation will be based on a pH shift. To ensure the eluate is compatible with the binding conditions of the subsequent polishing chromatography process, a sodium phosphate ($NaPO_4$) elution buffer at a pH of 6.8 will be used to change the net charge of the spike protein to negative and thus release it from the column. The complete 89-minute operating procedure is described in Table 3.2.7b. For all steps in this process, the buffer will be applied to the column at a linear velocity of 535.8 cm/hr. No studies could be found on the purification of the spike protein using the selected CEX resin, so the recovery and purity were assumed to be the same as for the AEX process: 80% and 90%, respectively.

Table 3.2.7b. CEX Chromatography Process

Phase	Buffer	Column Volume (CV)	Time (min)
1: Equilibrate	20 mM NaOAc pH 3.5	5	17.5
2: Load	20 mM NaOAc pH 3.5	9.55 L	15.63
3: Wash	20 mM NaOAc pH 3.5	5	17.5
4: Elute	5 mM $NaPO_4$ pH 6.8	5	17.5
5: CIP	1 mM NaCl → 1 mM NaOH	3 NaCl → 3 NaOH	21.0

3.2.8. Ceramic Hydroxyapatite Chromatography

The eluate from CEX chromatography will be directly fed to a final chromatography column for polishing. This polishing step is necessary because the two prior IEX processes can induce the formation of high molecular weight (HMW) aggregates and multimers due to changes in buffer concentration and/or pH during elution (Sun et al., 2011). It is critical to remove these HMW contaminants generated in the downstream process because they can adversely affect process economics and product safety. In addition to having the potential to induce an adverse immune response if injected into the body, aggregates hinder manufacturing processes by reducing product yield and activity. Size exclusion chromatography (SEC) was initially considered for this polishing step since it separates molecules based on differences in size. However, SEC typically requires long columns and low flow rates - factors which reduce productivity - and the small load volumes necessitate a preceding concentration step, which would increase the costs of the downstream process (Cibelli et al., 2021). To achieve the desired

separation of the spike protein from HMW impurities without the inefficiencies of SEC, ceramic hydroxyapatite (CHT) chromatography will be implemented.

CHT chromatography is a common application of mixed-mode chromatography. The unique selectivity of mixed-mode resins results from the contribution of multiple binding mechanisms. Hydroxyapatite surfaces feature three types of functional groups arranged in a repeating pattern: positively charged pairs of calcium ions (C-sites), clusters of negatively charged phosphate groups (P-sites), and hydroxyl groups. As illustrated in Figure 3.2.8a, the positively charged amino groups of proteins interact ionically with P-sites, while negatively charged carboxyl and phosphoryl groups of biomolecules bind to C-sites via the formation of coordination complexes, similar to those formed by the metal affinity interactions involved in IMAC. Hydrogen bonding to the hydroxyl groups can also occur. These three types of interactions operate cooperatively or individually, affording unique selectivity for bioseparation. Acidic and basic proteins - which carry a negative and positive charge, respectively, at the typical operating pH range of 6.5-7.5 for CHT chromatography (Vang & He, 2018) - interact with CHT resin through different mechanisms: acidic proteins chiefly bind to C-sites by metal affinity interactions, while basic proteins bind to P-sites by cation exchange interactions

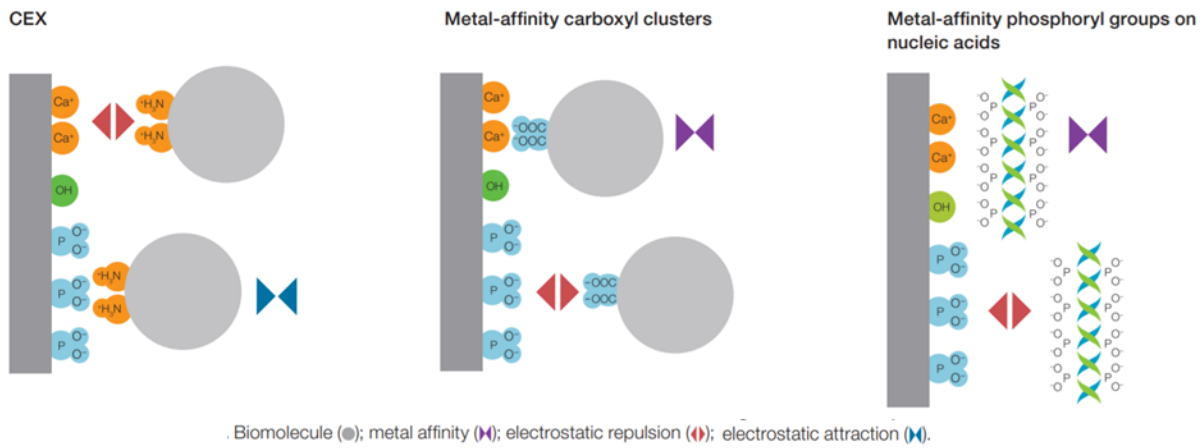


Figure 3.2.8a. Schematic representation of CHT binding mechanisms (*Bio-Rad, n.d.-b*)

In this chromatography process, the column will operate in bind-and-elute mode and will be packed with CHT™ Ceramic Hydroxyapatite Type I Media (40 μm) from Bio-Rad Laboratories. This resin was selected over the Type II Media since it has a higher binding capacity for acidic proteins, like the spike protein (Bio-Rad, n.d.-a). Previous studies employing Bio-Rad's CHT resins achieved high resolution of target proteins from aggregates using a column with a 13 cm bed height operated at a 300 cm/hr linear velocity (Bio-Rad, n.d.-b). It is common industrial practice to scale chromatography processes by increasing column diameter while keeping the same bed height and linear flow velocity - i.e., residence time (Cytiva, 2021). The CHT resin has a dynamic binding capacity of 25 g/L; therefore, 6.84 L of resin is needed to capture the 171 g of spike protein in the feed solution. To accommodate this volume while maintaining a residence time of 2.6 minutes, the column must have a diameter of 26 cm. The resin can support a maximum operating pressure of 100 bar, but the Carman-Kozeny equation, eq. (3.2.5a), predicts a pressure drop of 1 bar, so there is no risk of exceeding the pressure constraint. Key geometric features and operating conditions of the CHT chromatography column are presented in Table 3.2.8a.

Table 3.2.8a. CHT Column Specifications

Column Length, L (cm)	13
Column Diameter, d_c (cm)	26
Column Volume, V_c (L)	6.84
Flow Rate, Q (L/hr)	157.8
Linear Velocity, u (cm/hr)	300
Operating Pressure Drop, ΔP_{max} (bar)	1
Operating Temperature, T (°C)	25
Cycle Time, t_{cycle} (minutes)	95
Spike Protein Yield (%)	83

The CHT chromatography process will occur over 95 minutes in the six steps outlined in Table 3.2.8b. When the eluent from the CEX column is loaded at 300 cm/hr onto the pre-equilibrated CHT column, the resin will bind both the spike protein and aggregates. The strength of a molecule's interaction with the CHT resin is proportional to the size of the molecule (Saraswat et al., 2013). Therefore, the HMW aggregates will have a higher affinity for the resin, and thus will elute later than the target spike protein. At the binding conditions, the buffer pH of 6.8 is only slightly above the pI of the spike protein; therefore, the spike protein carries a weak net negative charge. Therefore, a minimal change in the ionic strength of the mobile phase should be sufficient to release the spike protein from the resin. The resin manufacturer recommends eluting with 20 CV of sodium phosphate buffer over a linear concentration gradient

of 5-500 mM. In a study of the effect of different binding and elution conditions on the purification of an acidic enzyme with a pI (6) similar to that of the spike protein, it was observed that recovery and purity can be simultaneously maximized when the operating pH is 6.8 by eluting up to a sodium phosphate concentration of 100 mM (Vang & He, 2018). Therefore, we have modified the recommended elution procedure to span a narrower sodium phosphate concentration gradient of 5 to 100 mM at pH 6.8. Ideally, the concentration gradient of the elution buffer would be optimized in the lab so just enough salt can be added to ensure only the spike protein elutes while the aggregates remain adsorbed. To reduce processing time, we have also decreased the elution CV to 10. Following elution, the column will be cleaned with a high concentration salt wash to strip away any species still bound to the resin. Finally, the column will be sanitized for reuse.

Table 3.2.8b. CHT Chromatography Process

Phase	**Buffer**	**Column Volume (CV)**	**Time (min)**
1: Equilibrate	5 mM $NaPO_4$ pH 6.8	10	26
2: Load	5 mM $NaPO_4$ pH 6.8	10.7 L	4
3: Wash	5 mM $NaPO_4$ pH 6.8	5	13
4: Elute	Gradient 5-100 mM $NaPO_4$ pH 6.8	10	26
5: Clean	500 mM $NaPO_4$ pH 6.8	5	13
6: Sanitize	1 M NaOH	5	13

In a study comparing the performance of different mixed-mode chromatography media in monoclonal antibody (pI = 6.9) aggregate removal, high recovery (83%) and high purity (99.5%)

of mAb monomers were simultaneously achieved using the same CHT resin (Type I, 40 µm) being employed in this spike protein purification process (Khandelwal & He, 2016). Without any publicly available data on the use of CHT resins to separate spike proteins from contaminating aggregates, we will assume the same yield and purity performance as observed in the mAb study.

3.2.9. Viral Filtration

The purpose of viral filtration is to remove residual viruses that may have been introduced to the solution during the filtration process. It does this through ultrafiltration. However, unlike past ultrafiltration in the process, the pore size will be larger than the spike protein, allowing the spike protein to pass through as the permeate. Viral particles are too big to pass through the membrane and will stay in the retentate.

For this viral filtration step, we will use the Cytiva ReadyToProcess hollow fiber filters at a size 750 kDa. We chose 750 kDa as a molecular weight cut-off because we thought it would be a good size to exclude particles greater than the size of our spike protein at 140 kDa. The Cytiva filter has a membrane surface area of 0.92 m^2, which, when combined with a flux of 30 LMH, takes 2.35 hours to run. The recovery was around 95%.

3.2.10. Final Ultrafiltration and Diafiltration

The last step in our downstream process right before formulation/filling is another ultrafiltration followed by a diafiltration. Following the same filtration design from before, a hollow fiber membrane is used and the ultrafiltration and diafiltration will be run using the same equipment. The goal of this final ultrafiltration step is to concentrate the spike protein for formulation. A protein solution volume of 65 L is coming from viral filtration at a protein concentration of 2.07 g/L, which is quite concentrated at this point. A molecular weight cut-off of 30 kDa was chosen due to the goal of wanting to retain as much protein as possible. There will

be a cross-flow pressure of 8 psi along the membrane (Cytiva, 2020). The filter has a membrane surface area of 1.15 m^2. With a flux of 30 LMH, the time it took was 1.8 hours. The recovery ended up being around 99.7%.

For the final diafiltration step, the same hollow fiber membrane was used with a goal of switching the buffer to formulation. The buffers used here include 150 mM NaCl in WFI and 2.5 mM $NaPO_4$ in WFI with a diafiltration volume of 22.4 L. The initial batch coming into this step is 3.25 L at a starting protein concentration of 41.4 g/L. The same molecular weight cut-off of 30 kDa was chosen. The filter has a membrane surface area of 1.15 m^2. With a flux of 30 LMH, the time it took was 0.65 hours. The recovery ended up being around 99.3%.

3.3. Formulation and Filling

3.3.1. Final Formulation

The final formulation of our vaccine was based on the Sanofi-GSK recombinant protein based vaccine being developed for COVID-19. Sanofi-GSK's monovalent SARS-Cov-2 vaccine contains 10 µg of antigen for a 0.5 mL dose. According to Sanofi's Phase 3 clinical trial results, two doses of the vaccine had 100% efficacy against severe COVID-19 disease, 75% efficacy against moderate COVID-19 disease, and 57.9% efficacy against any symptomatic COVID-19 disease (Sanofi, 2022). Therefore, our vaccine regimen will consist of two 0.5 mL vaccine doses each containing 10 µg of antigen. Unlike vials containing both adjuvant and antigen, our adjuvant and antigen will be filled in separate vials for storage and combined shortly before administration (Sanofi Pasteur, 2021). Although each antigen and adjuvant vial has a volume of 10 mL, they are only filled halfway to 5 mL. When combined, a pair of adjuvant and antigen vials yields 20 vaccine doses.

Formulation Mixing Tank

In total, 6675 L of solution will be formulated and mixed before filling into vials. We will use a single 2000 L Mixer. To accommodate for having only one mixer, formulation will be split into 4 rounds consisting of 1668.75 L each. The first two rounds will be for antigen formulation and the last two will be for adjuvant formulation. After a round of formulation is complete, it will be transferred directly to vial filling, and after those vials are filled, the next round of formulation begins in the same mixer.

Each formulation will be mixed in a Thermo Scientific Hyperforma Single-Use 2000 L Jacked Mixer. The mixer will operate at 350 RPM impeller agitation speed which will require 408.7 W of power. For each mixing round, the mixer will be maintained between 2 - 8°C, and

each formulation will be mixed for 2 hours to ensure homogeneity. Table 3.3.1a shows the required ingredient quantities to be added during an antigen solution mixing round and an adjuvant emulsion mixing round.

Table 3.3.1a. Ingredients for half of a batch

Antigen	
Total Volume (L)	1668.750
Protein Solution from Downstream	
Antigen (g)	66.750
NaCl (g)	98.350
$NaPO_4$ (g)	4.600
WFI (L)	1.625
Ingredients Added	
Polysorbate 20 (g)	183.563
PBS (L)	1667.125
2-phenoxyethanol (g)	8343.750

Adjuvant (AS03)	
Total Volume (L)	1668.75
Squalene (g)	71355.75
α-tocopherol (g)	79165.50
Polysorbate 80 (g)	32440.50
PBS (L)	1668.75
2-phenoxyethanol (g)	8343.75

Antigen Solution Formulation

From Downstream, 3.25 L of protein solution (per batch) containing 133.5 g of antigen, 196.7 g NaCl, and 9.20 g $NaPO_4$ is available for formulation. This amount will yield 13.35 million vaccine doses each containing 10 µg (0.2434 µL) of antigen. To properly formulate the vaccine, the antigen will be combined in aqueous solution with phosphate buffer, stabilizers, surfactant, and preservative. Based on a formulation of a vaccine developed using BEVS by Protein Sciences, approximately 27.5 µg of polysorbate 20 will be added as the surfactant (Protein Sciences, 2021). The purpose of the surfactant is to inhibit adsorption, denaturation, and aggregation of proteins at interfaces (Medi & Chintala, 2014). To prevent contamination in multidose vials, a preservative is required; therefore, 5 mg/mL of 2-phenoxyethanol will be

added to the formulation (OIDP, 2021; Geier et al., 2010). 2-phenoxyethanol was selected over thimerosal due to concerns that thimerosal might contribute to long-term accumulation of mercy in the body (Geier et al., 2010). Phosphate-buffered saline (PBS) will be used as the aqueous carrier to resuspend the antigen. PBS will contain sodium chloride, monobasic monohydrate sodium phosphate, and dibasic dodecahydrate sodium phosphate suspended in WFI; thus, the density and viscosity of this formulation is approximated to that of water (Sanofi Pasteur, 2021; Garçon et al., 2012). PBS is also used as a stabilizer to ensure that the vaccine is isotonic and to maintain a stable pH.

Adjuvant Emulsion Formulation

Prior to injection, the vaccine will be mixed with an adjuvant emulsion formulation. The formulation contains the Adjuvant System 03 (AS03) manufactured by GSK, which reduces the amount of antigen needed by enhancing the body's immune response to the vaccine through enhancing antigen persistence at the injection site. For a 0.5 mL vaccine dose, AS03 is composed of 10.69 mg squalene, 11.86 mg α-tocopherol, and 4.86 mg polysorbate 80 (Sridhar et al., 2022). This quantity of polysorbate 80 will act as the surfactant. Additionally, 5 mg/mL of 2-phenoxyethanol preservative will be added to the adjuvant formulation. The adjuvant formulation will be combined in aqueous solution of PBS. The PBS will be composed of sodium chloride, dibasic sodium phosphate, monobasic potassium phosphate, and potassium chloride suspended in WFI (Sanofi Pasteur, 2021).

Table 3.3.1b. Formulation of one 0.5 mL dose

Antigen		**Adjuvant (AS03)**	
Total Volume (mL)	0.25	**Total Volume (mL)**	0.25
Protein Solution from Downstream		**Squalene (mg)**	10.69
Antigen (µg)	10	**α-tocopherol (mg)**	11.86
NaCl (µg)	14.73	**Polysorbate 80 (mg)**	4.86
$NaPO_4$ (µg)	0.69	**PBS (mL)**	0.25
WFI (µL)	0.2432	**2-phenoxyethanol (mg)**	1.25
Ingredients Added			
Polysorbate 20 (µg)	~ 27.5		
PBS (mL)	0.2498		
2-phenoxyethanol (mg)	1.25		

3.3.2. Filling

Vials

After careful consideration, we chose to use multidose vials instead of single-dose vials. Single-dose vials would allow the vaccine to be preservative-free because there would be no risk for contamination since the vial would be discarded after use; however, multidose vials require a preservative to help limit the growth of bacteria after it is opened (CDC, 2019). Additionally, in countries with a lower vaccine demand, single-dose vials could eliminate concerns about vaccine wastage that comes from opening a multidose vial without enough people to administer the vaccine to (Eaton & Murphy, 2021). However, the large amount of single-dose vials required would be more expensive, would require a larger capacity for cold storage, and would require more time to fill. Since there are still many countries with a shortage of COVID-19 vaccines, it

was ultimately decided that 10 mL multidose vials will be used in order to maximize production efficiency.

Each batch will produce 13.35 million vaccine doses in total. When combined, a pair of antigen and adjuvant vials will yield 20 doses. Therefore, a total of 1.335 million vials will be needed per batch.

Vial Filling Process

The vial filling process will occur at a speed of 36,000 vials per hour using equipment purchased from Bausch+Ströbel. Thus, it will take 37.08 hours to fill the 1.335 million vials per batch.

An aseptic production environment will be maintained in order to ensure a sterile product. Therefore, the glass vials will first undergo vial cleaning and then vial depyrogenation. Vial cleaning is an internal and external washing of the vial, and is conducted on the Bausch+Ströbel FAU series cleaning machine. Vial depyrogenation, done with the Bausch+Ströbel DHT series sterilizing tunnel, conveys the vials through a heat tunnel (up to 600°F) to remove microbes from glass. These two steps ensure that the vials are sterile before filling. Next, the glass vials will be filled using the Bausch+Ströbel FFV series filling and closing machine to the appropriate dose from the formulation mixing tank and the vials are closed by the insertion of rubber stoppers. Then, the vials will be closed with crimp caps using the Bausch+Ströbel RVB series closing machine. Finally, the Bausch+Ströbel ME-8081 tray-loading unit will inspect for cracks, load vials onto a tray, and transfer them to storage. The filled vials will be stored as a liquid at a temperature of 2 - 8℃ for up to 6 months (Dunkle et al., 2022). The refrigeration process for storage and final packaging process is beyond the scope of this project.

3.4. Ancillary Equipment

3.4.1. Pumps

Peristaltic pumps will be used in this manufacturing plant to transport fluids between process equipment. These positive displacement pumps are inherently gentle and low-shear, ensuring sensitive insect cell and protein suspensions cannot be damaged by high velocities or contact with mechanical parts. By confining fluid to flexible, biocompatible tubing that can be discarded or sterilized between batches, peristaltic pumps are ideal for maintaining fluid path sterility, minimizing leaks and cross-contamination, and simplifying process validation (Markarian, 2017). The variety of tubing formulations and sizes allows the pumps to operate over a wide range of flow rates to meet the specific flow requirements of different unit operations.

In order to estimate the utility costs for operating the pumps, the hydraulic power required to drive the pump was determined by multiplying the volumetric flow rate (Q) by the total pressure differential (ΔP). All pumps were designed to account for a 0.5 atm pressure drop due to frictional losses during fluid transfer through tubing. Since tubing length and diameter are unknown, each flow stream will be equipped with a pump operating at a 1.0 atm pressure differential. This pressure is low enough to prevent unwanted damage to cells and proteins but high enough to supply the low volumetric flow rates needed for each process. For unit operations that require an applied pressure, such as membrane filtration, homogenization, and chromatography, a specific pressure requirement was used in the pump design. No significant elevation changes are expected in the manufacturing process so the gravity head contribution to differential pressure was ignored. For each pump, the total efficiency (η_{total}) in converting

electrical energy to kinetic energy and in transmitting power from shaft to fluid was taken to be 70%.

$$Power = \frac{Q \times \Delta P}{\eta_{total}} \quad (3.4.1a)$$

In total, the plant will require 32 operational pumps and 16 pumps on standby in case of system pump failure. The flow rates, differential pressures, and power requirements of the pumps involved in generating flow between process units and of the pumps required to apply specific pressures across process equipment are summarized in Tables 3.4.1a and 3.4.1b, respectively. A total of 15 kW of energy is required to power all the flow and pressure pumps in operation. The spare pumps do not consume power.

Table 3.4.1a. Power requirements for flow pumps

Pump ID	Source	Destination	Stream Contents	Flow Rate (L/min)	Pressure Differential (atm)	Power at 70% Total Efficiency (W)
P-101	JMT-101	SUB-101	Sf-900™ II SFM	1.42	1.50	5.13
P-102	JMT-101	SUB-102	Sf-900™ II SFM	2.73	1.50	9.90
P-201	SUB-102	SUB-201	Uninfected cell culture	2.47	1.50	8.93
P-301	SUB-102	SUB-301	Uninfected cell culture	1.67	1.50	6.03
P-302	SUB-201	SUB-301	BV inoculum	1.85	1.50	6.69
P-303a	JMT-301a	SUB-301a	Sf-900™ II SFM	3.44	1.50	12.45
P-303b	JMT-301b	SUB-301b	Sf-900™ II SFM	3.44	1.50	12.45
P-303c	JMT-301c	SUB-301c	Sf-900™ II SFM	3.44	1.50	12.45
P-303d	JMT-301d	SUB-301d	Sf-900™ II SFM	3.44	1.50	12.45
P-303e	JMT-301e	SUB-301e	Sf-900™ II SFM	3.44	1.50	12.45
P-401	SUB-301a/b/c/d/e	CEN-401	Fermentation broth	33.33	1.50	120.63
P-404	MT-401	ST-402	Diafiltration 1 buffer (20 mM Tris-HCl pH 7.4)	2.83	1.50	10.23
P-420	MT-406	ST-404	Diafiltration 2 buffer (150 mM NaCl, 2.5	0.75	1.50	2.71

			mM $NaPO_4$)			
P-501	ST-404	JMT-501	Purified API in diafiltration buffer 2	1.63	1.5	5.88

Table 3.4.1b. Power requirements for applied pressure pumps

Pump ID	Source	Destination	Stream Contents	Flow Rate (L/min)	Pressure Differential (atm)	Power at 70% Total Efficiency (W)
P-402	JMT-401	HPH-401	Resuspended cell pellet + protease inhibitors	16.67	272.68	10964.15
P-403	ST-401	DEF-401	Disrupted cells	420.00	2.54	2575.06
P-405	ST-402	UF-401/DF-401	Protein solution in WFI then in diafiltration buffer 1 (20 mM Tris-HCl pH 7.4)	224.00	1.04	564.38
P-406	ST-402	AEX-401	Protein solution feed to AEX	0.55	2.97	3.91
P-407	MT-401	AEX-401	Equilibration and wash buffer (20 mM Tris-HCl pH 7.4)	0.55	2.97	3.91
P-408	MT-402	AEX-401	Elution buffer (20 mM NaOAc pH 3.5)	0.55	2.97	3.91
P-409	MT-403	AEX-401	AEX cleaning buffer 1 (1 mM NaCl)	0.55	2.97	3.91
P-410	MT-404	AEX-401	AEX cleaning buffer 2 (1 mM NaOH)	0.55	2.97	3.91
P-411	JMT-402	CEX-401	Protein solution feed to CEX	0.61	2.97	4.37
P-412	MT-402	CEX-401	Equilibration and wash buffer (20 mM NaOAc pH 3.5)	0.61	2.97	4.37
P-413	MT-405	CEX-401	Elution buffer (5 mM $NaPO_4$ pH 6.8)	0.61	2.97	4.37
P-414	MT-403	CEX-401	CEX cleaning buffer 1 (1 mM NaCl)	0.61	2.97	4.37
P-415	MT-404	CEX-401	CEX cleaning buffer 2 (1 mM NaOH)	0.61	2.97	4.37
P-416	CEX-401	CHT-401	Protein solution feed to CHT	2.63	1.49	9.44
P-417	MT-405	CHT-401	Equilibration and wash buffer (5 mM $NaPO_4$ pH	2.63	1.49	9.44

			6.8) + elution buffer (5-500 mM $NaPO_4$ pH 6.8) + cleaning buffer (500 mM $NaPO_4$ pH 6.8)			
P-418	MT-404	CHT-401	CHT sanitization buffer (1 M NaOH)	2.63	1.49	9.44
P-419	ST-403	VF-401	Protein solution in $NaPO_4$	26.00	0.61	38.42
P-421	ST-404	UF-402/DF-402	Protein solution in $NaPO_4$ then in diafiltration buffer 2 (150 mM NaCl, 2.5 mM $NaPO_4$)	224.00	1.04	564.38

3.4.2. Tanks

Tanks will be used in the manufacturing plant for preparation and storage of process fluids such as buffer solutions, CIP chemicals, fermentation media and substrates, and liquid waste. Tanks will also be used to collect waste during downstream processing; these tanks and the protocol for disposing of their contents are discussed in section 3.5.1. Several different types of tanks will be used throughout the facility. Thermo Scientific™ HyPerforma™ Jacketed Single-Use Mixers (S.U.M) will be used for processes that require agitation and temperature control, such as Sf-900™ II SFM preparation, cooling of the resuspended Sf9 cell solution prior to high-pressure homogenization, viral inactivation, and final product formulation. HyPerforma™ Unjacketed S.U.M.s will be used to prepare buffers at room temperature for diafiltration and chromatography. The jacketed and unjacketed HyPerforma™ mixing tanks hold single-use bags within a stainless steel cylindrical shell. These tanks are available in sizes ranging from 50 L to 2000 L. For processes that require neither agitation nor temperature control, unjacketed, single-use, stainless steel storage tanks will be used. These storage tanks were sized by calculating the volume of fluid required per production batch and rounding up to

the nearest ten, hundred, thousand, or ten thousand liter increment. Table 3.4.2a. summarizes the purpose and capacity of the tanks used in the manufacturing process.

Table 3.4.2a. Description of holding tanks

Tank ID	Description	Working Volume of Tank (L)
JMT-101	Sf-900™ II SFM preparation for seed train	2000
JMT-301a/b/c/d/e	Sf-900™ II SFM preparation for protein production	5x2000
JMT-401	Post-centrifugation resuspension	2000
ST-401	Hold disrupted cell slurry following HPH	2000
MT-401	Tris-HCl buffer preparation	2000
ST-402	UF-401/DF-401 feed/recirculation tank	4000
MT-402	NaOAc buffer preparation	50
MT-403	NaCl buffer preparation	50
MT-404	NaOH buffer preparation	50
JMT-402	Viral inactivation	50
MT-405	$NaPO_4$ buffer preparation	250
ST-403	VF feed/recirculation tank	100
ST-404	UF-402/DfF-402 feed/recirculation tank	100
MT-406	NaCl & $NaPO_4$ buffer preparation	50
JMT-501	Formulate API for vial filling	2000
ST-405	Liquid waste collection	2x10000

3.4.3. Cooling Jackets

To maintain optimal process temperatures for certain agitated operations, cooling jackets will be implemented to actively remove heat from the process. By completely surrounding the outside of a tank, cooling jackets maximize the surface area over which heat can be exchanged. The even distribution of coolant over this surface area prevents hot or cold spots from forming within the tank. Cooling jackets also eliminate the risk of contamination posed by inserting a cooling rod or coil directly into the tank. The Thermo Scientific™ HyPerforma™ agitated

vessels that will be used in this facility feature an external cooling jacket, so the geometry of these jackets did not need to be designed. Instead, energy balances were formulated for all vessels requiring precise temperature control in order to determine the heat duty of the jackets and the associated mass flow rate of coolant through the jackets. These specifications will dictate the operating costs of using a utility-supplied coolant.

The heat duty of the agitated mixing tanks was determined by summing the heat inputs and outputs. Sources of heat for fermentations include substrate feeds (Q_S), air in-flow (Q_{Ae}), metabolic activity of the cells (Q_M), agitation of the impeller (Q_{Ag}), and recirculation pumps used to force coolant through the jacket (Q_P). Sources of cooling include evaporation of water from the fermentation broth (Q_E); heat loss from the vessel due to construction materials, geometry, wall thickness, insulation use, and other environmental conditions (Q_V); and the flow of coolant through the jacket (Q_C). At steady state, the sum of the heat inputs and outputs is zero, and the energy balance on the fermentation system is described by the following equation.

$$Q_S + Q_{Ae} + Q_M + Q_{Ag} + Q_P = Q_E + Q_V + Q_C \quad (3.4.3a)$$

It is common practice to simplify eq. 3.4.3a by assuming Q_V and Q_P are negligible (Georgescu et al., 2008). Since the bioreactors in this facility are being run in batch mode, there are no substrates being introduced during operation, so Q_S can be ignored. The fermentation broth will be sparged with pure oxygen entering at the same temperature as the broth; therefore, without a temperature difference to drive heat transfer, Q_{Ae} can also be ignored. Evaporation-induced heat losses are not significant in large-scale stirred tank reactors due to their favorable surface-to-volume ratio (Wiegmann et al., 2018). Additionally, the bioreactors in this facility are not being operated at a particularly elevated temperature, so the vapor inside the bioreactors should not be of high humidity. Thus, Q_E can be ignored.

During fermentation, heat is generated and lost. If the heat generation exceeds the heat loss, then the fermentation will require cooling to maintain a steady, optimum temperature (27 ℃) for the heat balance. Rearranging the simplified energy balance to solve for Q_C allows the heat duty to be determined, and this will serve as a basis for the design of the operating conditions of the cooling jackets surrounding each bioreactor.

$$Q_C = Q_M + Q_{Ag} \tag{3.4.3b}$$

The rate of heat removal from is related to the mass flow rate (m) of coolant through the jacket, the specific heat capacity of the coolant (C_P), and the difference between the coolant's temperatures (ΔT) at the jacket's inlet and outlet by the following equation:

$$Q_C = mC_P\Delta T \tag{3.4.3c}$$

Two types of coolants will be used in this facility. For the vessels used for fermentation and viral inactivation, the associated cooling jackets will be serviced with chilled water at 5 ℃. The high specific heat capacity of liquid water (4204 J/kg-K at the average of the coolant's inlet and outlet temperatures [Green & Southard, 2018]) ensures enough heat will be removed to maintain the optimum process temperature without a significant increase in the water's temperature. For the mixing tanks used for Sf-900™ II SFM preparation, post-centrifugation resuspension, and final product formulation, the cooling jackets will be supplied with a 50% (by volume) aqueous ethylene glycol solution. Although this solution has a lower heat capacity than pure water (3406 J/kg-K at the average of the coolant's inlet and outlet temperatures [Engineering ToolBox, 2003]), the lower freezing point (-37 ℃) ensures the coolant remains a liquid at a low enough temperature to maintain the colder temperatures required for these unit operations. The ethylene glycol solution will be fed to the appropriate jackets 30 ℃ above its freezing point in order to prevent slush formation. Both the chilled water and ethylene glycol

solution will exit the cooling jackets 10 ℃ warmer than they entered. The design specifications for the cooling jackets used in different unit operations are summarized in Table 3.4.3a.

Table 3.4.3a. Cooling jacket conditions for different unit operations

Unit Operation	**Optimum Vessel Temperature (℃)**	**Jacket Inlet Temperature (℃)**	**Jacket Outlet Temperature (℃)**	**Coolant**
Bioreactors (Count: 8)	27	5	15	Chilled Water
Cell Culture Media Preparation Mixers (Count: 6)	4	-7	3	50% Ethylene Glycol Sol.
Post-Centrifugation Resuspension Mixer	4	-7	3	50% Ethylene Glycol Sol.
Viral Inactivation Mixer	25	5	15	Chilled Water
Formulation Mixer	Between 2-8	-7	3	50% Ethylene Glycol Sol.

Combining eqs. 3.4.3b and 3.4.3c yields a relationship to determine the mass rate at which the coolant must flow through the jacket's channels in order to maintain the fermentation broth at a constant temperature:

$$m = \frac{Q_M + Q_{Ag}}{C_P \Delta T} \tag{3.4.3d}$$

While the heat input from the impeller was determined using eq. 3.1.1c, the heat input from the metabolic activity of the cells was determined using the following equation provided by Georgescu et al. (2008):

$$Q_M = 430 \times SOUR \times N \tag{3.4.3e}$$

where $SOUR$ is the specific oxygen uptake rate (mmol/cell/s) of the cells and N is the maximum number of cells in the fermentation broth. The empirical factor, 430, represents the amount of

heat that is released per mmol of oxygen that the cells consume. This empirical factor may vary slightly from cell line to cell line, but as seen in previous work, there does not appear to be much variation (Cooney et al., 1969).

The heat duty of the cooling jacket and the associated mass flow rate of the coolant for each bioreactor during a single production batch are outlined in Table 3.4.3b. The five 2000 L production bioreactors are reported twice in Table 3.4.3b because the sOUR, and thus the rate of metabolic heat generation, of Sf9 cells is different for the growth and infection stages.

Table 3.4.3b. Per-batch bioreactor cooling jacket heat duty and coolant mass flow rate

Bioreactor	**Metabolic Heat Generation, Q_M (W)**	**Agitation Power, Q_{Ag} (W)**	**Heat Duty of Associated Cooling Jacket, Q_C (W)**	**Mass Flow Rate of Coolant (kg/min)**
100 L Seed Train	6.97	1.99	8.95	0.01
2000 L Seed Train	121.26	36.25	157.51	0.22
1000 L BV Amplification	64.50	15.90	80.40	0.11
5x2000 L Production, Growth Phase	258.00	40.00	298.00	0.43
5x2000 L Production, Infection Phase	322.50	40.00	362.50	0.52

The heat duty for the mixing tank cooling jackets was determined using a similar approach. Since there is no metabolic heat generation contributing to the energy balance on these tanks, eq. 3.4.3b simplifies to:

$$Q_C = Q_{Ag} \tag{3.4.3f}$$

The heat duty of the cooling jacket and the required mass flow rate of the coolant for each mixing tank are summarized in Table 3.4.3c.

Table 3.4.3c. Per-batch mixing tank cooling jacket heat duty and coolant mass flow rate

Mixing Tank	Heat Duty of Associated Cooling Jacket, Q_C (W)	Mass Flow Rate of Coolant (kg/min)
2000 L Sf-900™ II SFM Preparation for Seed Train	256.64	0.452
5x2000 L Sf-900™ II SFM Preparation for Production	256.64	0.452
2000 L Post-Centrifugation Resuspension	256.64	0.452
50 L Viral Inactivation	1.80	0.003
2000 L Formulation	408.70	0.720

3.4.4. CIP & SIP

Clean-in-Place (CIP) is a method of cleaning the interior surfaces of pipes, vessels, process equipment, filters, and associated fittings, without disassembly. Steam-In-Place (SIP) is responsible for repeatedly steaming areas of product contact, including vessels, flow paths, and sample ports. This may be done to kill harmful materials at the end of a batch. Although most of the processes use single use systems (SUS), several unit operations do not and thus require CIP and SIP procedures. The CIP procedures are different for each of the AEX, CEX, and CHT chromatography operations and these protocols are discussed in sections 3.2.5., 3.2.7., and 3.2.8., respectively. For the disc stack centrifuge used, the CIP procedure is usually meant to do light rinsing in between uses. The centrifuge is typically flushed with clean water to prevent solids from collecting. WFI is sufficient to flush. It is an extra, low-speed flush that's programmed as a separate sequence used when the centrifuge is not running. For the high-pressure homogenizer for cell lysis, there are a number of preventive maintenance tasks that should be regularly completed to help it last for many years. This includes regular replacement of motor unit brushes, lubrication of bearings using a liquid sample/medium, and regular exterior cleaning of

the homogenizer using soap and water. The CIP for a homogenizer is usually similar to a centrifuge where water is used to flush through the system lines to keep it clean and prevent any buildup from occurring. SIP usually takes place following the final rinse after CIP. Every little part of the process, such as piping and vessels, come in direct or indirect contact with process inputs, process, and process outputs is sterilized to ensure that there is no microbiological activity in the system. Before every new run of AEX, CEX, and CHT chromatography, CIP will be conducted followed by SIP. CIP is conducted first in order to wash away and remove particulates that lay within the vessel. SIP then kills any microbes that survived CIP. SIP uses steam to sanitize the equipment. The amount of steam used, price of steam used, and logistics of steam production will not be discussed in this project.

4. Final Design

Figures 4a and 4b are the detailed process flow diagrams (PFD) for the upstream and downstream processes. The PFD contains labeled process equipment, streams, pumps, heat exchanges, tanks, and all other process equipment. Corresponding material balances are found in section 4.5.

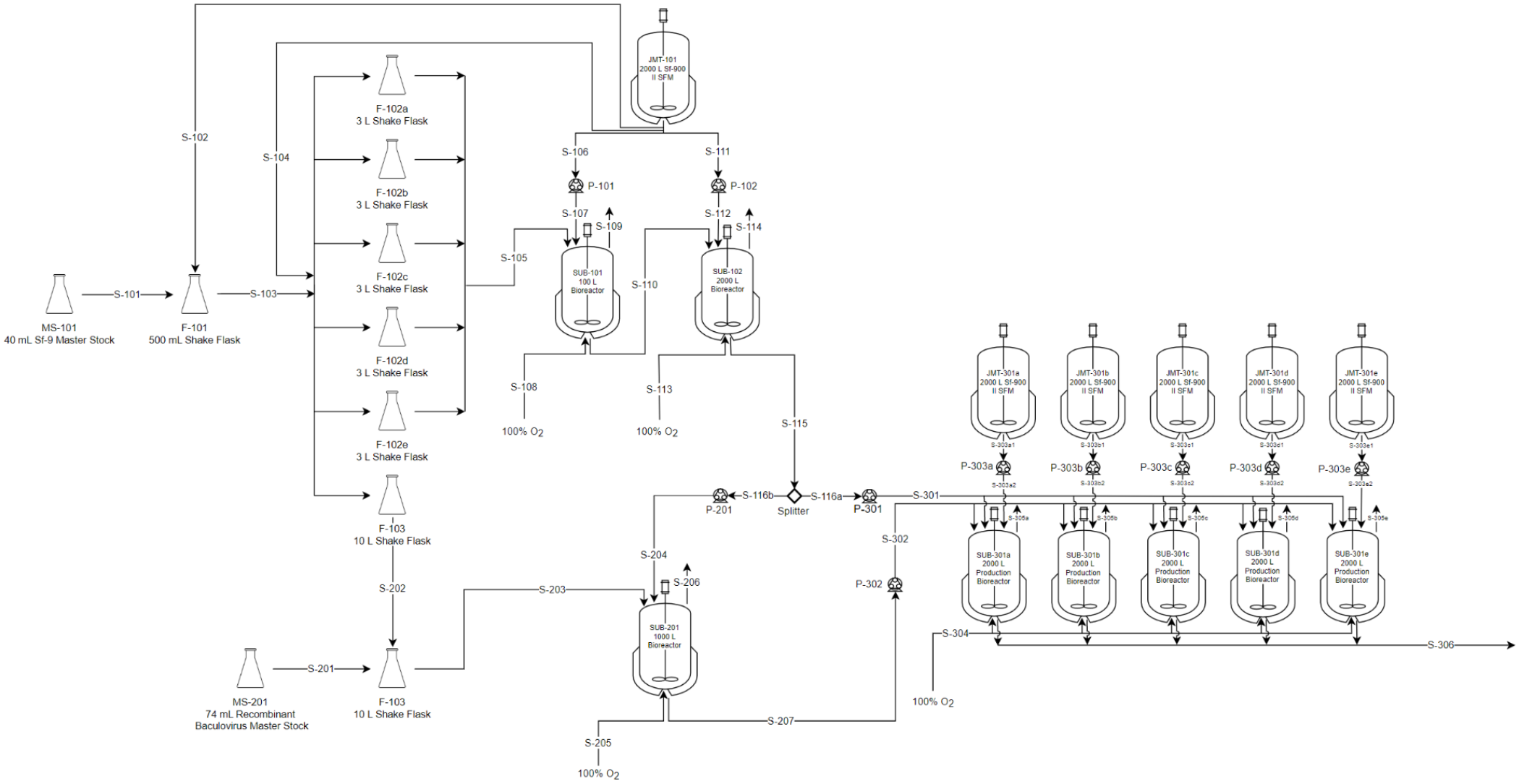


Figure 4a. Upstream process flow diagram

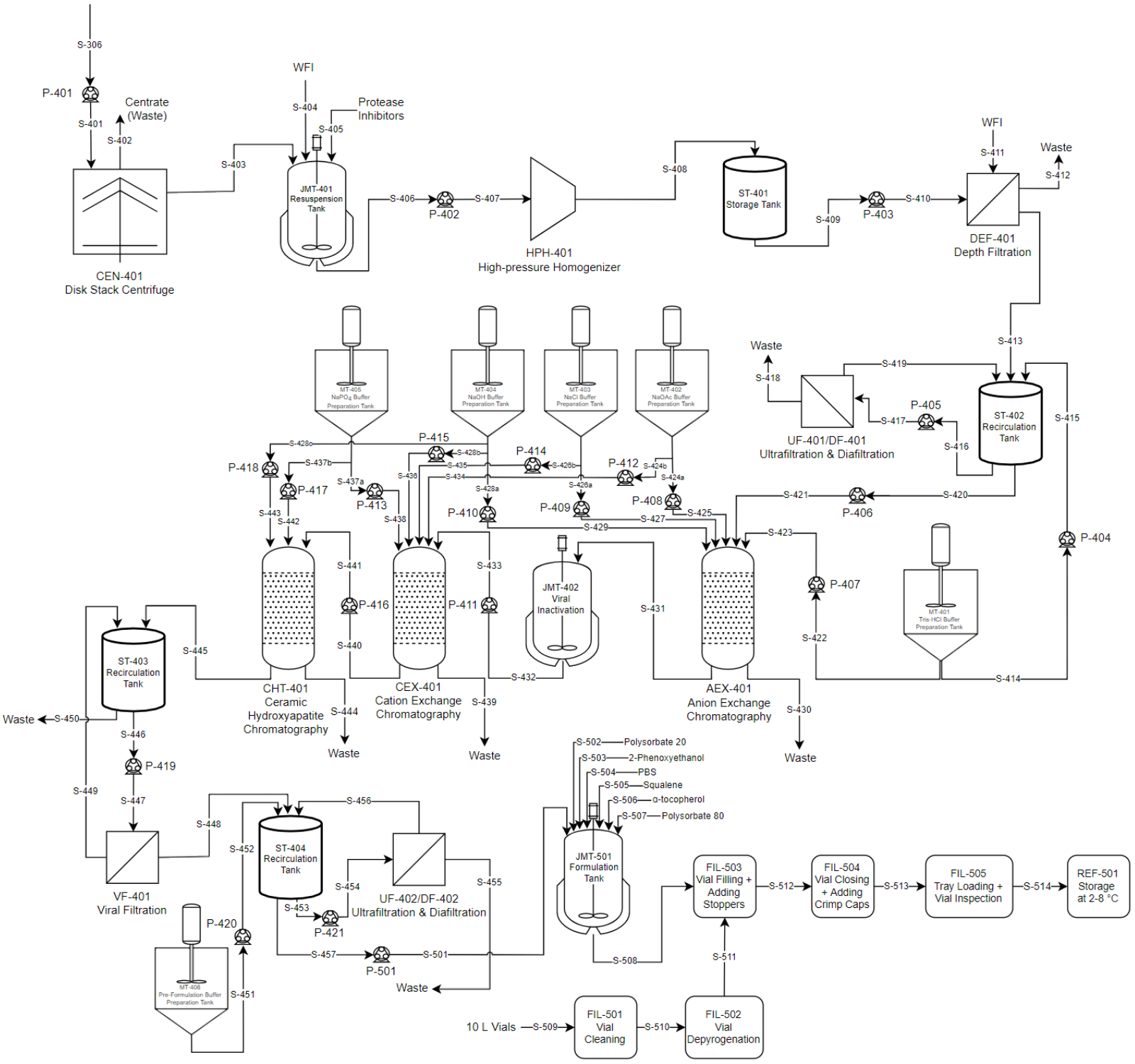


Figure 4b. Downstream process flow diagram

4.1. Upstream

4.1.1. Insect Cell Growth Seed Train

To start the seed train, we will defrost ten 4 mL vials of *Spodoptera frugiperda* (Sf9) insect cells in a water bath set at 37 ℃. This Sf-9 master stock (MS-101) will have a cell density of 4.00E+06 cells/mL. The 0.04 L master stock will be transferred along with 0.46 L of serum-free media (Gibco™ Sf-900™ II SFM) to a 500 mL shake flask (F-101) where it will grow for 4.08 days until a cell density of 8.10E+06 cells/mL is achieved. Next, the 500 mL will be transferred and split into five 3 L shake flasks (F-102a/b/c/d/e) and one 10 L shake flask (F-103). A total of 21.91 L of SFM will be added to these flasks. At this stage, the insect cells will grow for 4.8 days until a target cell density of 8.10E+06 cells/mL is reached. 7.4 L will be transferred to the baculovirus amplification train, and the rest, 15 L, will be fed into the 100 L bioreactor (SUB-101). The bioreactor will consist of a 45° pitched blade impeller with 3 blades, and the impeller will operate at 146 RPM which will require 2 W of power. The tank diameter will be 43.8 cm and the liquid height will be 66 cm. 85 L of SFM will be added, and the media will require 0.11 kg glucose and an aeration rate of 2 L/min of pure oxygen. The insect cells will grow in the 100 L bioreactor for 2.4 days, until a target cell density of 8.10E+06 cells/mL is achieved. The 100 L of material will feed into the 2000 L insect cell growth bioreactor (SUB-102). This bioreactor will consist of a 45° pitched blade impeller with 3 blades, and the impeller will operate at 72.5 RPM which will require 36 W of power. The tank diameter will be 119.4 cm and the liquid height will be 155.5 cm. 1640.7 L of SFM will be added, and the media will require 1.09 kg glucose and an aeration rate of 4 L/min of pure oxygen. The insect cells will grow for 3.61 days until they reach a target cell density of 8.1E+06 cells/mL. 740.7 L will

transfer to the baculovirus amplification train, and the rest, 1000 L, will feed into the five 2000 L (total of 10,000 L) protein production bioreactors (SUB-301a/b/c/d/e).

4.1.2. Baculovirus Amplification Train

A separate cell bank will be used to grow the recombinant baculovirus into a viral stock. The baculovirus will then be pumped into the flasks and bioreactors to infect the growing insect cells and produce the spike protein. The baculovirus amplification train starts by purchasing 74 mL of baculovirus master stock (MS-201) (AcNPV expressing the SARS-CoV-2 spike protein) at a virus titer of 8.1E+07 pfu/mL. 7.41 L of cell culture from the insect cell seed train 10 L shake flask, F-103, will be transferred to the baculovirus seed train. The cell culture in the shake flask will be infected with 74 mL of purchased baculovirus at an MOI value of 0.1 for optimal virus production. The baculovirus will then be incubated in the cell culture for 2 days at 27℃. The cells and virus will then be transferred to a 1000 L bioreactor (SUB-201), and amplified there with an MOI of 0.1. 740.7 L of grown cell culture will be taken from the 2000 L insect cell seed train growth bioreactor for viral infection. The 1000 L baculovirus bioreactor will consist of a 45° pitched blade impeller with 3 blades, and the impeller will operate at 78.5 RPM which will require 15.9 W of power. The tank will have a 95.9 cm diameter and a 105.3 cm liquid height. The 1000 L bioreactor requires an aeration rate of 3 L/min, and the baculovirus will be incubated by infecting the cell culture and amplified for another 2 days. After the growth in the 1000 L bioreactor at 27℃, the incubated baculovirus will remain in the bioreactor at room temperature. After 3 days, the contents of the tank will be pumped into the five 2000 L (total of 10,000 L) production bioreactors, SUB-301a/b/c/d/e, to infect the cells and produce recombinant proteins.

4.1.3. Antigen Production

Five 2000 L Thermo Scientific™ HyPerforma™ production single-use bioreactors, SUB-301a/b/c/d/e, will be used for antigen production. Each bioreactor will produce 60 g of our recombinant spike protein. In the first step, 200 L of cell media at a cell density of 8.75E+05 cells/mL will be transferred into each bioreactor along with 3.5 kg of glucose for supplemental nutrients. An additional 1652 L of Gibco™ Sf-900™ II SFM media will be added to each bioreactor. The bioreactor will consist of a 45° pitched blade impeller with 3 blades at an RPM of 75. The tank diameter will be 119.4 cm and the liquid height will be 155.5 cm. An aeration rate of 4 L/min of pure oxygen will be used. After a period of 1.56 days, the cells will reach a target cell density of 3.0E+06 cells/ml. Each bioreactor will then be inoculated with 148 liters of baculovirus from the baculovirus amplification train to infect the cells at an MOI of 2. An additional 4.8 kg of glucose will be added for supplemental nutrients. An aeration rate of 4 L/min of pure oxygen will be maintained. The liquid height within the tank will be 178.7 cm with an RPM of 75. After an infection period of 3 days, the contents of the bioreactors are ready for centrifugation.

4.2. Downstream

4.2.1. Centrifugation

After antigen production, the contents of the five production bioreactors will be fed to the Alfa Laval BTPX 305 disk stack centrifuge (CEN-401) at a flow rate of 2000 liters per hour. Centrifugation will take 5 hours to complete and will have an estimated recovery of 100%. The cell pellet will be collected on the sides of the centrifuge and is ejected horizontally into a 200 L mixing bag with the use of nozzles. The remaining 10,000 liters of supernatant will be ejected for disposal as solid waste. At completion, 18.3 kg of cell pellet will be transferred to a 2000 L jacketed mixer for cell lysis.

4.2.2. Cell Lysis

After centrifugation, 18.3kg of cell pellet will be mixed in a Thermo Scientific Hyperforma Single-Use 2000 L Jacketed Mixer which is our resuspension tank (JMT-401). This resuspended cell solution will have a volume of 1830 liters, consisting of cell pellet, WFI, and protease inhibitors: 10 μM chymostatin, 10 μM leupeptin, 1 μM pepstatin A, and 0.2 mM phenylmethylsulfonyl fluoride.

After resuspension, the cell solution will be homogenized using an EmulsiFlex-C1000 high pressure homogenizer (HPH-401) manufactured by Avestin. The cell solution will be homogenized at 4,000 psi and 4℃ for one single pass. When operated at a flow rate of 1000 L/h, this process will take 1.83 hours to homogenize all of the 1830 L of cell solution. The output stream will be fed into a storage tank (ST-401) prior to depth filtration stage.

4.2.3. Depth Filtration

After cell lysis, 1830 L will be fed into our depth filtration system (DEF-401). This system consists of Millipore's 3 rack, which holds thirty depth filter pods. We will be using

Millipore's Millistak+® CE50 Pod Depth Filters. These filters have a pore sizes between 0.6 and 0.1 microns and are made of cellulose. Each pod has a surface area of 1.4 m^2. To complete the washing and flushing stages, 2100 L of WFI will be used. In the filtering stage, the 1830 L of cell solution will be fed through the filters at a flow rate of 420 L/min (25,200 L/h). The depth filtration process will take 23.8 minutes. After depth filtration, 3,930 L will be fed to the tangential flow filtration systems for concentration and buffer replacement.

4.2.4. Ultrafiltration and Diafiltration for Anion Exchange Chromatography

From depth filtration, 3,930 L of protein solution will be fed at a spike protein concentration of 0.0687 mg/mL to the recirculation tank (ST-402) for the ultrafiltration (UF-401) and diafiltration (DF-401). A hollow fiber membrane will be used with a MWCO of 30 kDa. The Uniflux 400 system will have a total membrane area of 52 m^2 when equipped with 4 UFP-30-C-85 hollow fiber cartridges, each with an area of 13 m^2. For an optimal process, a concentration factor of 20, average flux of 30 LMH, and final volume of 196.5 L will be chosen. The ultrafiltration process will take 2.39 hours, where the protein solution will concentrate from 0.0687 mg/mL to 1.37 mg/mL. The protein recovery yield will be around 99.7% and the rejection coefficient can be assumed to be 0.999.

Continuous diafiltration will be used for our design. We want to remove 99.9% of the previous buffer and replace it with Tris-HCl. The diafiltration volume will be determined to be 1357.37 L and the time for diafiltration will be calculated to be 0.87 hours with a protein recovery of 99.3%. 20 mM of Tris-HCl will be added to the 1357.37 liters of WFI for a mass of 4.278 kgs. The same Uniflux 400 system used for ultrafiltration will be used for this diafiltration step. After diafiltration, 196.5 L of protein solution at a concentration of 1.36 mg/mL will be

transferred to anion exchange chromatography. For both diafiltration and ultrafiltration, a transmembrane pressure of 16 psid will be used as recommended by Cytiva.

4.2.5. Anion Exchange Chromatography

Following diafiltration, 267 g of spike protein suspended in 196.5 L of binding buffer (20 mM Tris-HCl at pH 7.4) will be loaded at a linear flow velocity of 510 cm/hr (32.7 L/hr flow rate) onto a 29.8 cm tall and 9 cm diameter (1.91 L volume) cylindrical column packed with Thermo Scientific™ POROS™ XQ Strong AEX Resin. The anion exchange column (AEX-401) will be operated in bind and elute mode at a temperature of 25 ℃ and a maximum pressure drop of 2.5 bar. Prior to loading the feed solution, the chromatography column will be equilibrated with 5 CV of binding buffer. The loading process will take 6 hrs. After washing the column with 5 CV of binding buffer, the bound spike protein and other positively-charged impurities will be eluted from the column by dropping the pH to 3.5 with 5 CV of 20 mM NaOAc. The spike protein recovery is 80%, so 213.9 g of protein is eluted in the 9.55 L elution buffer. The column will be cleaned by first pumping 3 CV of 1 mM NaCl at a flow rate of 32.7 L/min followed by 3 CV of 1 mM NaOH at the same flow rate.

4.2.6. Viral Inactivation

From anion exchange chromatography, 9.55 L of material will be fed to viral inactivation (JMT-402) at a flow rate of 32.7 L/hr. Viral inactivation will occur at a pH of 3.5 in the Thermo Scientific HyPerforma Single-Use 50 L Jacketed Mixer. To effectively inactivate the virus, the mixer will be maintained at 25℃ for 60 minutes. The mixer will operate at a 141 RPM impeller agitation speed which will require 1.80 W of power. After inactivation is complete, the material will be fed to cation exchange chromatography at a flow rate of 36.6 L/hr and a pH of 3.5.

4.2.7. Cation Exchange Chromatography

Following viral inactivation, the acidic protein solution will be loaded at a linear flow velocity of 535.8 cm/hr (36.6 L/hr flow rate) onto a 31.3 cm tall and 9.3 cm diameter (2.14 L volume) cylindrical column packed with Thermo Scientific™ POROS™ XS CEX resin. The cation exchange chromatography column (CEX-401) will be operated in bind and elute mode at a temperature of 25 ℃ and a maximum pressure drop of 2.5 bar. The equilibration and washing stages will each require 5 CV of 20 mM NaOAc at pH 3.5. To achieve an 80% recovery of the target product, 171 g of spike protein will be eluted from the column in 5 CV of 5 mM $NaPO_4$ at pH 6.8. The column will be cleaned by first pumping 3 CV of 1 mM NaCl at a flow rate of 32.7 L/min followed by 3 CV of 1 mM NaOH at the same flow rate.

4.2.8. Ceramic Hydroxyapatite Chromatography

The eluate from CEX chromatography will be fed at a linear flow velocity of 300 cm/hr (157.8 L/hr flow rate) into a 13 cm tall and 25.9 cm diameter (6.84 L volume) cylindrical column packed with CHT™ Ceramic Hydroxyapatite Type I Media from Bio-Rad Laboratories. The ceramic hydroxyapatite chromatography column (CHT-401) will be operated in bind and elute mode at a temperature of 25 ℃ and a pressure drop of 1 bar. The equilibration and washing steps will require 10 and 5 CV, respectively, of 5 mM $NaPO_4$ at pH 6.8. To achieve an 83% recovery, 141.9 g of spike protein will be eluted with 10 CV (68.4 L) of $NaPO_4$ spanning a linear concentration gradient of 5-100 mM. The column will be cleaned with 5 CV of 500 mM $NaPO_4$ and sanitized with 5 CV of 1 M NaOH. The eluent stream will be fed into a recirculation tank (ST-403) for viral filtration.

4.2.9. Viral Filtration

Following the final chromatography step, the Cytiva ReadyToProcess 750kDa hollow fiber will be used to process 68.4 L for viral filtration (VF-401). This hollow fiber filter has a membrane area of 0.92 m^2. With a flux of 30 LMH, this stage will take 2.35 hours. Recover will be about 95% and a final permeate volume of 65 L and a protein concentration of 2.07 g/L will be sent to the second ultrafiltration stage for concentration.

4.2.10. Final Ultrafiltration and Diafiltration

Following the same filtration designs from before, a hollow fiber membrane will be used and the ultrafiltration and diafiltration will be run using the same equipment. A protein solution volume of 65 L at a protein concentration of 2.07 g/L will be transferred to the recirculation tank (ST-404) for ultrafiltration (UF-402) and diafiltration (DF-402). A molecular weight cut-off of 30 kDa will be used. The filter has a membrane surface area of 1.15 m^2. With a flux of 30 LMH, the time the process will take is 1.8 hours. The protein recovery yield will be around 99.7%.

For the final diafiltration step, the same hollow fiber membrane will also be used. The buffers we will utilize include 150 mM NaCl in WFI and 2.5 mM $NaPO_4$ in WFI with a diafiltration volume of 22.4 L. The initial batch coming into this step will be 3.25 L at a starting protein concentration of 41.4 g/L. The same molecular weight cut-off of 30 kDa will be used here. The filter has a membrane surface area of 1.15 m^2. With a flux of 30 LMH, the time it will take is 0.65 hours and the recovery will end up being around 99.3%.

4.3. Formulation and Filling

4.3.1. Final Formulation

The final formulation for a single vaccine dose will include 0.25 mL of the antigen solution formulation and 0.25 mL of the adjuvant emulsion formulation. When these two

solutions are combined prior to injection, one vaccine dose will contain 10 µg of antigen, 27.5 µg of polysorbate 20, 4.86 mg polysorbate 80, 10.69 mg squalene, 11.86 mg α-tocopherol, 2.5 mg 2-phenoxyethanol, and 0.49976 mL PBS. A total of 4.005 kg antigen, 11.01 kg polysorbate 20, 1946.43 kg polysorbate 80, 4281.35 kg squalene, 4749.93 kg α-tocopherol, 1001.25 kg 2-phenoxyethanol, and 200.15 kL PBS will be needed each year to produce 30 batches each containing 13.35 million vaccine doses.

A total of 6675 L of solution per batch (3.25 L antigen and 6671.75 L PBS) will need to be formulated before filling. This volume will be split into four mixing rounds: two corresponding to the antigen solution formulation and two for the adjuvant emulsion formulation. Therefore, 1668.75 L will be mixed at a time, then that formulation will be sent to vial filling and the next mixing round will begin. Each formulation will be mixed in the 2000 L mixing tank (JMT-501), which will operate at 350 RPM impeller agitation speed which will require 408.7 W of power. The mixer will be maintained between 2 - 8°C, and each formulation will be mixed for 2 hours. It will take a total of 8 hours to formulate each batch.

4.3.2. Filling

After each formulation is mixed, it will be sent to vial filling (FIL-503). The antigen solution and adjuvant emulsion will be produced in two separate vials. Both of these vials will be 10 mL multidose vials filled with enough solution to yield 20 vaccine doses per pair of vials. 1.335 million vials will be needed per batch, and a total of 40.05 million vials will be needed each year.

The glass vials will first undergo vial cleaning (FIL-501) and then vial depyrogenation (FIL-502). Then the glass vials will each be filled with 5 mL of the appropriate formulation and will be closed with a rubber stopper. Next, the filled vials will be closed with crimp caps (FIL-

504), and sent to vial inspection (FIL-505). Once inspected, the approved vials will be loaded onto a tray and transferred to 2 - 8°C storage (REF-501) for up to 6 months. The refrigeration process is beyond the scope of this project. The vial filling process will occur at a speed of 36,000 vials per hour using equipment purchased from Bausch+Ströbel. It will take a total of 37.08 hours to fill each batch.

4.4. Production Schedule

Figure 4.4a shows that it will take 22 days to produce 133.5 grams of antigen and 2 days for formulation and filling of the final product.

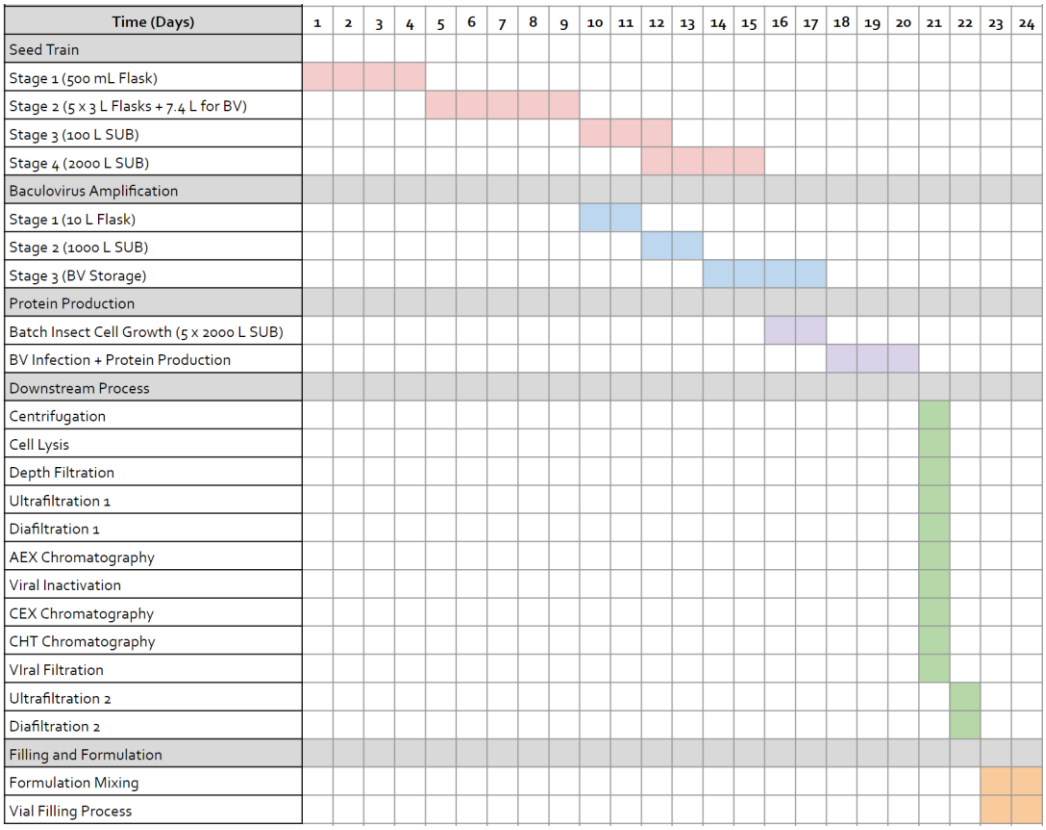


Figure 4.4a. Single upstream and downstream batch

In order to supply 400 million vaccine doses, we aim to produce 4 kg of antigen each year. Each batch will yield 133.5 grams of spike protein, as shown in Table 4.5c. Therefore, we need to produce 30 batches per year. In order to meet this requirement, we will begin the next

batch after 8 days of processing, as shown in Figure 4.4b and Figure 4.4c. These 8 days will provide sufficient time to clean equipment and address any manufacturing problems identified in the previous batch. Assuming 8 days between batches, we will operate 256 days a year. For calculations, see Appendix A.3.

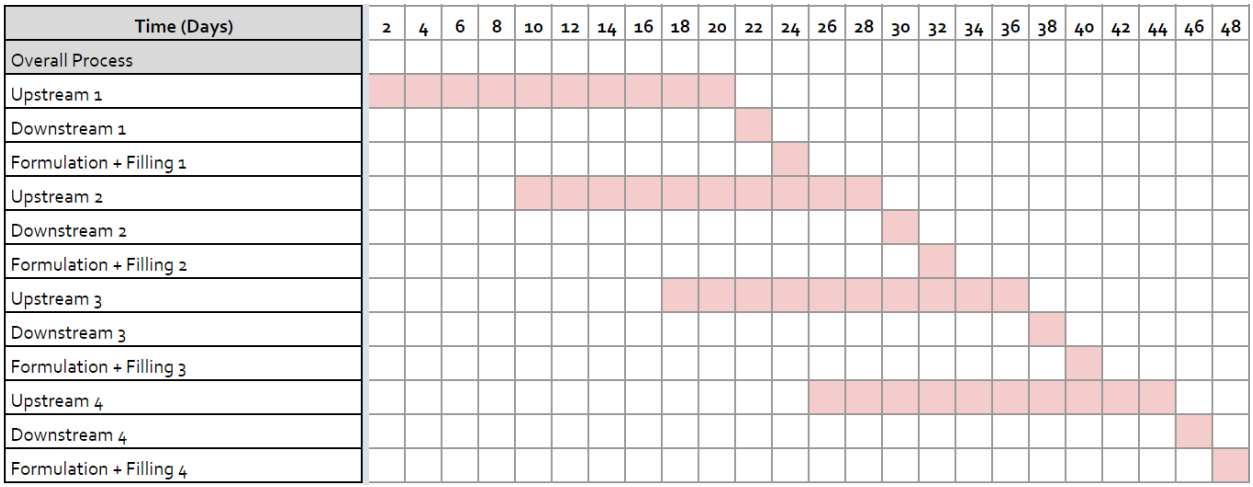


Figure 4.4b. Summarized multiple batch use diagram

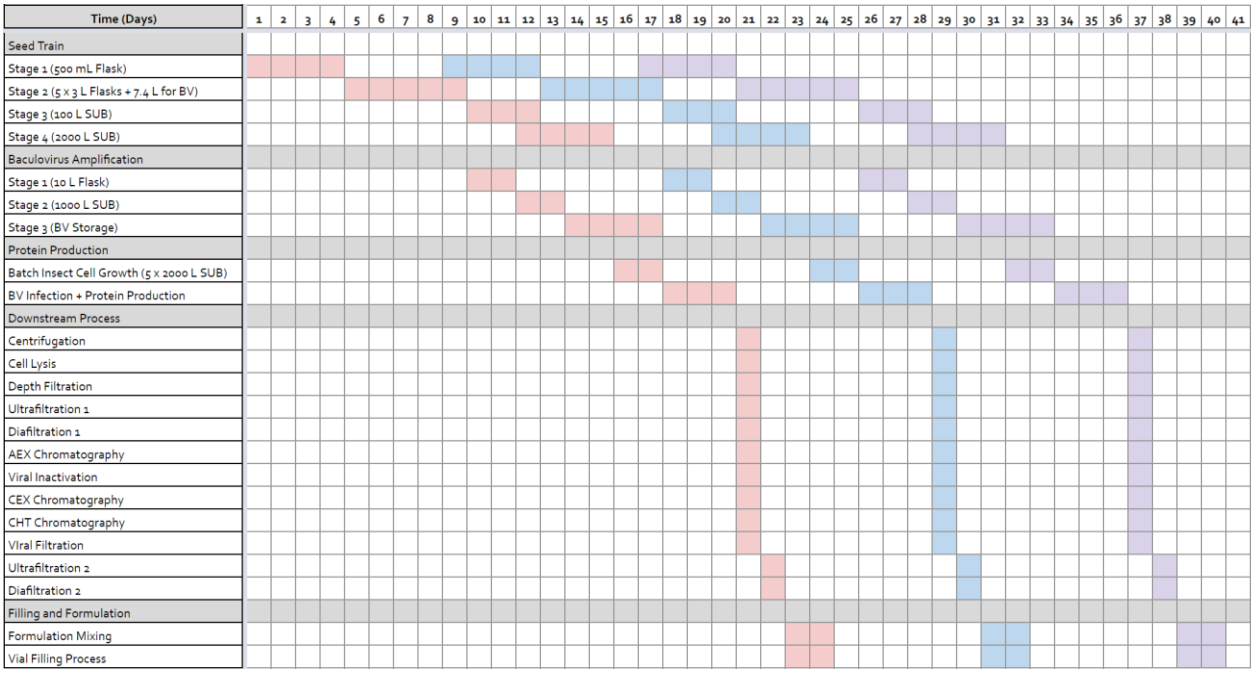


Figure 4.4c. Detailed multiple batches use diagram

4.5. Material Balances

Table 4.5a. Upstream seed train summary

Seed Train					
Stage	**Media Required (L)**	**Glucose Required (kg)**	**Aeration per Reactor (L/min)**	**Final Amount of Cells**	**Total Growth Time (days)**
Stage 0 (Stock)	0	0	0	1.60E+08	0
Stage 1 (500 mL Flask)	0.46	0	0	4.05E+09	4.08
Stage 2 (5 x 3 L Flasks + 7.4L for BV)	21.91	0	0	1.81E+11	4.80
Stage 3 (100 L Bioreactor)	85.00	0.11	2.00	8.10E+11	2.40
Stage 4 (2000 L Bioreactor)	1640.70	1.09	4.00	1.41E+13	3.61
Stage 5 (5 2000 L Production Bioreactor)	8259.26	41.30	4.00	3.00E+13	1.56
Total	**10007.33**	**42.50**		**3.00E+13**	**16.44**

Table 4.5b Upstream baculovirus train summary

Baculovirus Growth		
Stage	**MOI (virus/cells)**	**Time (days)**
Stage 1 (10 L Flask)	0.1	2
Stage 2 (1000 L Bioreactor)	0.1	2
Stage 3 (5 2000 L Production Bioreactor)	2	3
Total		**7**

Table 4.5c. Downstream time and recovery summary

Stage	Time	Antigen Out (g)	Antigen Recovery (%)
From Upstream	-	300.0	-
Centrifugation	5 hr 0 min	300.0	100
Cell Lysis	1 hr 50 min	300.0	100
Depth Filtration	14 min	270.0	90.00
Ultrafiltration 1	2 hr 24 min	269.2	99.70
Diafiltration 1	52 min	267.3	99.31
AEX Chromatography	7 hr 14 min	213.9	80.00
Viral Inactivation	1 hr 0 min	213.9	100.00
CEX Chromatography	1 hr 29 min	171.0	80.00
CHT Chromatography	1 hr 35 min	141.9	83.00
Viral Filtration	2 hr 21 min	134.8	94.98
Ultrafiltration 2	1 hr 47 min	134.4	99.70
Diafiltration 2	39 min	133.5	99.31
Total	**26 hr 25 min**	**133.5**	**44.49**

Table 4.5d. Upstream material balance

Section	Stage	Stream	Inlet or Outlet	Material	Amount	Units
Seed Train	Masterstock	S-101	Outlet	SFM	40	mL
				Cells	9.76E-02	g
	Sf9 Stage 1 (F-101)	S-101, S-102	Inlet	SFM	460	mL
				Cells	9.76E-02	g
		S-103, S-104	Outlet	Solution	500	mL
				Cells	2.47E+00	g
	Sf9 Stage 2 (F-102a-e, F-103)	S-103, S-104	Inlet	SFM	21.91	L
				Solution	500	mL
				Cells	2.47E+00	g
		S-105, S-202	Outlet	Solution	22.4074	L
				Cells	1.10E+02	g
	Sf9 Stage 3	S-105, S-107	Inlet	SFM	85	L
				Solution	15	L
				Glucose	1.10E-01	kg
				Cells	7.44E+01	g
		S-110	Outlet	Solution	100	L
				Cells	3.66E+01	g
	Sf9 Stage 4	S-110, S-112	Inlet	SFM	1640.7	L
				Solution	100	L
				Glucose	1.09E+00	kg
				Cells	4.94E+02	g

		S-115	Outlet	Solution	1740.7	L
				Cells	8.60E+03	g
	Sf9 Stage 5	S-301, S-303a2-e2, S-207	Inlet	SFM	8259.26	L
				Solution	1740.7	L
				Glucose	4.13E+01	kg
				Cell Debris	5.561	mg/L
				Baculovirus	6.00E+13	Virion number
				Cells	4.94E+03	g
		S-306, S-305a-e	Outlet	Solution	10000	L
				Cell Debris	26.384	mg/L
				Cells	1.83E+04	g
BV Train	BV Stage 0	S-201	Outlet	Solution	7.40E-02	L
				Baculovirus	6.00E+09	Virion number
	BV Stage 1	S-201, S-202	Inlet	Cells	3.66E+01	g
				Solution	7.407	L
				Baculovirus	6.00E+09	Virion number
		S-203	Outlet	Cell Debris	62.000	mg/L
				Solution	7.407	L
				Baculovirus	6.00E+11	Virion number
	BV Stage 2	S-203, S-204	Inlet	Cells	3.66E+03	g
				Cell Debris	62.000	mg/L

				Solution	740.740	L
				Baculovirus	6.00E+11	Virion number
		S-207	Outlet	Cell Debris	62.000	mg/L
				Solution	740.740	L
				Baculovirus	6.00E+13	Virion number

Table 4.5e. Downstream material balance

Stage	Inlet or Outlet	Stream	Stream flow (L/min)	Material	Amount	Units
Centrifugation	Inlet	S-401	2.67	Spike Protein	0.300	g/L
				Cell Debris	1.830	g/L
				Cell Solution	10000.000	L
	Outlet	S-403	2.67	Spike Protein	300.000	g
				Cell Debris	18300.000	g
	Waste Outlet	S-402	2.67	Supernatant	10000.000	L
Cell Lysis	Inlet	S-403, S-404, S-405	2.67	Spike Protein	0.164	g/L
				Cell Debris	10.000	g/L
				WFI	1830.000	L
				Chymostatin	11.121	g
				Leupeptin	7.807	g
				Pepstatin A	1.255	g
				PMSF	63.754	g
	Outlet	S-408	2.67	Spike Protein	0.164	g/L
				Cell Debris	10.000	g/L
				WFI	1830.000	L
				Chymostatin	11.121	g
				Leupeptin	7.807	g
				Pepstatin A	1.255	g
				PMSF	63.754	g
Depth Filtration	Pre-wash	S-411	420.00	WFI	2100.000	L

	Inlet	S-410	420.00	Same as Cell Lysis Outlet		
	Outlet	S-413	420.00	Spike Protein	0.148	g/L
				Cell Debris	0.500	g/L
				WFI	1830.000	L
				Chymostatin	11.121	g
				Leupeptin	7.807	g
				Pepstatin A	1.255	g
				PMSF	63.754	g
	Post-flush	S-413	420.00	WFI	2100.000	L
Ultrafiltration 1	Inlet	S-417	26.00	Same as Depth Filtration Outlet		
	Retentate	S-419	0.55	Spike Protein	1.370	g/L
				Cell Debris	2.328	g/L
				WFI	196.500	L
	Permeate	S-418	26.00	Chymostatin	11.121	g
				Leupeptin	7.807	g
				Pepstatin A	1.255	g
				PMSF	63.754	g
				Spike Protein	0.000	g/L
				WFI	1633.500	L
Diafiltration 1	Inlet	S-417	0.55	Same as Retentate above		
	Buffer Inlet	S-415	26.00	WFI	1357.370	L
				Tris-HCl	3.152	g/L
	Retentate	S-419	0.55	Spike Protein	1.360	g/L

				Cell Debris	1.164	g/L
				Tris-HCl	21.773	g/L
				WFI	196.500	L
	Permeate	S-418	26.00	Spike Protein	0.001	g/L
				WFI	1357.370	L
AEX Chromatography	Equilibrate	S-423	0.55	Tris-HCl	3.151	g/L
				WFI	9.540	L
	Load in	S-421	0.55	Spike Protein	1.360	g/L
				Cell Debris	1.164	g/L
				Tris-HCl	21.773	g/L
				WFI	196.500	L
	Load out	S-430	0.55	Cell Debris	1.048	g/L
				Tris-HCl	21.773	g/L
				WFI	196.500	L
	Wash in	S-423	0.55	Tris-HCl	3.151	g/L
				WFI	9.540	L
	Wash out	S-430	0.55	Tris-HCl	3.151	g/L
				WFI	9.540	L
				Cell Debris	1.199	g/L
	Eluate in	S-425	0.55	NaOAc	1.642	g/L
				WFI	9.540	L
	Eluate out	S-431	0.55	NaOAc	1.642	g/L
				WFI	9.540	L

				Spike Protein	22.400	g/L
				Cell Debris	0.060	g/L
	CIP	S-427, S-429, S-430	0.55	NaCl	1.000	mM
				NaOH	1.000	mM
				WFI	11.457	L
Viral Inactivation	Inlet	S-431	0.55	NaOAc	1.642	g/L
				WFI	9.540	L
				Spike Protein	22.400	g/L
				Cell Debris	0.060	g/L
	Outlet	S-432	0.61	Same as Inlet		
CEX Chromatography	Equilibrate	S-434	0.61	NaOAc	1.641	g/L
				WFI	10.687	L
	Load in	S-433	0.61	NaOAc	1.640	g/L
				WFI	9.550	L
				Spike Protein	22.400	g/L
				Cell Debris	0.060	g/L
	Load out	S-439	0.61	Cell Debris	0.054	g/L
				NaOAc	1.640	g/L
				WFI	9.550	L
	Wash in	S-433	0.61	NaOAc	1.641	g/L
				WFI	10.687	L
	Wash out	S-439	0.61	NaOAc	1.641	g/L
				WFI	10.687	L

				Cell Debris	0.003	g/L
	Eluate in	S-438	0.61	NaPO4	0.590	g/L
				WFI	10.687	L
	Eluate out	S-440	0.61	NaPO4	0.590	g/L
				WFI	10.687	L
				Spike Protein	16.000	g/L
				Cell Debris	0.003	g/L
	CIP	S-435, S-436	0.61	NaCl	0.029	g/L
				NaOH	0.020	g/L
				WFI	12.824	L
CHT Chromatography	Equilibrate	S-442	2.6306	NaPO4	0.590	g/L
				WFI	68.396	L
	Load in	S-441	2.6306	NaPO4	0.590	g/L
				WFI	10.687	L
				Spike Protein	16.000	g/L
				Cell Debris	0.003	g/L
	Load out	S-444	2.6306	Cell Debris	0.002	g/L
				NaPO4	0.590	g/L
				WFI	10.687	L
	Wash in	S-442	2.6306	NaPO4	0.590	g/L
				WFI	34.198	L
	Wash out	S-444	2.6306	NaPO4	0.590	g/L
				WFI	34.198	L

				Cell Debris	0.002	g/L
	Eluate in	S-442	2.6306	NaPO4	806.796	g
				WFI	68.396	L
	Eluate out	S-445	2.6306	NaPO4	806.796	g
				WFI	68.396	L
				Spike Protein	2.075	g/L
				Cell Debris	0.000	g/L
	CIP	S-442	2.6306	NaPO4	49.150	g/L
				WFI	41.037	L
	Sanitize	S-428c	2.6306	NaOH	39.997	g/L
				WFI	34.198	L
Viral Filtration	Inlet	S-447	0.46	Same as Eluate out above		
	Permeate	S-448	0.46	Spike Protein	2.075	g/L
				WFI	64.976	L
	Retentate	S-449	0.02	Spike Protein	2.076	g/L
				WFI	3.420	L
Ultrafiltration 2	Inlet	S-454	0.61	Same as Permeate above		
	Retentate	S-456	0.03	WFI	3.249	L
				Spike Protein	41.354	g/L
	Permeate	S-455	0.575	Spike Protein	0.006	g/L
				WFI	61.727	L
Diafiltration 2	Inlet	S-454	0.03	Same as Retentate		
	Buffer Inlet	S-452	0.575	WFI	22.442	L

				NaCl	8.766	g/L
				$NaPO_4$	0.410	g/L
	Retentate	S-456	0.03	Spike Protein	41.077	g/L
				NaCl	60.550	g/L
				$NaPO_4$	2.831	g/L
				WFI	3.249	L
	Permeate	S-455	0.575	Spike Protein	0.040	g/L
				WFI	22.442	L
Antigen Formulation	Inlet	S-501	0.575	Spike Protein	133.500	g
				NaCl	196.700	g
				$NaPO_4$	9.200	g
				WFI	3.250	L
		S-502	0.575	Polysorbate 20	367.125	g
		S-504		PBS	3334.251	L
		S-503	0.575	2-phenoxyethanol	16687.500	g
Adjuvant Formulation	Inlet	S-505		Squalene	142711.500	g
		S-506	0.575	α-tocopherol	158331.000	g
		S-507		Polysorbate 80	64881.000	g
		S-504	0.575	PBS	3337.500	L
		S-503		2-phenoxyethanol	16687.500	g

5. Economics

5.1. Summary

Table 5.1. Summary of first year profits

Description	Net effect
Revenue	$6,000,000,000
Fixed Capital Costs	-$10,000,000
Labor	-$7,411,415
Waste disposal	-$1,477,381
Raw Material Costs	-$133,419,633
Utility Cost	-$21,423
Miscellaneous (taxes)	-$1,219,898,557
Total in first year	**$4,619,659,417**

The yearly revenue is calculated by multiplying the number of doses we are selling each year by the wholesale price of $15, which is in the range of both Pfizer and Moderna COVID-19 vaccines prices (Cao, 2020). Four-hundred million doses multiplied by fifteen dollars yields $6 billion in revenue.

The following sections go into further detail about each cost and how they were calculated. In addition to the cost explanations, payout period and scenarios are discussed as well as the internal rate of return for this project.

5.2. Capital Costs

5.2.1. Fixed Capital Investment

The total cost of equipment to purchase $8,102,125. A summary of the breakdown of equipment costs are located in table 5.2.1a.

Table 5.2.1a. Summary of equipment costs

Total Equipment Cost	
Equipment	**Cost**
Bioreactors	$227,270
Downstream Processing	$406,125
Tanks	$279,140
Filling	$7,000,000
Ancillary	$189,590
Total Equipment Cost	**$8,102,125**

A list of the full equipment costs are located in table 5.2.1b. Equipment costs were estimated using the 2017 CAPCOST software, prices from online vendors, and previous capstone groups (Lin, 2021 and Barton, 2021). The prices from previous capstone projects were adjusted by using the 2021 CEPCI value of 708.

Table 5.2.1b. Total equipment costs

Type of Equipment	**ID**	**Cost**
Pump (Flow)	P-101	$3,445
Pump (Flow)	P-102	$4,080
Pump (Flow)	P-201	$4,080
Pump (Flow)	P-301	$3,445
Pump (Flow)	P-302	$3,445
Pump (Flow)	P-303a	$4,080
Pump (Flow)	P-303b	$4,080
Pump (Flow)	P-303c	$4,080
Pump (Flow)	P-303d	$4,080
Pump (Flow)	P-303e	$4,080

Pump (Flow)	P-401	$7,600
Pump (Flow)	P-404	$4,080
Pump (Flow)	P-420	$4,080
Pump (Flow)	P-501	$3,445
Pump (Applied Pressure)	P-402	$28,300
Pump (Applied Pressure)	P-403	$17,000
Pump (Applied Pressure)	P-405	$14,300
Pump (Applied Pressure)	P-406	$10,900
Pump (Applied Pressure)	P-407	$3,445
Pump (Applied Pressure)	P-408	$3,445
Pump (Applied Pressure)	P-409	$3,445
Pump (Applied Pressure)	P-410	$3,445
Pump (Applied Pressure)	P-411	$3,445
Pump (Applied Pressure)	P-412	$3,445
Pump (Applied Pressure)	P-413	$3,445
Pump (Applied Pressure)	P-414	$3,445
Pump (Applied Pressure)	P-415	$3,445
Pump (Applied Pressure)	P-416	$3,445
Pump (Applied Pressure)	P-417	$4,080

Pump (Applied Pressure)	P-418	$4,080
Pump (Applied Pressure)	P-419	$4,080
Pump (Applied Pressure)	P-421	$14,300
Holding Tanks	MT-101	$17,200
Holding Tanks	MT-301	$86,000
Holding Tanks	ST-404	$1,300
Holding Tanks	MT-401	$17,200
Holding Tanks	MT-402	$17,200
Holding Tanks	ST-401	$4,000
Holding Tanks	MT-403	$15,400
Holding Tanks	MT-404	$15,400
Holding Tanks	MT-405	$15,400
Holding Tanks	MT-406/VI-401	$15,400
Holding Tanks	MT-407	$15,400
Holding Tanks	ST-402	$4,000
Holding Tanks	ST-403	$1,300
Holding Tanks	ST-405	$21,340
Holding Tanks	MT-408	$15,400
Seed Train SUB	SUB-101	$6,670
Seed Train SUB	SUB-102	$32,900
Baculovirus SUB	SUB-201	$22,700
Antigen Production SUB	SUB-301	$165,000
Centrifugation	CEN-401	$37,400
Homogenizer	HOM-401	$260,000
Depth Filter Rack	/	$13,700
Ultrafiltration 1 Machine	UF-401	$59,400

Anion Exchange Chromatography	AEX-401	$11,875
Cation Exchange Chromatography	CEX-401	$11,875
Ceramic Hydroxyapatite Chromatography	CHT-401	$11,875
Vial Cleaning	VCE-501	$1,400,000
Vial Depyrogenation	VDP-501	$1,400,000
Vial Filling	VFG-501	$1,400,000
Vial Closing	VCO-501	$1,400,000
Tray Loading + Vial Inspection	TL-501	$1,400,000
Formulation (Tank)	MT-501	$17,200

Purchased equipment only makes up a small portion (20%) of the fixed capital investment. There are other costs associated with the equipment such as installation, instrumentation, piping, electrical, buildings, and land (Peters et al., 2003). There are other indirect costs that make up the total of the fixed capital investment that include engineering and supervision, construction expenses, legal expenses, contractor's fees, and contingency (Peters et al., 2003). The total fixed capital investment is $40,510,625. A summary of the total fixed capital costs is in Table 5.2.1c.

Table 5.2.1c. Fixed capital investment

Component	**% of Fixed Capital Investment**	**Cost**
Purchased Equipment	20%	$8,102,125
Installation	9%	$3,645,956
Instrumentation	7%	$2,835,744
Piping	6%	$2,430,638
Electrical	4%	$1,620,425

Buildings	12%	$4,861,275
Service Facilities	8%	$3,240,850
Land	1%	$405,106
Engineering and Supervision	12%	$4,861,275
Construction Expenses	8%	$3,240,850
Legal Expenses	1%	$405,106
Contractor's Fees	2%	$810,213
Contingency	10%	$4,051,063
Total Fixed Capital Cost		**$40,510,625**

5.3. Operating Costs

5.3.1. Materials

Thirty batches will be produced per year, and the total materials cost required is $128,712,527. The total materials cost to make one batch is $4,707,107. An extra batch of materials should be purchased to account for material losses during the manufacturing process. Therefore, the total annual cost that will be spent on materials is $133,419,633. Materials required consist of the raw materials such as reagents, solvents, buffers, and resins for chromatography, and single-use materials such as single-use bags, filter cartridges, and vials. For upstream, the cost of glucose, insect cells, and SFM broth were obtained from Thermo Scientific, and the cost of baculovirus was estimated from UPenn (Thermo Fisher, 2014; Adler et al., 2021). For downstream and formulation, the bulk raw material prices were obtained from Thermo Scientific, MilliporeSigma, Cayman Chemicals, and Bio-Rad. Cost for water for injection was obtained from Cytiva. The insect cells and baculovirus will be purchased once and a master stock will be maintained. All other raw materials will need to be replenished after each batch. To

produce 30 batches per year, the mass and volume requirements and corresponding costs of raw materials are shown in Table 5.3.1a.

Table 5.3.1a. Annual raw materials cost

Unit Operation	Material	Quantity per Batch	Units	Price/Unit	Cost per 30 Batches
Upstream	Sf9 insect cells	40.000	mL	$438.67	$17,547
	Thermo Fisher Scientific Gibco Sf-900 II SFM broth	10007.327	L	$72.50	$21,765,935
	AcNPV Baculovirus expressing the SARS-CoV-2 spike protein	74.000	mL	$112.50	$8,325
	Glucose	42.500	kg	$193.51	$246,720
Cell Lysis	Water for injection (WFI)	1830.000	L	$13.04	$715,896
	10 μM Chymostatin	11.121	g	$14,180.00	$4,730,835
	10 μM Leupeptin	7.807	g	$3,450.00	$808,002
	1 μM Pepstatin A	1.255	g	$3,780.00	$142,338
	0.2 mM PMSF	63.754	g	$3.84	$7,344
Depth Filtration	Water for injection (WFI)	4200.000	L	$13.04	$1,643,040
Diafiltration 1	20 mM Tris-HCl	4.278	kg	$338.00	$43,379
	Water for injection (WFI)	1357.370	L	$13.04	$531,003
AEX	Thermo Scientific™ POROS™ XQ Strong AEX Resin	1.910	L	$175,620.00	$335,358
	Water for injection (WFI)	40.101	L	$13.04	$15,687
	20 mM Tris-HCl	60.190	g	$338.00	$610,322
	20 mM NaOAc	15.664	g	$387.00	$181,861
	1 mM NaCl	0.335	g	$0.02	$0
	1 mM NaOH	0.229	g	$0.02	$0
CEX	Thermo Scientific™ POROS™ XS CEX Resin	2.137	L	$11,240.00	$24,024
	Water for injection (WFI)	44.885	L	$13.04	$17,559
	20 mM NaOAc	35.066	g	$387.00	$407,112

	5 mM $NaPO_4$	6.303	g	$0.28	$53
	1 mM NaCl	0.375	g	$0.02	$0
	1 mM NaOH	0.256	g	$0.02	$0
CHT	Bio-Rad Laboratories CHT™ Ceramic Hydroxyapatite Type I Media 40 µm	4308.933	g	$10.63	$45,804
	Water for injection (WFI)	239.400	L	$13.04	$93,653
	$NaPO_4$	2884.297	g	$0.28	$24,181
	1 mM NaOH	1367.813	g	$0.02	$985
Diafiltration 2	Water for injection (WFI)	22.442	L	$13.04	$8,779
	150 mM NaCl	196.700	g	$0.02	$105
	2.5 mM $NaPO_4$	9.200	g	$0.28	$77
Antigen Formulation	Polysorbate 20	367.125	g	$0.62	$6,775
	PBS	3334.251	L	$40.00	$4,001,101
	2-phenoxyethanol	16.688	kg	$67.44	$33,762
Adjuvant Formulation	Squalene	142.712	kg	$402.04	$1,721,272
	α-tocopherol	158.331	kg	$664.00	$3,153,954
	Polysorbate 80	64.881	kg	$125.46	$244,199
	PBS	3337.500	L	$40.00	$4,005,000
	2-phenoxyethanol	16.688	kg	$67.44	$33,762
Total					**$45,625,751**

Single-use materials will be replaced after each batch and are critical in order to maintain a single-use process. Costs of shake flasks and filters are from MilliporeSigma and Cytiva. Costs of the BioProcess Containers were estimated from prices listed by ThermoScientific. Costs of and single-use mixing bags and single-use tank bags were calculated from *BioProcess Design and Economics* (Petrides, 2015). Material costs for filling were obtained from MilliporeSigma.

Peristaltic pump tubing cost was obtained from a 2020 capstone (Xu et al., 2020). To produce 30 batches per year, the single-use material requirements and corresponding costs are shown in Table 5.3.1b.

Table 5.3.1b. Annual single-use material cost

Unit Operation	Material	Quantity per Batch	Units	Price/Unit	Cost per 30 Batches
Upstream	500 mL Shake Flask	1	Flask	$28.80	$864
	3 L Shake Flask	5	Flask	$65.00	$9,750
	10 L Shake Flask	1	Flask	$160.00	$4,800
	100 L BioProcess Container	1	BPC	$590.00	$17,700
	1000 L BioProcess Container	1	BPC	$1,328.00	$39,840
	2000 L BioProcess Container	6	BPC	$2,148.00	$386,640
Cell Lysis	2000 L Single-Use Mixer Bag	1	Bag	$2,083.00	$62,490
Depth Filtration	Millipore's Millistak+® CE50 Pod Depth Filters	30	Filter	$808.00	$727,200
Ultrafiltration 1	Cytiva UFP-30-C-85 Hollow Fiber Cartridge	4	Cartridge	$9,583.00	$1,149,960
Viral Inactivation	50 L Single-Use Mixer Bag	1	Bag	$549.00	$16,470
Viral Filtration	Cytiva ReadyToProcess 750kDa Hollow Fiber Cartridge	1	Cartridge	$4,648.00	$139,440
Ultrafiltration 2	Cytiva UFP-30-C-85 Hollow Fiber Cartridge	4	Cartridge	$9,583.00	$1,149,960
Formulation	2000 L Single-Use Mixer Bag	1	Bag	$2,083.20	$62,496
Filling	10 mL Vials	1,335,000	Vial	$1.28	$51,264,000
	Vial Rubber Stoppers	1,335,000	Stopper	$0.47	$18,743,400
	Vial Crimp Caps	1,335,000	Cap	$0.21	$8,210,250
Mixing Tanks	50 L Single-Use Mixer Bag	4	Bag	$549.00	$65,880
	500 L Single-Use Mixer Bag	1	Bag	$1,153.20	$34,596
	2000 L Single-Use Mixer Bag	7	Bag	$2,083.20	$437,472
Storage Tanks	100 L Single-Use Tank Bag	2	Bag	$421.60	$25,296
	2000 L Single-Use Tank Bag	1	Bag	$1,277.20	$38,316
	4000 L Single-Use Tank Bag	1	Bag	$2,219.60	$66,588
	10000 L Single-Use Tank Bag	2	Bag	$5,046.80	$302,808

Pumps	Peristaltic Pump Tubing	640	ft	$6.80	$130,560
Total					**$83,086,776**

5.3.2. Utilities

To power process equipment, electricity will be sourced from Eskom - the largest electricity public utility in Africa - at a rate of $0.092 per kWh (MyBroadband, 2021). The annual electrical power supply requirement of the manufacturing facility is organized by equipment in Table 5.3.2a. The total annual electricity cost is $21,065.

Table 5.3.2a. Annual electricity consumption costs

Equipment	Annual Electrical Power Requirement (kWh)	Annual Cost
Bioreactors	151301	$13,920
Mixers	75125	$6,911
Pumps	761	$70
Centrifuge	1140	$105
Homogenizer	412	$38
TFF System (Main Power)	225	$21
Total	**228964**	**$21,065**

To maintain optimum process temperatures, coolant will be circulated through the jackets surrounding the bioreactors and mixing tanks. The cost of each coolant is based on the process heat duty, which is discussed in Section 3.4.3. Chilled water at 5 ℃ will be sourced from a public utility at a price of $4.77 per GJ, and a 50% aqueous ethylene glycol solution at -7 ℃ costs $6.56 per GJ (Turton et al., 2018). The annual cooling requirement per jacketed vessel was determined by multiplying the heat duty (Tables 3.4.3b and 3.4.3c) by the total operation time across 30 annual batches, and these calculated values are presented along with the cost for each unit operation in Table 5.3.2b. The total annual cost to cool these vessels is $280.

Table 5.3.2b. Annual cooling requirement and cost per unit operation

Unit Operation	Coolant	Annual Cooling Requirement (GJ)	Annual Cost
100 L Seed Train Bioreactor	Chilled Water (5 ℃)	0.0556	$0.265
2000 L Seed Train Bioreactor	Chilled Water (5 ℃)	1.4726	$7.024
1000 L BV Amplification Bioreactor	Chilled Water (5 ℃)	0.4168	$1.988
5x2000 L Production Bioreactor	Chilled Water (5 ℃)	20.1039	$95.896
2000 L Sf-900™ II SFM Preparation for Seed Train	50% EG (-7 ℃)	10.0868	$66.169
5x2000 L Sf-900™ II SFM for Production	50% EG (-7 ℃)	16.0743	$105.448
2000 L Post-Centrifugation Resuspension	50% EG (-7 ℃)	0.1376	$0.903
50 L Viral Inactivation	Chilled Water (5 ℃)	0.0002	$0.001
2000 L Formulation	50% EG (-7 ℃)	0.3531	$2.316
Total		**22.05 (CW); 26.65 (EG)**	**$280.010**

Compressed, pure oxygen at 3.3 barg and 27 ℃ will be purchased for fermentation from an off-site vendor at a cost of $0.005 per std m^3 (Turton et al., 2018). Between the eight single-use bioreactors that will be used in this facility, 15700 std m^3 of oxygen will be required annually for a total cost of $79. The oxygen consumption and cost per bioreactor are summarized in Table 5.3.2c.

Table 5.3.2c. Annual oxygen consumption and cost per bioreactor

Equipment	Annual Oxygen Consumption (std m^3)	Annual Cost
SUB-101 (1x100 L)	647.10	$3.24
SUB-102 (1x2000 L)	1948.85	$9.74
SUB-201 (1x1000 L)	810.42	$4.05
SUB-301 (5x2000 L)	12307.99	$61.54
Total	**15714.36**	**$78.57**

The final major utility cost is associated with waste disposal. The downstream process will generate a total of 17,314 kg of aqueous liquid waste per batch. Flasks, single-use bags, impellers, TFF membrane filters, and pump tubing constitute the 7,309 kg of solid waste produced per batch. Both the liquid and solid waste come into contact with infectious species and caustic chemicals. Therefore, they are both classified as hazardous waste and disposal measures should be devised in accordance with guidelines established by the South African Department of Environmental Affairs. Turton et al. (2018) provides an upper limit disposal cost of $2 per kg of hazardous waste. Therefore, the total cost of disposing of the liquid and solid waste produced in one batch is $49,246, and the annual cost of disposal of $1,477,381.

The total annual cost of utilities, excluding expenses for powering facility lighting and HVAC systems, is $1,498,804 kg.

5.3.3. Labor

Labor costs were estimated using eq. 8.3 from Turton et al. (2018).

$$N_{OL} = (6.29 + 31.7P^2 + 0.23N_{np})^{0.5} \quad (5.3.1a.)$$

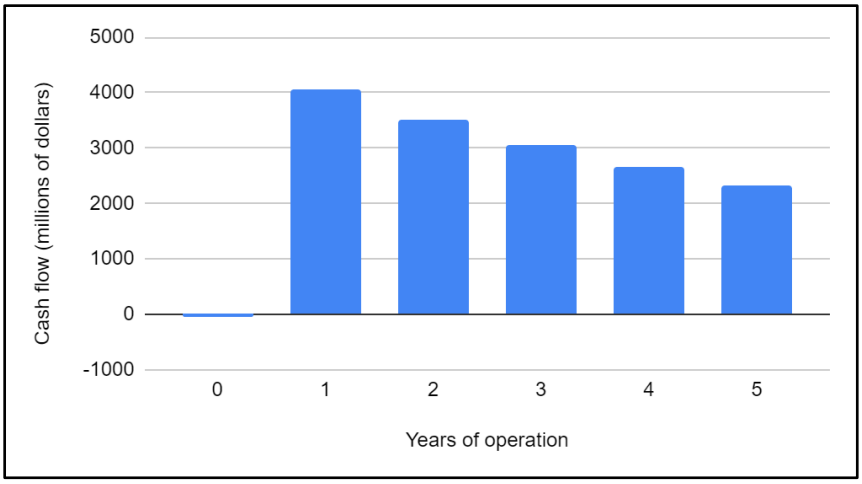
where N_{OL} represents the number of operators required to run the process unit per shift, P is the number of processing steps involving the handling of particulate solids, and N_{np} is the number of nonparticulate processing steps. In total, our manufacturing plant will have six particulate

processes and twenty-three non-particulate processes, as shown in Appendix 7. The number of operators required per shift under these conditions was determined to be 33.95. Turton et al. (2008) estimate that the total number of operators required to run our facility should be 4.5 times the number of operators needed in the plant at any given time. Multiplying these values and rounding up to the nearest integer yields a total of 153 operators (Turton, 2018). In Cape Town, South Africa, the mean annual wage for chemical plant and system operators is $20,000 (Salary Expert, 2022). Including a 30% fringe benefit rate, the total labor costs for operators is $3,640,743 a year. Peters (2003) estimates that the cost of administrative, engineering, and support personnel is 10-20% of the cost of operating labor. This averages to 15% of the cost of operating labor, yielding an annual supervisory and clerical labor cost of $546,111. Lastly, Peters (2003) estimates that the cost of maintenance and repairs (including both personnel and materials) is between 2-10% of the fixed-capital investment, with 7% being a reasonable value to assume. This calculation gives an annual maintenance cost of $2,836,715 (Peters, 2003). Therefore, the total annual labor cost is estimated to be $7,411,415.

5.4 Payout and Scenarios

The net present value (NPV) of this BEVS SARS-CoV-2 manufacturing plant is $15.6 billion dollars over the lifespan of 5 years. This means our plant will generate a $15.6 billion return on investment at an interest rate of 15% every year, taking into account the time value of money. To calculate this NPV, we split up the initial fixed capital investment of $41 million dollars into $8 million dollars per year in accordance with tax laws. The purpose for this tax law is to prevent companies from writing off all of their capital investment in the first year, so that the government can tax the company more over the lifespan of the plant. Figure 5.4.1 illustrates the discounted cash flow over the lifespan of the plant.

Figure 5.4.1. Discounted cash flow over the lifespan of the plant



The payout period for initial capital investment is 24 days into full plant operation, after the first batch has been produced, filled, and sold. Assuming a wholesale price of $15 per dose, one batch produces $195 million dollars in profit, paying in surplus the initial investment of $41 million dollars. This means our plant is extremely profitable, and investors will obtain their return on investment within the first year of plant operation. Refer to A.5 for detailed tables of Internal Rate of Return and Cash Flow calculations.

Many scenarios have been considered for the commercial viability of this plant. As of writing this, infection rates of COVID-19 have dropped significantly after the distribution of vaccines, most notably of mRNA technology, across rich nations. Nevertheless, Africa's vaccination rates still are low, meaning that we can still serve a large customer base in Africa. The first scenario to adjust to a poorer customer base is to reduce our wholesale vaccine to $1.5 per dose, 10% of the original price. Assuming we still sell 400 million doses annually, the first year profit is $350 million dollars, well above the initial capital investment of $41 million. This means our plant is still commercially viable, while still disseminating a vaccine that is sorely needed. The second scenario is that we will only sell 40 million doses annually, 10% of what we intended to sell. At $15 dollars a dose, this scenario will also give us a profit of $350 million

dollars, returning initial investment in the first year. The third scenario is that the raw materials, both equipment and chemical related, will cost twice as much. Given the high demand on single-use equipment, this scenario may be very likely. The total profit of the first year in this scenario is $4.5 billion dollars in the first year, which is similar to the control in Table 5.1. Whether it is a reduction in demand, price of dose, or affordability of materials, our plant will be very profitable. The fourth scenario is to lower our vaccine to the lowest possible price and still receive a 20% internal rate of return on investment. We found that price to be 39 cents per dose. For detailed calculations, refer to A.6.

6. Regulatory, Environmental, Health, and Safety Concerns

6.1 Regulatory Requirements

To ensure the final vaccine product is consistently high in quality, safety, and efficacy, from batch to batch, for its intended use, the manufacturing facility will comply with current Good Manufacturing Practices (cGMP) enforced by the South African Health Products Regulatory Authority (SAHPRA). Rather than relying on inspections or complaints to capture deviations or risks, a strong quality management system will be integrated into the manufacturing process through the use of documentation (batch sheets); validations of raw and in-process materials, systems equipment, and utilities; regular Kaizen events to identify and develop strategies for process improvement; and management of change (MOC) protocols. SAHPRA also emphasizes the importance of periodic retraining of all facility personnel in order to reinforce established skills and introduce new ones that are pertinent to the execution of their assigned functions (South African Health Products Regulatory Authority, 2019). Maintaining a cGMP facility will not only keep the plant workers safe but will also protect the patients receiving the vaccine.

6.2. Environmental Concerns and Waste Management

The environmental impact of the facility results from the consumption of WFI and the generation of waste. WFI production requires large amounts of energy that may leave a significant environmental footprint. The waste from this vaccine manufacturing process comes into contact with viral particles, cell debris, and caustic CIP solutions; therefore, it is classified as hazardous. Complying with local waste management guidelines will help to prevent the release of toxic chemicals and genetically modified organisms (GMOs) into the environment where they can disrupt the native flora and fauna.

WFI Consumption

Like most biomanufacturing plants, this facility needs a large amount of WFI for formulating media, buffers, and the final drug product as well as for cleaning the process equipment. WFI consumption is organized by unit operation in Table 6.2a. The total amount of WFI needed per batch is 24414 L, which translates to 183100 kg of WFI per kg of antigen. For reference, this water consumption ratio is 46 times higher than that for the large-scale manufacture of a monoclonal antibody (Idris et al., 2016). Due to a lack of confidence in emerging membrane-based purification technologies, most pharmaceutical manufacturers opt for distillation to produce WFI. These distillation processes consume a lot of energy and consequently may contribute to environmental pollution. To reduce the environmental impact of this vaccine manufacturing process with respect to WFI consumption, the water should be sourced from a vendor employing an energy-efficient purification process, such as vapor compression as opposed to multiple-effect distillation. Concentrating the aqueous protein solution earlier in the downstream process would also help to reduce the WFI requirements for many of the filtration and chromatography processes.

Table 6.2a. WFI requirements per batch

Process Unit	Per-batch Volume (L)
Fermentation	10008
Resuspension of cell pellet before homogenization	1830
Depth Filtration Washing + Flushing	4200
Diafiltration for Anion Exchange Chromatography	1357
Anion Exchange Chromatography	40
Cation Exchange Chromatography	45
Ceramic Hydroxyapatite Chromatography	239
Final Diafiltration	22
PBS for Formulation	6672
Per-batch Total Volume	**24414**

Liquid Waste

No liquid waste is produced in the upstream process. During downstream processing, a total of 17314 L of liquid waste is produced per batch as shown in Table 6.2b. This liquid waste is classified as hazardous according to South African national standards (South African Department of Environmental Affairs and Development Planning, 2006). Two 10,000 L single-use storage tanks will be used to capture this liquid waste during each production campaign. These tanks will be resistant to corrosion, labeled as biohazardous, and locked once filled. In an effort to protect groundwater reserves from toxic leachates, the South African Department of Forestry, Fisheries, and the Environment (DFFE), as of August 2019, prohibits the disposal of liquid waste at landfills (United Nations Environment Programme, 2020). Therefore, liquid

waste collection, treatment, and disposal will be outsourced to an authorized service in the community, such as the Vissershok Hazardous Waste Management Facility in Cape Town.

Table 6.2b. Liquid waste produced per batch

Source	Content in Waste Stream	Volume Produced (L)
Centrifugation	Fermentation broth	10000.00
Depth Filtration	Contaminated WFI	2100.00
Ultrafiltration/ Diafiltration 1	Protein solution in WFI + protein solution in diafiltration buffer 1	4894.37
AEX	Equilibration, feed, washing, and cleaning buffers	30.55
CEX	Equilibration, feed, washing, and cleaning buffers	34.20
CHT	Equilibration, feed, washing, cleaning, and sanitizing buffers	171.00
Viral Filtration	Protein solution remaining in recirculation tank	3.42
Ultrafiltration/ Diafiltration 2	Protein solution in NaPO4 + protein solution in diafiltration buffer 2	80.92
Total		**17314**

Solid Waste

Flasks, single-use systems used for fermentation and mixing, impellers, TFF membrane filters, and peristaltic pump tubing are considered solid biohazardous waste because they are exposed to a genetically modified viral vector and other adventitious agents that may be carried in the virus-infected insect cell culture (Stacey & Possee, 1996). The total solid waste produced per campaign is shown in Table 6.2c. The solid waste will be collected in biohazard bags, and, like the liquid waste, dispatched to an off-site facility for treatment and disposal. Incineration is the dominant method of treating solid bioprocess waste in South Africa (Cairncross & Nicol, 2005). In addition to greatly reducing the volume and toxicity of waste that must be discarded in landfills, this disposal option provides a return on investment in the form of the usable energy

that is recovered by the combustion process (Disposals Subcommittee of the Bio-Process Systems Alliance, 2008).

Table 6.2c. Solid waste produced per batch

Item	Quantity/Batch	Mass (kg)/Unit	Total Mass (kg)/Batch
500 mL Shake Flask	1	0.35	0.35
3 L Shake Flask	5	0.89	4.45
10 L Shake Flask	1	3.62	3.62
100 L S.U.B BPC	1	12.5	12.50
1000 L S.U.B. BPC	1	125	125.00
2000 L S.U.B. BPC	6	250	1500.00
50 L S.U.M. BPC	5	6.25	31.25
100 L ST BPC	2	12.5	25.00
500 L S.U.M. BPC	1	62.5	62.50
2000 L S.U.M. BPC	9	250	2250.00
2000 L ST BPC	1	250	250.00
4000 L ST BPC	1	500	500.00
10000 L ST BPC	2	1250	2500.00
Depth Filters	30	0.85	25.50
UF1/DF1 Filters	4	0.25	1.00
Viral Filters	1	0.25	0.25
UF2/DF2 Filters	4	0.25	1.00
Peristaltic Tubing	640 ft	0.0252 kg/ft	16.13
Total			**7308.55**

Gaseous Waste

During upstream fermentation, carbon dioxide gas is produced as a waste by-product of aerobic metabolism. In order to prevent dissolved carbon dioxide from accumulating in the culture medium and inhibiting Sf9 cell growth and protein production, oxygen will be sparged into the bioreactors to strip the excess carbon dioxide. To reduce greenhouse gas emissions, it is

standard industrial practice to vent the exhaust gas back into the atmosphere after the stream has passed through a filter (Adler et al., 2021). This gas contains negligible amounts of carbon dioxide and thus does not pose any threats to human health.

6.3. Safety and Health

To prevent the introduction of contaminants into the facility and the escape of infectious agents into the surrounding environment, a detailed hygiene program will be implemented. Although a viral vector system is being used to produce the SARS-CoV-2 spike protein, the recombinant baculoviruses only infect insect cell lines and thus do not pose any inherent risks to humans (Airenne et al., 2013). Therefore, the facility will be operated under Biosafety Level 1 (BSL-1) conditions. All persons (including visitors, maintenance staff, senior management, and inspectors) entering production areas must wear personal protective equipment (PPE) appropriate to the environment and the tasks being conducted. Placards will be posted at entrances to different areas of the facility to communicate the local PPE requirement. The minimum PPE requirement for all production areas includes a disposable lab coat, gloves, and safety glasses. Protective garments should never be worn outside the facility premises and must be discarded after every use.

In addition to mandatory PPE, the construction and layout of the facility will serve to balance cGMP and biosafety guidelines. Rooms will be equipped with sinks for hand washing. Ceilings, walls, and floors should be coated in a smooth, hard finish that can be easily cleaned. All systems equipment will be equipped with alarms and automatic shutdown procedures. To prevent cross-contamination or mix-ups between different process components, unit operations should be performed in separate and defined areas of adequate size. Several operations in the facility will be conducted under aseptic conditions, including the initial cell propagation steps

and the vial filling procedure. These areas will be ventilated by an air supply filtered through high-efficiency particulate air (HEPA) filters under positive pressure relative to surrounding corridors and adjacent production areas (Halkjær-Knudsen, 2007). These aseptic areas will only be accessible through a double door airlock system.

Apart from the biohazards, there are several chemical hazards that must be managed. The health risks posed by caustic NaOH CIP solutions and the strongly acidic acetate buffer used for chromatography processing and viral inactivation warrant the implementation of appropriate safety controls. All relevant chemicals used throughout the manufacturing process and their associated risks are summarized in Table 6.3a. MSDS documentation for these chemicals should be posted on the production floor where it will be easily accessible to plant operators.

Table 6.3a. Chemical hazard information

Chemical Name (linked to MSDS)	**OSHA PEL-TWA (mg/m^3)**	**Safety Concerns**
Sf-900™ II SFM	N/A	No known hazards
Glucose	N/A	No known hazards
Tris-HCl	N/A	Irritating to eyes, respiratory system, and skin. Incompatible with bases and strong oxidizing agents.
Sodium Acetate	N/A	Irritating to eyes, respiratory system, and skin. May be combustible at high temperatures.
Sodium Phosphate	N/A	Incompatible with strong acids and strong bases. Avoid excessive heating.
Sodium Chloride	N/A	Mildly irritating to eyes, respiratory system, and skin. Incompatible with strong oxidizing agents and strong acids.
Sodium Hydroxide	2	Severely irritating to skin, eyes, and mucous membranes. Causes second- and third-degree burns on short contact. Toxic by ingestion. Corrosive to metals and tissue. Unstable at elevated temperatures and

		pressures.
Chymostatin	N/A	Incompatible with strong acids, bases, and oxidizing agents. Combustible solid.
Leupeptin	N/A	No known hazards
Pepstatin A	N/A	No known hazards
PMSF	N/A	Causes severe skin burns and eye damage. Toxic if swallowed. Incompatible with acids, strong oxidizing agents, and strong bases.
Polysorbate 20	N/A	Ingestion may cause irritation of the GI tract. Incompatible with strong oxidizers.
Polysorbate 80	N/A	Mild skin and eye irritation. Incompatible with bases, heavy metal salts, and strong oxidizing agents.
PBS	N/A	May cause irritation to eyes, skin, respiratory and digestive tracts. Incompatible with strong acids and strong bases.
2-Phenoxyethanol	N/A	Harmful if swallowed. Causes serious eye irritation. Incompatible with strong oxidizing agents, acid anhydrides, and acid chlorides.
Squalene	N/A	May be fatal if swallowed and enters airways. Incompatible with strong oxidizing agents.
α-tocopherol	N/A	May cause an allergic skin reaction
Water for Injection	N/A	No known hazards
Pure oxygen	N/A	May cause or intensify fire, and the container may explode if heated

To design an inherently safer process with respect to the storage of hazardous chemicals, a minimization strategy will be employed such that no more than a month's worth of raw materials will be stored on-site at any given time. Minimizing chemical inventory reduces the risk of a toxic release, contamination with the process, and exposing reactive chemicals to incompatible materials. All storage tanks will be situated within dikes to contain spills, and the

tanks containing potentially reactive chemicals will be located sufficiently apart to avoid accidental mixing. Each room of the production area will be equipped with emergency eyewash and shower stations to mitigate the adverse consequences of chemical exposures. Operators handling the strong base NaOH and the strong acid NaOAc during the downstream diafiltration, chromatography, and viral inactivation operations will be required to wear a chemical apron and goggles with a full face shield. The tightly-controlled ventilation in the facility should keep airborne NaOH levels below the permissible exposure limit (2 mg/m^3 is the PEL enforced by OSHA), so respiratory protection is not necessary.

7. Societal Impact

Our manufacturing facility will be located in Cape Town, South Africa. The site would provide 153 jobs as operators. More jobs would be created for construction of the facility. Over $1.2 billion in revenue as taxes over a period of 5 years will be earned by the government of South Africa. This site will serve as a manufacturing and distribution hub of COVID-19 vaccines for the African continent. In total, 400 million doses of our recombinant SARS-CoV-2 recombinant protein vaccine will be manufactured and distributed to countries within Africa each year. The societal impact of this is significant as only 11% of the population within Africa has been vaccinated as of February, 2022 (United Nations, 2022). Establishing vaccine production in an African country will help to reduce the vast inequities in global vaccine distribution. It is necessary to reach the WHO's target for 70% of the global population to be vaccinated in order to help prevent the emergence of viral variants and reduce the burden of the pandemic on economies and healthcare systems around the world (WHO, 2021c). Furthermore, from an ethical standpoint, the population within Africa are just as deserving of vaccinations against this disease that other people around the world have received. This manufacturing facility is a step

towards that goal. Another important impact is the development of a localized biotechnology and pharmaceutical industry that involves the training of workers with cGMP's and sterile techniques. Currently, more than 80% of Africa's pharmaceutical consumables are imported from other countries (Byaruhanga, 2020). By establishing this site, countries in Africa can become more self-sufficient in providing pharmaceutical products for its domestic population. This will not only help the African continent rapidly respond to disease outbreaks in the future but will also facilitate the rollout of improved vaccines for diseases such as Malaria

8. Conclusions and Recommendations

As COVID-19 continues to spread around a world marked by massive inequalities in vaccine provision, increasing vaccine production capacity remains as urgent as ever. Drawing inspiration from an established vaccine manufacturing process, a biopharmaceutical production facility has been designed that will produce 400 million doses per year of an adjuvanted, recombinant spike protein-based SARS-CoV-2 vaccine using the BEVS platform. The facility will achieve an IRR of $15.6 billion at 15% interest rate. With a fixed capital investment of $41 million, a payout can be expected starting after just 24 days of operating the plant. Charging $15 per dose of vaccine will result in $6 billion in revenue. Accounting for fixed capital costs, operating costs (labor, materials, utilities), and other taxes, our net profit after the first year will be $4.6 billion. Thus, the project is extremely profitable and we recommend the building of this facility to increase the availability of COVID-19 vaccines in the African continent. Nevertheless, if we were to switch our strategy to sell our vaccine at the lowest possible price and still reach 20% internal rate of return on investment, then we would price our vaccine at $0.39 per dose.

The facility will use single-use bioreactors to produce 0.3 kg of intracellular spike protein per batch in baculovirus-infected Sf9 insect cells. Downstream processing will have an overall

recovery of 44.5%, yielding 0.1335 kg of purified and formulated spike protein per batch. Between the upstream, downstream, formulation and filling processes, a single batch takes 24 days to complete. To successfully meet our annual production target, 30 batches will be completed each year, with each manufacturing campaign staggered eight days apart. The facility will be operational for 256 days each year, leaving more than enough time to accommodate out-of-spec batches, malfunctioning equipment, and other process deviations.

Many of the unit operations in this process were designed based on experimental data provided in equipment manuals and outdated research. We recommend conducting laboratory experiments to determine the spike protein's specific biochemical interactions with the downstream processing technologies we have decided to use, such as chromatography resins and membrane filters. Additionally, our cell growth model neglected the effects of substrate consumption and protein production on the specific growth rate. Therefore, running tests to acquire the data to accurately model uninfected and infected Sf9 cell growth kinetics would be valuable. This additional kinetic data would also permit the design of a fed batch fermentation process, which would supply the nutrients to support higher levels of protein production than the batch operation implemented in the present design. One of the major environmental impacts of this facility is the significant amount of WFI required, especially for fermentation. Replacing several of the shake flask and small-scale bioreactor steps in the seed train with a single-use WAVE bioreactor system may help to reduce both WFI and electricity consumption

The BEVS manufacturing process has already proven useful in developing vaccines for influenza, HPV, and now COVID-19, and its many advantages can be exploited to produce vaccines for other diseases. With the goal of trying to help low-income countries, this will

provide greater vaccine distribution to those countries who lack access to cold-chain infrastructure and biopharmaceutical production capacity.

9. Acknowledgements

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11. Appendix

A.1 Kolmogorov Eddy Length Scale

The turbulent flow produced by the agitation in the bioreactors may generate eddies which can damage or even kill the cells. The Kolmogorov eddy length scale calculates the smallest eddies present in the flow. The Kolmogorov eddy size model states that eddies that are smaller than the cell diameter can cause damage. Therefore, this value was calculated to ensure we operate the bioreactors in such a way that eddies are larger than the Sf-9 cell diameter: 30 μm (Üstün-Aytekin et al., 2013). The Kolmogorov eddy length scale is used to determine if the specified agitation rate is safe to use. This length scale was calculated using the following equation:

$$\eta = (\frac{\nu^3}{\varepsilon})^{1/4} \quad \text{(A.1)}$$

where ν is the kinematic viscosity of the culture medium and ε is the energy dissipation rate (m^2/s^3) which is the ratio of agitation power (P) to the spherical mass of the media that directly contacts the impeller.

A.2 Homogenous Mixing Time

The homogeneous mixing time indicates how long the cell culture suspension needs to be stirred, at the specified agitation rate, in order to achieve 95% homogeneity. A mixing time of less than 1 minute is good since a short time indicates better mixing properties of the impeller and a balance of shear force within the reactor (Godoy-Silva et al., 2010). The mixing time was calculated using the following equation:

$$\theta_m = 3.3 \cdot [N^{-1} N_P^{-1/3} (D_T/D_i)^{2.43} \quad] \quad \text{(A.2)}$$

where N is the agitation rate, N_p is the impeller power number, D_T is the tank inner diameter, and D_i is the impeller diameter. The above equation applies when the batch aspect ratio (H_L/ D_T) is

greater than 1. The liquid height, H_L, was calculated using the ratio of the maximum liquid height to the maximum fluid volume.

A.3 Production Schedule calculations

- To produce 4 kg of antigen per year, we will need to produce 30 batches each year. We plan to start a batch after every 8 days. Therefore, as shown below, we will require 256 days of manufacturing.
    - $Days\ operating\ per\ year = 16 + 8 * (\#\ of\ batches)$ $256\ days$ $= 16 + 8 * (30\ batches)$

A.4 Additional Cost Analysis

Table A.4a. Compressed oxygen consumption per bioreactor

Equipment	Quantity	Aeration Rate (L/min)	Operation Time (hr)	Volume (m^3)	Pressure (barg)	Temperature (°C)	STD Volume (m^3)	Cost Per Batch	Annual Cost
SUB-101 (100 L)	1	2.00	57.49	6.90	3.30	27.00	21.57	$0.11	$3.24
SUB-102 (2000 L)	1	4.00	86.57	20.78	3.30	27.00	64.96	$0.32	$9.74
SUB-201 (1000 L)	1	3.00	48.00	8.64	3.30	27.00	27.01	$0.14	$4.05
SUB-301 (2000 L)	5	4.00	109.35	131.22	3.30	27.00	410.27	$2.05	$61.54

Sample calculation: Converting m^3 to std m^3

$$V_{STD} = V(\frac{P}{P_{STD}})(\frac{T_{STD}}{T}) \quad \text{(A.4)}$$

where Turton et al. (2018) defines standard pressure (P_{STD}) as 1.013 bar and standard temperature (T_{STD}) as 15 °C. For SUB-101, the std volume of oxygen consumed per batch is computed as follows:

$$V_{STD} = (6.90\ m^3)(\frac{3.30\ bar}{1.013\ bar})(\frac{15\ +\ 273.15\ K}{27\ +\ 273.15\ K}) = 21.57\ std\ m^3$$

A.5 Rate of Return and Cash Flow

Table A.5a. Rate of return

Interest:	15.00%			
Year	**Net after-tax cash flow (millions)**	**cum cash position**	**Discount factor**	**Present-Value of cash flow**
0	-40.00	-40	1	-40
1	4660	4,620	0.87	4052.17
2	4660	9,280	0.76	3523.63
3	4660	13,940	0.66	3064.03
4	4660	18,600	0.57	2664.37
5	4660	23,260	0.50	2316.84
			Net present value:	**15581.04276**

Table A.5b. Cash flow

Year	**Investment (millions)**	**Revenue**	**Expenses**	**Gross Profit**	**Taxes**	**Net After-tax cash flow**
0	-40.00	0	0	0	0	-40.00
1		6000	140	5860	-1200	4650
2		6000	140	5860	-1200	4650
3		6000	140	5860	-1200	4651
4		6000	140	5860	-1200	4660
5		6000	140	5860	-1200	4660

A.6 Scenario Calculations

Table A.6a. Scenario #1: very affordable vaccines (10% of price)

Summary of yearly profits	
Description	Net effect
Revenue	$600,000,000
Labor	-$7,411,415
Waste disposal	-$1,477,381
Raw Material Costs	-$133,419,633
Utility Cost	-$21,317
Miscellaneous (taxes)	-$85,898,579
Total per year	$363,239,501

Table A.6b. Scenario #1: revenue calculation

Revenue Calculation	
number of doses	400000000
wholesale price per dose ($)	1.5
Total revenue	$600,000,000.0

Table A.6c. Scenario #2: saturated market, low demand (10% of projected revenue)

Summary of first year profits	
Description	Net effect
Revenue	$600,000,000
Labor	-$7,411,415
Waste disposal	-$1,477,381
Raw Material Costs	-$133,419,633
Utility Cost	-$21,317
Miscellaneous (taxes)	-$94,430,753
Total per year	$363,239,501

Table A.6d. Scenario #2: revenue calculation

Revenue Calculation	
number of doses	40000000
wholesale price per dose ($)	15
Total revenue	$600,000,000

Table A.6e. Scenario #3: expensive materials (2x as expensive)

Summary of yearly profits	
Description	Net effect
Revenue	$6,000,000,000
Labor	-$7,411,415
Waste disposal	-$1,477,381
Raw Material Costs	-$266,839,266
Utility Cost	-$21,317
Miscellaneous (taxes)	-$1,200,412,630
Total per year	$4,523,837,991

Table A.6f. Scenario #3: revenue calculation

Revenue Calculation	
number of doses	400000000
wholesale price per dose ($)	15
Total revenue	$6,000,000,000

Table A.6g. Scenario #4: Minimum price dose

Summary of yearly profits	
Description	Net effect
Revenue	$144,000,000
Labor	-$7,411,415
Waste disposal	-$1,477,381
Raw Material Costs	-$133,419,633
Utility Cost	-$21,317
Miscellaneous (taxes)	-$1,190,753
Total per year	$156,000,000

Table A.6h. Scenario #4: revenue calculation

Revenue Calculation	
number of doses	400000000.00
wholesale price per dose ($)	0.39
Total revenue	$ 156,000,000.00

Table A.6i. Scenario #4 minimum price dose rate of return

Interest:	20.00%			
Year	**Net after-tax cash flow (millions)**	**cum cash position**	**Discount factor**	**Present-Value of cash flow**
0	-40.00	-40	1	-40
1	1	$14	-26	0.83
2	2	$14	-13	0.69
3	3	$14	1	0.58
4	4	$14	15	0.48
5	5	$14	28	0.40
			Net present value:	**0.88242822**

Table A.6j. Scenario #4 minimum price dose cash flow

Year	Investment (millions)	Revenue	Expenses	Gross Profit	Taxes	Net After-tax cash flow
0	-40.00	0	0	0	0	-40.00
1		$156	$142	$14	0	$14
2		$156	$142	$14	0	$14
3		$156	$142	$14	0	$14
4		$156	$142	$14	0	$14
5		$156	$142	$14	0	$14

A.7 Labor

Table A.7a. Particulate process equipment

Particulate (P) Process Equipment	**Number of Equipment**
Centrifugation	1
Homogenizer	1
Pre-homogenization Resuspension	1
Viral Inactivation	1
Antigen Solution Formulation tank	1
Adjuvant Emulsification Tank	1
Total, P	**6**

Table A.7b. Non-particulate process equipment

Non-particulate (NP) Process Equipment	**Number of Equipment**
Exchangers	1
Bioreactors	8
Depth Filtration	1
Ultrafiltration/Diafiltration	2
Viral Filtration	3
Chromatography	3
Vial cleaning	1
Vial depyrogenation	1
Vial Filling + Closing (Rubber Stoppers)	1
Vial Closing (Crimp Caps)	1
Vial inspection, tray-loading, transfer to storage	1
Total, N_{np}	**23**