DESIGN AND OPTIMIZATION OF A LENTIVIRAL VECTOR THROUGH CAR-T CELL THERAPY PLATFORM FOR AGC BIOLOGICS

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Executive Summary

Cell therapies are crucial, advancing tools used in the treatment for diseased and damaged cells. This project aims to contribute to this developing field by proposing an efficient process to produce autologous chimeric antigen receptor (CAR) T-cells for the treatment of CD19 malignancies. Simply put, CAR-T cell manufacturing involves the isolation and expansion of patient T-cells. The cells are then genetically engineered to express the desired CAR, which recognizes the malignancy of interest. Lentiviral vectors are often used for CAR-T transduction, and are produced via transient transfection of HEK293-T cells.

Herein, we describe the design, optimization, and economic analysis of lentiviral vector (LVV) and CAR-T manufacturing processes. Guided by our project description, technical background, and material balances, we first developed both visual and SuperPro process flow diagrams of LVV and CAR-T manufacturing processes. We developed revival and suspension protocols of HEK293-T cells, which occur prior to bioreactor inoculation. We recommended the use of Cytiva Xcellerex XDR-50 bioreactor for cell transient transfection with the viral rector. This unit utilizes single use materials, minimizing the possibility of contamination and time required for cleaning. We recommended a benzonase endonuclease treatment for effective removal of nucleic acid contaminants. We selected filters for tangential flow depth filtration, tangential flow filtration, and sterile filtration. In order to remove further impurities, we also designed an anion exchange column. The downstream separations train was optimized to provide adequate removal of impurities while maintaining high product concentration and purity.

Once the LVV process design was concluded, we shifted our focus to the design of the CAR-T manufacturing process. We developed a protocol for leukopak thawing and recommended the use of CliniMACS Prodigy unit to automate the CAR-T production process. Lastly, a controlled rate freezer was designed to cryopreserve the final CAR-T product prior to transportation back to the patient.

We finally performed a full economic analysis on the entire end-to-end process. Our base case analysis using a 12.5% IRR yielded a CAR-T treatment sale price of $749,091.26, a year 3 ROI of 23.9%, a payback period of 4.2 years, and a NPV of $2,443,000 for period-end cash flows and $2,553,000 for period-beginning cash flows. Through modulation of the target % IRR, we discovered that modest increases in sale price of the treatment accompany quite significant improvements in ROI and payback period; providing opportunities for AGC to balance a competitive sale price against higher profitability.

Due to the successful and profitable design of LVV and CAR-T manufacturing processes, we can confidently recommend that this would be a process investment well worth the money. Beyond the notable profitable profits generated by these processes, they as a whole show promise to advance the production of CAR-T therapeutics and make them more accessible to the average patient. An investment in this plant would be an investment into a life-saving therapy which has the potential to improve quality of life and longevity of countless patients suffering from CD19 malignancies.

1. Project Description, Scope, and Background

The Immune System: The immune system is a complex bodily system for which knowledge and understanding of it is constantly changing. With that being said, it is known that the cells of the immune system are able to recognize pathogens present in the human body by recognizing specific proteins, called antigens, on the surface of the invading cells (American Cancer Society, 2022).

The primary immune cell type relevant to current cancer immunotherapies is the effector T cell. Effector T cells use a variety of methods to eradicate pathogens, but most notably possess the capability to induce apoptosis, or programmed cell death, in self-cells which present pathogenic antigens on their surface (Sompayrac, 2019). Effector T cells achieve this through a transmembrane protein known as the Fas ligand which, when bound to a Fas receptor present on all human cell types, triggers an internal death mechanism in the affected cell (Sompayrac, 2019). Naturally, this makes effector T cells an attractive target for cancer therapies, as they could be manipulated to clear tumors by inducing the death of cancerous cells. However, T cells typically undergo negative selection during formation to ensure that they will not become activated by antigens from self-cells, thereby preventing them from becoming activated in response to cancers as these do not produce any pathogenic antigens (Sompayrac, 2019). Furthermore, T cells normally require co-stimulation by other immune cells for full activation (Sompayrac, 2019). Therefore, T-cell-based cancer therapies such as CAR-T therapy focus on modifying the method of activation of T cells to allow them to recognize cancerous cells and become activated without co-stimulation.

CAR-T Cell Therapy Overview: The chimeric antigen receptor (CAR) T-cell therapy is a cellular immunotherapy which engineers a person's T cells to recognize and fight cancer (American Cancer Society, 2022). The basic concept behind CAR-T cell therapies is to genetically engineer T-cells to both recognize surface proteins on cancerous cells and to become activated without a co-stimulatory signal, thereby initiating a targeted attack of the immune system to eradicate the cancer (American Cancer Society, 2022). To ensure immune compatibility, cells are taken from the patient and laboratory techniques are used to add the gene for a particular receptor–called a chimeric antigen receptor (CAR)–which recognizes specific cancer cell antigens and contains all the necessary intracellular signaling domains to single handedly activate the cell (American Cancer Society, 2022). The engineered T-cells are then re-infused back into the patient's body, equipping them with the cellular capabilities to attack specific malignancies (American Cancer Society, 2022). Each CAR-T cell therapy is engineered such that the CARs expressed are specific for a particular cancer antigen (American Cancer Society, 2022). Thus, this is a powerful therapy with the capability to treat a variety of cancers (American Cancer Society, 2022).

Specific Applications of CAR-T: CAR-T cell therapies are perhaps most widely known for their ability to treat specific kinds of leukemia and lymphoma, for which the cancerous cells express an antigen known as CD19. In this particular case, the T-cells would be engineered to express receptors which recognize and bind to the CD19 antigen (American Cancer Society, 2022). CAR-T cell therapies which target CD19 comprise most FDA licensed CAR-T therapies, including Kymriah from Novartis and Yescarta from Kite.

CAR-T General Process Steps: A successful CAR-T cell therapy begins with collecting blood product from the patient. After being shipped to and received at the facility, the product is separated–typically via leukapheresis–and washed (American Cancer Society, 2022; Patel et al., 2011). Once T cells have been isolated, they undergo transduction, usually via a lentiviral vector, wherein the gene for the desired CAR is added into the cells. Both cytotoxic–CD8–and marker–CD4–T cell populations are transduced to target the desired cells. The cells are then grown and multiplied in the lab before being dosed and frozen. Lastly, the CAR-T cells are shipped back to the patient's facility for infusion. CAR-T manufacturing processes typically take between 8 and 15 days and can be either fully automated or a combination of manual and automated processes, the latter of which is most common.

Lentiviral Vector CAR Gene Transduction: Viral vectors are a safe and effective way to deliver modificable genetic material into cells. They are a popular choice for gene delivery in many areas due to their high transfer efficiency and the comparatively short amount of time needed to reach successful numbers of genetically modified and cultured cells. Successful delivery of genetic information via viral vectors requires intact delivery of the genetic material into the nucleus of the targeted cell(s), where it can be stably expressed without harmful toxicological and/or immunogenic responses ("Lentiviral Vectors - the Application for CAR-T Therapies"). Lentiviral vectors are a commonly-used type of retrovirus which can infect both dividing and nondividing cells ("Lentiviral Vectors - the Application for CAR-T Therapies"). They are safer than other forms of viral vectors–for example, gammaretroviral vectors–and are the vector of choice in the manufacturing processes of several CAR-T therapies in clinical trials. In the case of lentiviral vectors (LVVs), transgenes can be delivered into cells and tissues which have long had stable genetic expression and these transgenes have been shown to exhibit long-term expression ("Lentiviral Vectors - the Application for CAR-T Therapies"). In the case of CAR-T therapies, lentiviral vectors are utilized to deliver the genetic information for T cells to express the chimeric antigen receptor and as well as intracellular signaling and costimulatory domains (Patel et al., 2011). During the gene transduction process, the patient's T cells are incubated with the relevant viral vector. During this incubation period, the vector uses its inherent viral machinery to introduce the relevant genetic material into the T cells. The genetic information is transcribed and translated by the T cells, resulting in expression of the CAR on the cell surface and the costimulatory and signaling domains intracellularly (Patel et al., 2011). While CAR-T cells must be generated individually for each patient receiving the therapy, the

lentiviral vector remains constant for cancers with the same malignancies. Thus, lentiviral vectors can be produced in large quantities and studies have shown that frozen quantities can be stable over long periods of time ("Lentiviral Vectors - the Application for CAR-T Therapies").

Lentiviral Vector Production: Clinical-grade lentiviral vectors are most commonly produced via transfection of Human Embryonic Kidney (HEK) 293 or 293-T cells (Levine et al., 2017). The production of LVVs involves the use of a cell line, referred to as the packaging cell, to generate the viral vector particles. This is accomplished via expansion of the packaging cells followed by transfection with plasmid DNA which encodes the proteins for lentiviral vector production (Merten et al., 2016). During production, the packaging cell line produces the lentiviral vector particles, which can then be isolated from the culture medium to be used for T cell transduction to generate CAR-T cells (Merten et al., 2016). A number of purification steps, including chromatography and flow filtration, are performed to isolate the LVV particles. Following purification, the working media undergoes sterilization steps to remove cellular debris and lastly, the vector stacks can be cryopreserved prior to further use (Merten et al., 2016).

Large-scale, industrial LVV manufacturing typically handles cell cultures in large bioreactors. Each purified bulk product run is quality controlled separately, with the bulk purified, pooled, and sterile filtered prior to final vailing (Levine et al., 2017). HEK 293 cells are a popular packaging cell choice, as they grow readily in suspension culture and can be expanded in numerous kinds of vessels, including shake flasks and stainless steel bioreactors (Levine et al., 2017). The major problem to overcome in large-scale LVV production is the transfection step, which requires large amounts of plasmid DNA to generate sufficient levels of LVV product (Levine et al., 2017). One way to overcome this challenge is the use of genetically modified stable cell lines which do not require transient transfection.

CAR-T Cell Production: As briefly outlined above, the manufacturing of CAR-T cells is a process which requires several steps, with quality control testing being performed throughout the process.

T Cell Harvest and Isolation - The first step in any CAR-T manufacturing process is the harvesting of the patient's T cells. Typically, blood is obtained from the patient and a process called leukapheresis separates the leukocytes, or white blood cells (WBCs), from the other blood components ("Lentiviral Vectors - the Application for CAR-T Therapies"). After sufficient WBCs have been harvested, T cells are isolated. Typically this process involves washing the cells out of the buffer used in leukapheresis, followed by a counterflow centrifugal elutriation, a process which separates the leukocytes by size and density while maintaining cell viability ("Lentiviral Vectors - the Application for CAR-T Therapies"). An additional step which can be performed is the separation of CD4/CD8 T cell subsets via antibody bead conjugation.

T Cell Activation and Transduction - Following T cell isolation, it is typical to run an activation and transduction process. For T cell activation, it is common practice to use beads coated with anti-CD3/anti-CD23 antibodies alone or in combination with growth factors such as interleukin-2 (IL-2) ("Lentiviral Vectors - the Application for CAR-T Therapies"). More recently, it has been shown that the use of cell-based artificial antigen-presenting cells (aAPCs) in combination with IL-2 has demonstrated superior activation and ex vivo expansion of the T cells ("Lentiviral Vectors - the Application for CAR-T Therapies"). These beads or aAPCs can be removed from the cell culture via magnetic separation after the activation, transduction, and expansion process is complete. In the case of activation using IL-2 and aAPCs, it has been shown that T cells will grow logarithmically in bioreactors for several weeks ("Lentiviral Vectors - the Application for CAR-T Therapies"). Lastly, it should be noted that the culturing conditions during T cell activation can be refined further to polarize T cells to a particular phenotype ("Lentiviral Vectors - the Application for CAR-T Therapies").

To genetically engineer the patient's T cells to express the desired CAR, T cells are then incubated with the chosen viral vector encoding that CAR ("Lentiviral Vectors - the Application for CAR-T Therapies"). After several days, the viral vector is washed out of the cell culture, typically via dilution or medium exchange. Recently, bioreactor culture systems have been optimized to provide the conditions (e.g., gas exchange and culture mixing) best suited to cell growth ("Lentiviral Vectors - the Application for CAR-T Therapies"). While some methods use separate units for cell preparation, activation, transduction, expansion, and final formulation, others are designed to perform all steps within a single unit. For instance, the CliniMACS Prodigy is a single device which accomplishes all, and it has recently been shown that this unit is an effective way to automate the CAR T cell production process.

CAR-T Cell Concentration and Final Formulating - After the expansion process is complete, the cell culture is often concentrated to a volume which can feasibly be infused into the patient. Typically, the concentrated cells are cryopreserved in an infusible medium before being transported to and thawed at the patient's treatment center. The final formulation steps, including the cell washing and concentration steps, can be semi-automated to improve process throughput.

Project Description: As the number of CAR-T cell therapies moving into later-phase clinical trials increases and the therapy becomes a more viable treatment option for a variety of cancers, developing a functional, compliant, and rapid CAR-T cell manufacturing process becomes an increasingly important task ("Lentiviral Vectors - the Application for CAR-T Therapies"). Here, we will design a cell therapy platform to produce autologous CAR-T products, meaning that the therapy is one which uses the patient's own cells. Moreover, the design of this platform will focus on CD19 CAR-T products to be used for B-Cell Lymphoma treatments.

This CAR-T production process has a starting material of frozen human peripheral blood mononuclear cells (PBMCs), with approximately 100 x 10^6 cells as the starting number of patient cells. The cells will be processed over 10 days to yield the desired CAR-T cell therapy. The cells will be dosed and frozen prior to being shipped back to the patient's facility for infusion. We will also be designing the viral vector production process, which will be accomplished via batch manufacturing in a 50L suspension vessel. HEK293-T cells will be used to produce the vector, starting with approximately 1 x 10^7 cells per vial. Cell expansion (Media A) and transfection (Media B) medias will be used during the processing, which takes place over 14 days. The target vector concentration post-fill is 1,724 ng/mL.

For the lentiviral vector process, the upstream cell expansion and suspension portion will begin with cell revival and shake flash expansion, followed by further cell expansion and viral vector production. Next the cells will be harvested and processed via salt addition and endonuclease treatment. Depth and sterile filtration filtration will be performed before the product moves into the downstream purification and fill/finish processing portion of this process. As part of the downstream processing and fill/finish operations, the cells will undergo two separate column chromatography steps separated by a tangential flow filtration operation. This will be followed by a sterile filtration step before final fill of the product.

2. Technology Background

2.1. Lentiviral Vector Technology Background

LVV Large-scale manufacturing overview

The large-scale manufacturing of lentiviral vectors for clinical use has a number of obstacles, including lack of widely available stable vector packing systems, lot size limitation, and lot-to-lot variability, the latter of which is a direct result of the currently preferred multi-plasmid transient transfection procedure.

Expansion of an aliquot of the packing cell line in culture (e.g., HEK293-T cells)

Production of clinical-grade LVVs are most often produced via transient transfection of HEK293 or HEK293-T cell lines, the latter of which is most common (Merten et al.). HEK293-T cells are often the packaging cell of choice due to the presence of an SV40 T-antigen in the cells, rendering them more efficient for viral vector production. They also demonstrate increased cell growth and transfection efficiency when compared to other packing cell lines, notable HEK293 cells (Merten et al.). HEK293-T cells are also advantageous due to their ability to undergo suspension growth in serum-free media, which is particularly useful for large-scale LVV production (Merten et al.). The primary expansion of the packing cell line can be accomplished

in a variety of vessels, including Petri dishes, T-flasks, and multi tray systems (Merten et al.). Studies have shown that there are no notable or observable differences between these cell culture systems (Merten et al.). For this process, we will start with 1×10^7 HEK293-T cells in vial. Prior to cell expansion the concentration will be 2×10^2 cells/mL and after expansion will be 5.4×10^5 cells/mL. The cells must be doubled 11.4 times, so the total expansion time for this step will be the cell doubling time × 11.4.

Transient transfection of packaging cells with plasmid DNA encoding the necessary proteins for production of the LVV

The LVV systems used today are typically four-plasmid systems, which includes three helper plasmids and one gene transfer vector plasmid (Merten et al.). All accessory, non-necessary genes of HIV-1 are removed, decreasing the probability of recombination and host-cell integration events (Merten et al.). The gene transfer vector encoding the CAR of interest is the only genetic material which is transferred to target cells (Merten et al.). In total, the HEK293 or HEK293-T cells are transfected with four plasmids which encode the following: the *gag-pol* genes, the *rev* gene, the VSV-g envelope gene, and a SIN lentiviral gene transfer gene plasmid with an internal promoter for transgene expression (Merten et al.). Co-transfection of these four plasmids enables the packaging cell line to produce the desired functional vector particles (Milone et al.). To transfect the packaging cells, transfection agents such as calcium phosphate and cationic polymers are needed. Over the course of a few days, the packing cells produce the desired LVV particles. The large-scale harvesting of the packaging cell line can be accomplished via scale-up or scale-out methods, the latter of which is more common. For a scale-up approach, the industrial standard is to use a large bioreactor (Merten et al.). Production of LVV in bioreactors requires that the packaging cells be expanded in suspension (Merten et al.). Studies have demonstrated efficient and successful expansion, transfection, and LVV production using HEK293-T cells in 50L single use bioreactors, a setup which we will use in our design of this process (Merten et al.). We will start with 5.4×10^5 cells/mL entering the reactor and end with a final concentration of 3.8×10^7 transfer units (TU) of virus per mL.

Bioreactor Harvest

The LVV particles are then harvested from the culture medium. Techniques such as flow filtration harvest the clarified vector while other post-processing techniques remove contaminating productions (e.g., viral DNA) (Milone et al.). The concentration prior to harvest will be 3.8×10^7 TU/mL. We anticipate an approximate yield of 70% in this step, yielding a post-harvest concentration of 2.7×10^7 TU/mL.

Downstream Processing

Sophisticated downstream processing protocols ensure that undesirable contaminants, such as plasmids and host-cell DNA or proteins, are removed from the working LVV product (Merten et al.). These steps typically follow those traditionally used in the biotechnology industry and include membrane filtration and chromatography methods. First off, the harvested volume undergoes a salt / endonuclease treatment to remove any functional DNA particles. The next aim in downstream processing is to capture the target LVV from cell culture, eliminating major contaminants. This is accomplished via a series of filtration units, including both depth and sterile filtration. The next step is to remove specific impurities (proteins, DNA, endotoxins) via chromatography and ultrafiltration techniques. These polishing steps remove contaminants and impurities, generating a safe and active product which is almost ready for formulation and packaging. Lastly, the working product undergoes another sterile filtration step for sterilization purposes, removing any remaining impurities (e.g., DNA and cellular debris). This step is vital as these impurities can cause adverse responses in vivo. (Milone et al.). The final viral stocks can then be cryopreserved, a form in which they remain stable for upwards of 2 years (source 1 from project intro, Milone et al.). The final product will be a vector stock at a concentration of around 4.30×10^8 TU/mL, or 1724 ng/mL of lentiviral vector.

2.2. CAR-T Technology Background

CAR-T cell therapies rely on the stable expression of chimeric antigen receptors (CARs) specific to the cancer being targeted. A CAR contains three distinct domains important for T cell function: antigen recognition, co-stimulatory signaling, and T-cell signaling domains (Milone et al.). The stable expression of a CAR can be achieved via gene delivery by viral gene transfer systems. These gene transfer systems–referred to as viral vectors–are a relatively safe and efficient way to modify eukaryotic cell gene expression (Milone et al.). One of the most common stable gene expression vectors used for clinical applications, and which will be the genetic delivery method of choice for this process, are lentiviral vectors (Wang et al.). Lentiviral vectors, which are derived from HIV-1, are advantageous due to their ability to deliver transgenes to nondividing cells and as a whole display safer genomic integration profiles. (Wang et al., Milone et al., Merten et al.). In fact, the use of lentiviral vectors to engineer CAR T-cell therapies for B cell malignancies demonstrated noteworthy success and led “to regulatory approval of the first genetically engineered cellular therapy using lentiviral vectors” (Milone et al.).

PBMC Sample Processing

Following leukapheresis, a raw patient PBMC sample must be processed to remove contaminants and isolate the desired T cell populations prior to transduction. Washing and filtration to remove contaminants and red blood cells can be accomplished using devices such as

the Terumo COBE2991 and Fresenius Kabi Lovo, which were both designed for laboratory use to automate washing and filtration processes (Wang et al.). The COBE2991 includes a built-in protocol for the preparation of a concentrated WBC sample, which is achieved through centrifugation of the whole blood sample, which separates WBCs from other blood components according to the differing specific gravities of these components (Terumo). The Lovo, meanwhile, achieves PBMC washing and RBC removal using spinning-membrane filtration, which has the advantage of allowing for continuous processing (Fresenius Kabi).

Blood cell fractionation for the further isolation of lymphocytes can be achieved by the Terumo Elutra and BioSafe Sepax, both of which employ centrifugation to separate blood cells based on size and density (Wang et al., Terumo, Surplus Solutions).

Isolation of T cells is commonly done by immunolabeling these cells using antibodies conjugated with magnetic particles, then separating these cells magnetically. This separation method has the advantage of a high specificity, as unique surface markers can be chosen for antibody labeling. Examples of processes which implement these techniques include magnetic-activated cell sorting (MACS), EasySep, and magnetic separation using Dynabeads (Lopez Munoz et al.).

T Cell Activation and CAR-T Production

Activation of the T cells is required both for effective transduction via viral vectors and for the subsequent expansion of the CAR-T cells produced by transduction. In natural immune functions, T cells are activated by antigen-presenting cells (APCs), which interact with both the T cell receptor and co-stimulatory surface proteins such as CD28. However, due to the autologous nature of using APCs to activate T cells, this method can be complicated by variations in APC frequency and efficacy between patients (Wang et al.). Therefore, artificial antigen-presenting cells (AAPCs) are often employed for T cell activation in a manufacturing environment. Both cell-based and bead-based systems are available. Cell-based systems rely on insect cell, murine fibroblast, or human leukemia cell lines which have been modified to express multiple costimulatory surface proteins commonly found on APCs. Bead-based systems typically consist of beads conjugated with anti-CD3 and anti-CD28 antibodies. Since CD3 is the signaling protein associated with TCR activation and CD28 is the costimulatory surface protein most commonly targeted by human APCs, these beads are capable of providing both a primary and costimulatory signal to activate T cells (Kim et al.). Examples of bead-based AAPCs available for manufacturing purposes include Dynabeads CD3/28 and Miltenyi MACS GMP TransAct CD3/28 beads, the latter of which has the advantage of being biodegradable so their removal is not necessary prior to CAR-T therapeutic formulation (Wang et al.).

Modification of T cells to express the CAR can be done in a variety of ways, but the technology relevant to this project is transduction by a lentiviral vector. The viral vector enters the T cell and integrates its DNA vector into the T cell genome. This method results in high transduction efficiency and a high stability of the transgene, yielding T cells which will express the CAR long-term (Godecke et al.).

CAR-T cell expansion is typically performed by culturing the cells in a bioreactor or shake flask. Examples include the GE WAVE 25L bioreactor or G-Rex flask system. CAR-T cells can also be expanded by continual stimulation by the human leukemia cell lines described above, since T cells will naturally proliferate when activated against an antigen (Wang et al.).

Final Formulation and Cryopreservation

Depending on the techniques employed for T cell separation and activation, a cell washing step could be required in addition to the final dosing and formulation. Both of these processes can be accomplished by the same instruments which would be used for the initial PBMC processing steps, the Terumo COBE2991 and Fresenius Kabi Lovo, both of which were discussed in the "PBMC Sample Processing" section above (Wang et el.).

All parts of the CAR-T process, up through the formulation step, could also be accomplished using a single instrument: the CliniMACS Prodigy system. The Prodigy includes a pre-loaded automated CAR-T cell manufacturing program. This program first performs PBMC washing and fractionation through the instrument's built-in centrifuge. Then, T cells are isolated via MACS, as mentioned in the "PBMC Sample Processing" section above. T cells are activated by passing them through a polymer matrix conjugated to CD3 and CD28 agonists. The instrument further supports transduction via lentiviral vectors. The Prodigy system finally allows for cell expansion through cell culturing using the TexMACS GMP Medium and MACS GMP Cytokines (Miltenyi).

Cryopreservation of CAR-T cells must be done using a controlled-rate freezer to avoid ice crystal formation and subsequent damage of the cells. Examples of controlled-rate freezers suitable for this application include the CBS CRF2101, Thermo Scientific CryoMed, and for larger-scale applications, the Biolife High-Capacity Freezer (Cryosolutions, Thermo Fisher Scientific, Biolife Solutions).

3. Environmental, Health, and Safety Considerations

3.1. Safety Issues

3.1.1. Facility Design, Safety Equipment, and Safe Operating Procedures

The facility's design must prioritize physical segregation of unit processes, taking into consideration the required operating space and sanitization needs. This containment strategy should include the movement of employees through various stages of the facility, personal protective equipment (PPE) policies for each suite, and sterilization requirements. It is crucial to manage airflow in a way that prevents air from flowing between suites with different sterilization requirements.

To ensure the plant's safe operation, the following guidelines must be established as a foundation:

1. All operators working with unit operations must receive proper training and obtain relevant qualifications in the safe operation of the unit, as well in all related safety protocols
2. Operators must familiarize themselves with the operating procedures and safety guidelines of the unit before initiating any operation
3. Personnel should receive regular, comprehensive training in PPE usage, and PPE should be easily accessible to all workers.
4. Regular safety drills, inspections, and continuous monitoring should be conducted to ensure personnel familiarity with safety protocols and maintain the process in a safe working condition.
5. All units must undergo thorough and regular inspections for any defects, such as leaks or corrosion, and to confirm the proper functioning of safety devices and countermeasures.
6. Appropriate measures must be implemented to minimize exposure to hazardous or biohazardous materials as much as possible, including proper ventilation and sterilization procedures.
7. Supervising personnel should always be present on-site and must be informed of any deviations from standard operating procedures
8. In the event of an emergency, the unit must be shut down immediately, personnel should be evacuated, first responders notified, and any additional appropriate emergency protocols should be followed.

By adhering to these guidelines, the facility will maintain a safe environment for its employees and ensure a high degree of quality control for the manufacturing process.

3.1.2. Ventilation

As discussed in the upcoming Biosafety section, current good manufacturing practices (cGMP) establish regulations for airborne particles and pathogens. Therefore, it is essential to ensure that

the air quality in the workspace adheres to these standards, providing a safe environment for operators.

To maintain compliance with cGMP guidelines, proper ventilation systems must be designed and implemented throughout the facility. This will help control and minimize the spread of airborne contaminants, ensuring a clean and safe working atmosphere. Attention should be given to air filtration, circulation, and temperature control, as well as to the regular maintenance of the ventilation systems. By doing so, the facility will promote the well-being of its employees and maintain the integrity of the LVV/CAR-T cell therapy manufacturing process.

3.1.3. Biosafety and Biocontainment

Cell and gene therapy facilities inherently expose workers to biohazards, such as cells, vectors, and plasmids. It is crucial to implement appropriate measures to minimize risks and ensure the health and safety of operators.

The Occupational Safety and Health Administration (OSHA) established the 29 CFR 1910.1030 standard, outlining regulations for handling bloodborne pathogens. Moreover, for employees directly interacting with human-origin biologics, AGC Biologics is required to offer Hepatitis B vaccines at no cost.

Current good manufacturing practices (cGMP) facilities employ four grades (A-D) to classify the degree of sterilization stringency, with Grade A being the most stringent. The facility layout should enable each suite to operate at a grade appropriate for the process steps occurring in that area.

Our process includes the following Biosafety Cabinet (BSC) environment grades:

1. Grade A: Cell expansion in flasks, media preparation, and any additional manual CAR-T open processing steps.
2. Grade B: Cell Therapy and finishing viral vector suites.
3. Grade C: Cell expansion suites for the LVV process and the purification suite. This grade is justified by the assessed risk due to the absence of cells and minimal growth-promoting products in purification solutions.
4. Grade D: Bioreactor suites, as they incorporate sterile welding and aseptic connectors

All other units can operate in Grade C or D environments, according to the specific requirements of each unit. By adhering to these biosafety and biocontainment guidelines, the facility will maintain a safe working environment and uphold the integrity of the LVV/CAR-T cell therapy manufacturing process.

3.1.4. Chemical Safety

It is essential to evaluate all chemicals used in the facility for potential hazards to human health. Appropriate countermeasures must be provided to address chemical hazards and should be easily accessible throughout the facility. Specific areas must be designated for the storage of corrosive, flammable, toxic, and biohazardous materials, ensuring safe handling and segregation. Additional guidance and regulations can be found in OSHA 29 CFR 1910.119, which outlines the "Process Safety Management of Highly Hazardous Chemicals" (OSHA). This regulation serves to prevent or minimize the consequences of a catastrophic release of toxic, reactive, flammable, explosive, or otherwise hazardous chemicals.

To maintain chemical safety in the CAR-T/LVV process, the following measures should be considered:

1. Implement a chemical inventory management system to track chemical usage, storage locations, and expiration dates.
2. Develop and maintain Safety Data Sheets (SDS) for all chemicals used within the facility, ensuring easy access for employees. A preliminary MSDS has been included in Table 3.4.1 of the report.
3. Conduct regular training sessions for personnel on the proper handing, storage, and disposal of chemicals, including emergency response procedures.
4. Equip storage areas with appropriate containment and ventilation systems to prevent accidental release or exposure to hazardous chemicals.
5. Establish a routine inspection program to check the integrity of chemical containers and storage facilities, ensuring prompt identification and correction of any potential hazards.

By implementing these chemical safety measures, the facility will minimize risks associated with chemical hazards, protect employees, and maintain compliance with relevant regulations in the context of the CAR-T/LVV process.

3.1.5. Personal Protective Equipment (PPE) and Ergonomics

OSHA 29 CFR 1910.132-138 outlines the appropriate PPE, such as gloves, masks, and eye protection, necessary to protect workers from potential hazards in the workplace. As cell and gene therapy manufacturing can introduce additional hazards (e.g. repetitive strain from heaving lifting) (DiGiandomenico et al.), it is important to account for such hazards and mitigate these risks. At a minimum, employees must wear PPE in accordance with their level of direct contact with biological material.

The basic requirements (DiGiandomenico et al.) include:

1. Single-use, full-coverage, disposable gowning

2. Safety glasses
3. Gloves
4. Protection from aerosols (aprons, face shields, and respirators)
5. Hearing protection (as appropriate)

It is essential to thoroughly determine the specific type of each PPE item on a case-by-case basis, tailored to the needs of each situation. Hazards can range from physical risks, such as transporting excessively heavy objects or falling objects, to ergonomic strains caused by repetitive actions.

In order to prevent ergonomic strain, OSHA recommends providing operators with adjustable chairs, footrests, and work surfaces to minimize the strain on their backs, necks, and legs. ("Ergonomics: The Study of Work." Occupational Safety and Health Administration, U.S. Department of Labor, www.osha.gov/SLTC/ergonomics/index.html) ("Workstations and Seating." Occupational Safety and Health Administration, U.S. Department of Labor, www.osha.gov/SLTC/etools/computerworkstations/components_seating.html.)

Furthermore, OSHA advises employers to provide a regular 15-minute break every 4 hours for workers performing standing work to prevent fatigue. By implementing these PPE and ergonomic measures, the facility will ensure the safety and well-being of its employees during the CAR-T/LVV process.

3.1.6. Waste Management and Disposal

The Colorado Department of Public Health and Environment has established several regulations pertaining to hazardous waste management in Article 15 of Title 25 of the Colorado Revised Statutes (C.R.S.). These regulations are particularly relevant to the management of infectious waste generated during the operation of a CAR-T/LVV platform.

Part 2 of Article 15 of the C.R.S. details the regulatory requirements for creating and using designated waste disposal sites. Part 3 outlines the guidelines for collaborating with the State Hazardous Waste Management Program. In the context of CAR-T/LVV processes, working with infectious material is inevitable. C.R.S. Title 25, Article 15, Part 4 (specifically 25-15-401 through 25-15-406) outlines Colorado State regulations regarding the handling of infectious waste.

Additional articles of the C.R.S. that outline standards the process should adhere to include Article 16, which provides further guidance for hazardous waste sites, and Article 16.5, which focuses on pollution prevention.

To ensure compliance with these regulations, the CAR-T/LVV process should incorporate the following waste management and disposal measures:

1. Implement waste segregation protocols to separate infectious, hazardous, and non-hazardous waste streams.
2. Develop a waste handling and disposal plan that aligns with state regulations and guidelines for infectious and hazardous waste management.
3. Train employees on proper waste handling procedures, including the use of appropriate PPE and waste container labeling.
4. Establish partnerships with licensed waste disposal facilities to ensure proper treatment and disposal of hazardous and infectious waste.
5. Regularly audit waste management practices to verify compliance with state regulations and identify opportunities for improvement or pollution prevention.

By incorporating these waste management and disposal measures, the facility will maintain compliance with state regulations, protect the environment, and ensure the safety of employees and the surrounding community during the CAR-T/LVV process.

3.1.7. Emergency Preparedness and Response

Emergencies that may occur on-site include common accidents such as spills, releases, medical emergencies, and severe weather events (DiGiandomenico et al.). It is crucial to develop emergency response plans and infrastructure to swiftly and appropriately address such incidents, aiming to minimize potential harm to human health and the facility. The facility should implement continuous monitoring to ensure prompt alerts in case of any process deviations that could jeopardize operator health.

The NIH provides a document outlining guidelines for research involving recombinant or synthetic nucleic acid molecules (NIH, Appendix K). Section III (Experiments Covered by the NIH Guidelines) and Appendix K (Physical Containment for Large Scale Uses of Organisms Containing Recombinant or Synthetic Nucleic Acid Molecules) offer valuable guidance for assessing the risks associated with various components of the CAR-T/LVV platform.

To ensure a comprehensive emergency preparedness and response plan, the facility should consider the following measures:

1. Establish clear communication channels for reporting incidents and alerting relevant personnel.
2. Develop site-specific emergency response procedures that address potential risks and scenarios, including spills, medical emergencies, and severe weather events.
3. Train employees on emergency response procedures, evacuation routes, and the use of emergency equipment such as fire extinguishers and spill kits.

4. Ensure easy access to emergency response staff, with first responders able to reach the site quickly, and retain appropriate emergency personnel on-site.
5. Regularly update and document emergency preparedness protocols, taking into account new risks, changes in facility layout, or updated regulations.
6. Conduct frequent safety drills for various emergency situations, involving all workers to reinforce familiarity with procedures and improve response times.

3.2. Occupational Health and Worker Training

To ensure a safe working environment during the CAR-T/LVV process, all workers must be approved to work in biohazardous suites. To gain approval, they must demonstrate a thorough understanding of the countermeasures implemented in the facility to mitigate biohazard risks. Additionally, workers should receive frequent training to maintain awareness of occupational health and safety procedures. Key training topics include, but are not limited to:

1. Medical information: Ensure workers are informed about potential health risks associated with handling biohazardous materials and the importance of adhering to safety guidelines.
2. Mandated use of Personal Protective Equipment (PPE): Train workers on the proper selection, use, and disposal of PPE to minimize exposure to hazards.
3. Emergency preparedness: Provide training on emergency response procedures, evacuation routes, and the use of emergency equipment.
4. Safety control measures: Educate workers about engineering controls, administrative controls, and safe work practices designed to reduce exposure to hazards.

In addition to these training topics, the facility should consider implementing the following measures to promote occupational health and worker training:

1. Develop and maintain a comprehensive training program that covers all relevant safety guidelines and protocols.
2. Ensure new hires receive thorough orientation and training before starting work in biohazardous suites.
3. Conduct regular refresher training sessions to reinforce safety procedures and update workers on any changes to protocols or regulations.
4. Track and document individual training records, ensuring all workers have completed necessary training modules.
5. Foster a culture of safety by encouraging open communication about safety concerns and promoting continuous improvement in safety practices.

Additionally, various aspects of occupational health should also be considered and addressed to create a comprehensive safety program that minimizes risks for workers in this specialized field.

1. Exposure assessment and monitoring: Regularly assess and monitor potential exposures to hazardous materials, including chemicals, biohazardous agents, and physical hazards. Implement an exposure assessment plan, which should include air sampling, surface sampling, and personal monitoring to ensure that exposure levels are within acceptable limits.
2. Immunization and medical surveillance: Provide employees with access to immunizations, such as the Hepatitis B vaccine, as well as routine medical surveillance to monitor potential health effects related to exposure to biohazardous materials. Early detection and intervention can help prevent or minimize adverse health outcomes.
3. Ergonomic assessments: Evaluate workstations, equipment, and tasks for ergonomic risks, such as repetitive motion injuries, awkward postures, and excessive force requirements. Implement ergonomic interventions, including equipment adjustments, work process modifications, and employee training to reduce these risks.
4. Stress and mental health: Address psychosocial factors that may contribute to stress and mental health issues in the workplace, such as high workload, tight deadlines, or unclear job expectations. Promote a supportive work environment, encourage open communication, and provide access to mental health resources and counseling services.
5. Incident reporting and investigation: Establish a system for reporting and investigating incidents, near misses, and workplace hazards. Analyze data to identify trends and areas for improvement, and implement corrective actions to prevent future incidents.
6. Health promotion and wellness programs: Encourage employee well-being by offering health promotion and wellness programs that focus on nutrition, exercise, and stress management. These programs can help improve overall health, reduce absenteeism, and increase job satisfaction.
7. Regulatory compliance: Stay informed about the latest local, state, and federal regulations related to occupational health and safety in CAR-T/LVV manufacturing. Ensure that the facility complies with all relevant standards, guidelines, and best practices.

3.3. Environmental Impact and Sustainability

The nature of manufacturing for biologics treatment demands stringent sterility throughout the process. This requirement often necessitates the use of disposable or single-use equipment, which can have significant environmental consequences. It is essential to minimize the environmental impact without compromising the quality and sterility of the final product, while also taking into account the financial burden of the entirety of the manufacturing process in mind.

Sustainability must be considered not solely in terms of the impact on the environment, but also in relation to the economical implications of the manufacturing process (Whitford et al.). A

scientifically rigorous and systematic approach must be taken for the analysis of the final process, to understand the totality of the environmental impact imposed by both single-use and permanent equipment. Several metrics can be evaluated on the process including Process Mass Intensity (PMI), Life Cycle Assessment (LCA), Cumulative Energy Demand (CED), and Global Warming Potential (GWP). These metrics should be considered when evaluating the environmental impact and sustainability of the final process.

As single-use technologies undergo continuous improvement, the designed process should be regularly updated and reviewed for its environmental impact. This ensures that as newer, more environmentally friendly technologies emerge, they can be integrated into the process. Interestingly, in certain biomanufacturing processes, the energy consumption associated with single-use materials is lower than traditional bioprocessing, due to the high energy and emissions costs of sterilization, cleaning, and materials in conventional bioprocessing (Whitford et al.).

Appropriate end-of-life management for single-use components is critical, as outlined in the Waste Management section. Potential areas for reducing operational costs and environmental impacts include minimizing packaging, establishing contracts with recycling firms for applicable products, and implementing strict record-keeping and adherence to time limits (Whitford et al.).

Furthermore, it is important to evaluate the environmental impact of each waste stream generated during the process. Special attention should be given to ensuring that wastewater treatment systems are present on-site at the facility, in compliance with the Colorado Revised Statutes (CRS) Title 25, Article 10 (the On-site Wastewater Treatment Systems Act).

3.4. Regulations to Prevent Replication-Competent Lentiviruses (RCL)

As mentioned earlier, CAR-T/LVV manufacturing processes must adhere to stringent regulations set forth by federal agencies. The use of replication-competent lentiviruses in the manufacturing process introduces the risk of insertional mutagenesis (integration of undesired foreign genetic material into the patient's genome) in patients receiving the therapy. To avoid such risks, it is crucial to follow regulations outlined by the US Food and Drug Administration (FDA) and other relevant guidelines (Milone et al.):

1. Extensive RCL Testing: Conduct thorough testing for RCLs at multiple stages of the manufacturing process, including the packaging cell line, the purified vector product, and the genetically modified cellular therapy product. Ensure that all tests meet the required sensitivity and specificity criteria before administering the therapy to patients.
2. Assay Utilization: Employ the S+L- assay (Sarcoma positive, leukemia negative) to test for RCLs, as it is considered the gold standard for detecting replication-competent lentiviruses. The assay should be performed on samples taken from various stages of the manufacturing process to ensure the absence of RCLs.

3. Quality Control and Documentation: Maintain a comprehensive quality control system to track the testing and handling of lentiviral vectors and genetically modified cells. Document all test results and maintain detailed records to demonstrate compliance with regulatory requirements.
4. Employee Training: Ensure that all personnel involved in the manufacturing process are adequately trained in handling lentiviral vectors and genetically modified cells. Regular training sessions should cover biosafety practices, contamination prevention, and proper handling of biohazardous materials.
5. Facility Design and Equipment: Design the manufacturing facility to minimize the risk of cross-contamination and ensure that all equipment used in the production process is maintained and operated according to established guidelines. Implement strict cleaning and decontamination protocols to maintain aseptic conditions throughout the facility.

Although additional regulations, such as post-infusion monitoring and long-term follow-up with patients, are outlined by the US FDA, these regulations may fall outside the scope of responsibilities of a Contract Development and Manufacturing Organization (CDMO) like AGC. Nevertheless, it is essential for all parties involved in CAR-T/LVV manufacturing to collaborate and communicate effectively to ensure the safety and efficacy of the final therapeutic product.

3.5. Worst Case Scenario and How to Prevent It

The substances and units in this process have very low to no flammability scoring, as well as operate at low pressures. One of the most hazardous parts of this process is working with a product that is highly susceptible to contamination. This requires an equally sterile process. For this reason single use materials are utilized to reduce risk of contamination between batches, and ensure the system directly in contact with the product is constantly maintained. There comes a risk in the handling of the single use materials, where they may be improperly implemented or be defective. In this case the product may come in contact with the system it is in therefore contaminating it. Additionally this poses a hazard to the employees who may come into contact with the cells requiring them to follow proper guidelines as described above.

Additionally many steps in the process require temperature control to be implemented. If some malfunction or emergency were to occur there could be a loss in temperature control. This would result in a large loss in product. Since the products are specialized therapeutics an event with this loss of product could create a large backlog in production as well as revenue. Improper regulation of the process can damage the product, no longer allowing the customers to receive their therapeutic. The therapeutic is often a last resort effort of the patients that receive it as only a single sample can be taken from each person to individualize the final product. If the product created is damaged and no longer usable then the single sample is lost along with the patient's

ability to receive this crucial therapeutic. Not only is this detrimental to the patient but also to the company as there is no room for this error in such important work.

3.6. HAZOP

The HAZOP study was done for both the Cytiva Xcellerex XDR-50 bioreactor as well as the CliniMACS Prodigy in table 3.3.1 and table 3.3.2 respectfully. The analysis summarizes the process parameter, deviations, cause, consequence to the deviation, safeguards, and recommendations to mitigate the risk.

Table 3.3.1: HAZOP study *-Cytiva Xcellerex XDR-50 bioreactor.*

Project Name: Lentiviral Vector Production					**Date:** 2023/April/09
Process: Produce LVV cells					
Process Parameter	**Deviation**	**Cause**	**Consequence**	**Safeguards**	**Recommendation**
Pressure	High	● Outlet blockage ● Blocked rupture disk ● Malfunctioning pressure transmitter ● Pump Failure	● Increased pressure inside unit that may cause harm to LVV production ● Pressure buildup cause bioreactor to rupture	● Rupture disk	● Have failsafe rupture disk ● Install check valve on inlet to prevent backflow in case of overpressurization ● Routine check on safeguard
	Low	● Leak ● Malfunctioning pressure transmitter	● Decreased pressure inside the unit which can be	● Pressure regulator	● PT routine checks ● Pressure Regulator maintenance

			detrimental to the product		
Temperature	High	• Malfunctioning temperature controller • Malfunctioning temperature transmitter • Malfunctioning jacket	• Increased temperature could decrease cell viability	• TC, TT • Reactor jacket	• Routine checks on temperature controllers and sensors • Routine check and maintenance on the jacket • Check on exiting cell viability
	Low	• Malfunctioning temperature controller • Malfunctioning temperature transmitter • Malfunctioning jacket	• Reduced temperature may significantly impact cell metabolism and production	• TC, TT • Reactor jacket	• Routine checks on temperature controllers and sensors • Routine check and maintenance on the jacket • Check on exiting cell concentration

Table 3.3.2: HAZOP study *-CliniMACS Prodigy*

Project Name: CAR- T					**Date:** 2023/April/09
Process: CliniMACS Prodigy					
Process Parameter	**Deviation**	**Cause**	**Consequence**	**Safeguards**	**Recommendation**
Temperature	High	• Malfunctioning temperature controller • Malfunctioning temperature transmitter • Malfunctioning jacket	• Increased temperature could decrease cell viability	• TC, TT • Reactor jacket	• Routine checks on temperature controllers and sensors • Routine check and maintenance on the jacket • Check on exiting cell viability
	Low	• Malfunctioning temperature controller • Malfunctioning temperature transmitter • Malfunctioning jacket	• Reduced temperature may significantly impact cell metabolism and production	• TC, TT • Reactor jacket	• Routine checks on temperature controllers and sensors • Routine check and maintenance on the jacket • Check on exiting cell concentration
Contamination		• Hole in tubing • Improper filtration	• Contaminate final product	• Filtration at end of process • Routine check of tubes	• Routine replacement of tubing

Flow	High	• Malfunction cell selection • Malfunctioning pressure controller	• Cell shearing • Incorrect cell reading	• FT, FC • PT, PC	• Routine replacement of tubing • Routine checks on FT, FC • Routine checks on PT, PC
	No Flow	• Malfunction cell selection • Malfunctioning pressure controller	• Jam in lines • Pressure buildup that may rupture tubing	• FT, FC • PT, PC	• Routine replacement of tubing • Routine checks on FT, FC • Routine checks on PT, PC

3.7. Material Safety Data Sheet

Table 3.4.1 below summarizes key chemical components in the system, including flash points, flammability ratings, reactivity, health considerations, and any special considerations. The chemicals were identified in the respective simulations for LVV and CAR-T in SuperPro.

Table 3.4.1: Chemical Safety Data Sheet (MSDS) Summary Table. *Values taken from the ThermoFisher Scientific database.*

Chemical	Flash Point (° C)	Flammability (NFPA Rating)	Reactivity	Health (NFPA Rating)	Special Considerations
4-Isopropylbenzoic acid	N/A	0	0	2	Store sealed in cool, dark well ventilated room

Chemical	Flash Point (° C)	Flammability (NFPA Rating)	Reactivity	Health (NFPA Rating)	Special Considerations
Amino Acids	N/A	0	0	0	Store sealed in cool, dark well ventilated room
Ammonium Acetate	136	1	1	1	Store sealed in cool, dark well ventilated room
Benzonase Nuclease	N/A	0	0	0	Store sealed in cool, dark well ventilated room
Carbon dioxide	75	2	0	2	Combustible material
D-Sucrose	N/A	1	0	0	Store sealed in cool, dark well ventilated room

Chemical	Flash Point (° C)	Flammability (NFPA Rating)	Reactivity	Health (NFPA Rating)	Special Considerations
DMSO	87	2	1	2	Store sealed in cool, dark well ventilated room
Domiphen Bromide	N/A	1	1	2	Store sealed in cool, dark well ventilated room
Doxycycline Hydrochloride	N/A	1	0	2	Store sealed in cool, dark well ventilated room
Ethyl alcohol	16.6	3	0	2	Store sealed in cool, dark well ventilated room
Formaldehyde	50	2	0	3	Flammable, vapors may form explosive mixture with air

Chemical	Flash Point (° C)	Flammability (NFPA Rating)	Reactivity	Health (NFPA Rating)	Special Considerations
Glucose	N/A	1	0	1	Store sealed in cool, dark well ventilated room
Leukopak	N/A	0	0	0	Store sealed in cool, dark well ventilated room
Magnesium Chloride	N/A	0	0	1	Combustion products may include carbon oxides or other toxic vapors
Nitrogen	N/A	1	0	2	Store sealed in cool, dark well ventilated room
Nucleic Acids	N/A	0	0	1	Store sealed in cool, dark well ventilated room

Chemical	Flash Point (° C)	Flammability (NFPA Rating)	Reactivity	Health (NFPA Rating)	Special Considerations
Oxygen	N/A	0	0	2	Store sealed in cool, dark well ventilated room
Phosphate Buffered Saline Solution	N/A	0	0	1	Store sealed in cool, dark well ventilated room
Phosphoric Acid	N/A	0	1	3	Possibility of irritating gas and vapors
Polysorbate 80	150	1	0	0	Store sealed in cool, dark well ventilated room
Potassium Chloride	N/A	0	1	1	Store sealed in cool, dark well ventilated room

Chemical	Flash Point (° C)	Flammability (NFPA Rating)	Reactivity	Health (NFPA Rating)	Special Considerations
Potassium Dihydrogen Phosphate	N/A	0	1	0	Possibility of irritating gas and vapors
Sodium Chloride	N/A	0	0	0	Combustion products may include carbon oxides or other toxic vapors.
Sodium Hydrogen Phosphate	N/A	0	1	1	Store sealed in cool, dark well ventilated room
Sodium Hydroxide	N/A	0	0	3	Store sealed in cool, dark well ventilated room
Sucrose	N/A	1	0	0	Store sealed in cool, dark well ventilated room

Chemical	Flash Point (° C)	Flammability (NFPA Rating)	Reactivity	Health (NFPA Rating)	Special Considerations
Triton X-100	251	1	1	2	Possibility of irritating gas and vapors
Tris hydrochloride	N/A	1	1	2	Store sealed in cool, dark well ventilated room

4. Impact of the Project

The impact of this project could be broken into four main categories: societal impact, environmental impact, strengths and weaknesses, and costs and benefits. This project aimed to design a new process that would be used to manufacture CAR-T cells and LVVs for AGC Biologics. The short-term applications of this project include greater access to CAR-T therapies since AGC Biologics is a CDMO that provides CAR-T cells for other biotechnology companies. AGC Biologics could enter the gene therapies market with this process, making the product more accessible to other companies. For long-term applications, this project would make CAR-T therapies more accessible. Currently, CAR-T therapies are extremely expensive and are used as a last resort, but if more companies could manufacture CAR-T cells and LVVs, it could drive the cost down. CAR-T cells have been approved to be used in six blood cancers, so this project could make those cancers easier to treat by expanding access to CAR-T therapies (*National Cancer Institute*, 2022). The societal impact of this project is that this project will provide individualized medicine for cancer patients in need. Often, CAR-T therapies are last resort therapies, so people would seek out treatment when nothing else worked (Ledford, 2022).

The environmental impact of this project would have a somewhat negative impact because of the sterilization requirements. Since the CAR-T cells and LVVs must be produced in a highly sterile environment, many components must be single-used and disposed of afterward. All the materials being disposed of increase the waste that AGC Biologics would produce. However, this is not unique to this specific process, as many bioprocesses include single-use systems that have to be disposed of.

The strengths of this specific project include that we were able to implement the CliniMACS Prodigy to assist in automating the process and optimizing parts of the process. The CliniMACS system allowed for the majority of the CAR-T process to be done in one piece of equipment, making it more efficient for operator use and time. Additionally, we optimized the type of filters that would be used and the timing of the LVV production, since it is a lengthy process. Since this project could be optimized, it was a strength because it could benefit more patients than initially expected. The weakness of this project is the inability to scale up the process. This project deals with patients' cells which must be kept highly sterile throughout the entire process so only one patient can be processed at a time. Also, the processing time is lengthy so it makes it more difficult to manufacture different parts of the LVV at the same time.

Lastly, the costs of this project include the high cost of the CAR-T cells, the high specificity of treatment, and the immunosuppressive behavior of the treatment. First, CAR-T therapies are extremely expensive and could cost over $500,000 for one treatment so it limits the people that could afford the treatment (*National Cancer Institute*, 2022). Additionally, CAR-T therapies do

not have success for solid tumors and only treat specific blood and lymphoma cancers where the CAR-T cell can reach the cancer cell. Patients that take CAR-T therapies also remain immunosuppressed for the rest of their life since the CAR receptors permanently alter the T-cells the body produces (Sterner and Sterner, 2021). Although this therapy can save many lives, it limits the quality of life that the patients have post-treatment. The benefits of CAR-T and LVV production include increased access to treatment and improvement in quality of life. As previously mentioned, this project can increase access to CAR-T therapies since AGC Biologics would be an additional manufacturer. The quality of life would also increase since this project provides the tools to treat cancers that would otherwise be fatal.

5. Approach & Premises

5.1. Overview of Approach

We began our work on this project by roughling outlining all the tasks to be completed in a Gantt chart created in Google Sheets. We divided up the work accordingly so that we knew which team members would be responsible for which aspects of the project. However, these assignments were relatively fluid and naturally changed throughout the duration of the project. Nevertheless, they were helpful in enabling team members to have guidance from the start on what tasks they needed to focus on. Our work with the project officially began with writing out the project description, scope, and background and technology backgrounds to guide us throughout our work on the project. We also outlined relevant battery limits, expanding on the initial information that AGC Biologics had provided us for the project, and built process flow diagrams in Canva and SuperPro to help us visualize our process design. Environmental, health, and safety (EH&S) considerations were built upon throughout the course of our work on the project.

We first focused our efforts on the design and model of the lentiviral vector (LVV) production process. This included process modeling in SuperPro Designer, mathematical modeling in MATLAB, and unit designing through literature research and exploring available external resources. We divided up the LVV process design into sections, with different team members focusing on different aspects of the process. LVV process design included establishing cell revival, suspension expansion, bioreactor, and endonuclease protocols as well as making decisions on the reactor, viral vector, filters, chromatography columns, and freezers to use. Once the LVV process design was completed, we transitioned to focusing on other tasks. Certain team members focused on design of the CAR-T process while others focused on economic analysis and others on wrapping up EH&S considerations via HAZOP analysis. We came together as a team in the end to blend our work together into this report and our final presentation.

The softwares used in our approach to this project includes Google Sheets (Gantt chart, material balances, suspension expansion protocol, and economics), MATLAB (mathematical modeling of

50L suspension bioreactor), and SuperPro Design (PFD and simulations of LVV and CAR-T processes)

5.2. Project Management

From the start of our work on this project, we assigned group members different tasks and responsibilities which were organized via the use of a Gantt chart, shown below in Figures 5.2.1, 5.2.2, and 5.2.3. In terms of timelines, we sought to follow those which were suggested by our instructors. This included having the Gantt chart, project background, battery limits, and project premises completed as well as the process conceptualized by February 9. By February 23, we aimed to define our PFDs, finalize battery limits based on liaison feedback, and start material balances. Between February 23 and March 23 we planned to dedicate time to finalizing material balances, complete process simulations, work on mathematical modeling, define our homework problem, and work on equipment design. Between March 23 and the end of the project, we hoped to finalize our process designs and perform economic analysis and process optimization. It is important to note that the aforementioned timeline was that which we tried to adhere to throughout the course of our work on this project. Our work was fluid and certain areas took more or less time to complete depending on any issues faced during the completion of the relevant tasks. The precise days over which we completed each section is reflected in the Gantt chart below. The Gantt chart also makes note of task assignments, although there were some instances in which other group members assisted on tasks in which they were not assigned and were not the main person involved in completing the task.

	Start Date	End Date	Start on Day	End on Day	Duration	Team Member(s)
Oral Presentation	Repeated	Repeated				All
Cover Page & Table of Contents	2/6	5/3	1	87	86	Hannah
Executive Summary and Recommendation	5/1	5/3	84	86	2	All
Project Description, Scope, and Background	2/6	2/9	1	4	3	Hannah
Technological Background/Process Outline	3/4	3/23	26	45	19	Hannah & Nigel
Environmental, Health & Safety Consideration	2/6	4/6	1	60	59	Ashish & Alexis
HAZOP	3/23	4/6	46	60	14	Alexis
Impact of Project	4/28	5/2	81	85	4	Maya
Process and Economic Battery Limits	2/6	2/10	1	5	4	Maya, Nigel
Process Flow Diagram	2/9	3/2	3	24	21	Alexis, Maya, Hannah
Material Balances	2/12	3/23	7	46	39	Nigel
Reactor Protocol	3/21	3/25	43	47	4	Nigel
Superpro Simulations	3/2	4/3	24	56	32	Hannah & Maya
Mathematical Modelling	3/9	3/23	31	45	14	Maya
Endonuclease & LV Design	4/6	4/20	59	73	14	Hannah & Maya
Filters Design	4/3	4/21	56	74	18	Ashish & Alexis
Chromatography Columns Design	3/27	4/4	49	57	8	Nigel
Cell Expansion Design	3/23	4/20	45	73	28	Hannah
CliniMACS Protocol / Design	3/25	5/1	47	84	37	Hannah & Alexis
Freezers Design	4/11	4/18	65	72	7	Nigel
Estimation of Capital Investment and Costing comparison	4/22	5/2	76	86	10	Nigel
Variable and Fixed Operating cost estimate	4/22	5/2	76	86	10	Nigel
Profitability anaysis (IRR, NPV, ROI)	4/22	5/2	76	86	10	Nigel
Economic Sensitivity Analysis	4/22	5/2	76	86	10	Nigel
HW Problem	4/8	4/20	61	73	12	Ashish
References, Nomenclature, Appendices	2/6	5/3	1	87	86	All
Final Deliverables	2/6	5/3	1	87	86	All

Figure 5.2.1: Gantt Chart: *This is a snapshot of the Gantt chart generated for this project containing all tasks as well as their start dates and end dates and which team members contributed to their completion and authorship.*

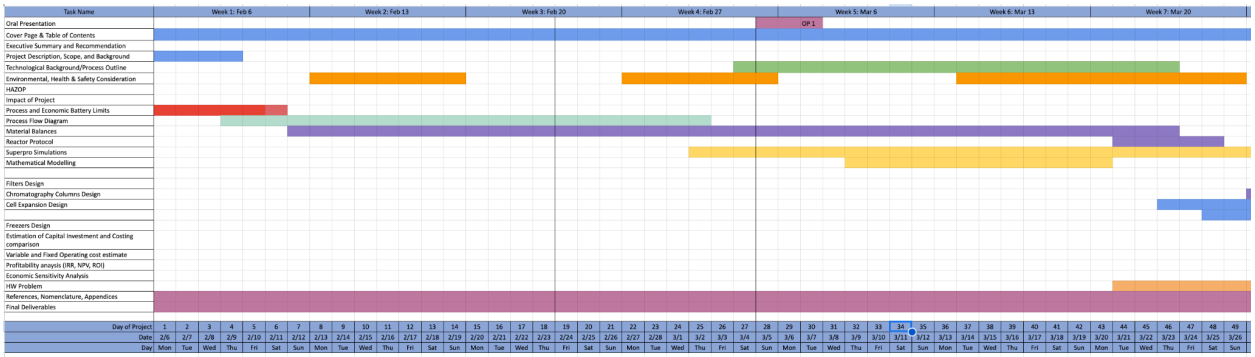

Figure 5.2.2: Gantt Chart: *This is a snapshot of weeks 1 to 7 in the Gantt chart generated for this project containing a visual diagram of when all tasks were worked on and who contributed to that work.*

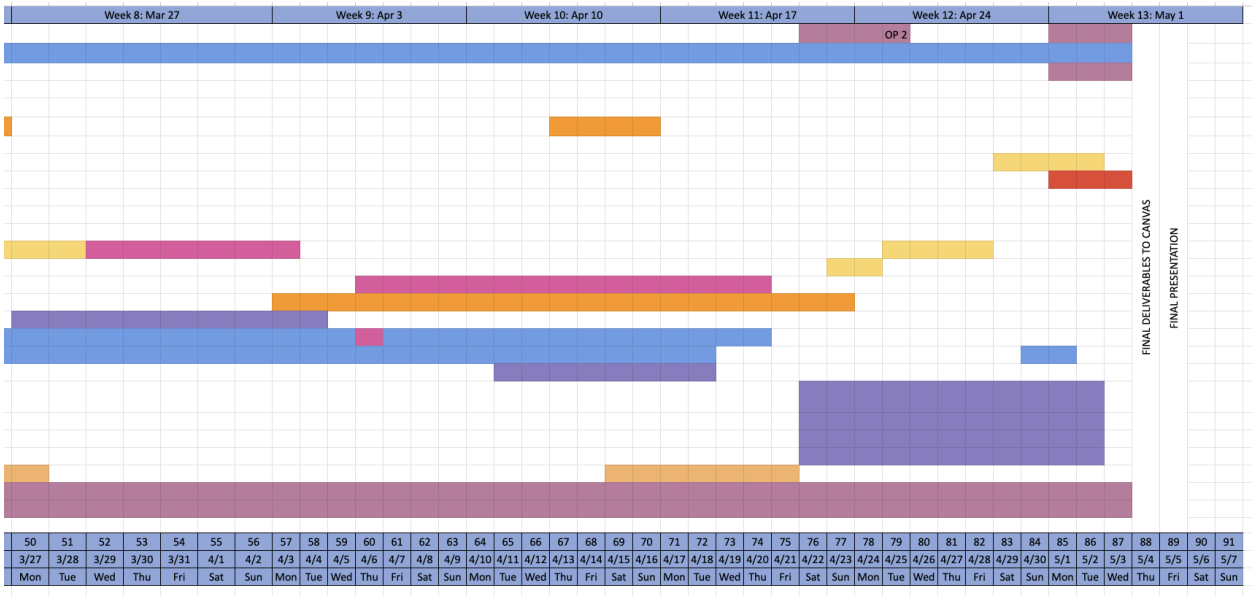

Figure 5.2.3: Gantt Chart: *This is a snapshot of weeks 8 to 13 in the Gantt chart generated for this project containing a visual diagram of when all tasks were worked on and who contributed to that work.*

A few zoomed-in snapshots of the Gantt chart timeline shown above in Figures 5.2.2 and 5.2.3 were captured for biweekly timelines and can be found in Appendix B Figures B-1 to B-4 of this report.

Nigel Highhouse was responsible for working on the following tasks:

- Technological background and process background of CAR-T process
- Process and economic battery limits
- Project and economic premises
- Material balances
- Reactor protocol
- Designed overall downstream process flow
- Chromatography column design
- Assisted with filter design
- Freezer protocol
- Economics: estimation of capital investment and costing comparison, profitability analysis (IRR, NPV, ROI), economic sensitivity analysis
- Report and presentation editing
- Assisted with Table of Content

Hannah Padgette was responsible for working on the following tasks:

- Creating the cover page / table of contents / report outline
- Project description, scope, and background
- Technological background and process background of LVV process

- Process flow diagram of LVV process in SuperPro
- Assisted with the setup of LVV and CAR-T process for simulation in SuperPro
- Endonuclease protocol development
- LVV cell revival and suspension expansion protocol development
- CliniMACS protocol, design, and writeup
- Overview of approach
- Project management writeup
- Report and presentation editing
- Initial data and assumptions made

Maya Singh was responsible for working on the following tasks:

- Process and economic battery limits
- Impact of project
- Process flow diagram of CAR-T process in SuperPro
- Setup and building of LVV and CAR-T process for simulation in SuperPro
- Assisted with cell suspension expansion protocol
- Mathematical modeling of 50 L bioreactor
- Lentiviral vector choice
- Report and presentation editing
- Assisted with Table of Content

Ashish Srivastava was responsible for working on the following tasks:

- Environmental, health, and safety considerations
- Filters design
- Homework problem

Alexis Walker was responsible for working on the following tasks:

- HAZOP & MSDS
- Worst case scenario
- Assisted with filters design
- Assisted with evaluating CliniMACS Prodigy considerations
- Assisted with battery limits
- Assisted with Environmental, health, and safety considerations
- Utilities write up
- Visual process flow diagram for LVV and CAR-T processes
- Report and presentation editing
- Assisted with executive summary

All group members were responsible for the oral presentations, executive summary, final recommendations, references, appendices, and final deliverables. The group maintained consistent communication via text and met in person two to three times per week on average.

5.3. Process Description and Flow Diagram

5.3.1. Visual Process Flow Diagrams

Herein, we detail lentiviral vector (LVV) and CAR-T cell manufacturing processes. In order to better visualize these processes, a process flow diagram was constructed in Canva, as displayed in Figure 5.3.1.1. Separate sections of the overall process are color coded, with the LVV upstream process shown in a light blue outline, the LVV downstream process shown in teal, and the CAR-T process shown in dark blue. Steps to be conducted by the CliniMACS Prodigy system have an additional dark gray border.

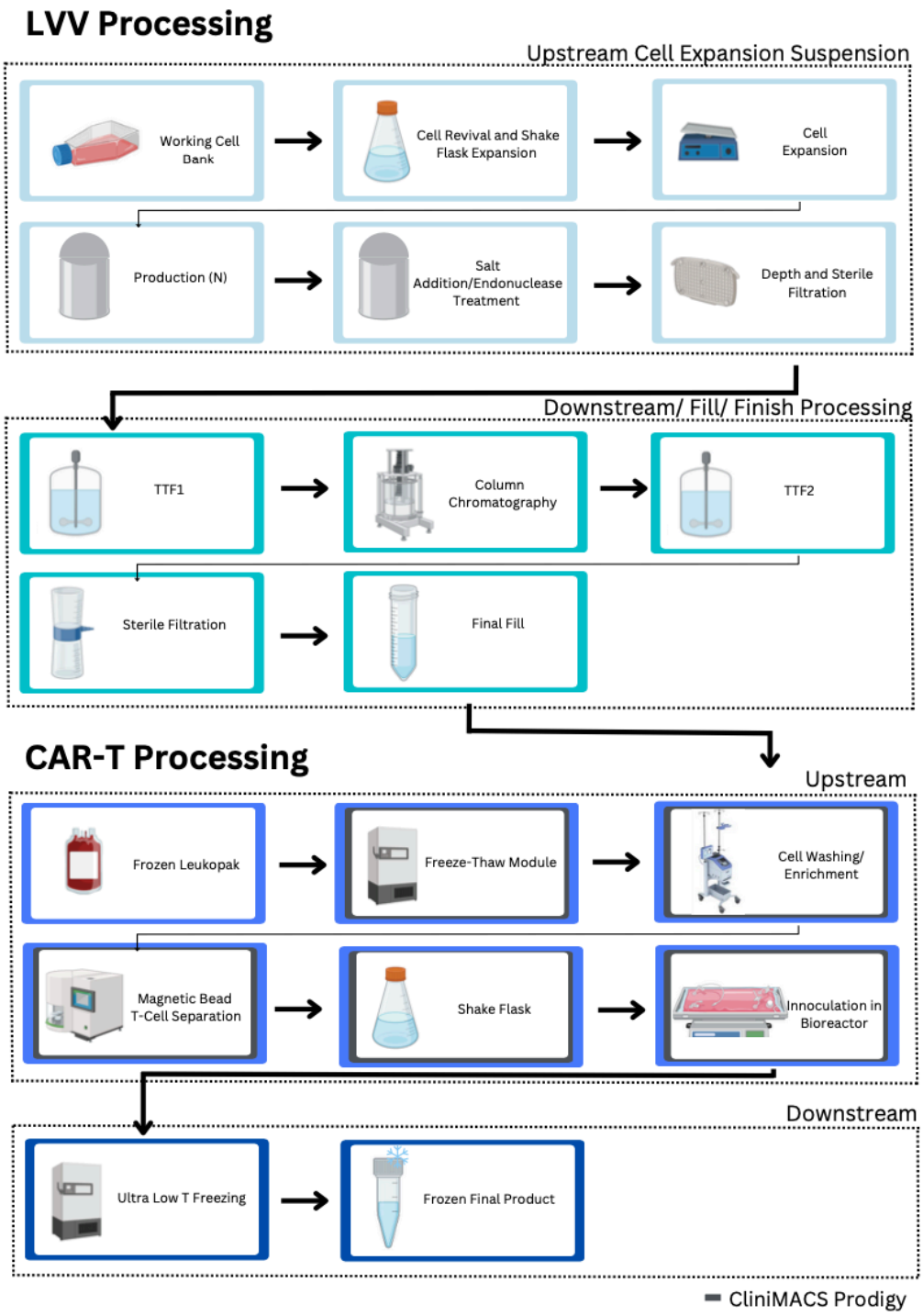

Figure 5.3.1.1: LVV through CAR-T Manufacturing Process Flow Diagram. *The overall digram depicts the process from LVV production to CAR-T manufacturing.*

Individual units such as the bioreactor, filters, CliniMACS Prodigy, and freeze modules are described further through the report.

Lentiviral Vector Visual Process Flow Diagram

The lentiviral vector production process is broken down into upstream and downstream processes, as shown below in Figure 5.3.1.2.

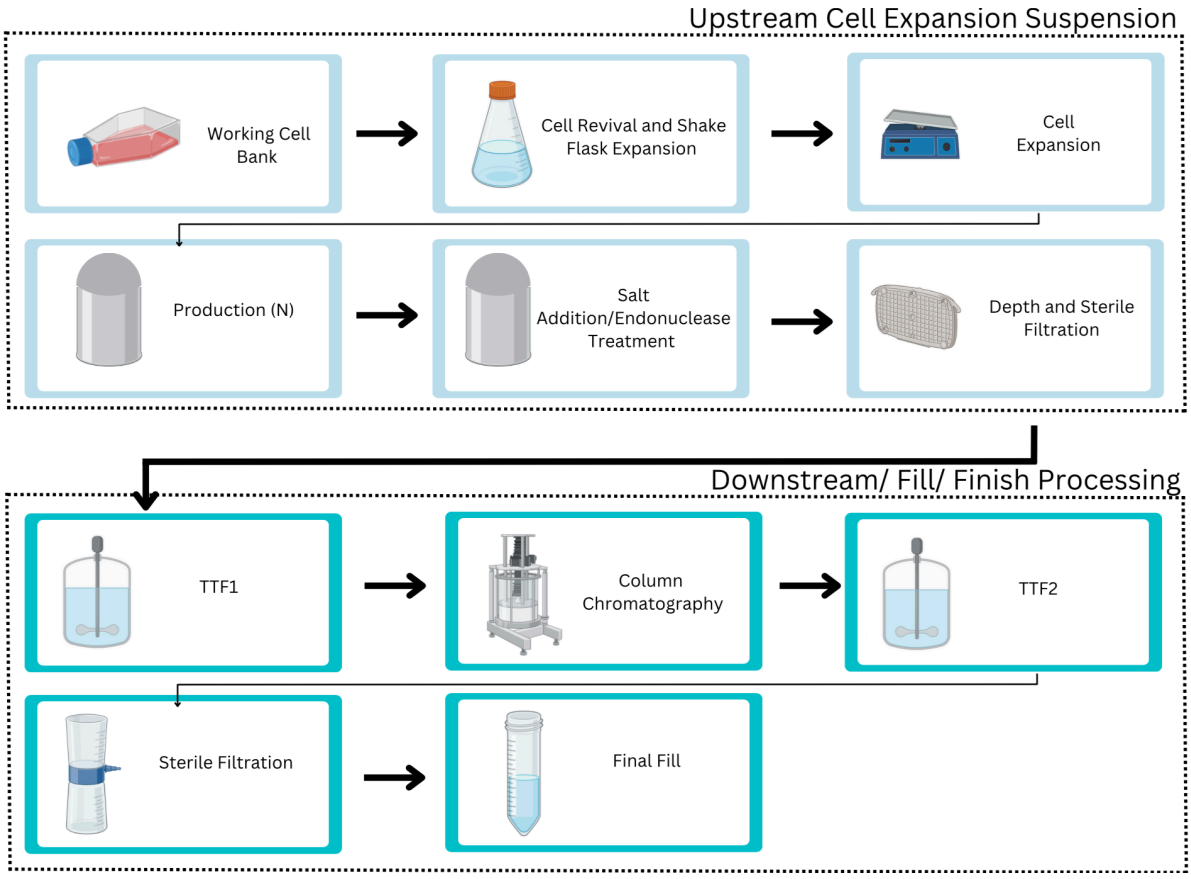

Figure 5.3.1.2: LVV Process Flow Diagram. *Lentiviral vector process including both upstream and downstream steps.*

The LVV begins with the working cell bank of HEK293-T cells. The T cells are revived before transitioning to expansion. After these steps, the cells undergo further expansion, transient transfection, and endonuclease treatment in the bioreactor. Then, the working volume undergoes depth and sterile filtration steps. Downstream processing includes tangential flow filtration, sterile filtration, and column chromatography. The LVV production process ends with a final fill and cryopreservation.

CAR-T Visual Process Flow Diagrams

The production of the CAR-T cells occurs as shown below in Figure 5.3.1.3.

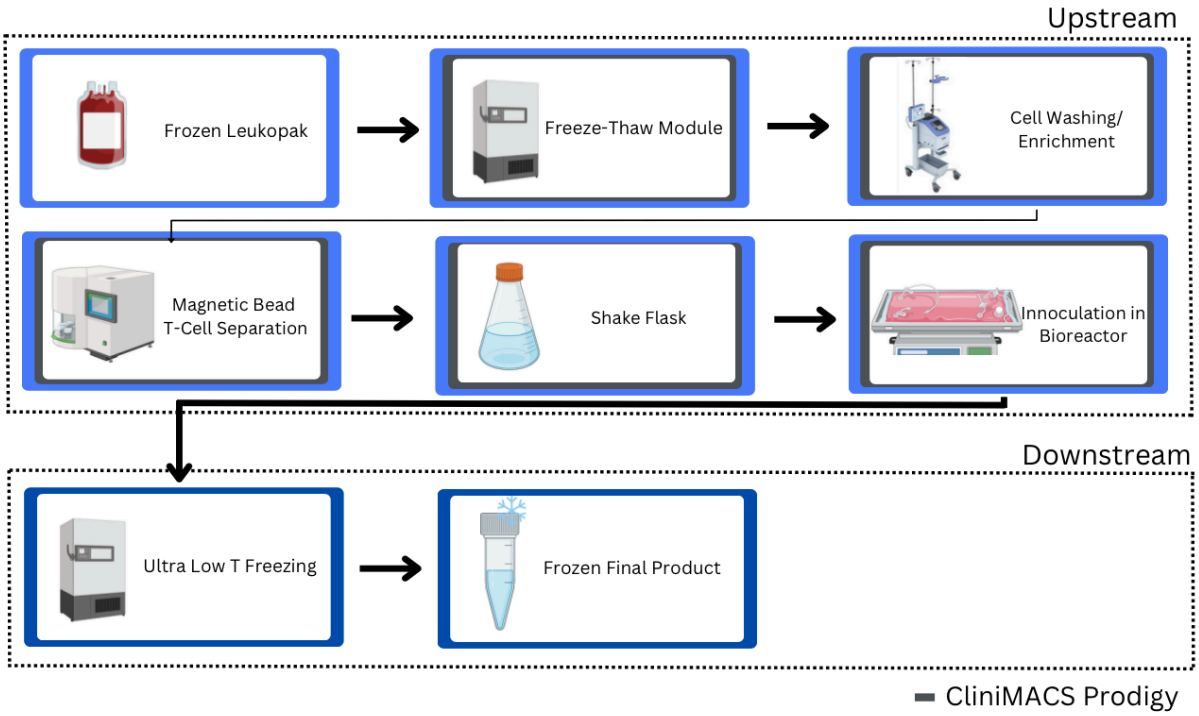

Figure 5.3.1.3: CAR-T cell Process Flow Diagram. *CAR-T cell process including both upstream and downstream steps.*

The CAR-T production process begins with a frozen leukopak, which must first be thawed. The general thawing occurs in a water bath. The following manufacturing steps include cell washing, T-cell separation, transient transfection, and CAR-T cell expansion, which can be automated by the CliniMACS Prodigy system. Finally, the CAR-T cells undergo controlled rate freezing, yielding a cryopreserved therapy which can then be transported back to the patient.

5.3.2. SuperPro Process Flow Diagrams

Beyond the aforementioned visual process flow diagrams, we also sought to generate process flow diagrams (PFDs) that outlined more specifics of the individual process units and streams. We utilized SuperPro Designer, a batch and continuous process modeling software in various industries. We constructed two separate diagrams, one for the lentiviral vector (LVV) production process and another for the CAR-T cell manufacturing process. We modeled the SuperPro PFDs based on preliminary process information given by AGC Biologics and the process flow diagrams published by da Gama Gerreira *et al.,* who modeled similar gene therapy manufacturing processes using SuperPro.

Lentiviral Vector SuperPro Process Flow Diagram

The LVV upstream process PFD begins with cell revival and expansion. As shown in Figure 5.3.2.1 below, this portion of the process begins with HEK293-T cells. They are first revived and before undergoing a shake flask expansion process. This suspension expansion grows the cells up to a number and concentration needed to seed the bioreactor, also shown in the figure below. The cells will undergo additional expansion in the bioreactor as well as transient transfection with the LVV of choice. Additional supporting units are found in this portion of the PFD, including storage for growth media, air filtration, and gas compression units.

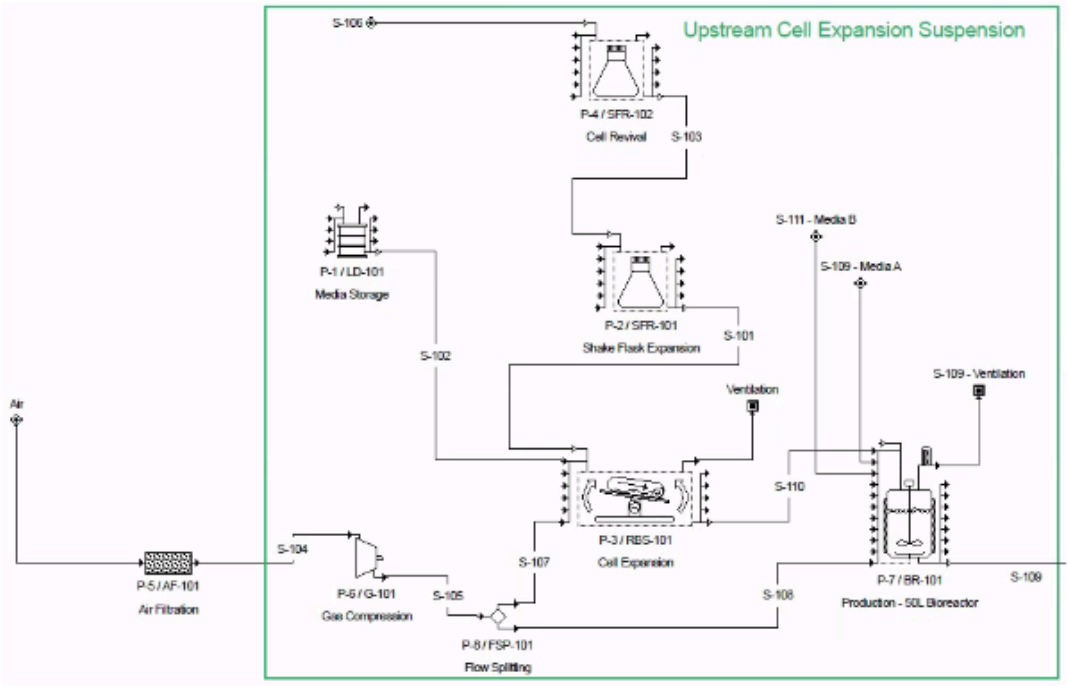

Figure 5.3.2.1: LVV Upstream Cell Expansion Suspension PFD. *The upstream portion of the LVV manufacturing process revolves around HEK293-T cell expansion. Processes in this portion of the process include cell revival, shake flask expansion, and 50L bioreactor suspension expansion and vector transfection.*

Next, the HEK293-T cells and viral vectors are harvested from the bioreactor and undergo a series of processing steps before the working volume is fed to the downstream portion of the process. As shown in Figure 5.3.2.2, harvest from the bioreactor includes a salt / endonuclease treatment, a vital step in lentiviral vector processing. Depth and sterile filtration steps also occur with harvest and aim to minimize the presence of cellular and other contaminants in the final vector product. A retentate storage unit is also depicted here to demonstrate flexibility in operation timing with upstream and downstream processes. If desired, AGC Biologics may hold the vector product prior to release for downstream processing and fill/finish operations.

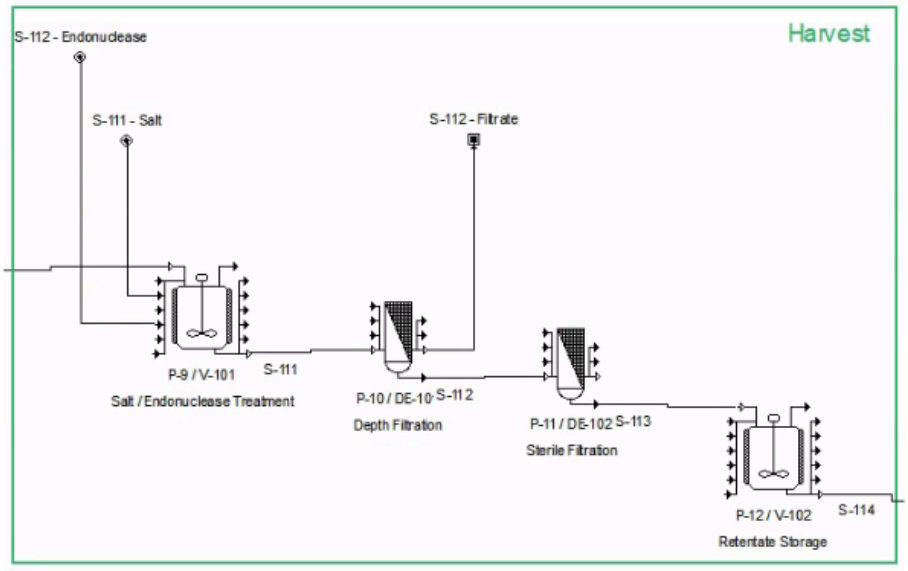

Figure 5.3.2.2: LVV Harvest PFD. *The harvest portion of the LVV manufacturing process revolves around pulling the vector product out of the bioreactor and performing some preliminary processing and filtration steps. Specifically, an endonuclease treatment is completed to remove nucleic acids from the working volume. Depth and sterile filtration units are then used to separate the viral vectors from cells and cellular contaminants.*

The final portion of LVV production consists of downstream processing and fill/finish operations. As shown in Figure 5.3.2.3, a series of chromatography and filtration–both tangential flow and sterile–units are employed to ensure sterility and purity of the final vector product. Final fill and storage of the vectors is the last step in this process and makes the lentiviral vector available for use in CAR-T manufacturing as needed.

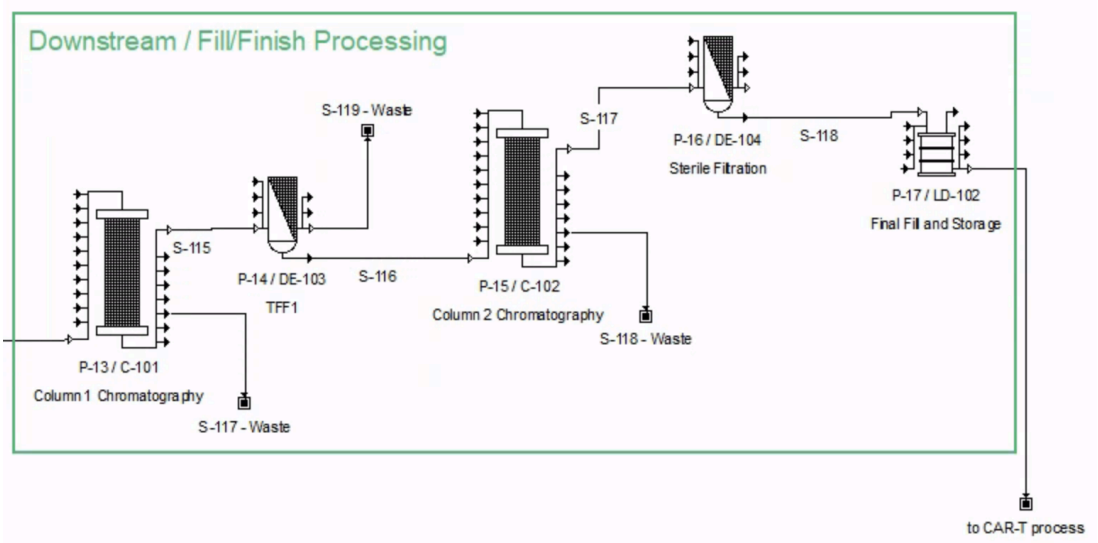

Figure 5.3.2.3: LVV Downstream and Fill/Finish Processing PFD. *The downstream portion of the LVV manufacturing process consists of a series of chromatography, tangential flow filtration,*

and sterile filtration steps. Final fill and storage generate a high-quality vector product that is available as needed for CAR-T manufacturing.

CAR-T SuperPro Process Flow Diagram

The CAR-T PFD can be split into the upstream and downstream sections. Figure 5.3.2.5 shows the upstream portion of the CAR-T PFD. The process begins with a thawing unit, modeled by a freeze-thaw module, which thaws frozen leukopak to room temperature. Next, a cell washing unit was added to wash the cells with two buffers modeled as a general box to give flexibility in the design. The flow splitter was used to reach a specific value that would be fed into the shake flask unit, where the rest was discarded as waste. The shake flask unit modeled the transduction and cell activation, which included the specific media and mixing of the LVV and media. Once the cells were transduced, they entered a shaking bioreactor, modeled by an inoculum preparation in SuperPro. Suggested media was added to the bioreactor to display the required material. Finally, a microfiltration system was used to purify the product before it went to the downstream portion.

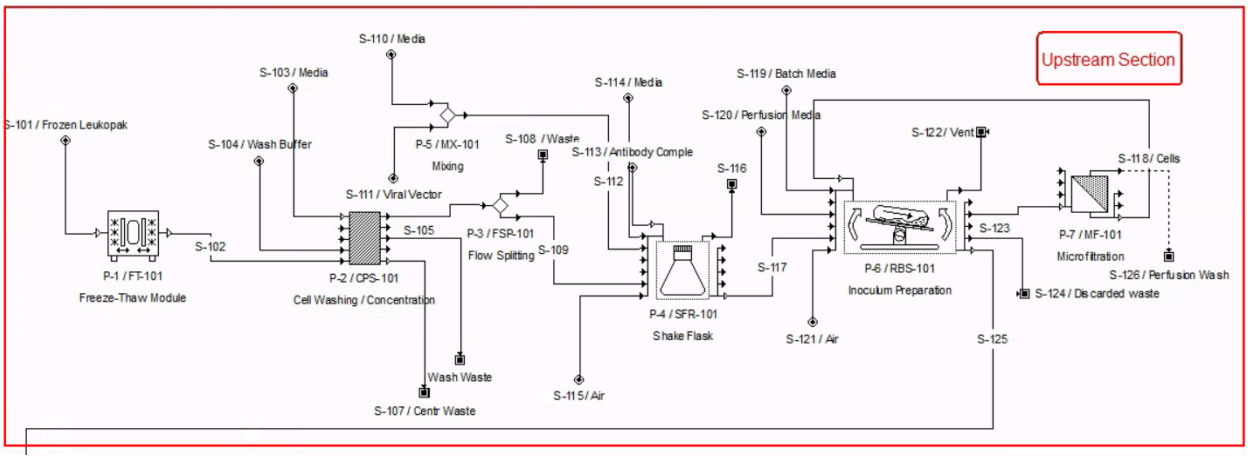

Figure 5.3.2.4: CAR-T Upstream PFD. *The upstream portion of the CAR-T manufacturing process consists of a thaw module, a cell washing unit, a shake flask, a shaking bioreactor, and a filtration unit.*

The next portion of the CAR-T PFD was the downstream portion which can be seen in Figure 5.3.2.5. This included a cell washing unit, a check for quality control, packaging, and a freezing unit. Similarly to the upstream portion of the CAR-T PFD, the cells were washed with the same setup. The main difference was that for the downstream portion, a cryopreservation buffer was used instead of media. Before the final product was packaged, a sample was sent for quality control which was indicated by the flow splitting unit. Next packaging was modeled as a generic procedure that converts the batch process to a discrete process. The freezing was modeled as a freezer module and the final product was split into a specific quantity of product.

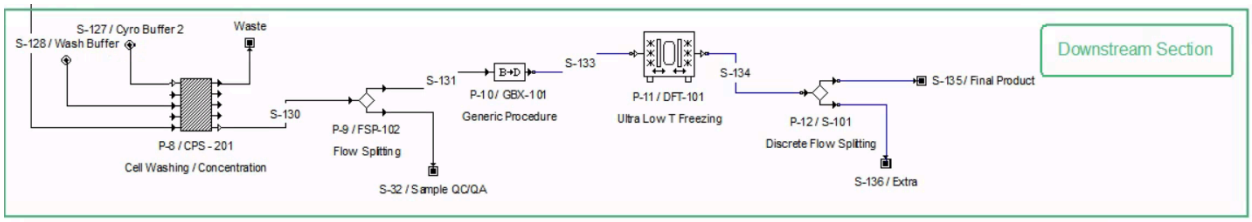

Figure 5.3.2.5: CAR-T Downstream PFD. *The downstream portion of the CAR-T manufacturing process consists of a cell washing unit, packaging, and a freezing unit.*

5.4. Initial Data & Assumptions Made

In our first presentation with the AGC Biologics team, we were given a presentation on some of the initial things we needed to consider during our work on the project. They outlined for us what cell therapy is, which human cells are targeted/used for cell therapy, and outlined what autologous CAR-T therapies are and how they are manufactured. They also outlined the general CAR-T process steps and gave us some initial process information for the lentiviral vector manufacturing process. Lastly, they provided us with some initial data to take into consideration when doing material balances and when designing the processes.

From this initial information provided by AGC Biologics, we assumed that we would be manufacturing a CD19 targeted CAR-T product over the course of 10 days, starting with a frozen PBMC vial with 100×10^6 cells. The T cells would be transduced with the in-house lentiviral vector, produced via a 50L suspension process. We assumed that 1 vial of HEK293-T cells to start this process would contain 1×10^7 cells. We assumed that cell expansion and transfection medias provided by AGC Biologics were sufficient and did not require supplementation with any additional materials. We assumed that we would achieve a vector concentration of $\geq$ 1,724 ng/mL ($\geq 4.3 \times 10^8$ TU/mL) post-fill and aimed for a process time of around 14 days. For LVV manufacturing, we assumed the following yields for downstream processing: clarification - 95-105%, chromatography - 70%, tangential flow filtration - 85-95%, and sterile filtration - 70%. We assumed all other yields were 95% unless otherwise specified. For CAR-T, we assumed that the patient capacity to design for was 52 patients per year, with the average patient weight is 170 pounds. We assumed that all units and materials being used at the facility would be single-use, so considering the cleaning of units between runs was not within the scope of our design. This includes single use flasks, bioreactor bags, mixing bags, and flow kits. Moreover, all of the equipment described herein does not have any direct product contact parts so there is no need for cleaning between uses. Instead of considering the cleaning of equipment, the thing that we did have to consider in our design was that all materials and equipment being used are sterilized and have the correct sterility assurance level for their associated use. Per the information we obtained from AGC Biologics, we also assumed that space was not a constraint, so our design and choice of units were made according to what we thought was best. We assumed that cell growth and

expansion media contained all components required for cell growth and transfection and that the HEK293-T cell line was already adapted to suspension expansion.

With regards to economics, we assumed a process lifetime of 15 years and standard IRR. We assumed that, given AGC Biologics is a CDMO, the material costs would be a pass-through cost that the clients pay for but for the purposes of economic analysis factored in an entire cost of all materials at $500k per run for both the LVV and CAR-T processes. Moreover, we assumed that salaries were $40/hr per employee, with all processing requiring a minimum of 3 staff. We assumed that they would work no longer than 10-hour days and no more than 8 hours doing processing. We assumed there would be 1 supervisor per shift per stream (i.e., upstream has one and downstream has one if working on the same day) with a salary of $55/hr. We assumed that 7 days a week coverage would be allowed if needed

5.5. Battery Limits and Design & Economic Premises

5.5.1. Battery Limits

Battery limits are essential because they split up various areas into different sections, called functional units. The main units are inside battery limits (ISBL), which consist of the necessities for plant operation, and outside battery limits (OSBL), everything not included in ISBL. The units that are part of ISBL are the equipment directly related to the production of the product, the CAR-T cell therapy in this case. A few examples of OSBL include external utilities, workers' common spaces, management workspace, maintenance workshops, and material storage.

For this project, ISBL was divided into two main functional sections: lentiviral vector (LVV) production and CAR-T cell therapy manufacturing. The LVV ISBL can be further broken down into the suspension portion of the LVV production, the harvest of the LVVs, and the purification of the LVVs to yield a product suited for transduction in T cells. The upstream process can be further broken down into units including cell revival, cell expansion, and production. Next, the harvest is composed of endonuclease treatment, and depth and sterile filtration. The downstream portion is divided into a depth filtration step, two different tangential flow filtration steps, a chromatography column, sterile filtration, and the final fill. The CAR-T process ISBL can be broken down into initial cell processing, CAR-T cell production, and CAR-T processing steps. Initial cell processing includes the collection of patient blood samples and isolation of non-differentiated T cell populations using CD4 as a marker. The initial T cell processing can be accomplished by an automated CliniMACS Prodigy system, or using separate antibody labeling and magnetic bead separation steps. The isolated T cells must be activated prior to transduction, which can be done using an artificial antigen-presenting cell (APC) platform or CD3-specific antibodies. CAR-T cell production revolves around the transduction step, where T cells are modified to express the desired chimeric antigen receptor via DNA

transduction by the LVV in a shake flask. Finally, in the CAR-T cells undergo processing in the form of cellular expansion, dosing, and freezing in a controlled-rate freezer. The CAR-T manufacturing process can take approximately 8 to 15 days.

Next, the first OSBL of the upstream process is the process of collecting the cells from the patient and shipping the cells to the facility. The following OSBL includes any external utilities, such as water and electricity that could be used to produce the product. Since all of the production is done in one location, there are no transportation OSBLs needed during the upstream and downstream processes. Since the LVV and CAR-T cells need to be frozen to a very low temperature after production, a utility such as liquid nitrogen has to be used. After the downstream production, the next OSBL transports the cells back to the facility where they can be infused.

5.5.2. Design Premises

- Company: AGC Biologics
- Annual Capacity: 30 CAR-T treatments/year
- Goal: Site expansion
- Location: Longmont, CO
- Continuous vs. Batch: Batch operation for all reactors
- Single-use materials are desired for all process stages
- Average patient treated is 170 lb
- Starting Material Specifications:
    - LVV WCB is $1x10^7$ HEK293-T cells in 20 mL vial
    - CAR-T patient PBMC sample of $100x10^6$ cells
- Size of Bioreactor Harvest Pool: 50 L
- Product Specifications:
    - 0.500-1.00 L LVV formulation at $\geq 4.30x10^8$ TU/mL
    - CAR-T treatment at 10^6 CAR-T cells/kg of patient body weight
- AGC Proposed Times:
    - LVV: 14 days
    - CAR-T: 10 days

5.5.3. Economic Premises

- Operating Hours/Year: 8000 hours/year
- Cytiva XDR-50 bioreactor is available at no additional cost
- Lab-scale cell revival expansion materials are available at no additional cost
- Controlled-rate freezer is available at no additional cost
- Miscellaneous Equipment Cost: 10% of total purchased price of all equipment
- Site Type: Project addition
    - Cost of Site Preparation: 5% of total purchased price of all equipment

    - Cost of Service Facilities: 5% of total purchased price of all equipment
- Contingency and Contractor's Fee: 18%
- Inflation: 1.9%
- Working Capital: 2.5% of Fixed Capital Investment
- Startup Date: January 1, 2026
- Project Life: 15 years
    - 2-year construction period
- Cost of raw materials:
    - $500,000.00/run all utilities, reagents, media, and waste disposal
    - $26,000.00/run CliniMACS Prodigy kit
    - $165.51/run Cytiva Supor EKV filter
    - $5,800.00/run Sartorius 12" Bioproducer TFF modules
    - $5,700.00/run Sartorius Sartobind Q AEX chromatography column
- Operator Salary: $40/hr
    - Operating Supervisor Salary: $55/hr (138% of operator salary)
    - Employee Benefits: 40% of salary
    - General Overhead: 125% of operator and supervisor salary
- Processing requires a minimum of 3 staff
- Operators work no longer than 10-hour days, with no more than 8 hours spent on processing
- 1 supervisor per shift per stream is required
- Processing occurs 24 hr/day (3 shifts), 7 days/week
- Tax Rates
    - Total Effective Tax Rate: 24%
    - Insurance and Local Taxes: 1.5%
- Cost of Capital: 4.5%
- Depreciation Method: 5-year MACRs
- Salvage Value: 0% of purchased cost
- Accounts Receivable: 45 days
- Base Case Sales Price of Products: $749,091.26 per CAR-T treatment
- Base Case Target IRR: 12.5%
- Base Case Target ROI: 23.9%

5.6. Material Balances

Beginning the material balances for the lentiviral vector production process, we identified a starting working cell bank (WCB) of 1.00×10^7 cells in 20 mL of medium. Furthermore, we identified a target product concentration of 4.50×10^8 TU/mL LVVs in a target volume of 500 mL. We have further developed a set of typical recoveries for the different steps of the downstream processing on which we based the material balances. These typical recoveries are summarized below in Table 5.6.1:

Table 5.6.1: Typical recoveries of LVV downstream processing steps. *Recoveries are given as a percentage. Values are based on Moreira, et al. (2020) and Valkama, et al. (2020).*

Processing Stage	Typical/Approximate Recovery (%)
Clarification	95
Harvest/Depth Filtration	70
Tangential Flow Filtration 1	90
Chromatography	30
Tangential Flow Filtration 2	90
Sterile Filtration	70

Using these typical recoveries, we worked backwards from the target concentration of 4.50×10^8 TU/mL LVVs to determine the necessary inlet and outlet concentrations at each processing step. Since we assumed no changes to the process volume occur in the chromatography, TFF2, and sterile filtration steps, the material balances around these steps were as follows:

$$C_{LVV,\,Ster\,Out} = \left(C_{LVV,\,Ster\,In}\right)(\%\,Rec.)$$
$$4.50 \times 10^8\,TU/mL = \left(C_{LVV,\,Ster\,In}\right)(70\%)$$
$$C_{LVV,\,Ster\,In} = 6.43 \times 10^8\,TU/mL = C_{LVV,\,TFF2\,Out}$$

$$C_{LVV,\,TFF2\,Out} = \left(C_{LVV,\,TFF2\,In}\right)(\%\,Rec.)$$
$$6.43 \times 10^8\,TU/mL = \left(C_{LVV,\,TFF2\,In}\right)(90\%)$$
$$C_{LVV,\,TFF2\,In} = 7.14 \times 10^8\,TU/mL = C_{LVV,\,Chrom\,Out}$$

$$C_{LVV,\,Chrom\,Out} = \left(C_{LVV,\,Chrom\,In}\right)(\%\,Rec.)$$
$$7.14 \times 10^8\,TU/mL = \left(C_{LVV,\,Chrom\,In}\right)(30\%)$$
$$C_{LVV,\,Chrom\,In} = 2.38 \times 10^9\,TU/mL = C_{LVV,\,TFF1\,Out}$$

Since the TFF1 processing step involves the concentration of the process volume from the 50 L harvested from the bioreactor down to 500 mL of target product volume, the material balances around the TFF1 step were as follows:

$$C_{LVV,\,TFF1\;Out} = \left(C_{LVV,\,TFF1\;In}\right)(\%\;Rec.)\left(\frac{V_{initial}}{V_{final}}\right)$$
$$2.38\;*\;10^{9}\;TU/mL = \left(C_{LVV,\,TFF1\;In}\right)(90\%)\left(\frac{50.0\;L}{0.500\;L}\right)$$
$$C_{LVV,\,TFF1\;In} = 2.65\;*\;10^{7}\;TU/mL = C_{LVV,\,Har\;Out}$$

The harvest and depth filtration step does not involve any change in process volume. Therefore, the material balances around this step were as follows:

$$C_{LVV,\,Har\;Out} = \left(C_{LVV,\,Har\;In}\right)(\%\;Rec.)$$
$$2.65\;*\;10^{7}\;TU/mL = \left(C_{LVV,\,Har\;In}\right)(70\%)$$
$$C_{LVV,\,Har\;In} = 3.78\;*\;10^{7}\;TU/mL = C_{LVV,\,BRX\;Out}$$

In the bioreactor, HEK293-T cells are seeded, cultured, and production of LVVs is performed. Based on the experimental methods and resultant data from Cytiva Life Sciences (2019), we determined a specific LVV production of 3.6 TU/cell over the production period, and a HEK293-T cell expansion factor of 19.4 over the course of the preceding bioreactor cell culture. These data allowed for the determination of the required cellular concentration for bioreactor inoculation. Therefore, the material balances around the bioreactor were as follows:

$$C_{LVV,\,BRX\;Out} = \left(C_{Cells,\,BRX,\,Ind}\right)(Specific\;LVV\;Production)$$
$$3.78\;*\;10^{7}\;TU/mL = \left(C_{Cells,\,BRX,\,Ind}\right)(3.6\;TU/cell)$$
$$C_{Cells,\,BRX,\,Ind} = 1.05\;*\;10^{7}\;cells/mL$$

$$C_{Cells,\,BRX,\,Ind} = \left(C_{Cells,\,BRX\;In}\right)(Expansion\;Factor)$$
$$1.05\;*\;10^{7}\;cells/mL = \left(C_{Cells,\,BRX\;In}\right)(19.4)$$
$$C_{Cells,\,BRX\;In} = 5.40\;*\;10^{5}\;cells/mL = C_{Cells,\,Exp\;Out}$$

Given this required cellular concentration for bioreactor inoculation and the starting WCB of 1.00×10^7 cells, the required number of times the HEK293-T cells must be doubled during the suspension expansion in the upstream, n_{double}, could be determined. The initial WCB must be diluted to a 50 L process volume, as this is the working volume of the bioreactor. Therefore, the material balances for suspension expansion were as follows:

$$C_{Cells,\,Exp\,In} = \left(C_{Cells,\,WCB}\right)\left(\frac{V_{WCB}}{V_{Process}}\right)$$
$$C_{Cells,\,Exp\,In} = \left(\frac{1.00*10^{7}\ cells}{20\ mL}\right)\left(\frac{0.020\ L}{50.0\ L}\right)$$
$$C_{Cells,\,Exp\,In} = 2.00 * 10^{2}\ cells/mL$$

$$\left(C_{Cells,\,Exp\,In}\right)\left(2^{n_{double}}\right) = \left(C_{Cells,\,Exp\,Out}\right)$$
$$\left(2^{n_{double}}\right) = \left(\frac{C_{Cells,\,Exp\,Out}}{C_{Cells,\,Exp\,In}}\right)$$
$$n_{double}(ln(2)) = ln\left(\frac{C_{Cells,\,Exp\,Out}}{C_{Cells,\,Exp\,In}}\right)$$
$$n_{double} = ln\left(\frac{C_{Cells,\,Exp\,Out}}{C_{Cells,\,Exp\,In}}\right)/ln(2)$$
$$n_{double} = ln\left(\frac{5.40*10^{5}\ cells/mL}{2.00*10^{2}\ cells/mL}\right)/ln(2)$$
$$n_{double} = 11.4\ times$$

Given the literature doubling time of HEK293-T cells of 23.4 hours, the initial estimated time required for suspension expansion based on our material balances is 11.1 days (Bauler, *et al.*, 2020). The relevant inlet and outlet concentrations for each process stage are summarized in Table 5.6.2 below.

Table 5.6.2: Summary of inlet and outlet concentrations for each LVV process stage.

Process Stage	Value	Units	Nomenclature
Starting WCB	$1.00*10^{7}$	cells	$C_{Cells,\,WCB}$
Expansion Inlet	$2.00*10^{2}$	cells/mL	$C_{Cells,\,Exp\,In}$
Expansion Outlet	$5.40*10^{5}$	cells/mL	$C_{Cells,\,Exp\,Out}$
Bioreactor Inlet	$5.40*10^{5}$	cells/mL	$C_{Cells,\,BRX\,In}$
Bioreactor at Induction	$1.05*10^{7}$	cells/mL	$C_{Cells,\,BRX\,Ind}$
Bioreactor Outlet	$3.78*10^{7}$	TU/mL	$C_{LVV,\,BRX\,Out}$
Harvest Inlet	$3.78*10^{7}$	TU/mL	$C_{LVV,\,Har\,In}$
Harvest Outlet	$2.65*10^{7}$	TU/mL	$C_{LVV,\,Har\,Out}$

TFF1 Inlet	$2.65*10^7$	TU/mL	$C_{LVV,\ TFF1\ In}$
TFF1 Outlet	$2.38*10^9$	TU/mL	$C_{LVV,\ TFF1\ Out}$
Chromatography Inlet	$2.38*10^9$	TU/mL	$C_{LVV,\ Chrom\ In}$
Chromatography Outlet	$7.14*10^8$	TU/mL	$C_{LVV,\ Chrom\ Out}$
TFF2 Inlet	$7.14*10^8$	TU/mL	$C_{LVV,\ TFF2\ In}$
TFF2 Outlet	$6.43*10^8$	TU/mL	$C_{LVV,\ TFF2\ Out}$
Sterile Filter Inlet	$6.43*10^8$	TU/mL	$C_{LVV,\ Ster\ In}$
Sterile Filter Outlet	$4.50*10^8$	TU/mL	$C_{LVV,\ Ster\ Out}$

Beginning the material balances for the CAR-T cell production process, we identified a starting peripheral blood mononuclear cell (PBMC) sample of 1×10^8 cells taken from patients. Based on experimental data from Löffler, *et al.* (2003), we determined an average T-cell frequency in the PBMC sample of a patient with chronic lymphocytic B cell leukemia of 6.76%. Based on Wang & Rivière (2016), Micucci, *et al.* (2006), and López-Muñoz & Méndez-Montes (2013), we estimated T-cell recoveries of 95% for each process stage in the CAR-T process. Therefore, the material balances around the T cell selection, activation, and transduction stages were as follows:

$$N_{T\ Cells,\ PBMC} = \left(N_{Cells,\ PBMC}\right)(T\ cell\ frequency)$$
$$N_{T\ Cells,\ PBMC} = \left(1.00\ *\ 10^8\ cells\right)(6.76\%)$$
$$N_{T\ Cells,\ PBMC} = 6.76\ *\ 10^6\ T\ cells = N_{T\ Cells,\ Sel\ In}$$

$$N_{T\ Cells,\ Sel\ Out} = \left(N_{T\ Cells,\ Sel\ In}\right)(\%\ Rec.)$$
$$N_{T\ Cells,\ Sel\ Out} = \left(6.76\ *\ 10^6\ T\ cells\right)(95\%)$$
$$N_{T\ Cells,\ Sel\ Out} = 6.42\ *\ 10^6\ cells = N_{T\ Cells,\ Act\ In}$$

$$N_{T\ Cells,\ Act\ Out} = \left(N_{T\ Cells,\ Act\ In}\right)(\%\ Rec.)$$
$$N_{T\ Cells,\ Act\ Out} = \left(6.42\ *\ 10^6\ cells\right)(95\%)$$
$$N_{T\ Cells,\ Act\ Out} = 6.10\ *\ 10^6\ cells = N_{T\ Cells,\ Trd\ In}$$

$$N_{CAR-T\ Cells,\ Trd\ Out} = \left(N_{T\ Cells,\ Trd\ In}\right)(\%\ Rec.)$$
$$N_{CAR-T\ Cells,\ Trd\ Out} = \left(5.80\ *\ 10^{6}\ cells\right)(95\%)$$
$$N_{CAR-T\ Cells,\ Trd\ Out} = 5.80\ *\ 10^{6}\ cells = N_{CAR-T\ Cells,\ Exp\ In}$$

Based on Dasyam, *et al.* (2020), we determined an average desired CAR-T cell dose of 1.00×10^6 cells/kg patient body weight. Using a basis of a 170 lb (77.11 kg) patient, the required number of times the CAR-T cells must be doubled during the final expansion step in the downstream, n_{double}, could be determined. Therefore, the material balances for CAR-T expansion were as follows:

$$N_{CAR-T\ Cells,\ Exp\ Out} = (Dosage)(Patient\ BW)$$
$$N_{CAR-T\ Cells,\ Exp\ Out} = \left(1.00\ *\ 10^{6}\ cells/kg\right)(77.11\ kg)$$
$$N_{CAR-T\ Cells,\ Exp\ Out} = 7.71\ *\ 10^{7}\ cells$$

$$\left(N_{CAR-T\ Cells,\ Exp\ In}\right)\left(2^{n_{double}}\right) = \left(N_{CAR-T\ Cells,\ Exp\ Out}\right)$$
$$\left(2^{n_{double}}\right) = \left(\frac{N_{CAR-T\ Cells,\ Exp\ Out}}{N_{CAR-T\ Cells,\ Exp\ In}}\right)$$
$$n_{double}(ln(2)) = ln\left(\frac{N_{CAR-T\ Cells,\ Exp\ Out}}{N_{CAR-T\ Cells,\ Exp\ In}}\right)$$
$$n_{double} = ln\left(\frac{N_{CAR-T\ Cells,\ Exp\ Out}}{N_{CAR-T\ Cells,\ Exp\ In}}\right)/ln(2)$$
$$n_{double} = ln\left(\frac{7.71*10^{7}\ cells}{5.80*10^{6}\ cells}\right)/ln(2)$$
$$n_{double} = 3.73\ times$$

Given the literature doubling time of CAR-T cells of 4 hours, the initial estimated time required for CAR-T cell expansion based on our material balances is 0.62 days (Yoon, *et al.*, 2010). The relevant inlet and outlet cell counts for each process stage are summarized in Table 5.6.3 below.

Table 5.6.3: Summary of inlet and outlet concentrations for each CAR-T process stage.

Process Stage	Value	Units	Nomenclature
Starting PBMC	$1.00*10^8$	cells	$N_{Cells,\ PBMC}$
PBMC T Cells	$6.76*10^6$	cells	$N_{T\ Cells,\ PBMC}$

Selection Inlet	$6.76*10^6$	cells	$N_{T\ Cells,\ Sel\ In}$
Selection Outlet	$6.42*10^6$	cells	$N_{T\ Cells,\ Sel\ Out}$
Activation Inlet	$6.42*10^6$	cells	$N_{T\ Cells,\ Act\ In}$
Activation Outlet	$6.10*10^6$	cells	$N_{T\ Cells,\ Act\ Out}$
Transduction Inlet	$6.10*10^6$	cells	$N_{T\ Cells,\ Trd\ In}$
Transduction Outlet	$5.80*10^6$	cells	$N_{T\ Cells,\ Trd\ Out}$
Expansion Inlet	$5.80*10^6$	cells	$N_{T\ Cells,\ Exp\ In}$
Expansion Outlet	$7.71*10^7$	cells	$N_{T\ Cells,\ Exp\ Out}$

6. Process Description & Simulation

6.1. SuperPro Simulations

We utilized the SuperPro Designer software to build working simulations of both LVV and CAR-T processes. SuperPro's capabilities for plant scheduling, economics, and optimization provide insight into the systems which are valuable in supporting the design of pharmaceutical processes. Our team obtained a tutorial document directly from Intelligen, the company responsible for the SuperPro software, which included a full, working simulation of viral vectors and autologous cell manufacturing processes. We built our own simulations using this tool as a guide, especially regarding inputting raw material information.

6.1.1. Lentiviral Vector Process Simulation

We completed the LVV Simulation using the Intelligen tutorial and our material balances as a guide. Using these tools as a guide, we rebuilt all the process units and compared them to the initial PFD we built to ensure we accurately modeled the process. The main differences between the PFD and the finalized simulation are air filtration, downstream chromatography, and media storage. First, the air filtration system was excluded from the upstream harvest portion as it broke our simulation and did not provide the correct amount of air. We recommend that when this process is built, a filtration unit be added to the process to ensure that the air coming into the process is adequately sterilized. Next, we had to increase the number of chromatography columns within the finalized simulation to incorporate cell washing, cleaning steps, and

filtration. Lastly, we removed most media storage units and added the media input to the process as streams directly fed into individual units.

The first portion of this simulation, shown below in Figure 6.1.1.1, includes HEK293-T cell revival and shake flask expansion units. Blending and storage of growth media are also depicted here. The inoculum preparation unit serves the purpose of further expanding the HEK293-T cells to the appropriate bioreactor seeding concentration and provides cells with fresh growth media.

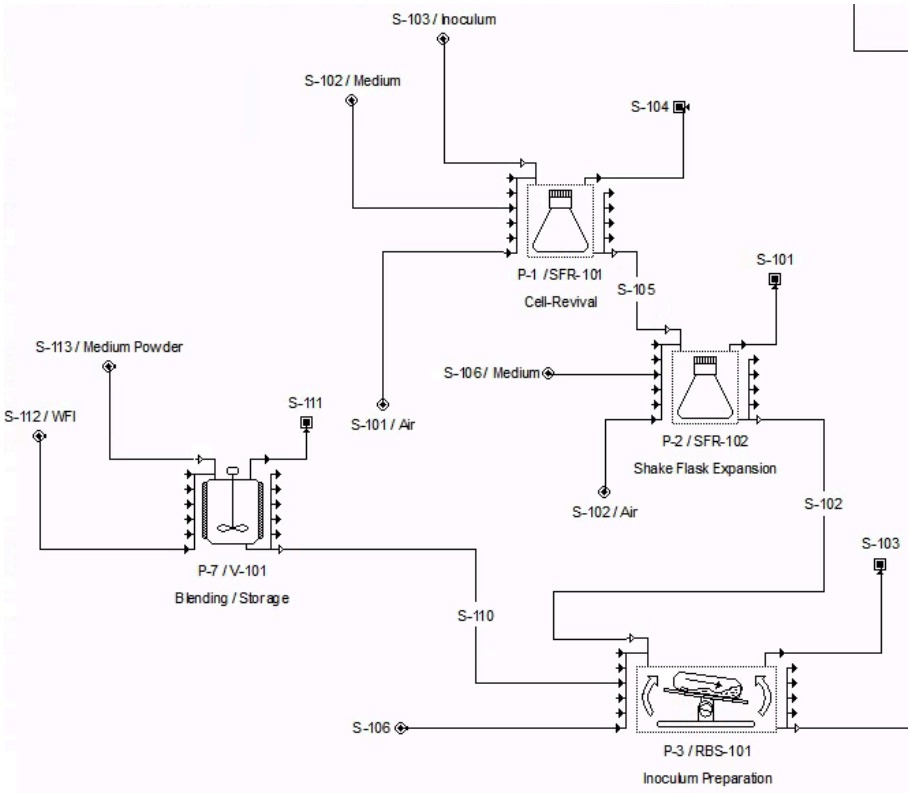

Figure 6.1.1.1: LVV SuperPro Simulation - HEK293-T Cell Revival Suspension and Expansion. *SuperPro Designer was used to model the revival and suspension of HEK293-T cells prior to seeding in the production bioreactor.*

The next part of the simulation included the bioreactor where the HEK293-T cells were transfected, labeled “cell culture” in Figure 6.1.1.2. As previously mentioned, we decided to exclude the air system, so the bottom stream represents the air for the bioreactor. Media, doxycycline, and cumic acid were added to the HEK293-T cells, which can be seen in their respective streams. The media storage tanks can be found in Figure 6.1.1.3. Stream S-108 designates the vent stream which includes vapors that were not needed for the rest of the process. To remove the excess particles, a microfiltration step was added where the cells and media were filtered through and recycled back to the bioreactor. The media was created by mixing medium powder and Water for Injection (WFI), the highest quality water used for cell culture. Medium powder and WFI were added to a blending

tank to mix together before it was transferred to a filtration system. After the filtration step, the media was collected in a storage tank that was used for the bioreactor system.

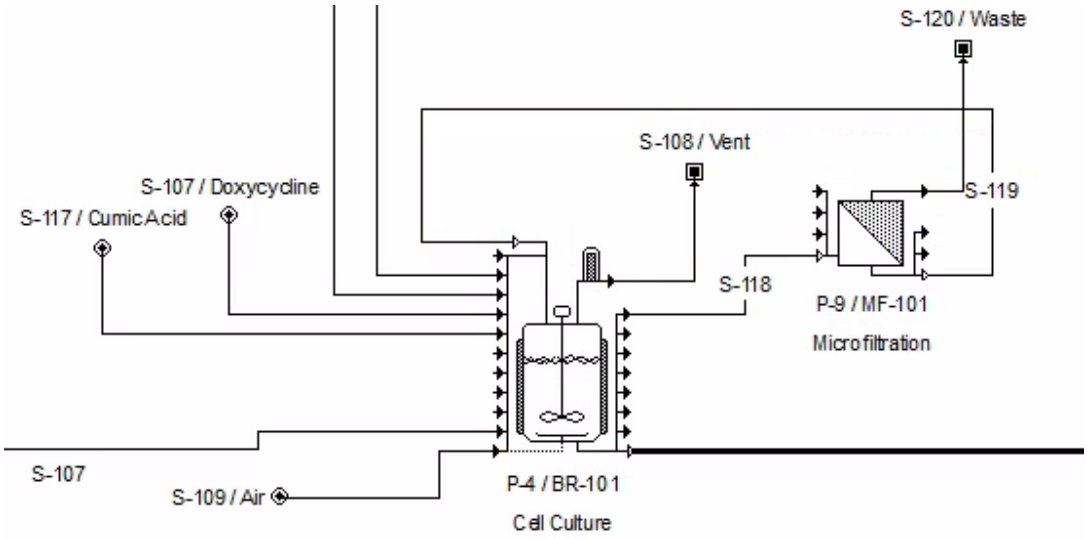

Figure 6.1.1.2: LVV SuperPro Simulation - Bioreactor and Filtration. *SuperPro Designer was used to model the transfection of HEK293-T cells and follow with filtration steps.*

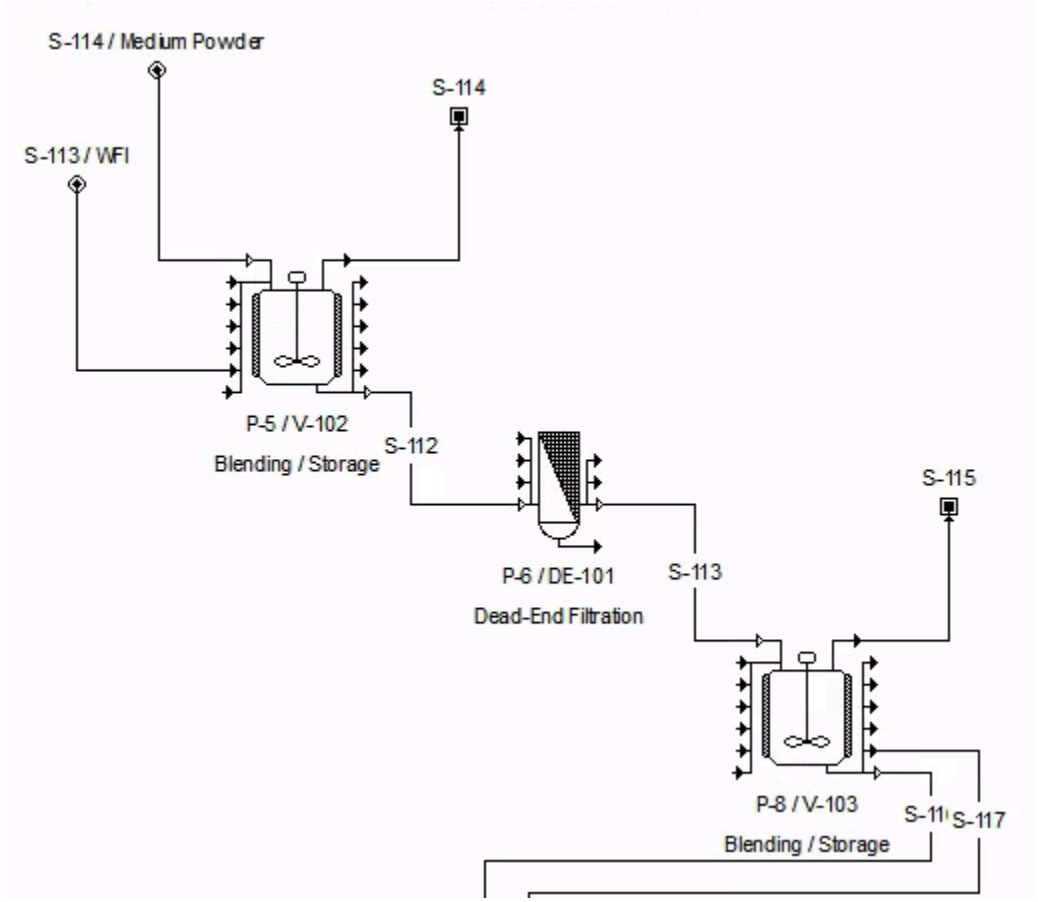

Figure 6.1.1.3: LVV SuperPro Simulation - Media Production for the Bioreactor. *SuperPro Designer was used to model the production of the media that was used for the bioreactor.*

Once the viral particles were created, cells and cell debris were removed from the main stream through three filtration steps as seen in Figure 6.1.1.4. The first two filtration processes were dead-end filters that removed the majority of the cells and some viral particles. The final filtration step was a membrane filter that removed all the remaining cell debris and kept the majority of the viral particles. Table 6.1.1.1 displays the components removed from each filtration step. For all of the filtration steps, media was used with the filtration steps. Once the viral particles were filtered, an endonuclease was added to degrade any leftover DNA in the stream. After the endonuclease was added, the LVV was purified in the downstream portion.

Table 6.1.1.1. Stream components removed in the filtration steps. *Filtration steps that display what components are filtered out to purify the viral particles.*

Component	Depth Filter #1	Depth Filter #2	Membrane Filter
Cells	80%	95%	100%
Viral Particles	10%	10%	1%

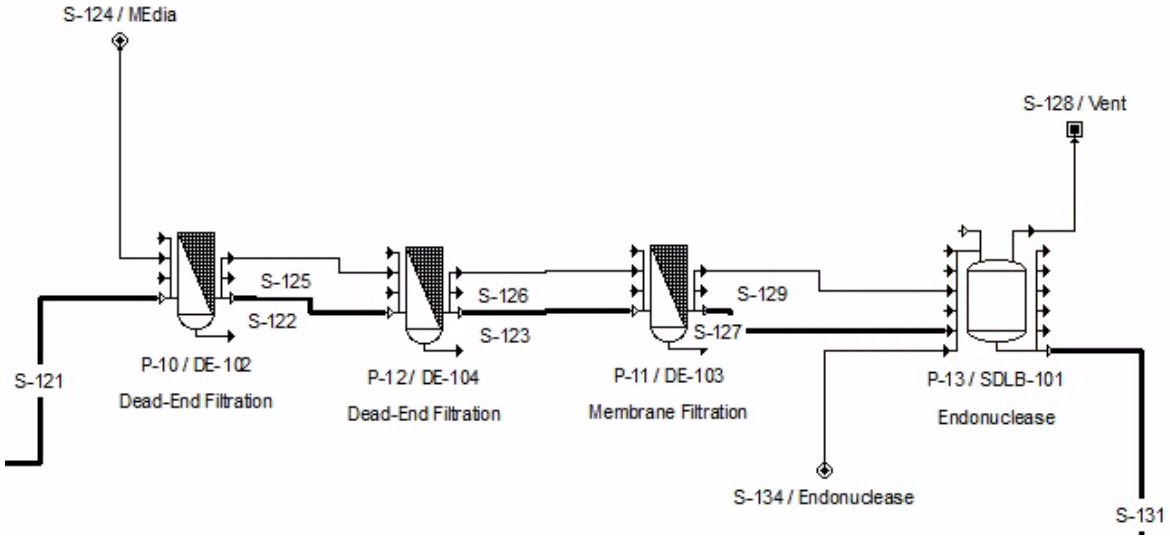

Figure 6.1.1.4: LVV SuperPro Simulation - Filtration. *SuperPro Designer was used to model the filtration process after the LVV production.*

In the downstream portion of the LVV production, an anion-exchange chromatography column was used to remove residual DNA impurities and proteins, as seen in Figure 6.1.1.5. Since the columns in SuperPro were not large enough to include all of the steps, two separate columns were created for cleaning (P-16) and washes (P-17). These columns were linked to the main column (P-15) so that they occurred simultaneously. The five main steps of the chromatography process include equilibration, loading, washing, elution, and regeneration. Between every step, the column was flushed and sanitized with P-17 using the listed media and was cleaned with

P-18 after the flushing process. The media on P-15 were used for the specific steps that originally came from a media storage tank, but because of the previously mentioned issues, they were directly added to the chromatography column.

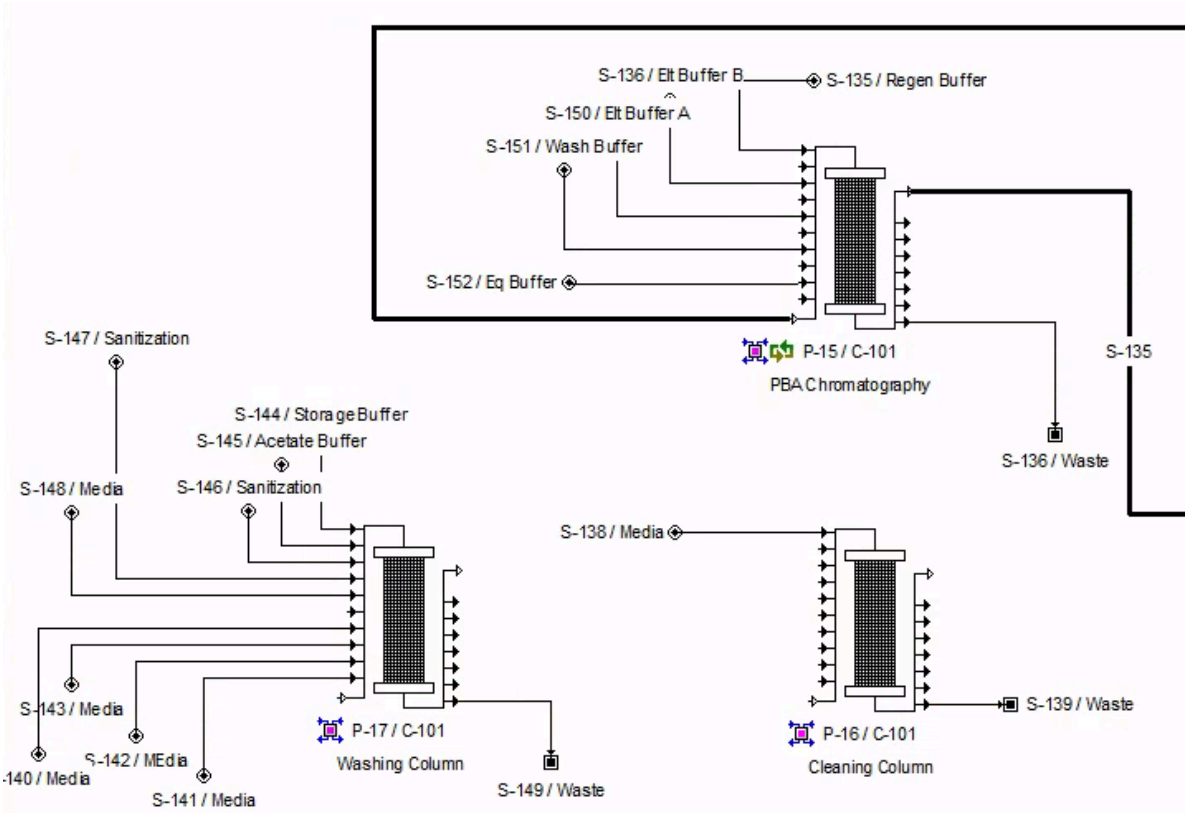

Figure 6.1.1.5: LVV SuperPro Simulation - Anion-Exchange Chromatography. *SuperPro Designer was used to model the purification of the LVV through an anion-exchange chromatography column.*

After the LVVs were purified, they were stored in a storage tank before they went through an ultrafiltration step. The process can be found in Figure 6.1.1.6. To ensure that the anion exchange column could produce enough product, it was kept in the storage tank until it went through the ultrafiltration step. The ultrafiltration process was used to replace the column elution media with a formulation buffer that the LVV can be resuspended in. After the ultrafiltration, the LVV was stored in a storage tank until it was ready to be used for CAR-T production.

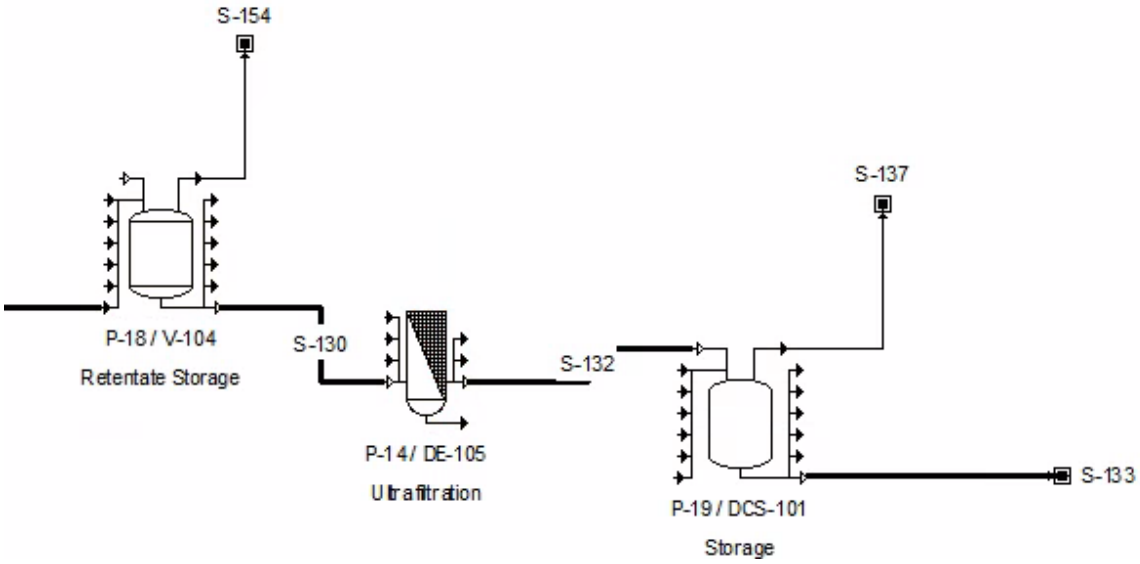

Figure 6.1.1.6: LVV SuperPro Simulation - Storage and Filtration. *SuperPro Designer was used to model the final steps of the LVV production which include storage and filtration steps.*

One important thing to note for this simulation is that we had significant struggles manually adjusting the amount of HEK293-T cells fed into the initial cell revival step. As such, the simulated process starts with 1.6 x 10^{11} cells per batch instead of 1.7 x 10^{7} cells per batch. While this is not ideal, we still feel the simulation provides significant insight into several aspects of this process, including economics and scheduling considerations. The executive summary for this project which provides this information can be below in Figure 6.1.1.7. The simulation tells us that the maximum number of batches achieved per year would be 17, totaling a 46.1-week utilization period from our restriction of a process operating 48 weeks per year.

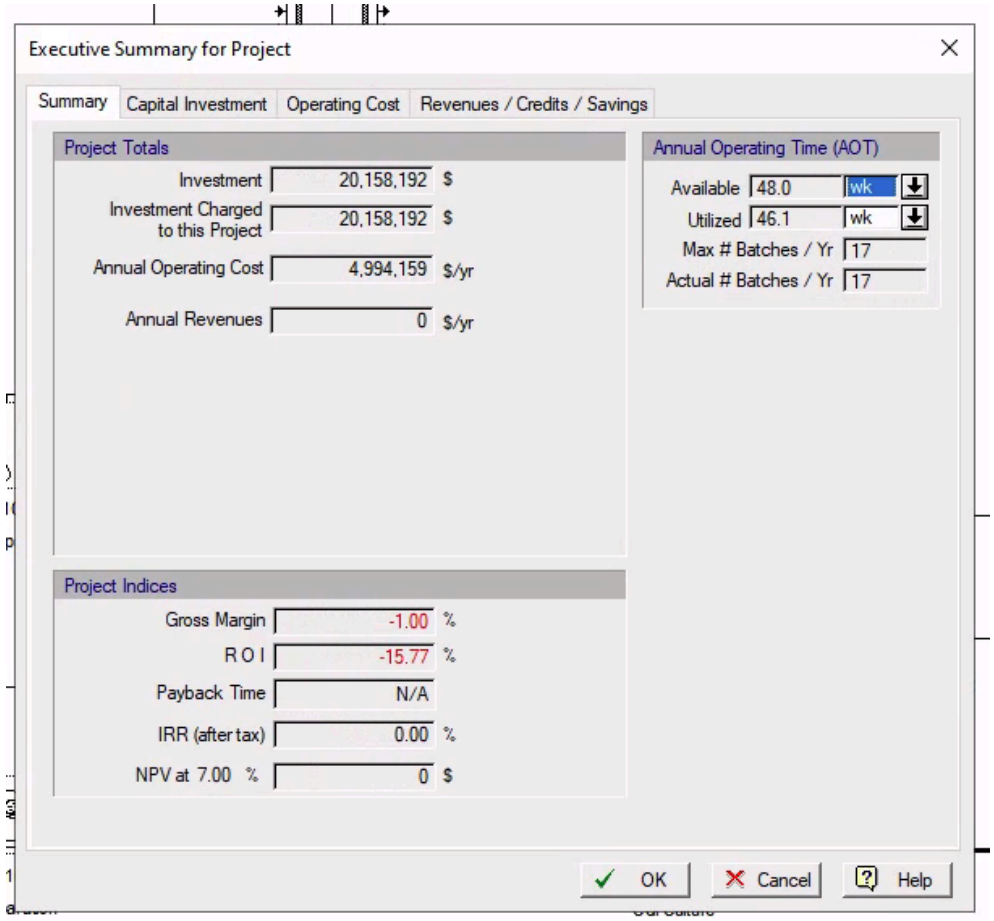

Figure 6.1.1.7: LVV SuperPro Simulation - Executive Summary. *SuperPro Designer gave the final executive summary which outputted information such as time and costs.*

6.1.2. CAR-T Process Simulation

We completed the CAR-T Simulation using the Intelligen tutorial and our material balances as a guide. Using these tools as a guide, we rebuilt all the process units and compared them to the initial PFD we built to ensure we accurately modeled the process. For the CAR-T simulation, we accurately matched the material balances to the simulation. Additionally, it was comparable to the initial SuperPro PFD. Although we proposed the CliniMACS Prodigy to automate part of the process, the CAR-T simulation included all individual units.

The first portion of the simulation includes thawing frozen leukopak and washing the leukopak, which can be seen in Figure 6.1.2.1. The thawing unit was created to behave like a water bath used in actual settings. After the leukopak was thawed to room temperature, the cells were washed with a buffer containing phosphate-buffered saline (PBS) and Ethylenediaminetetraacetic acid (EDTA). Platelets and red blood cells were removed, leaving just white blood cells that

could be resuspended in cell growth media. Finally, the flow splitter split the flow so that 7.5 mL went into the transduction step.

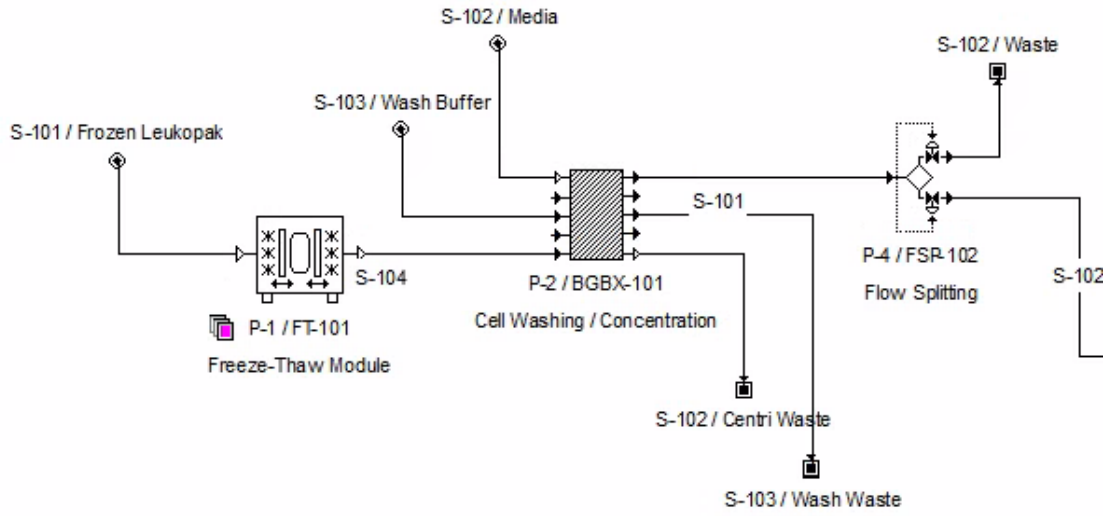

Figure 6.1.2.1: CAR-T SuperPro Simulation - Leukopak Preparation. *SuperPro Designer was used to model the thawing of frozen leukopak and the washing of the leukopak.*

The next part of the CAR-T simulation includes the transduction of the T-cells and cell expansion, as seen in Figure 6.1.2.2. In the Shake Flask unit (P-3), transduction and activation of the T-cells were done. Specific T-cell media was added along with the antibody complex, including antibodies to activate the T-cells. After 48 hours, the T-cells were activated, and the LVV created in the LVV process was added to transduce the T-cells. After transduction, the T-cells leave the Shake Flask reactor to a Rocking Bioreactor (P-5), where they undergo cell expansion. The cell expansion was modeled for six days total, where the first two days were the batch phase and the last four days were the perfusion phase. During the perfusion phase, a microfiltration system was used to purify the T-cells before the T-cells were sent downstream.

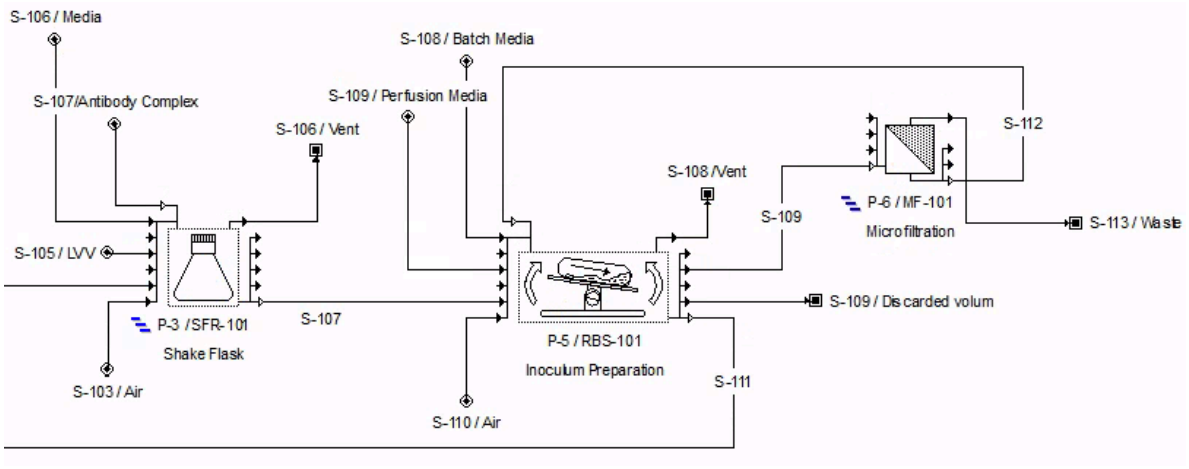

Figure 6.1.2.2: CAR-T SuperPro Simulation - T-cell transduction and expansion. *SuperPro Designer was used to model the T-cell transduction and expansion that adds the LVV to the T-cell.*

Once the T-cells were expanded, they were washed and then individually frozen, as seen in Figure 6.1.2.3. In the cell washing stage, they were washed to remove the previous media and resuspended in a new media. Before they could be frozen, they were washed twice with a wash buffer and then resuspended in a cryopreservation buffer. The flow was then split into a safety check (S-115) and the primary stream (S-116). Since the process up until this point was batch, the cell suspension was filled for each patient, which can be seen in the following unit (P-9). Next, the cryogenic bags were frozen to -135ºC before they were split into the final product (DS-103) and extra product as a backup.

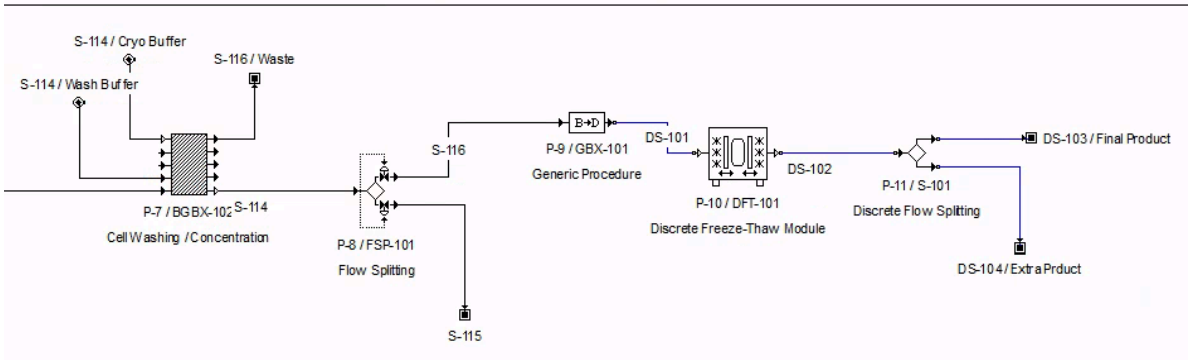

Figure 6.1.2.3: CAR-T SuperPro Simulation - Leukopak Preparation. *SuperPro Designer was used to model the thawing of frozen leukopak and the washing of the leukopak.*

The simulation provides significant insight into several aspects of this process, including economics and scheduling considerations. The executive summary for this project which provides this information, can be below in Figure 6.1.2.4. The simulation tells us that the maximum number of batches achieved per year would be 33, totaling a 47.9-week utilization period from our restriction of a process operating 48 weeks per year.

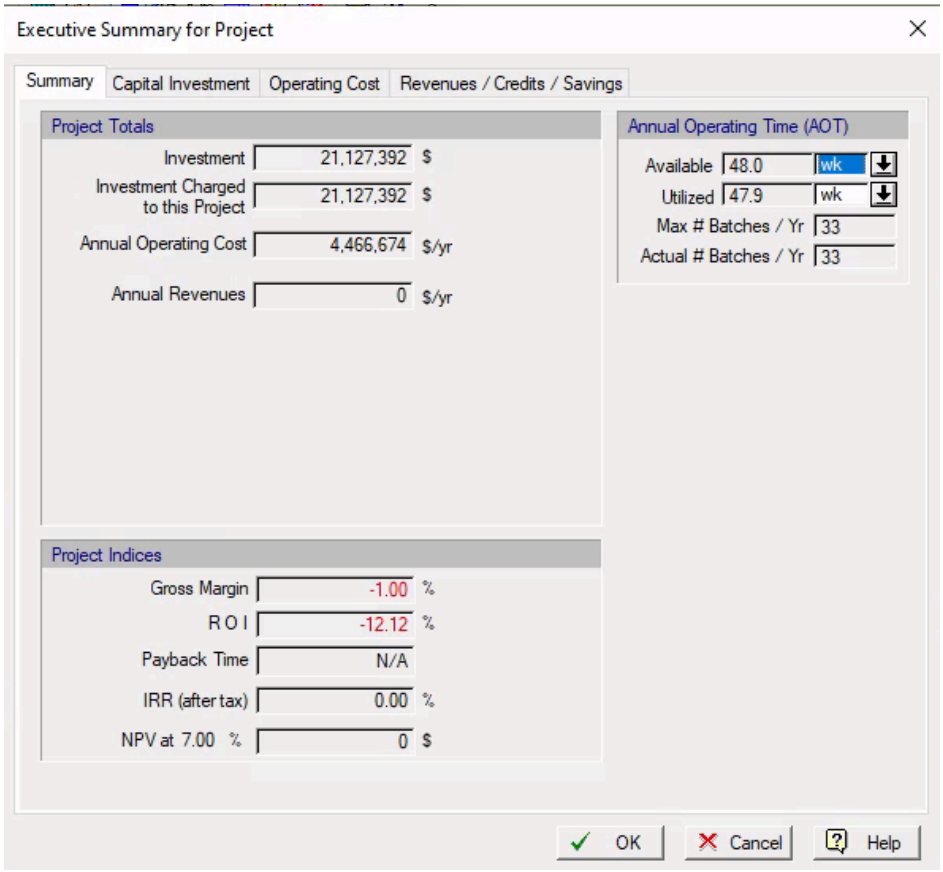

Figure 6.1.2.4: CAR-T SuperPro Simulation - Executive Summary. *SuperPro Designer gave the final executive summary, which outputted information such as time and costs.*

6.2. Cell Expansion Mathematical Modeling

Throughout the LVV process, the HEK293-T cells were expanded until they could be transfected with the lentiviral vector. We used Monod kinetics to model the cell expansion since it was an accurate model of cell growth and HEK293-T cells. Figure 6.2.1 displays the four main phases of cell growth, and Monod kinetics depicts cell growth within the log, or exponential, phase. While this process is displayed as one unit in SuperPro, realistically, it is modeled by many shake flasks in series that progressively get larger.

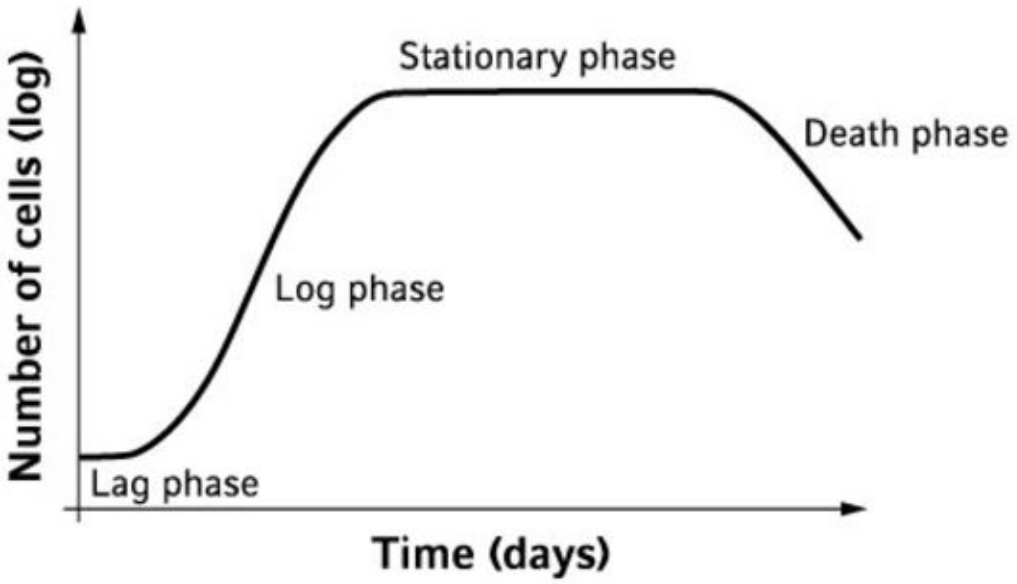

Figure 6.2.1: Cell Growth Graph. *Diagram of the four phases of cell growth, with the lag phase as the first stage, the log phase where the majority of cell growth occurs, the stationary phase where the cells remain at the same concentration, and the death phase where the cells begin to die (Everyday Culture Practice - Eppendorf Handling Solutions, n.d.).*

Monod kinetics related the growth rate of cells with the substrate concentration as the limiting factor, which can be found in Equation (6.2.1).

$$\frac{dX}{dt} = \frac{\mu_{max} C_s C_x}{K_s + C_s} \tag{6.2.1}$$

Each cell type has specific Monod constants and specific initial conditions that determine the cell growth. Weber *et al.* found the specific Monod constants and initial conditions that can be found in Table 6.2.1 (Weber *et al*., 2009).

Table 6.2.1. Monod kinetics constants. *These are the specific constants for HEK293-T cells*

Name	Value
μ_{max}	$6.97 \times 10^{-4} \frac{1}{hr}$
K_c	$0.178 \times 10^{-6} \frac{mol}{L}$
$C_{s,0}$	$800 \frac{g}{mL}$
$C_{x,0}$	$5 \times 10^{5} \frac{cells}{mL}$

Next, we found an expression for the substrate rate expression to accommodate for the substrate growth as the cells grew, which can be seen in Equation 6.2.2. The constants can then be imputed into Equation 6.2.1 to get the final rate expression, as seen in Equation 6.2.3.

$$\frac{dS}{dt} = 20 \tag{6.2.2}$$

$$\frac{dX}{dt} = \frac{(6.97 \times 10^{-4})C_s C_x}{0.178 \times 10^{-4} + C_s} \tag{6.2.3}$$

For the substrate growth expression, we opted for positive growth of substrate instead of the typical Monod kinetics because the ratio of substrate, or media, to HEK293-T cells was 20:1 (Moosemiller, n.d.). Additionally, the cells would be frequently changed with fresh media throughout the expansion time so the media must grow with the cells.

To perform the mathematical modeling, we employed MATLAB to solve Equations 6.2.2 and 6.2.3. The model was allowed to run until it reached the final cell concentration of 5.4×10^5 $\frac{cells}{mL}$, calculated from the material balances, which was indicated with a dotted line in Figure 6.2.2.

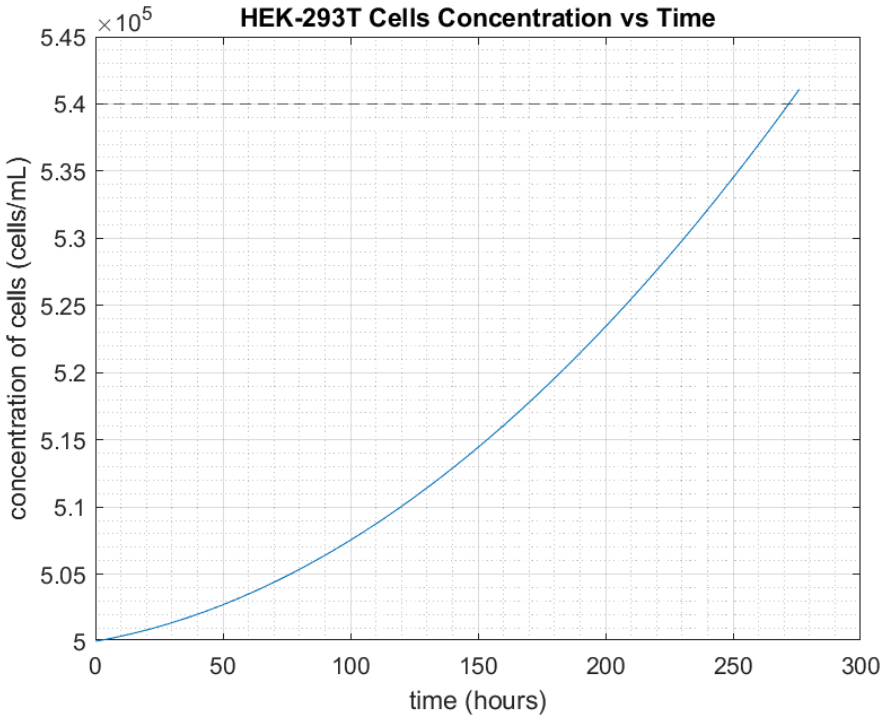

Figure 6.2.2: Mathematical Modeling for Cell Expansion. *Cell expansion of HEK293-T cells over 300 hours. The cells grew until they reached 5.4 x 10^5 cells/mL as the final concentration.*

The mathematical model took 11.33 days to reach $5.4 \times 10^5 \frac{cells}{mL}$, and the actual cell expansion time took 11.5 days, so our model was reasonably accurate. A table of nomenclature used for modeling the cell expansion can be found below in Table 6.2.2.

Table 6.2.2. Nomenclature for Cell Expansion. *Contains valuable information such as concentrations, constants, and material properties.*

Symbol	Definition	Value	Units
μ_{max}	Maximum Cell Growth Rate	6.97×10^{-4}	$\frac{1}{hr}$
K_c	Monod Constant	0.178×10^{-6}	$\frac{mol}{L}$
$C_{s,0}$	Initial Substrate Concentration	800	$\frac{g}{mL}$
$C_{x,0}$	Initial Cell Concentration	5×10^5	$\frac{cells}{mL}$
C_x	Cell Concentration	-	$\frac{cells}{mL}$
C_s	Substrate Concentration	-	$\frac{g}{mL}$

7. Process Details & Equipment Specifications

7.1. Lentiviral Vector Manufacturing

7.1.1. HEK293-T Cell Revival

Given that the cell revival step must be accomplished manually, we have established a protocol for HEK293-T cell revival using resources from ThermoFisher Scientific ("Growth and Maintenance of the 293FT Cell Line") on thawing of 298FT cell lines. This protocol requires the following materias: 1 vial of frozen HEK293-T cells (stored in liquid nitrogen until ready to use), water, ethanol, PBS, and growth medium. For this portion of the process, it is important to ensure that all solutions and equipment which come in contact with the cells are sterile. Moreover, it is vital that employees are using proper sterile technique and work in a laminar flow hood. The HEK293-T cells should be handled as potentially biohazardous materials under Biosafety Level 2 containment at least. The freezing medium of the cells will contain DMSO, a hazardous material, so care should be taken prior to handling. The materials required for the growth medium are outlined below in Table 7.1.1.1

Table 7.1.1.1. Materials required for preparation of growth medium. *Contains information about the components required to synthesize fresh growth medium for HEK293-T cell revival.*

Material	Product Name and Supplier	Price per Unit ($)
D-MEM (high glucose)	Gibco BenchStable DMEM (500 mL), ThermoFisher Scientific ("Bench Stable Media")	40.47
Fetal Bovine Serum (10%)	Gibco Fetal Bovine Serum (500 mL), ThermoFisher Scientific ("Fetal Bovine Serum")	676.00
MEM Non-Essential Amino Acids (NEAA, 0.1 mM)	Gibco MEM Non-Essential Amino Acids Solution (100X, 100 mL), ThermoFisher Scientific ("MEM Non-Essential Amino Acids Solution (100X)")	24.86
L-glutamine (6 mM)	Gibco L-Glutamine (200 mM, 100 mL), ThermoFisher Scientific ("L-Glutamine (200 mM)")	34.79
MEM Sodium Pyruvate (1 mM)	Gibco Sodium Pyruvate (100 mM, 100 mL), ThermoFisher Scientific ("Sodium Pyruvate (100 mM)")	14.13
Penicillin-Streptomycin (Pen-Strep, 1%)	Gibco Penicillin-Streptomycin-Glutamine (100X, 100 mL), ThermoFisher Scientific ("Pencillin-Streptomycin-Glutamine (100X)")	39.13
Gibco Geneticin (500 µg/mL)	Gibco Geneticin Selective Antibiotic (50 mg/mL, 100 mL), ThermoFisher Scientific ("Geneticin Selective Antibiotic (G418 Sulfate) (50 mg/mL)")	620.00

To prepare the growth medium, first combine 900 mL of D-MEM with 100 mL of fetal bovine serum. The D-MEM listed in Table 7.1.1.1 above is only available in 500 mL volumes, so two bottles will be needed to prepare one batch of complete medium. Add the following materials to

the mixture: 10 mL of 200 mM L-glutamine (100X), 10 mL of 10 mM MEM NEAA (100X), 10 mL of MEM Sodium Pyruvate (100X), and 10 mL of Pen-Strep (100X). Filter the medium using a 0.45 µm filtration device and store at 4ºC until ready to use. This medium is stable for 6 months at the aforementioned temperature. Prior to use, add 500 µg/mL of Geneticin. One important thing to note is that the D-MEM contains 4 mM L-glutamine, which is enough to support cell growth, but its concentration slowly decays over time so the medium needs to be supplemented with additional L-glutamine.

To thaw the HEK293-T cells:

1. Remove the frozen vial from liquid nitrogen.
2. Thaw in a 37ºC water bath until the ice is completely gone.
3. Decontaminate the outside of the vial with 70% ethanol.
4. Transfer the cells to a sterile 15 mL conical tube containing PBS.
5. Centrifuge the cells at 150-200 x g, allowing a pellet to form.
6. Resuspend the cells in 2 mL of complete medium without Geneticin.
7. Transfer the cells to a T-75 cm^2 flask containing 10 mL of complete medium without Geneticin.
8. Incubate overnight at 37ºC

One thing to note is that this cell revival protocol requires incubation of the cells in a T-flask overnight to allow them to recover from the thawing process. Given that the HEK293-T cell line being used by AGC Biologics for this process is already adapted to suspension expansion, this protocol could theoretically be adapted to use a shake flask instead of a T-flask. If a T-flask is used in this revival step, then the cells can be detached prior to suspension expansion using an EDTA solution.

7.1.2. HEK293-T Cell Suspension Expansion

We have consulted literature to build a protocol for HEK293-T cell suspension expansion prior to bioreactor inoculation. For each passage we estimated cell viability to be 95% (Chahal et al., 2014). We designed the protocol such that cells are initially seeded at 5×10^5 cells/mL, with cell cultures kept between 3×10^5 and 3×10^6 cells/mL to maximize cell growth and viability (Bauler et al., 2020). Our timing calculations are based on a literature HEK293-T cell doubling time of 23.4 hours. For each passage, the shake flask volumes used will be progressively increased and the working volume will not exceed 25% (Allman, 2021; “Shake Flasks for Suspension Culture”). Multitron Shaker Incubators should be used during this expansion process at 80% humidity and 5% CO_2 (Grieger et al., 2015). These units can fit 5L shake flasks are may be stacked to increase capacity without taking up any additional benchtop space.

The suspension expansion protocol will begin directly after cell revival, with the HEK293-T cells first being detached from the T-75 cm^2 flask using an EDTA solution. The cells will be centrifuged (150-200 × g) down into a pellet and resuspended in 20.0 mL of growth media in a 125 mL shake flask. The cells are now ready for the first suspension growth passage. Between each passage, the cells will be centrifuged down to a pellet and resuspended in a volume fresh growth medium equal to the working volume needed in the subsequent passage. A summary of the cell, timing, and flask information for each passage can be found below in Tables 7.1.2.1 - 7.1.2.5. A total of five passages are needed to reach a final cell number of 2.75×10^{10} cells, which exceeds our goal of 2.41×10^{10}, ensuring that should any additional cell death occur that the bioreactor would still be seeded with a sufficient number of cells.

Table 7.1.2.1. HEK293-T cell suspension growth - first passage. *Contains information about the starting and ending number of cells, timing, and flasks needed for the first passage of HEK293-T cell suspension expansion.*

First Passage - 125 mL Shake Flask	
Number of Shake Flasks Needed	1
Working Volume of Shake Flask	20.00 mL
Starting # of Cells	1.00×10^7 cells
Starting Concentration of Cells	5.00×10^5 cells per mL
Ending # of Cells	3.94×10^7 cells
Ending Concentration of Cells	1.97×10^6 cells per mL
# of Times Cells are Doubled	2.05
Hours for First Passage	48.00
Days for First Passage	2.00

Table 7.1.2.2. HEK293-T cell suspension growth - second passage. *Contains information about the starting and ending number of cells, timing, and flasks needed for the second passage of HEK293-T cell suspension expansion.*

First Passage - 250 mL Shake Flask	
Number of Shake Flasks Needed	2
Working Volume of Shake Flask	62.50 mL
Starting # of Cells	1.97×10^7 cells per flask 3.94×10^7 cells total
Starting Concentration of Cells (per flask)	3.15×10^5 cells per mL
Ending # of Cells	1.58×10^8 cells per flask 3.16×10^8 cells total
Ending Concentration of Cells (per flask)	2.53×10^6 cells per mL
# of Times Cells are Doubled	3.08
Hours for First Passage	72.00
Days for First Passage	3.00

Table 7.1.2.3. HEK293-T cell suspension growth - third passage. *Contains information about the starting and ending number of cells, timing, and flasks needed for the third passage of HEK293-T cell suspension expansion.*

First Passage - 500 mL Shake Flask	
Number of Shake Flasks Needed	4
Working Volume of Shake Flask	125.00 mL
Starting # of Cells	7.89×10^7 cells per flask 3.16×10^8 cells total

Starting Concentration of Cells (per flask)	6.31×10^5 cells per mL
Ending # of Cells	3.11×10^8 cells per flask 1.24×10^9 cells total
Ending Concentration of Cells (per flask)	2.49×10^6 cells per mL
# of Times Cells are Doubled	2.05
Hours for First Passage	48.00
Days for First Passage	2.00

Table 7.1.2.4. HEK293-T cell suspension growth - fourth passage. *Contains information about the starting and ending number of cells, timing, and flasks needed for the fourth passage of HEK293-T cell suspension expansion.*

First Passage - 1000 mL Shake Flask	
Number of Shake Flasks Needed	8
Working Volume of Shake Flask	250.00 mL
Starting # of Cells	1.55×10^8 cells per flask 1.24×10^9 cells total
Starting Concentration of Cells (per flask)	6.21×10^5 cells per mL
Ending # of Cells	6.12×10^8 cells per flask 4.89×10^9 cells total
Ending Concentration of Cells (per flask)	2.45×10^6 cells per mL
# of Times Cells are Doubled	2.05

Hours for First Passage	48.00
Days for First Passage	2.00

Table 7.1.2.5. HEK293-T cell suspension growth - fifth passage. *Contains information about the starting and ending number of cells, timing, and flasks needed for the fifth passage of HEK293-T cell suspension expansion.*

First Passage - 2000 mL Shake Flask	
Number of Shake Flasks Needed	10
Working Volume of Shake Flask	500.00 mL
Starting # of Cells	4.89×10^{8} cells per flask 4.89×10^{9} cells total
Starting Concentration of Cells (per flask)	9.79×10^{5} cells per mL
Ending # of Cells	2.75×10^{9} cells per flask 2.75×10^{10} cells total
Ending Concentration of Cells (per flask)	5.50×10^{6} cells per mL
# of Times Cells are Doubled	2.56
Hours for First Passage	60.00
Days for First Passage	2.50

After cell revival suspension and expansion, cell viability and counts should be completed to ensure that cell viability is at least 90% and that the bioreactor will be seeded at the proper concentration. The trypan blue exclusion method should be used to test the viability of the cells while a hemocytometer should be used to count the number of cells.

7.1.3. Bioreactor

The bioreactor which will be used for the lentivirus production stage of the LVV process is a Cytiva Xcellerex XDR-50 bioreactor which has already been purchased. The XDR-50 has a minimum working volume of 22 L and a maximum working volume of 50 L. The reactor makes use of a single-use bag system to eliminate the need for cleaning-in-place and steam-in-place. These single-use bags include an 8.5-inch diameter impeller, allowing for continuous stirring of the reaction medium, and also include ports to allow for aseptic connections to feed lines, dissolved oxygen sensors, and pH sensors. The reactor is jacketed, allowing it to maintain a constant temperature via an external temperature control unit. Monitoring and process controls are provided by an accompanying X-Station mobile control console, which consists of a touchscreen computer with mouse and keyboard, a programmable logic controller, and a programmable automation controller. The controller and reactor system is capable of running in batch, fed-batch, or perfusion modes. The reactor is constructed of 304-grade stainless steel, providing corrosion resistance against typical biological mediums (Cytiva, n.d.). The Cytiva Xcellerex XDR-50 is depicted in Figure 7.1.3.1 below.

Figure 7.1.3.1: *The Cytiva Xcellerex XDR-50 bioreactor, complete with I/O cabinet and X-Station mobile controller.*

For our LVV production process, the reactor will be operated in batch mode. Based on our material balances and following the protocols established by Cytiva Life Sciences (n.d.) and Manceur, *et al*. (2017), we recommend that the HEK293-T cells are inoculated at 5.4×10^5 cells/mL into 44.6 L of HyClone HyCell TransFx-H medium, supplemented with 4 mM L-glutamine, 0.1% (w/v) of Kolliphor P 188, 1 unit of anti-clumping agent, and 1 unit of HyClone LS250 lipid supplement. We recommend the culture is controlled at a temperature of 37℃, a pH of 7.15, a dissolved oxygen content of 40% (Cytiva, 2019; Manceur *et al*., 2017). The impeller speed required to maintain a constant power-to-volume ratio compared to the procedure developed by Cytiva Life Sciences at a 5 L scale is calculated as follows:

$$N_2 = N_1\left(\frac{1}{R}\right)^{2/3}$$

$$R = \left(\frac{V_2}{V_1}\right)^{1/3} = \left(\frac{50\ L}{5\ L}\right)^{1/3} = 2.15$$

$$N_2 = (90\ rpm)\left(\frac{1}{2.15}\right)^{2/3} = 53.95\ rpm \cong 54\ rpm$$

Therefore, the appropriate impeller speed for the 50 L scale is 54 rpm (Cytiva, n.d.). Cells are cultured for 5 days under these conditions, at which time LVV production is induced by adding doxycycline and cumate in 5.4 L of medium for final concentrations of 1μg/mL doxycycline and 30 μg/mL cumate. LVV harvest occurs 3 days post-induction, resulting in a total bioreactor processing time of 8 days (Cytiva, n.d.; Manceur *et al*., 2017). These protocols yield around 3.6 TU/cell of LVVs, which would result in the desired viral titer of 3.2×10^7 TU/mL when the reactor is inoculated with the aforementioned concentration of HEK293-T cells (Cytiva, n.d.).

7.1.4. Endonuclease Treatment

During lentiviral vector production, one of the vital downstream processing steps will be adding an RNA-DNA endonuclease to degrade / reduce / remove any remaining contaminating DNA (Bauler, 2020). Benzonase, a promiscuous endonuclease which attacks and degrades DNA and RNA in all forms, is one of the most popular materials used for this portion of lentiviral vector downstream processing ("Benzonase Nuclease Q&A"). Most established lentiviral vector production protocols make use of a benzonase treatment step to remove residual cellular and plasmid-derived nucleic acid contaminants (Merten et al., 2016). The use of benzonuclease early in the downstream processing of viral vectors is advantageous to break down large DNA contaminants; smaller DNA pieces and the benzonase itself can be removed in other downstream processing steps (Merten et al., 2016). One of the drawbacks of using benzonase, however, is

that large quantities of the nuclease must be added to be efficient in breaking down contaminant DNA and RNA (Merten et al., 2016).

A lentiviral vector production protocol using suspension HEK293-T cells recommends adding the benzonase to the HEK293-T vector producing cells 24 hours post-transfection for optimal results. Their data demonstrates that even with low levels of benzonase added at this time post-transfection, significant reduction of contaminant cellular and plasmid DNA can be achieved. Benzonase should be added to the cell culture to achieve a final concentration of 6.25 U/mL (Bauler et al., 2020).

According to Millipore Sigma, benzonase can be reversibly inhibited using EDTA and irreversibly inhibited only in extreme conditions (100 mM NaOH at 70oC for 30 minutes) ("Benzonas Nuclease Q&A"). It can be inhibited by monovalent cation concentrations greater than 50%, phosphate concentrations greater than 20 mM, and ammonium sulfate concentrations greater than 25 mM. Another important thing to note is that Benzonase requires 1-2 mM Mg^{2+} for activity. Benzonase can be separated from target products via chromatography steps. Therefore, it can be concluded that the use of chromatography steps after the endonuclease addition will be effective in removing this treatment from the final product.

Millipore Sigma's Benzonase nuclease is available in a purity grade I (>99% purity) high concentration (HC) formulation with an endonuclease concentration of 250 U/µL. For this particular formulation, 1.25 mL of benzonase should be added to achieve the final desired endonuclease concentration, calculated as follows:

$$C_1V_1 = C_2V_2$$

$$250\ \frac{U}{\mu L} \cdot \frac{1000\ \mu L}{1\ mL} \cdot V_1 = 6.25\ \frac{U}{mL} \cdot 50.0\ L\ \cdot\ \frac{1000\ mL}{L}$$

$$V_1 = 1.25\ mL$$

In summary, 1.25 mL of MilliPore Sigma Benzonase Nuclease (Purity grade I HC) should be added at 24 hours post-transfection of HEK293-T vector producing cells. For endonuclease activity, Mg^{2+} should also be added at this time to a concentration of 1-2 mM. Magnesium chloride powder is also available from MilliPore Sigma ("Magnesium chloride"). EDTA is available in a variety of forms, also from MilliPore Sigma, and can be added to temporarily and reversibly inhibit the endonuclease prior to chromatography steps ("Ethylenediaminetetraacetic acid").

7.1.5. Filtration

For the downstream portion of the LVV process, based on the design that we've developed, we recommend a process flow that follows these approximate steps: depth filtration (for harvest), tangential flow filtration (for concentration), AEX membrane chromatography (for purification), a second tangential flow filtration step (to remove residual impurities and replace the buffer to make the working solution suitable for final formulation), and then a final sterile filtration step (Merten et al.).

For the harvest depth filtration we recommend, using Repligen's KrosFlo tangential flow depth filtration (TFDF) system utilizing 0.5mm internal diameter hollow-fiber polypropylene depth filters (with a 500kDa cutoff and a channel depth of 2- to 4-μm) (Repligen). For larger-than-lab-scale bioprocesses tangential flow depth filtration provides higher filter capacities than depth filtration without build-up of biological residue within the filter (Tona et al.). This selected depth filtration system has a higher filter capacity, extends functional life, and improves the efficiency of separation of lentiviral vectors from producer cells (Tona et al.).

For the concentration tangential flow filtration (TFF) step (the first tangential flow filtration step), we recommend using the Cytiva ÄKTA readyflux system, which has the capability of operating with both membrane filter cassettes, and hollow fiber cartridges (CYtiva). We recommend using the Sartorius' Single-use Hollow Fiber TFF module (either the 100kDa and 300kDa options would be viable: both result in the same level of recovery after the first tangential flow filtration step (Cooper et al.). As outlined in Geraerts et al. the general protocol for the concentration step involves recirculating the fluid over a membrane with pores smaller than 0.1μm at a pressure of 1 bar so that the lentivirus is retained by the membrane.

For the final formulation and medium replacement tangential flow filtration step (the second tangential flow filter), we recommend using the same tangential flow depth filter from the depth filtration step: the Repligen KrosFlo TFDF system, but the total area of filtration can be significantly smaller due to the concentration of the biomedium in the previous TFF step (Repligen). The hollow fibers should have the same 500kDa cutoff.

For the final sterile filtration step, industrial LVV production processes often use 0.2μm or 0.45μm filters. As lentiviruses are typically 80-100 nm our recommendation is using the 0.2μm filter as it will lead to a higher level of purification and a higher quality final product. Based on the high volume that the filter will have to process, our recommendation is Millipore Sigma's Opticap® XL10 Durapore (sterile) (KVGLS10TH1) (Sigma Aldrich). The sterile Durapore filter is a capsule filter, which is capable of production scale processing.

7.1.6. Chromatography

Chromatography is a commonly-used method to remove some impurities, such as host cell DNA and secreted proteins, which cannot be efficiently separated on the basis of particle size through filtration. Chromatography has the advantage of being more scalable than alternatives such as centrifugation, and as such is frequently used in industrial-scale bioseparations design (Moreira *et al.*, 2020). The viral envelope of lentiviruses limits their ability to diffuse through the pores of chromatography beads, so membrane and monolith chromatography resins have been the primary focus of experimentation and LVV purification design (Moreira *et al.*, 2020; Valkama *et al.*, 2020).

Two alternative types of chromatography have been the focus of studies surrounding the purification of lentiviruses: including Anion Exchange (AEX) Chromatography and Affinity Chromatography. Affinity Chromatography has been found to give the advantage of better purification of active LVVs, but due to the relatively low recovery of LVVs inherent to this method and the lack of research supporting an industrial scale-up of this method, Affinity Chromatography will not be used in our design (Moreira *et al.*, 2020).

Valkama, *et al*. (2020) compared different AEX and HIC resins based on their ability to effectively purify lentiviruses and the recovery of lentivirus. AEX proved to provide acceptable purification but with much higher LVV recoveries than HIC. Additionally, it was found that AEX resins with a high ionic strength provided better isolation of LVVs, but at the expense of very low yields; therefore, weaker AEX resins were recommended as the optimal choice for the purification of LVVs (Valkama *et al.*, 2020). Specifically, the Sartorius Sartobind Q series chromatography membranes were recommended as attractive options for balancing effective purification with relatively high yields (Moreira *et al.*, 2020; Valkama *et al.*, 2020).

The relevant specifications of the Sartorius Sartobind Q membrane chosen for the remainder of the design are summarized in Table 7.1.6.1 below:

Table 7.1.6.1: Sartorius Sartobind Q specifications for chromatography column design and calculations (Sartorius, n.d.).

Parameter	Value	Units	Nomenclature (If Applicable)
Size	20	inches	-
Bed Length	4	mm	-
Column Volume	4477	cm^3	V

Membrane Volume	400	mL	$V_{membrane}$
Membrane Area	14600	cm^2	A
Void Volume	1080	mL	V_{void}
Pore Size	3	μm	d_p
Recommended Flow Rate	8	L/min	Q_{rec}

To begin, the column is loaded with the process medium which is recovered from the first tangential flow filtration unit. The time required for column loading is calculated as follows:

$$t_{loading} = \frac{Process\ Volume}{Q_{rec}}$$

Using a basis of 750 mL of concentrated process volume exiting the first tangential flow filter:

$$t_{loading} = \frac{0.750\ L}{8\ L/min} \times \frac{60\ sec}{min} = 5.63\ sec$$

Now, the time required for viral particle diffusion is calculated using the Einstein diffusion equation (Harrison *et al.*, 2015):

$$t_{diffusion} = \frac{\delta^2}{2\mathcal{D}_p}$$

Here, δ is the diffusion length and $\mathcal{D}_p$ is the pore diffusion coefficient. Due to limitations on the available information for the diffusion coefficient of lentiviral particles in HEPES or Tris-HCl buffers, the pore diffusion coefficient was estimated as being equal to the diffusion coefficient of virus in water at 20℃, 15.5 μm^2/sec (Bockstahler & Kaesberg, 1962). To determine the diffusion length, it is first necessary to determine the type of convective flow in the membrane by calculating the Reynolds number:

$$Re = \frac{d_m\left(\frac{\nu}{\varepsilon}\right)\rho}{\mu}$$

Here, d_m is the pore diameter of the membrane, ν is the velocity of the mobile phase, ε is the porosity of the membrane, ρ is the medium density, and μ is the medium kinematic viscosity. The medium properties were estimated to be equal to those of water for this calculation. The porosity of the column was determined as follows:

$$\varepsilon = \frac{V_{void}}{V} = \frac{1080\ cm^3}{4477\ cm^3} = 0.241$$

The mobile phase velocity was calculated as follows:

$$\nu = \frac{Q_{rec}}{A} = \frac{8\ L/min}{14600\ cm^2} \times \frac{1000\ cm^3}{L} = 0.55\ cm/min$$

Therefore, the Reynolds number was calculated as follows:

$$Re = \frac{(3\ \mu m)\left(\frac{0.55\ cm/min}{0.241}\right)\left(1\ g/cm^3\right)\left(10^{-4}\ cm/\mu m\right)(1\ min/60\ sec)}{0.01\ g/cm{-}s} = 0.0011$$

Since the Reynolds number is less than 0.1, the convective flow in the membrane is characterized as a laminar, creeping flow. Therefore, the maximum diffusion length is half the pore diameter, or 1.5 μm (Harrison *et al.*, 2015). The diffusion time is therefore calculated as:

$$t_{diffusion} = \frac{(1.5\ \mu m)^2}{15.5\ \mu m^2/sec} = 0.15\ sec$$

As is evident, the diffusion time for lentiviral particles in the Sartobind Q membrane is very small, which is the advantage in using membrane chromatography over bead chromatography, where small pore sizes inhibit any diffusion of the lentivirus into the porous beads (Moreira *et al.*, 2020; Valkama *et al.*, 2020).

For elution, NaCl solution is used since it is a more effective elution buffer than Tris-HCl (Valkama *et al.*, 2020). NaCl has one primary disadvantage as an elution buffer: lentiviruses are labile, so exposure to a high salt concentration results in virus inactivation; for example, a loss of 50% of lentivirus activity after one hour of exposure to 1 M NaCl is possible (Moreira *et al.*, 2020). Therefore, it is critical that eluted fractions are rapidly processed to the second tangential flow filtration unit for dilution and buffer replacement to avoid excessive loss of lentivirus activity. Elution with NaCl solutions in excess of 0.650 M NaCl have been shown to provide a higher lentivirus recovery compared to more dilute elution buffers, but this must be balanced with the increased impurity concentration found with increasing elution buffer strength. Therefore, an elution buffer of 0.444 M NaCl is recommended to provide effective removal of impurities while minimizing the loss of lentivirus into fractions which have significant impurity concentration (Valkama *et al.*, 2020).

Valkama, *et al.* (2020) eluted the Sartobind Q column with a total elution volume approximately equal to 80 times the membrane volume. Therefore, the total elution time is calculated as follows:

$$t_{elution} = \frac{80\left(V_{membrane}\right)}{Q_{rec}} = \frac{80(400\ mL)}{8000\ mL/min} \times \frac{60\ sec}{1\ min} = 240\ sec$$

The total time required for the AEX chromatography step is therefore calculated as:

$$t_{total} = t_{loading} + t_{elution} = (5.63\ sec\ +\ 240\ sec) \times \frac{1\ min}{60\ sec} = 4.1\ min$$

Based on this result, the AEX chromatography step can be performed relatively rapidly and does not contribute significantly to the processing time. This step can easily be performed within a single day alongside the rest of the downstream processing steps.

7.1.7. Lentiviral Vector Selection

The lentiviral vector (LVV) we have chosen is the LTG-1563 LVV sourced from Lentigen Technology Inc. The LTG-1563 is the most promising LVV because it has been used in preclinical and clinical trials for CAR-T therapies. It has also been used with the CliniMACS Prodigy, which we plan to use in our process. The LTG-1563 LVV contains a second-generation anti-CD19 chimeric antigen receptor (CAR) with an scFv FMC63 CD19 targeting domain, a CD8 hinge region, TNFRSF19-derived transmembrane region, 4-1BB costimulatory domain, and CD3ζ chain intracellular signaling domain (Palani et al., 2022).

A second-generation anti-CD19 CAR is a newer development of CARs that have enhanced T cell proliferation and cytotoxicity (Tokarew et al., 2018). Single-chain variable fragments (scFv) are a type of antibody that contain fusion proteins of variable regions of the heavy and light chains and are typically produced in bacterial cell culture. The FMC63 scFv is an IgG2a mouse monoclonal antibody with CD19 expression to target the CD19 domain (Zola et al., 1991). The hinge region on the antibody is the region between the fragment antigen-binding (Fab) and fragment crystallizable (FC) regions that increases the antibody's flexibility. The CD8 hinge region used in the LVV is used to help regulate the CAR signaling pathways (Fujiwara et al., 2020)4. The TNFRSF19-derived transmembrane region is a transmembrane protein part of the tumor necrosis factor receptor superfamily (TNFRSF) responsible for binding to tumor necrosis factors with anti-tumor effects. When the TNRFSF19 is present in the CAR-T cells, it increases the anti-tumor activity of the CAR(Schneider et al., 2017). In addition, the 4-1BB has anti-cancer effects that can induce activation of cytotoxic T-cells, so having a 4-1BB costimulatory domain makes the CAR-T cell more effective at killing the tumor cells (Cappell & Kochenderfer, 2021) (Vinay & Kwon, 2014). CD3ζ is part of the T-Cell Receptor (TCR) CD3 complex responsible for activating helper and cytotoxic T-cells. The specific function of CD3ζ is to assemble the TCR-CD3 complex and transport it to the cell surface (Deswal & Schamel,

1970). For these reasons, we recommend that AGC Biologics use the LTG-1563 LVV for the HEK293-T cell transfection.

7.2. CAR-T Manufacturing

7.2.1. Leukopak Thaw

We have established a protocol for thawing of the cyropreserved leukopak. This step is necessary as the CliniMACS Prodigy unit being used for CAR-T processing cannot take in frozen materials. It is important that the thawing protocol is strictly followed to ensure consistent cell quality, cell viability, and purity of the final product ("Cryopreserved Leukopak Thawing Protocol"). Leukopaks are typically frozen in a special cryopreservation medium containing DMSO (10%) using a controlled rate freezer to maximize cell viability ("Human Peripheral Blood Leukopak Thawing Protocol"). Given that DMSO is toxic to cells, an important part of this thawing process is to remove the cells from this media and resuspend them in the necessary growth media ("Human Peripheral Blood Leukopak Thawing Protocol"). When thawing, it is important to work carefully and quickly due to the toxicity of DMSO and general fragility of cells ("Human Peripheral Blood Leukopak Thawing Protocol").

Simply put, the leukopak will be thawed in a 37^{o}C water bath and will undergo a series of centrifugation steps to remove the freezing media and replace it with fresh growth media.9-12 Following this thawing process, cells should be held in an incubator overnight to allow them to recover before being fed into and processed in the CliniMACS Prodigy system.

Working in a laminar flow hood, follow this protocol to thaw incoming leukopak (Mock et al., 2016; "Protocol – Thawing Cyropreserved Leukopak"):

1. Prepare the growth media as follows:
    a. Warm in a 37^{o}C water batch.
    b. Remover from water and dry the container.
    c. Sterilize the exterior of the container with 70% ethanol.
2. Submerge the leukopak in a 37^{o}C water bath without movement.
3. Once the leukopak has thawed to the extent that there is only a sliver of ice remaining, remove carefully from the water bath
4. Gently clean the exterior of the bag with 70% alcohol
5. Using sterile scissors, cut the leukopak bag port and transfer to sterile 50 mL conical tubes, filling ⅓ of the way.
6. Rinse the leukopak bag with less than 50 mL of growth media and gently add remnants to an additional 50 mL conical tube.
7. Slowly add equal volumes of growth media to each of the conical tubes until filled.
8. Centrifuge cell suspensions at 300 × g at room temperature for 15 minutes.

9. Using a pipette, remove most of the supernatant and save in a separate conical tube.
    - It is important to leave a small amount of supernatant behind so that the cell pellet is not disturbed.
    - Since DMSO is typically heavier than the growth medium it is important to aspirate and discard the supernatant very soon after centrifugation ends.
10. While gently shaking the conical tube, add 15-20 mL of fresh medium to resuspend the cell pellet into a uniform suspension.
11. Centrifuge the cell suspension at 300 × g at room temperature for 15 minutes.
12. Using a pipette, carefully remove most of the supernatant, leaving a small amount behind so that the cell pellet is not disturbed.
13. While gently shaking the conical tube, add fresh growth medium to resuspend the cell pellet into a uniform suspension.
    - The amount of growth medium added here should be appropriate to be able to count the cells.
14. Count cells and determine viability using a hemocytometer and the trypan blue method ("Counting Cells in a Hemocytometer"; "Trypan Blue Exclusion").
15. If the cell count is lower than expected, centrifuge the supernatant saved in Step 10 at a slightly higher speed, count, and combine.
16. At this point cells are ready to go, although they might need to be recovered in medium for 24 hours prior to use in downstream applications depending on the cell count and viability obtained in Step 14.

For use of leukopak in a CliniMACS Prodigy unit, the appropriate growth media would be the TexMACS GMP Medium supplemented with 10% HS (GemCell Human Serum AB, premium grade) to a final concentration of 3% (Mock et al., 2016).

7.2.2. CliniMACS Prodigy

In designing the CAR-T manufacturing process, we had the option of either designing individual units for processing or utilizing an automated processing unit such as the CliniMACS Prodigy. The CliniMACS Prodigy system from Miltenyi Biotec enables automated CAR-T cell manufacturing in a closed, standardized GMP-compliant manner ("Automated cell manufacturing on a closed and scalable platform").

Although the use of an automated process has its advantages and disadvantages, outlined below, we ultimately decided on the use of a CliniMACS Prodigy unit for several reasons. The alternative would be the design and use of numerous complex procedures for each step in the manufacturing process. One of the major challenges of this is that it would be a labor intensive process that would elevate operational costs for AGC Biologics. Moreover, there would be a greater number of areas where operators would interface with the biological product, increasing

operator hazard and risking contamination of the final product. The CliniMACS Prodigy system would be entirely closed, reducing cleanroom requirements, increasing operator safety, reducing labor requirements, and preventing contamination of the product ("Clinical-grade TCR engineered T cells and CAR T cell manufacturing via viral transduction").

The CliniMACS Prodigy can be used for two applications which accommodate different aspects of T cell manufacturing. Both come with all reagents and components which would be needed for the entire T cell manufacturing workflow ("Clinical-grade TCR engineered T cells and CAR T cell manufacturing via viral transduction"). The CliniMACS Prodigy application relevant for this project would be a TCT system, which is specifically designed for CAR-T cell manufacturing, shown in Figure 7.2.2.1 below ("Clinical-grade TCR engineered T cells and CAR T cell manufacturing via viral transduction"). Use of a CliniMACS Prodigy T cell Transduction (TCT) process would automate all steps of the CAR-T process, from cell isolation and activation to transduction, expansion, harvest, and formulation of the final cell product. The TCT system takes in whole blood, PBMCs, or fresh leukapheresis as the starting material. Given that the incoming leukopak for this process will be frozen, a thawing protocol will first need to be employed prior to being set up in the CliniMACS unit. The culture capacity of the TCT system is 250 mL and it uses a CliniMACS Prodigy TS 520 tubing set ("Clinical-grade TCR engineered T cells and CAR T cell manufacturing via viral transduction"). The stimulation reagent is a MACS GMP T Cell TransAct and the unit can reach final T cell counts of approximately 5×10^9 cells ("Clinical-grade TCR engineered T cells and CAR T cell manufacturing via viral transduction"). For full CAR-T cell processing using this automated unit, AGC Biologics would need the following components: CliniMACS Prodigy Instrument, CliniMACS Prodigy Tubing Sets, various accessories, and Reagents and Buffers ("Automated cell manufacturing on a closed and scalable platform").

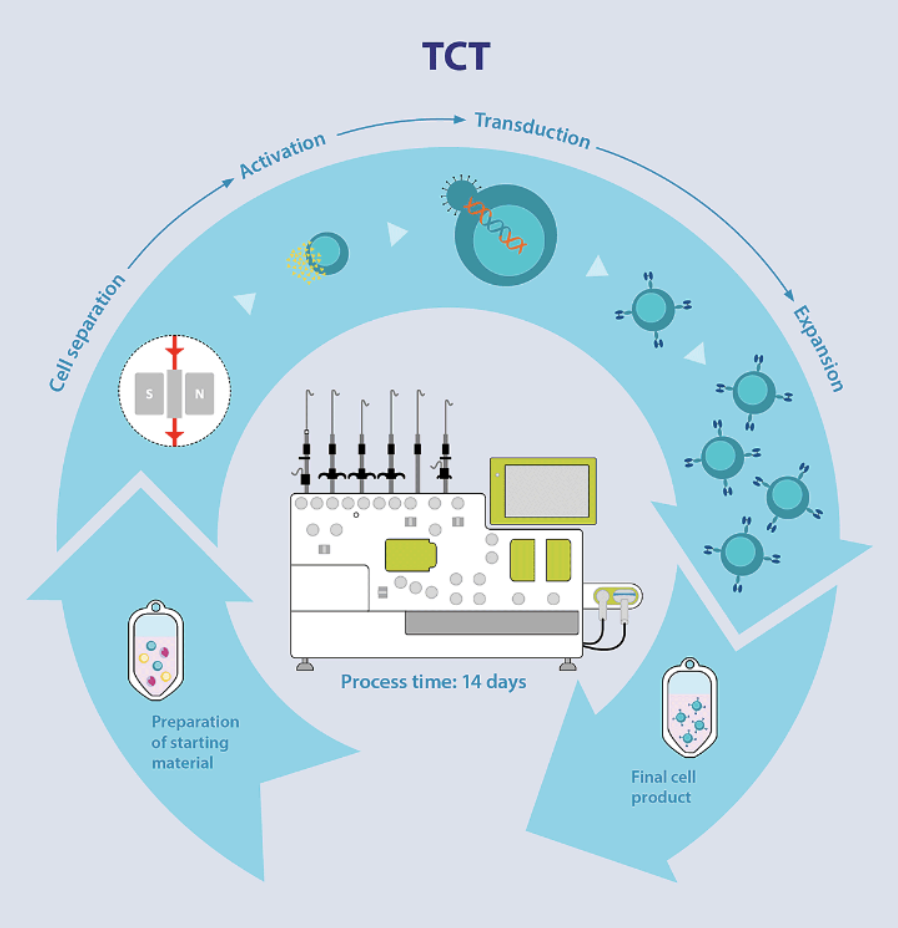

Figure 7.2.2.1: The CliniMACS Prodigy TCT system. *Utilizing a CliniMACS Prodigy TCT for CAR-T cell production would automate cell separation, activation, transduction, and expansion ("Automated cell manufacturing on a closed and scalable platform").*

The CliniMACS Prodigy Unit has a cost around $150,000 and consists of the following ("CliniMACS Prodigy Instrument"; "Point-of-Care Gene Therapy Is Out of the Box"):

- CentriCult unit (CCU) - temperature controlled unit for cell processing and cultivation
- Peristaltic pump - directs accurate volumes of liquids through the unit tubing
- Pinch valves - ensure controlled fluid pathways within the tubing set
- Touchscreen - software which guides the operator
- Magnet unit - houses the separation column for magnetically labeled cell enrichment
- Gas mix unit - to mix up to three gasses for cell cultivation

As shown in Figure 7.2.2.1 above, CliniMACS Prodigy TCT employs a four-step workflow which proceeds as follows: 1) Separation of T cells, 2) T cell activation, 3) Viral transduction, and 4) CAR T cell expansion. A summary of all medias necessary for these steps can be found below in Table 7.2.2.1.

Separation of T cells: The CliniMACS Prodigy employs magnetic isolation to perform T cell separation with high purities achieved ("Clinical-grade TCR engineered T cells and CAR T cell manufacturing via viral transduction"). This enables efficient separation of both $CD4^+$ and $CD8^+$ T cells without inducing premature activation. Moreover, it removes contaminating cells, enhancing T cell transduction efficiency, and requires no magnetic bead removal.

T cell activation: The CliniMACS Prodigy systems use a MACS GMP T Cell TransAct media to perform T cell activation. This is a colloidal polymeric nanomatrix which is conjugated to humanized, recombinant CD3 and CD28 agonists (""Clinical-grade TCR engineered T cells and CAR T cell manufacturing via viral transduction). This ready-to-use reagent has been specifically designed to activate and enrich T cell populations. Due to its nanoscale structure, it can be sterile filtered and the excess reagent can be removed via washing of TransAct activated cells ("Clinical-grade TCR engineered T cells and CAR T cell manufacturing via viral transduction").

Viral transduction: The CliniMACS Prodigy TCT system supports both gamma-retroviral and lentiviral vectors for T cell transduction. For this step, the MACS GMP Vectofusin-1 media is used as a viral transduction enhancer. It is a fully synthetic, non-toxic, histidin-rich, cationic amphipathic peptide which is compatible for enhancing viral transduction capacity in automated processes. It specifically enhances viral entry into target cells when added to the culture medium ("Clinical-grade TCR engineered T cells and CAR T cell manufacturing via viral transduction").

CAR T cell expansion: CAR-T cell expansion takes place within the cell cultivation chamber of the unit. Flexible programming is allowed in determining cell culture duration as well as medium supply and exchange ("Clinical-grade TCR engineered T cells and CAR T cell manufacturing via viral transduction"). Cultivation and expansion of CAR-T cells is enabled through the use of TexMACS GMP Medium, MACS GMP Cytokines, and MACS GMP T Cell TransAct media. The TexMACS GMP Medium was specifically designed for T cell activation. It is a serum- and xeno-component free media with pharmaceutical-grade human serum albumin that undergoes quality control testing with each batch ("Clinical-grade TCR engineered T cells and CAR T cell manufacturing via viral transduction"). MACS GMP Cytokines include Recombinant Human IL-2, IL-7, and IL-15.

Table 7.2.2.1: Materials required to run the CliniMACS Prodigy unit for CAR-T cell manufacturing.

Material Name	Amount	Cost ($)	Notes
CliniMACS CD4 Product Line • GMP MicroBeads • Reagent	• 7.5 mL • 7.5 mL	• 3,965.00 • Price available upon request	• Accessory molecule • Murine anti-CD4 monoclonal antibodies conjugated to

Material Name	Amount	Cost ($)	Notes
			superparamagnetic iron dextran particles
CliniMACS CD8 Product Line ● GMP MicroBeads ● Reagent	● 7.5 mL ● 7.5 mL	● 3,965.00 ● Price available upon request	● Murine anti-CD8 monoclonal antibodies conjugated to superparamagnetic iron dextran particles
MACS GMP T Cell TransAct	4 mL	2,400.00	-
MACS GMP Vectofusin-1	1 mg	1,030.00	-
TexMACS GMP Medium	2000 mL	375.00	Optimized for cultivation of human T cells
MACS GMP Recombinant Human IL-2	500 µg	2,940.00	● Lymphoid cell growth factor that plays an important role in the activation and maintenance of immune responses and lymphocyte development ● Promotes proliferation and differentiation of T cells
MACS GMP Recombinant Human IL-7	25 µg	820.00	● Pleiotropic cytokine with roles in modulating T cell development and homeostasis ● T cell growth factor and anti-apoptotic survival factor
MACS GMP Recombinant Human IL-15	25 µg	770.00	● Lymphoid cell growth factor ● Stimulates the proliferation of activated T cells and cytotoxic T lymphocyte generation

Following these four main steps, the CAR-T cells are harvested in a 100 mL buffer of choice ("Clinical-grade TCR engineered T cells and CAR T cell manufacturing via viral transduction"). A CliniMACS Formulation solution could be used and a CliniMACS Formulation unit could be employed in combination with the CliniMACS Prodigy unit to enable automated final filling and cryopreservation of the final product ("CliniMACS Formulation & Cryo Product Line"; "CliniMACS Formulation Set").

Integrated sampling pouches on the CliniMACS Prodigy tubing sets enable collection and subsequent testing of samples at any time throughout the cell manufacturing process. Miltenyi Biotec also has a variety of instruments available for automated and standardized testing, including MACSQuant Instruments for flow cytometry ("Clinical-grade TCR engineered T cells and CAR T cell manufacturing via viral transduction").

A summary of the manufacturing workflow of CD19 CAR-T Cells using a CliniMACS Prodigy unit is shown below in Figure 7.2.2.2. The entire process can be achieved in the unit using a single closed-tubing set in a 12 day timeframe (Palani et al., 2023). On Day 0, the samples are prepared and the T cells are both separated and activated (Palani et al., 2023). On Day 1, the T cells are transduced using the appropriate lentiviral vector. Between Days 1-12, the T cell expansion is completed (Palani et al., 2023). The process comes to an end with final formulation on Day 12 (3Palani et al., 2023 Quality control and in-process control samples can be analyzed at the time points indicated in the figure below (Palani et al., 2023).

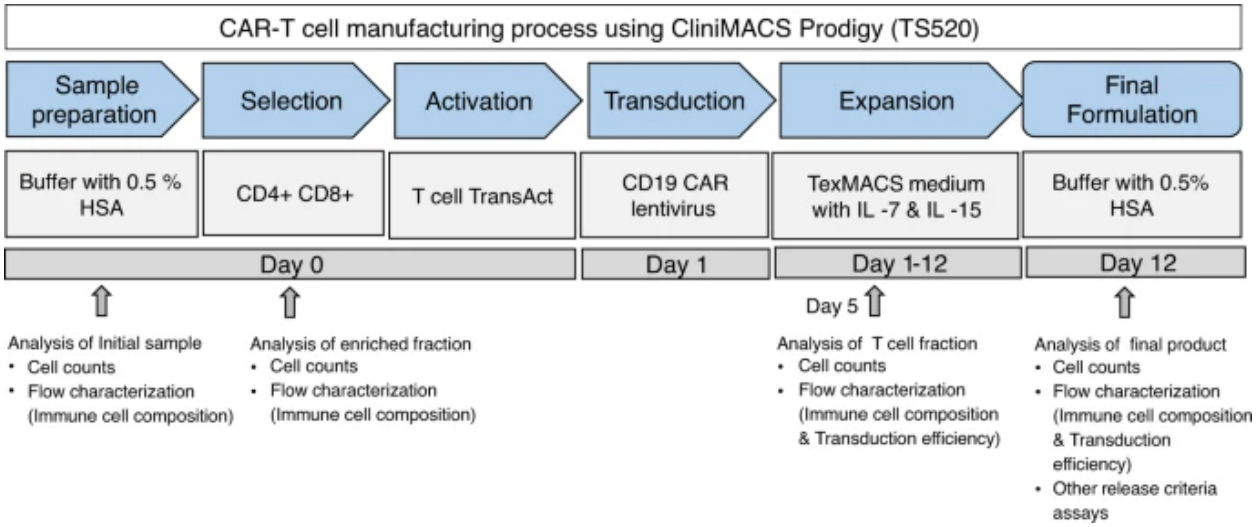

Figure 7.2.2.2: The CliniMACS Prodigy TCT automated CAR-T manufacturing timeline. *Palani et al. (2023) optimized production to occur over a 12 day time period, starting with sample preparation and ending with final formulation of the CAR-T product.*

Studies have demonstrated that the most significant costs of this process are associated with the reagents and consumables needed for CAR-T cell manufacturing in the CliniMACS Prodigy unit, as shown below in Figures 7.2.2.3 and 7.2.2.4.

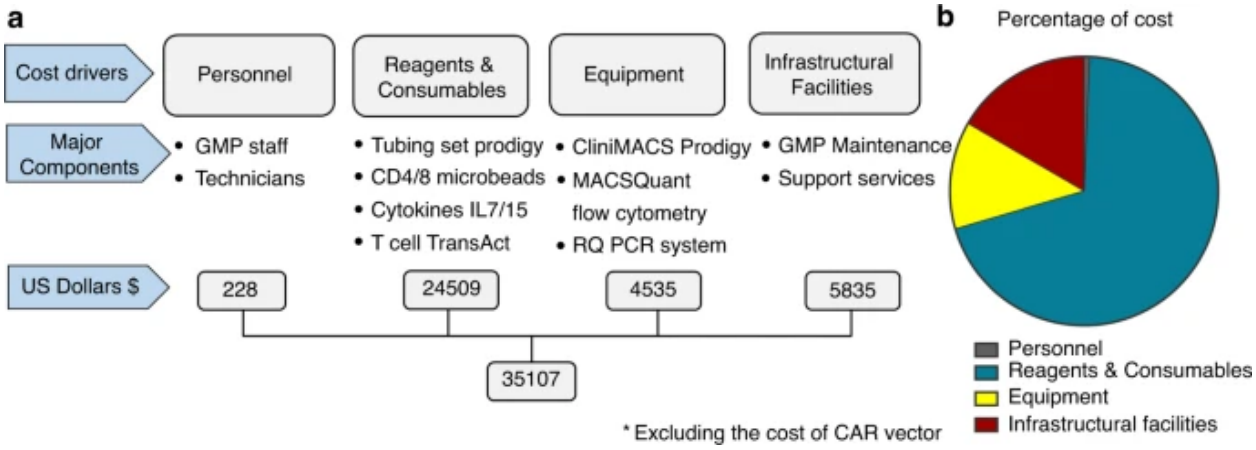

Figure 7.2.2.3: Economic analysis of automated CAR-T manufacturing using a CliniMACS Prodigy unit, as performed by Palani et al. (2023).

No	Category	Cost (Indian Rupees)	Cost (US Dollars)	Cost Percentage
1	Personnel	17,100	228	0.65%
2	Consumables/ disposables	6087	81.16	0.23%
3	Chemicals and reagents	1,832,103	24,428	69.57%
4	Electricity maintenance	6388	85.17	0.24%
5	Equipment maintenance	66,757	890.09	2.53%
6	Other test (apheresis and T cell subsets by flow cytometry)	32,664	435.52	1.24%
7	Depreciation of equipments	267,024	3560.32	10.14%
8	Infrastructural facilities (Including GMP)	154,275	2057	5.85%
9	GMP maintenance & support services	250,697	3342.62	9.52%
	Total	Rs. 2,633,095	USD. 35,107.88	100%

Figure 7.2.2.3: Breakdown of economics associated with automated CAR-T manufacturing using a CliniMACS Prodigy unit, as performed by Palani et al. (2023). *A total cost of CAR-T manufacturing per therapy would be around $35,000. The most significant costs are associated with chemicals and reagents, which account for nearly 70% of the overall cost.*

Advantages: Use of the CliniMACS Prodigy unit for automated CAR-T cell manufacturing has a number of benefits. First, the entire cell manufacturing platform occurs in a closed system, starting with the incoming leukopak and ending with the final CAR-T cell product. This reduces cleanroom requirements and decreases areas where operators interface with biological product,

increasing operator safety and reducing contamination risks. Moreover, the entire system is automated which would reduce labor requirements and ensure that the manufacturing process is both reproducible and standardized ("Clinical-grade TCR engineered T cells and CAR T cell manufacturing via viral transduction").

Disadvantages: Although the CliniMACS Prodigy unit has a number of great advantages, its drawbacks should not be overlooked. To start off, the unit's software provides protocol flexibility for different cell types, so an important part of using such a device in GMP cell therapy manufacturing would be to ensure that a protocol is established to reduce batch to batch variability and prevent any errors in processing due to operator error in software programming. Moreover, an important part of GMP manufacturing of cell therapies is 21 CFR Part 11 FDA regulations, which requires that automated systems need certain controls and access levels. For instance, operators would have different access levels than engineers would, enabling them to have more control over the process. The Prodigy currently does not employ any particular access levels or alarm functions, so a challenge with using it here is making sure that it is compliant with this GMP manufacturing requirement. However, this could be achieved manually through recording of data in logbooks. Another drawback to the CliniMACS Prodigy unit is that, since it is one centralized piece of equipment, it must be shut down entirely if one part of the process goes wrong. This would ultimately cause a decrease in production because the entire process would have to be offline while the part is fixed. This could also potentially lead to loss of patient product, which would likely have been a last treatment option for them. Additionally, there is limited throughput of the process. Given the scale-out and not scale-up nature of CAR-T cell manufacturing, there are limited capabilities for large-scale production of CAR-T cell therapies using a CliniMACS system. For a 12-day manufacturing protocol, a maximum of 36 patient therapies could be produced per year for each CliniMACS device (Palani et al., 2023). In order to achieve the desired number of therapies per year (plan to treat 52/yr), a minimum of two devices would be needed (Palani et al., 2023). This significantly increases the costs associated with the process, but given that processes which are not automated would still be scale out instead of scale up due to the autologous nature of the therapy, this is not necessarily a drawback of the CliniMACS Prodigy system itself but rather a hurdle in large-scale CAR-T therapy manufacturing as a whole. Lastly, the reagents and consumables costs of the Prodigy have a high cost of around $26,000 per round. Again, this is not necessarily a drawback to the CliniMACS Prodigy unit specifically but rather with a single-use approach to CAR-T manufacturing in general.

Although to the best of our knowledge, a CliniMACS Prodigy unit has not been employed for the manufacturing of an FDA-approved CAR-T therapy thus far, it does show significant potential. The process unit, reagents, and materials are all GMP-compliant to the best of our knowledge and by employing the use of data logbooks, the issue with 21 CFR Part 11 compliance can be overcome. Moreover, the feasibility of manufacturing clinically viable and

patient-specific anti-CD19 CAR-T cells has been established (Palani et al., 2023). A study published in Nature's *Bone Marrow Transplantation* journal demonstrates that anti-CD19 CAR-T cells can be manufactured using a CliniMACS Prodigy unit which achieves significant cell expansion and transduction efficiency and viability, meeting requirements for clinical applications (Palani et al., 2023). We therefore recommend the use of a CliniMACS Prodigy system for CAR-T cell manufacturing, but stress the implementation of logbooks to ensure all GMP requirements are met for the process.

7.3. Cryopreservation

Cryopreservation follows a similar general protocol for lentiviral vectors and CAR-T cells. In both cases, the product vials are frozen at 1℃/min to 4℃, at which point any applicable cryoprotectant is added, then the product is frozen at 1℃/min to the final storage temperature (Cytiva, n.d.; Rahman *et al.*, 2013). A balance needed to be struck in the ideal freezing rate for these products, with a rate of 10℃/min being effective for quicker freezing but with great potential for loss of viability/activity in the product, while a rate of 0.10℃/min is optimal for product stability with the downside of long freezing times. Generally, a rate of 1℃/min has been found to provide an appropriate balance between a reasonable freezing time and minimal damage to the product (Cytiva, n.d.). The industry-standard final storage temperature of lentiviral vector formulations is -80℃ (Rahman *et al.*, 2013). For CAR-T formulations, some protocols suggest freezing to a temperature as low as -120℃, however Cytiva Life Sciences (n.d.) has found minimal improvement in cell viability when CAR-T cells are cryopreserved at temperatures below their intracellular glass transition temperature of -47℃. Therefore, CAR-T cells are recommended to be frozen to a final storage temperature of -60℃, to account for temperature fluctuations during the freezing process. The approximate processing time for freezing of lentiviral vector products is calculated as follows:

$$t = (\Delta T)/(-1 \times Freezing\ Rate) = (T_{storage} - T_{ambient})/(-1 \times Freezing\ Rate)$$
$$t = (-80℃ - 20℃)/(-1℃/min) = 100\ min$$

Following the same calculation procedure, the approximate processing time for freezing of CAR-T cells is:

$$t = (\Delta T)/(-1 \times Freezing\ Rate) = (T_{storage} - T_{ambient})/(-1 \times Freezing\ Rate)$$
$$t = (-60℃ - 20℃)/(-1℃/min) = 80\ min$$

For lentiviral vectors, a potential cryopreservation medium found beneficial for the maintenance of viral activity during freezing and storage is comprised of 88-97% of tris-HCl buffer, 1-10% of B27 serum-free additive, 1-6% of CD-Lipid concentrated solution, and 1-8% of Human Serum

Albumin solution, by volume (Zhen & Zhang, 2017). The tris-HCl buffer contains tris-HCl and NaCl in molar concentrations of 0.8-20 mM and 0.08-0.15 M, respectively, yielding a pH of 7.0-8.0 (Zhen & Zhang, 2017). CAR-T cells are typically cryopreserved in a medium containing up to 10% dimethyl sulfoxide (DMSO) or glycerol as a cryoprotective agent. Since these components are toxic to human cells, it is essential that time for which cells are exposed to the cryopreservation medium pre-freeze and post-thaw is limited to 30 minutes each (Li *et al.*, 2019).

For the cryopreservation step, we recommend the CBS CRF2101 controlled-rate freezer or a similar unit. This freezer is 21 CFR Part 11 compliant, has a 28 L capacity, an effective temperature range of +50℃ to -180℃, and is capable of delivering freezing rates between 0.01 to 99.9℃/min (Cryosolutions, n.d.). These specifications make this an effective vessel for the desired lentivirus and CAR-T freezing applications. The CBS CRF2101 is depicted in Figure 7.3.1 below.

Figure 7.3.1: The CBS CRF2101 controlled-rate freezer. *The CRF2101 uses liquid nitrogen to provide for the controlled-rate freezing of biological samples, with freezing data being collected according to 21 CFR Part 11 requirements by the onboard laptop controller.*

8. Utility Summary

Since we are using a cell-based process, there are specific restrictions that the process must be maintained. For example, the temperature of the cells must be maintained at 37ºC and atmospheric pressure. The various substrates were not all at 37ºC originally, so various utilities were used to change the temperature. The bioreactor also operated at 5 MPa, so the atmospheric air needed to be pressurized to be used in the process. Our process is centered around cells' survival and growth, so using utilities to maintain proper temperature and dissolved oxygen conditions for cell cultures is essential. Cooling utilities used in the process include cooling water and chilled water. The heating utilities used in the process include hot water, steam, high-pressure steam, and air. The inlet and outlet temperatures, mass-to-energy factor, and the cost per megaton of utility are displayed in Table 8.1 below.

Table 8.1 Utilities Summary*. The relevant utility properties were gathered from our SuperPro simulations.*

Utility	Temperature In (℃)	Temperature Out (℃)	Mass-to-Energy Factor (kcal/kg)	Cost ($/ MT)
Cooling Water	25	30	4.9971	0.05
Chilled Water	5	10	5.0221	0.40
Hot Water	40	30	9.9904	0.05
Steam	152	152	503.6826	12.00
Steam (High P)	242	242	419.6322	20.00

9. Economics

In determining the required sale price of the CAR-T therapy to achieve the desired base case internal rate of return (IRR) of 12.5%, the first step we took was to determine pricing for our process equipment. For this, we sourced pricing information on the three pieces of non-single-use equipment: the Miltenyi CliniMACS Prodigy, the Repligen Krosflo, and the Cytiva AKTA Readyflux. The purchased price of the CliniMACS Prodigy was estimated based on a 2016 article from the Fred Hutchinson Cancer Center (Rabinovitz and Hudson, 2016). The purchased price of the Repligen Krosflo was gathered directly from Repligen's website (Repligen, 2023). Finally, the purchased price of the Cytiva AKTA was conservatively estimated as approximately 200% of used prices (LabX, 2023). The estimated purchased prices of these units are tabulated in Table 9.1 below.

Table 9.1: Estimated purchased price of non-single-use process equipment.

Equipment	Estimated Cost
Miltenyi CliniMACS Prodigy	$150,000.00
Repligen KrosFlo	$20,196.00
Cytiva AKTA Readyflux	$65,000.00

Next, we estimated the number of operators required for the entire end-to-end process. As a constraint, three operators per shift were required for all processing. Therefore, assuming three operators per shift were required for LVV processing, three operators per shift were required for CAR-T processing, and processing was done in three shifts per day, a total of eighteen operators were required. At a $40/hr wage, these operators' annual wages totaled $83,000.00. As a base case approximation in the economics spreadsheet, employee benefits were assumed to cost an additional 40% of the employee's annual wages. Two operating supervisors were required for the LVV process, with one supervising upstream processes while one supervises downstream processes. One operating supervisor was required for the CAR-T process. At a $55/hr wage, the cost of operating supervision was 138% of the operator wages. General overhead was assumed to be 125% of total operator wages. Insurance and local taxes were assumed to cost 1.5% of the fixed capital investment.

Next, to estimate the variable cost, we determined the approximate pricing of reagents, media, and single-use equipment per run of the full end-to-end process. As an approximation, we made the assumption that all utilities, reagents, and media for the end-to-end process cost $500,000.00. The estimated cost per run for the single-use CliniMACS prodigy kit was $26,000.00 (Rabinovitz and Hudson, 2016). The estimated cost of the Cytiva Supor EKV filter was $496.52 per three-pack of filters (Fisher Scientific, 2023). The cost of the 12" Bioproducer TFF filters from Sartorius AG, as quoted by a representative of Sartorius, was $8700.00 per three-pack of filters. The cost of the Sartobind Q AEX chromatography column from Sartorius AG, as quoted by a representative of Sartorius, was $5700.00 per column. The variable costs input into the economics spreadsheet are tabulated in Table 9.2 below.

Table 9.2: Raw material and single-use equipment cost information.

Item	Price ($ / unit)	Unit	Units per CAR-T therapy (units / component)
Misc. Reagents, Ingredients, Utilities	500,000.00	Run	1
CliniMACS Prodigy Kit	26,000.00	Kit	1
Supor EKV Filter	496.52	3-Pack Filters	0.33

Item	Price ($ / unit)	Unit	Units per CAR-T therapy (units / component)
TFF Filters (12" Bioproducer)	8700.00	3-Pack Filters	0.67
Sartobind Q Column	5700.00	Column	1

The information displayed in Tables 9.1 and 9.2 above, along with the economic premises listed in Section 5.5.3 of this report, were entered into the CHEN 4520 course economic spreadsheet as displayed in Appendix Figures A-1, A-2, and A-3. For the parameters described above, the total FCI required for construction of our process was estimated at $400,000.00. The results of a base-case analysis at 12.5% Internal Rate of Return (IRR) are displayed in Appendix Figure A-4, with non-cumulative and cumulative cash flows for every year displayed in Appendix Figures A-5 and A-6, respectively. Cash flows for each year were computed depending on the operation mode in that year by the spreadsheet using the following formulas:

$$Cash\ Flow_{Construction} = -f_{FCI,expended} FCI - WC - Land\ Cost$$

$$Cash\ Flow_{Startup} = ATE + D - R - Startup\ Costs$$

$$Cash\ Flow_{Operating} = ATE + D - R$$

$$Cash\ Flow_{Final} = ATE + D - R + WC + Salvage$$

Cumulative cash flows for a specific year were computed by taking the sum of cash flows for that year and all prior years. Return on Investment, ROI, was calculated based on year 3 by the following relationship:

$$\%\ ROI = 100\%(ATE)/TCI$$

The TCI is the Total Capital Investment and includes the WC and the FCI. For the base case 12.5% target IRR, the % ROI was computed as 23.9%. This indicated that profits in year 3 of operation totaled 23.9% of the total capital invested into constructing and starting the plant. Based on this measure, Payback Period, or PBP, was calculated as follows:

$$PBP = 1/ROI$$

For the base case 12.5% target IRR, the payback period was determined as 4.2 years, meaning 4.2 years of plant operation would be required for the plant's cumulative profits to equal the initial capital invested into construction and startup of the plant. Net Present Value (NPV), another indicator of plant profitability, was determined using the following relationship:

$$NPV = \left[\sum_{j=1}^{n} \frac{(Cash\ Flow)_j}{(1+Cost\ of\ Capital)_j}\right] + (WC + Salvage)\left[\frac{1}{(1+Cost\ of\ Capital)^n}\right] - TCI$$

The NPV is the difference between the present value of the cumulative cash flows at the end of operation and the initial cost of construction and startup for the plant. For the base case, the NPV was computed as $2,443,000.00 assuming cash flows are counted at the end of each period and $2,553,000.00 assuming cash flows are counted at the beginning of each period. The choice of when to count cash flows affects how the compounding cost of capital will be applied to these cash flows, with the "end of period" case being a more conservative estimate.

From the NPV, the IRR was determined as the cost of capital for which the NPV is equal to zero. In essence, the IRR is the maximum possible interest rate achieved by the investors assuming their investment is exactly repaid by the profits from the process across the process lifetime. It is desirable to have an IRR greater than the cost of capital, else the plant would not be considered profitable enough to be worth the initial capital investment. For the base case, the target IRR was 12.5%, which was 8% higher than the cost of capital used, indicating profitability for the plant. The sale price of the CAR-T therapy was set by the spreadsheet such that the plant achieves the target % IRR. For the base case 12.5% IRR, this yielded a CAR-T therapy sale price of $749,091.26 per treatment.

We then modulated the target % IRR to determine the effects on the various parameters described above. To achieve a 15% IRR, the required sale price of the CAR-T therapy was $753,230.13 per treatment, with results displayed in Appendix Figure A-7. This higher target IRR increased the ROI to 26.5% and reduced the payback period to 3.8 years, indicating that the plant was more profitable and achieved those profits faster at this higher sale price. The NPV for the investment in this case was $3,286,000.00 for cash flows counted at the end of each period and $3,434,000.00 for cash flows counted at the beginning of each period, confirming a greatly increased overall profitability for the plant compared to the base case. Non-cumulative cash flows for this case are displayed in Appendix Figure A-8, and cumulative cash flows for this case are displayed in Appendix figure A-9.

To achieve a 20% IRR, a CAR-T therapy selling price of $761,869.98 per treatment was required, with results displayed in Appendix Figure A-10. This increased IRR again increased the ROI to 31.9% and reduced the payback period to 3.1 years. The NPV was increased to $5,045,000.00 for period-end cash flows and $5,272,000.00 for period-beginning cash flows.

Non-cumulative cash flows for this case are displayed in Appendix Figure A-11, and cumulative cash flows for this case are displayed in Appendix figure A-12.

To achieve a 25% IRR, a CAR-T therapy selling price of $770,814.82 per treatment was required, with results displayed in Appendix Figure A-13. This increased IRR further increased the ROI to 37.4% and reduced the payback period to 2.7 years. The NPV was increased to $6,865,000.00 for period-end cash flows and $7,174,000.00 for period-beginning cash flows. Non-cumulative cash flows for this case are displayed in Appendix Figure A-14, and cumulative cash flows for this case are displayed in Appendix figure A-15.

While a higher target IRR seemed like an appealing choice from a profitability standpoint, these calculations all assumed that the number of patients treated per year was independent of the sale price of the therapy. To further elucidate the effects of target IRR on the required sale price of the CAR-T treatment, we performed a sensitivity analysis on the sale price of the CAR-T treatment by modulating the target IRR, with results displayed in Appendix Figure A-16. In general, the required sale price of the CAR-T therapy appeared to increase as a shallow exponential function with changes in target IRR. Using this model, the appropriate target % IRR can be chosen based on the maximum % IRR which maintains a competitive selling price of the CAR-T therapy.

Notably, the process has a payback period of 4.2 years for the base case 12.5% IRR. To investigate methods for the reduction of this payback period, a further sensitivity analysis was performed by modulating the % IRR to elucidate the effect on payback period for the process. The results of this sensitivity analysis are displayed in Appendix Figure A-17. The payback period for the process was found to decrease with an increase in % IRR, with the decrease following a shallow exponential function. Based upon these results, the payback period could be reduced by more than a year by increasing the % IRR from 12.5% to 20%. This sensitivity analysis provided further evidence that increasing the % IRR improved profitability for the process, but these improvements in profitability come with increases in treatment cost for the patient and lower accessibility of CAR-T therapy.

Next, a sensitivity analysis was performed using the base case IRR of 12.5% to determine the effect of variability in the assumed $500,000.00 reagent, raw material, and utility cost on the required selling price of the CAR-T therapy. The results of this sensitivity analysis are displayed in Appendix Figure A-18. The sale price of the CAR-T therapy increased roughly linearly with the assumed cost of raw materials, so if reagents, media, and utilities were able to be sourced for $250,000.00 per end-to-end process run rather than $500,000.00 per run, for example, the required sale price of the CAR-T therapy would be reduced to $496,186.56 per treatment. This change would allow the targeting of a significantly higher IRR while still maintaining a more accessible sale price. Therefore, investigation into the optimization of acquisition of these raw materials could provide avenues to dramatically improve profitability of the process.

The economic analysis of the viability of the CliniMACS Prodigy system compared to individual process units was a major consideration when investigating the viability of the Prodigy. Therefore, we investigated the sensitivity of the % ROI to the purchased price of the CliniMACS Prodigy, with results in Appendix Figure A-19. The % ROI of the process decreased in a stepwise fashion with the purchased price of the CliniMACS Prodigy, and this decrease was approximately 2.3% across the range of Prodigy costs tested. The use of the CliniMACS Prodigy led to an approximate 0.6% decrease in ROI compared to the approximate purchase price of individual process units, not factoring in savings in labor and prevention of product contamination. Therefore, the use of the CliniMACS Prodigy over the individual units was determined to have a very minor effect on the profitability of the overall end-to-end process, with other production costs such as the per-run cost of reagents, media, and utilities having a much greater effect on process profitability.

We further determined the effects of the annual inflation rate on the selling price of the treatment and the % ROI. The results of these sensitivity analyses are displayed in Appendix Figures A-20 and A-21, respectively. The sale price of the CAR-T therapy was observed to decrease linearly with increases in inflation rate, and this decrease was roughly $2,200 per treatment across a range of inflation rates from 0% to 3.8%. The % ROI, meanwhile, increased linearly with increases in inflation rate, and this increase was roughly 0.2% across the range of inflation rates. For this sensitivity analysis, the selling price of the treatment was assumed to be locked to the same inflation rate as the raw materials, hence the improvement in ROI with inflation. These results indicated that changes in the rate of inflation will not significantly impact the profitability of the process.

10. Process Optimization

10.1. LVV Process Time Optimization

The goal with developing this CAR-T process with transduction of an in-house lentiviral vector is to be able to provide potentially life-saving CAR-T therapies to patients. Specifically, we aimed to design processes which could support 52 patients per year.

The lentiviral process takes a total of 22.5 days, broken down as follows: 1 day cell revival, 11.5 days cell suspension expansion, 8 days bioreactor expansion and transfection, and 2 days downstream processing. Assuming an annual operating time of 8,000 hours and that each run generates enough viral vector to support one CAR-T cell therapy, the LVV portion of the process would be able to support a maximum of 14 patients per year, as calculated below, which is well below the goal of 52 patients per year. We therefore think the LVV production process is one of the greatest areas of process improvement within this project. Specifically, the most timely areas

of that process are the suspension expansion and bioreactor steps. Given a doubling time of nearly one full day, there is not much that can be done to expedite these steps. The throughput of this process, however, can still be optimized by working with more than one batch at a time. For instance, working three batches at a time with one in the suspension expansion phase, one in the bioreactor, and one in the downstream process, enabling optimization of LVV process timing and throughput.

$$8,000\ \frac{operating\ hours}{year} \times \frac{1\ operating\ day}{24\ operating\ hours} \times \frac{1\ LVV\ process\ cycle}{22.5\ operating\ days} = 14.8\ LVV\ process\ cycles\ per\ year$$

10.2. LVV Filtration Optimization

The high cost associated with CAR-T/lentiviral vector (LVV) therapy poses a significant barrier to patients seeking this revolutionary treatment (citation needed). Often, patients only resort to CAR-T therapy as a last resort when conventional treatments have failed. In response to AGC Biologics' request to identify process improvements for optimization during each run, our team has focused on enhancing the efficiency of the process, aiming to reduce the overall cost and make the treatment more financially viable for a broader population.

In industrial lentiviral vector production, filtration unit operations play a critical role in the concentration and purification of lentiviral vectors. However, filtration of lentiviral vectors at an industrial scale often leads to considerable inefficiencies (Cooper et al., 2011). Cooper et al. found that tangential flow filtration (TFF) offers a superior alternative to centrifugation at larger scales, providing a wide range of concentration factors (66-fold to 1800-fold concentration) (Geraerts et al., 2005).

In our proposed design, we employ depth filtration and tangential flow filtration in the downstream LVV process to capture the target LVV from cell culture and eliminate major contaminants. The process starts with depth filtration, followed by two tangential flow filtration steps: the first TFF step for concentration, and the second TFF step for removing residual impurities and replacing the buffer to make the working solution suitable for final formulation, after the chromatography step. We propose replacing the depth filtration and the tangential flow filtration concentration steps with tangential flow depth filters in both areas to intensify lentiviral vector production.

Tangential flow depth filters provide several advantages over traditional tangential flow filters. Tona et al. (2023) sought to improve the efficiency of tangential flow filtration by investigating the feasibility of tangential flow depth filtration. Their research demonstrated that equivalent tangential flow depth filtration systems had high filter capacity, extended functional life, and efficient separation of LVVs from producer cells.

By implementing tangential flow depth filters in our design, we expect to achieve the following benefits:

1. Improved filtration efficiency: The integration of tangential flow depth filters will result in higher filter capacity and more efficient separation of LVVs from producer cells (Tona et al., 2023).
2. Enhanced process robustness: Tangential flow depth filters offer extended functional life, which contributes to a more reliable and robust filtration process, reducing downtime and maintenance requirements.
3. Cost reduction: The use of tangential flow depth filters will lead to improved process efficiency and reduced operational costs, ultimately lowering the overall cost of CAR-T/LVV therapy, making it more accessible to patients.

In conclusion, our proposed design of incorporating tangential flow depth filters in the downstream LVV process seeks to optimize the filtration process, thereby reducing the cost of CAR-T/LVV therapy and making this life-saving treatment more accessible to a wider population

10.3. Chromatography Opportunity

A major drawback to chromatography operations involving lentiviral vectors is the relatively low recovery of purified lentivirus (Moreira *et al.*, 2020). This is related to overlap between the elution profiles of lentiviruses and cDNA and other common impurities (Valkama *et al.*, 2020). Affinity Chromatography is an alternative chromatography technique which has been found to give the advantage of better separation of active LVVs from common impurities at a lab scale. However, there is a lack of research supporting an industrial scale-up of this method (Moreira *et al.*, 2020). We believe affinity chromatography is a promising avenue for examination, as employment of this technique could result in improved yields over AEX chromatography, and therefore recommend further experimentation into industrial scale-up of this technique.

11. Homework Problem

A crossflow filtration system is used to concentrate and diafiltrate a 5.5L lentiviral vector (LVV) containing medium derived from HEK293 cells. A firm is planning a process that will use multiple flow paths (multiple cross-flow filtration systems) to handle different parts of the concentration and diafiltration. In flow path 1, the goal is to concentrate the medium down to 50 mL and the inlet pressure is maintained below 6 psi for the entirety of the process. After being concentrated, the vector is diafiltrated with 1000 mL of diafiltration mix (DPBS and 2.5 mL of FCS) and concentrated again down to 50 mL using flow path 1. Finally, flow path 2 is used to

further concentrate the 50 mL of medium to 1 mL (with the inlet pressure maintained below 9 psi)

a) Calculate the concentration factor achieved in each stage of the process (FP1
b) Determine the overall concentration factor for the entire process
c) Estimate the time required to complete the concentration and diafiltration process using

[Process referenced from Cooper, A. R., Patel, S., Senadheera, S., Plath, K., Kohn, D. B., & Hollis, R. P. (2011). Highly efficient large-scale lentiviral vector concentration by tandem tangential flow filtration. *Journal of virological methods*, *177*(1), 1–9. https://doi.org/10.1016/j.jviromet.2011.06.2019]

Solution:

a) Consider a diafiltration unit

FP1 Concentration:

- Volumes

  $V_i = 5.5L$

  $V_f = 50\ mL = 0.05\ L$
- Concentration Factor:

$$CF_{1,C} = \frac{V_i}{V_f} = \frac{5.5L}{0.05L} = 110$$

FP1 Diafiltration:

- The final volume after diafiltration remains the same (50 mL). Thus, the diafiltration step doesn't change the concentration of the cells and hence $CF_{1,D} = 1$

FP2 Concentration:

- Volumes

  V_i = 50mL = 0.05L

  V_f = 1 mL = 0.001 L
- Concentration Factor:

$$CF_{2,C} = \frac{V_i}{V_f} = \frac{0.05L}{0.001L} = 50$$

b) Overall concentration factor is just a multiple of each of the individual concentration factors

$$CF_{tot} = CF_{1,C} \times CF_{1,D} \times CF_{2,C} = 110 \times 1 \times 50 = 5500$$

Thus, we are concentrating the cells by a total factor of 5500 in this hypothetical process

c) Want: time to complete concentration and diafiltration processes in FP1 (given that $J = 20\ \frac{L}{m^2 h}$ and $A = 0.5 m^2$)

- Concentration Step:

- Volume removed:
  $\Delta V = V_i - V_f = 5.5 - 0.05L = 5.45L$
- Calculating filtration rate:
  $Rate = J \times A = 30 \frac{L}{m^2 h} \times 0.5 m^2 = 15\ L/h$
- Estimate the time required for the concentrating process
  $t_C = \frac{\Delta V}{Q} = \frac{5.45L}{15 \frac{L}{h}} = 0.363\ hr = 21.8\ min$

- Diafiltration step:
  - Adding 1L of diafiltration mix to 50 mL concentrated medium and then concentrating it back to 50 mL
  - Volume removed:
    $\Delta V = 1L - 0.05L = 0.95L$
  - Time required:
    $t_D = \frac{\Delta V}{Q} = \frac{0.95L}{15 \frac{L}{h}} = 0.063\ hr = 3.8\ min$

- Total time
  $t_{tot} = t_C + t_D = 21.8 + 3.8\ min = 25.6\ min$

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Appendices

Appendix A: Economics

CAPITAL COST ESTIMATE

		=User Input		=Calc by Computer			
Title:	AGC Biologics CAR-T Process				Date:	5/2/23	
Product	Infusable CAR-T Therapy						
Units of Capacity	Component						
Operating Hours per Year	8,000						
Capacity:	30	Component per Year			Site:	US West Coast	
Capacity:	0	Component per Hour					
						Item Cost ($k)	Subtotal ($k)
Enter cost of Land into cell B22 on Cash Flow sheet.							
Study Estimate/Factored Estimate Method							
Engineered Equipment/Purchased & Delivered							
Total Engineered Equipment/Purchased&Delivered							
Misc Equipment			10%				
Subtotal/Purchased Equipment&Delivered							
Lang Factor Approach due to Peters, Timmerhaus and West, 2003, from Table 22.17 in Seider, et al.							
Processing Plant Type	Fluids						
	Lang Factor						
Delivered Cost of Process Equipment							
Installation	47						
Instrumentation and control	36						
Piping	68						
Electrical	11						
Buildings (including services)	18						
Yard improvements	10						
Service facilities	70						
Total Direct Plant Cost							
Engineering and supervision	33						
Construction expenses	41						
Total Direct and Indirect Plant Costs							
Contractor's fee and legal expenses	26						
Contingency	44						
Fixed Capital Investment							
==========							
Bare Module Cost Method (PE w/ FBM factors)		PE Cost	FBM Factor				
Equipment at Bare Module Level							
CliniMACS Prodigy		150	1			150	'Gene therapy in a box' effective, reports Na | EurekAlert!
Repligen KrosFlo		20	2			40	
Cytiva AKTA readyflux		45	1			45	
Subtotal (DIC from Total Bare Module Cost w/FBM Factors)						235	
Misc. Equipment			10%			24	
Subtotal (DIC Equipment from Bare Module Costs)							259
Subtotal (DIC Equipment Costs)							259
Total Bare-Module Costs for Fabricated Equipment							
Total Bare-Module Costs for Process Machinery							
Total Bare-Module Costs for Spares							
Total Bare-Module Costs for Storage and Surge Tanks							
Total Costs for Initial Catalyst Charges							
Total Bare-Module Costs for Computers & Instrumentation							
Total Bare-Module Investment (TBM)						259	
Site Type	Project Addition	Cost of Site Preparation	5%			13	
		Cost of Service Facilities	5%			13	
		Allocated Costs For Utility Plants & Related Facilities					
Total Direct Permanent Investment (DPI)						285	
Cost of Contingencies and Contractor's Fee			18%			51	
Bare Module Total Depreciable Capital (TDC)						336	
Sum of FCI and TDC						336	
							EQUIP
Total (Current $$, USGC)							336
Site Factor			100%	of USGC Total			336
Inflation			1.9%	for	2.0 yrs		349
Scope Growth							

	Total Project-Level Cost										349	
							SAY				400	
	TOTAL DEPRECIABLE CAPITAL or FIXED CAPITAL INVESTMENT (TDC OR FCI).....								$	400	k

WORKING CAPITAL

Start-up Raw Materials Inventory

Quantity	Units	Price				
			/			k
			/			k
			/			k
			/			k
				Total		k

Start-up Spare Parts:	2.5%	of Investment		$10	k
TOTAL WORKING CAPITAL.....				$10	

Site factor table	
US Gulf Coast	1.00
US Southeast	0.95
US Northeast	1.10
US Midwest	1.15
US West Coast	1.25
Western Europe	1.20
Mexico	0.95
Japan	1.15
Pacific Rim	1.00
India	0.85

Project Type	Site	Services
New Project	15%	20%
Project Addition	5%	5%

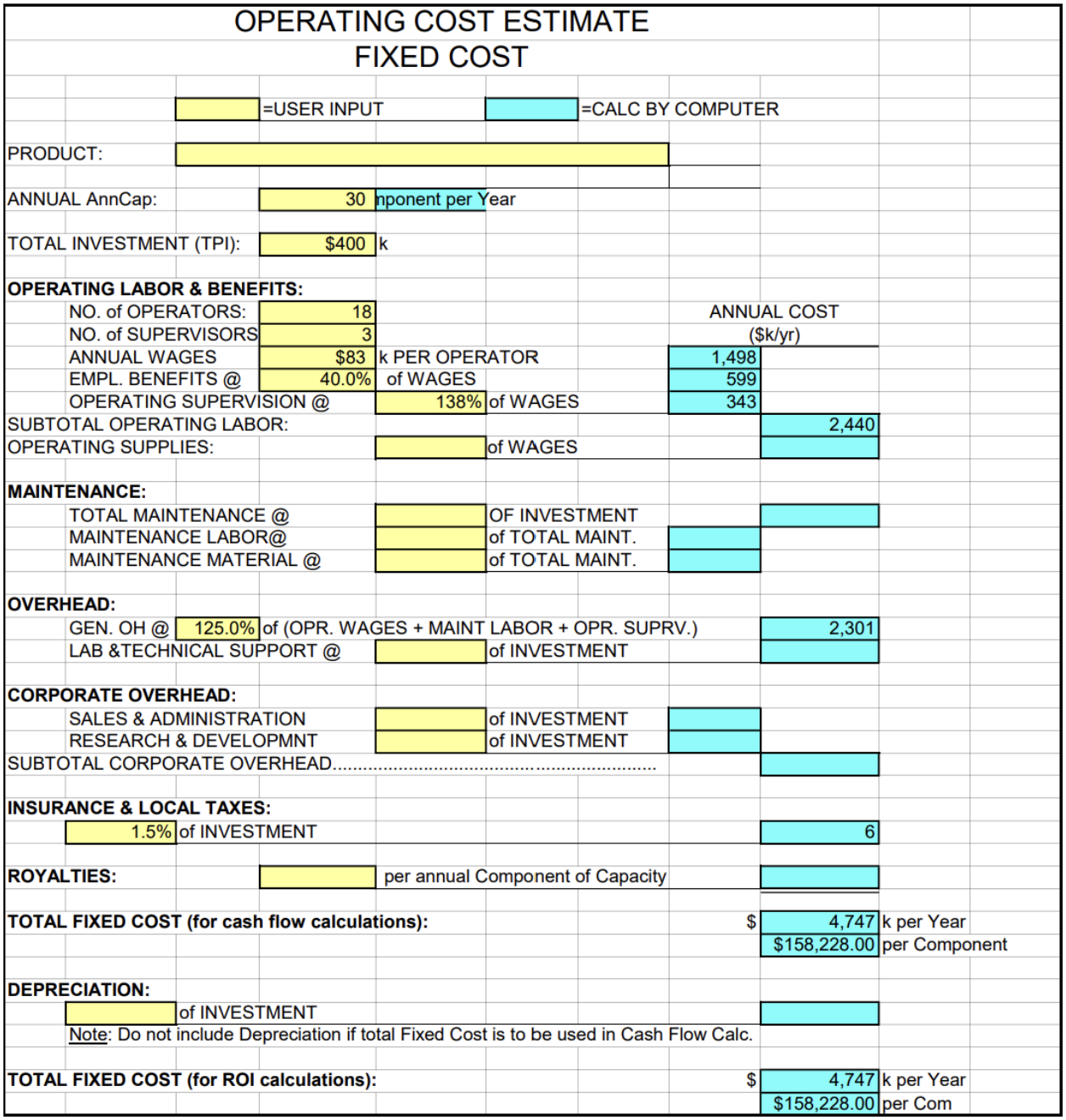
Processing Type	Column No.
Solids	2
Solids-Fluids	3
Fluids	4

Lang Factor Table	Solids	Solids-Fluids	Fluids
Delivered Cost of Process Equipment	100	100	100
Installation	45	39	47
Instrumentation and control	18	26	36
Piping	16	31	68
Electrical	10	10	11
Buildings (including services)	25	29	18
Yard improvements	15	12	10
Service facilities	40	55	70
Total Direct Plant Cost	269	302	360
Engineering and supervision	33	32	33
Construction expenses	39	34	41
Total Direct and Indirect Plant Costs	341	368	434
Contractor's fee and legal expenses	21	23	26
Contingency	35	37	44
Fixed Capital Investment	397	428	504

Figure A-1: Base case capital cost estimate printout from economics spreadsheet. *This page of the economics spreadsheet contains all inputs and calculated totals relevant to calculation of the fixed capital investment and working capital for the entire end-to-end process.*

OPERATING COST ESTIMATE VARIABLE COST						
	=USER INPUT		=CALC BY COMPUTER			
PRODUCT:	Infusable CAR-T Therapy					
ANNUAL CAPACITY:	30	nponent per Year				
INGREDIENTS:	UNIT OF MEASURE	COST PER UNIT ($)	UNITS OF INGRED/ nponent PRODUCT	COST PER Component of PRODUCT ($)		
Material cost	run	500000.00	1.00000	500000.000		
Prodigy kit	kit	26000.00	1.00	26000.000	'Gene therapy in a box' effective, reports Na | EurekAlert!	
Supor EKV filter	3-pack filter	384.00	0.33	128.000		
TFF1 Module	module	8700.00	1.00	8700.000		
TFF2 Module	module	8700.00	1.00	8700.000		
Sartobind Q	capsule	5700.00	1.00	5700.000	Sartorius SARTOBIND Q 200 ML 4 MM, Quantity: Each of 1 | Fisher Scientific	
SUBTOTAL INGREDIENTS					549228.000	
UTILITIES:						
HP STEAM	lb					
LP STEAM	lb					
PROCESS WATER	gal					
COOLING WATER	gal					
INERT GAS	CF					
ELECTRICITY	kWh					
COMPRESSED AIR	CF					
BOILER FEEDWATER	lb					
WASTE TREATMENT						
SUBTOTAL UTILITIES						
CATALYSTS & CHEMICALS						
CATALYST	mg					
CHEMICAL 1						
CHEMICAL 2						
CHEMICAL 3						
SUBTOTAL CATALYSTS & CHEMICALS						
PACKAGING MATERIALS						
PACKAGING LABOR						
BYPRODUCT CREDIT	MMBtu					
OTHER VARIABLE COSTS						
TOTAL VARIABLE COST					$549,228.000	per Component
					$16,477	k per Year

Figure A-2: Base case variable cost estimate printout from economics spreadsheet. *This page of the economics spreadsheet contains all inputs and calculated totals relevant to calculation of the variable cost for the entire end-to-end process.*

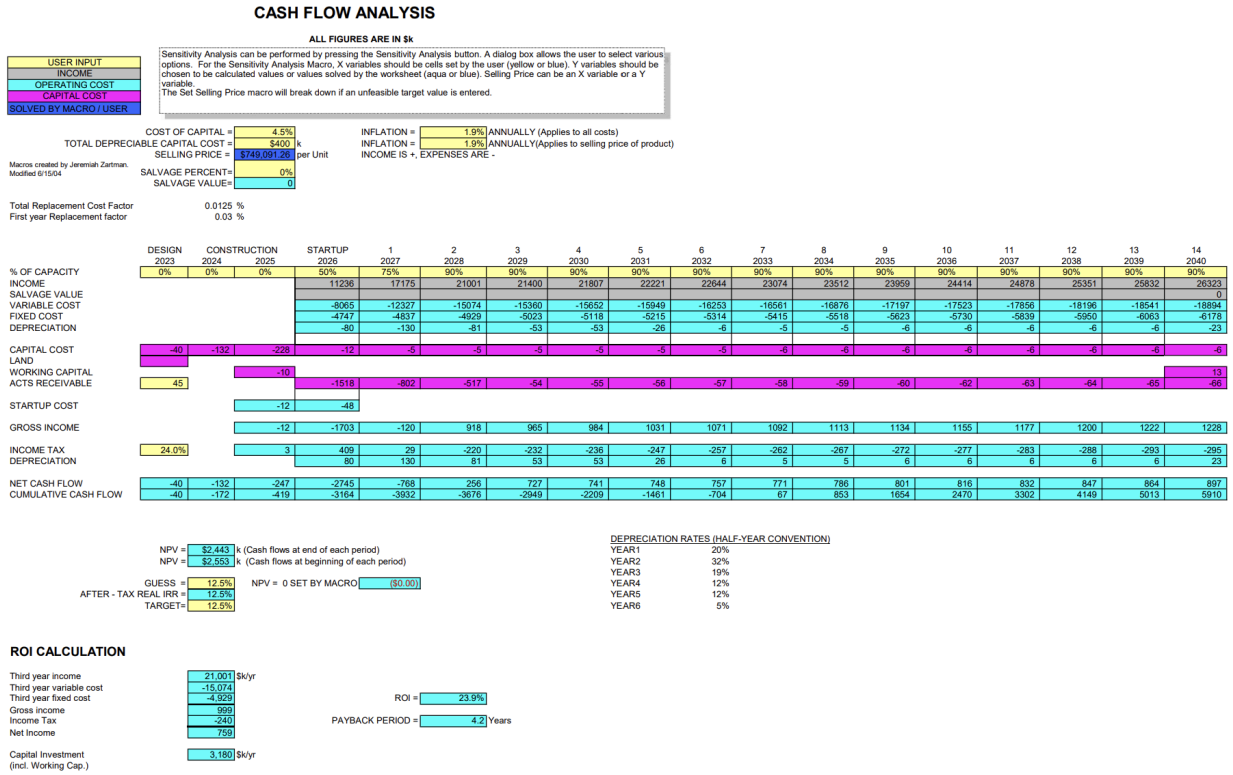


OPERATING COST ESTIMATE
FIXED COST

=USER INPUT | =CALC BY COMPUTER

PRODUCT:					
ANNUAL AnnCap:	30	nponent per Year			
TOTAL INVESTMENT (TPI):	$400	k			
OPERATING LABOR & BENEFITS:				ANNUAL COST ($k/yr)	
NO. of OPERATORS:	18				
NO. of SUPERVISORS	3				
ANNUAL WAGES	$83	k PER OPERATOR		1,498	
EMPL. BENEFITS @	40.0%	of WAGES		599	
OPERATING SUPERVISION @	138%	of WAGES		343	
SUBTOTAL OPERATING LABOR:					2,440
OPERATING SUPPLIES:		of WAGES			
MAINTENANCE:					
TOTAL MAINTENANCE @		OF INVESTMENT			
MAINTENANCE LABOR@		of TOTAL MAINT.			
MAINTENANCE MATERIAL @		of TOTAL MAINT.			
OVERHEAD:					
GEN. OH @	125.0%	of (OPR. WAGES + MAINT LABOR + OPR. SUPRV.)			2,301
LAB &TECHNICAL SUPPORT @		of INVESTMENT			
CORPORATE OVERHEAD:					
SALES & ADMINISTRATION		of INVESTMENT			
RESEARCH & DEVELOPMNT		of INVESTMENT			
SUBTOTAL CORPORATE OVERHEAD.....					
INSURANCE & LOCAL TAXES:					
1.5%	of INVESTMENT				6
ROYALTIES:		per annual Component of Capacity			
TOTAL FIXED COST (for cash flow calculations):				$	4,747 k per Year
					$158,228.00 per Component
DEPRECIATION:					
	of INVESTMENT				
Note: Do not include Depreciation if total Fixed Cost is to be used in Cash Flow Calc.					
TOTAL FIXED COST (for ROI calculations):				$	4,747 k per Year
					$158,228.00 per Com

Figure A-3: Base case fixed cost estimate printout from economics spreadsheet. *This page of the economics spreadsheet contains all inputs and calculated totals relevant to calculation of the fixed cost for the entire end-to-end process.*



CASH FLOW ANALYSIS

ALL FIGURES ARE IN $k

USER INPUT
INCOME
OPERATING COST
CAPITAL COST
SOLVED BY MACRO / USER

Sensitivity Analysis can be performed by pressing the Sensitivity Analysis button. A dialog box allows the user to select various options. For the Sensitivity Analysis Macro, X variables should be cells set by the user (yellow or blue). Y variables should be chosen to be calculated values or values solved by the worksheet (aqua or blue). Selling Price can be an X variable or a Y variable.
The Set Selling Price macro will break down if an unfeasible target value is entered.

COST OF CAPITAL = 4.5%
TOTAL DEPRECIABLE CAPITAL COST = $400 k
SELLING PRICE = $749,091.26 per Unit
SALVAGE PERCENT= 0%
SALVAGE VALUE= 0

INFLATION = 1.9% ANNUALLY (Applies to all costs)
INFLATION = 1.9% ANNUALLY(Applies to selling price of product)
INCOME IS +, EXPENSES ARE -

Macros created by Jeremiah Zartman. Modified 6/15/04

Total Replacement Cost Factor 0.0125 %
First year Replacement factor 0.03 %

	DESIGN 2023	CONSTRUCTION 2024	2025	STARTUP 2026	1 2027	2 2028	3 2029	4 2030	5 2031	6 2032	7 2033	8 2034	9 2035	10 2036	11 2037	12 2038	13 2039	14 2040
% OF CAPACITY	0%	0%	0%	50%	75%	90%	90%	90%	90%	90%	90%	90%	90%	90%	90%	90%	90%	90%
INCOME				11236	17175	21001	21400	21807	22221	22644	23074	23512	23959	24414	24878	25351	25832	26323
SALVAGE VALUE																		0
VARIABLE COST				-8065	-12327	-15074	-15360	-15652	-15949	-16253	-16561	-16876	-17197	-17523	-17856	-18196	-18541	-18894
FIXED COST				-4747	-4837	-4929	-5023	-5118	-5215	-5314	-5415	-5518	-5623	-5730	-5839	-5950	-6063	-6178
DEPRECIATION				-80	-130	-81	-53	-53	-26	-6	-5	-5	-6	-6	-6	-6	-6	-23
CAPITAL COST	-40	-132	-228	-12	-5	-5	-5	-5	-5	-5	-6	-6	-6	-6	-6	-6	-6	-6
LAND																		
WORKING CAPITAL			-10															13
ACTS RECEIVABLE	45			-1518	-802	-517	-54	-55	-56	-57	-58	-59	-60	-62	-63	-64	-65	-66
STARTUP COST			-12	-48														
GROSS INCOME			-12	-1703	-120	918	965	984	1031	1071	1092	1113	1134	1155	1177	1200	1222	1228
INCOME TAX	24.0%		3	409	29	-220	-232	-236	-247	-257	-262	-267	-272	-277	-283	-288	-293	-295
DEPRECIATION				80	130	81	53	53	26	6	5	5	6	6	6	6	6	23
NET CASH FLOW	-40	-132	-247	-2745	-768	256	727	741	748	757	771	786	801	816	832	847	864	897
CUMULATIVE CASH FLOW	-40	-172	-419	-3164	-3932	-3676	-2949	-2209	-1461	-704	67	853	1654	2470	3302	4149	5013	5910

NPV = $2,443 k (Cash flows at end of each period)
NPV = $2,553 k (Cash flows at beginning of each period)

GUESS = 12.5%
AFTER - TAX REAL IRR = 12.5%
TARGET= 12.5%
NPV = 0 SET BY MACRO ($0.00)

DEPRECIATION RATES (HALF-YEAR CONVENTION)

YEAR1	20%
YEAR2	32%
YEAR3	19%
YEAR4	12%
YEAR5	12%
YEAR6	5%

ROI CALCULATION

Third year income	21,001 $k/yr
Third year variable cost	-15,074
Third year fixed cost	-4,929
Gross income	999
Income Tax	-240
Net Income	759
Capital Investment (incl. Working Cap.)	3,180 $k/yr

ROI = 23.9%
PAYBACK PERIOD = 4.2 Years

Figure A-4: Base case resultant cash flow analysis from economics spreadsheet. *This page of the economics spreadsheet contains all final inputs and calculated totals relevant to NPV, ROI, PBP, sale price of the CAR-T therapy, and year-by-year cash flows for the base case 12.5% IRR. These calculations build on inputs entered in Figures ? through ? above.*

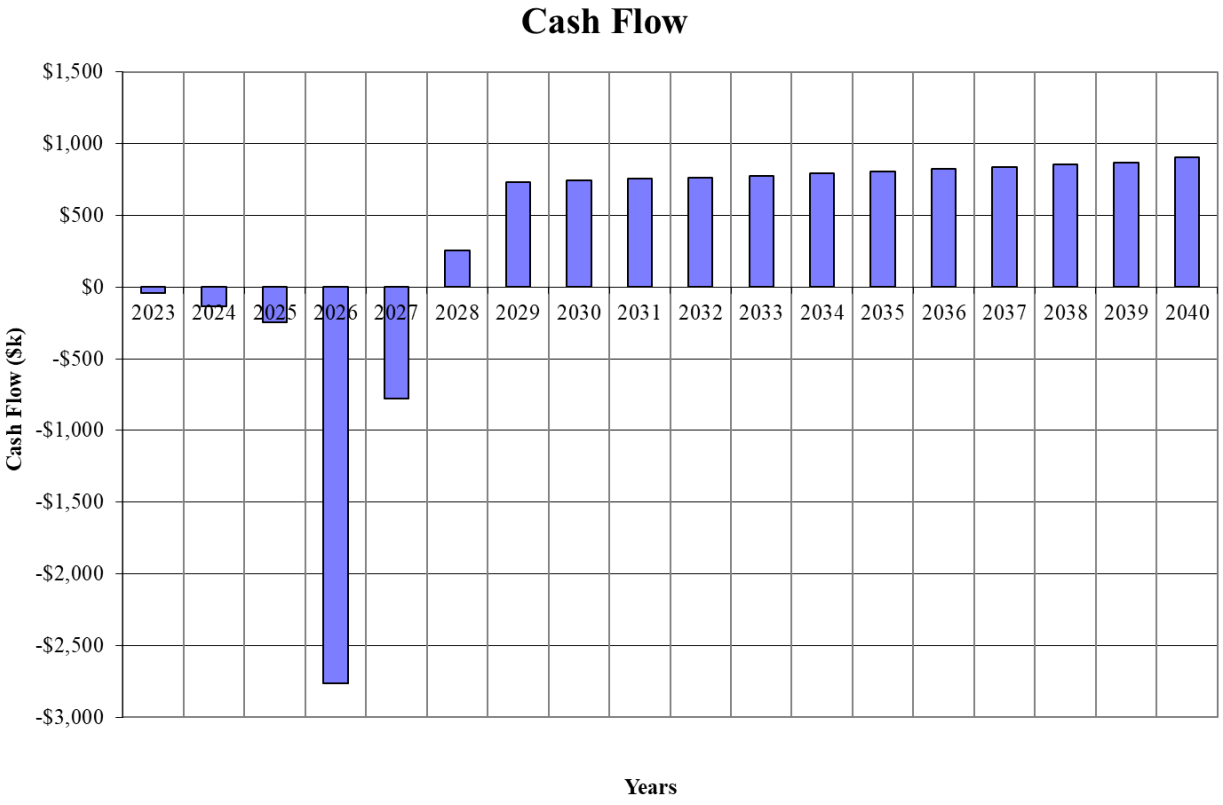


Figure A-5: Year-by-year net cash flow chart for base case analysis from economics spreadsheet. *This chart displays year-by-year net cash flows as calculated in the spreadsheet page displayed in Figure ? above.*

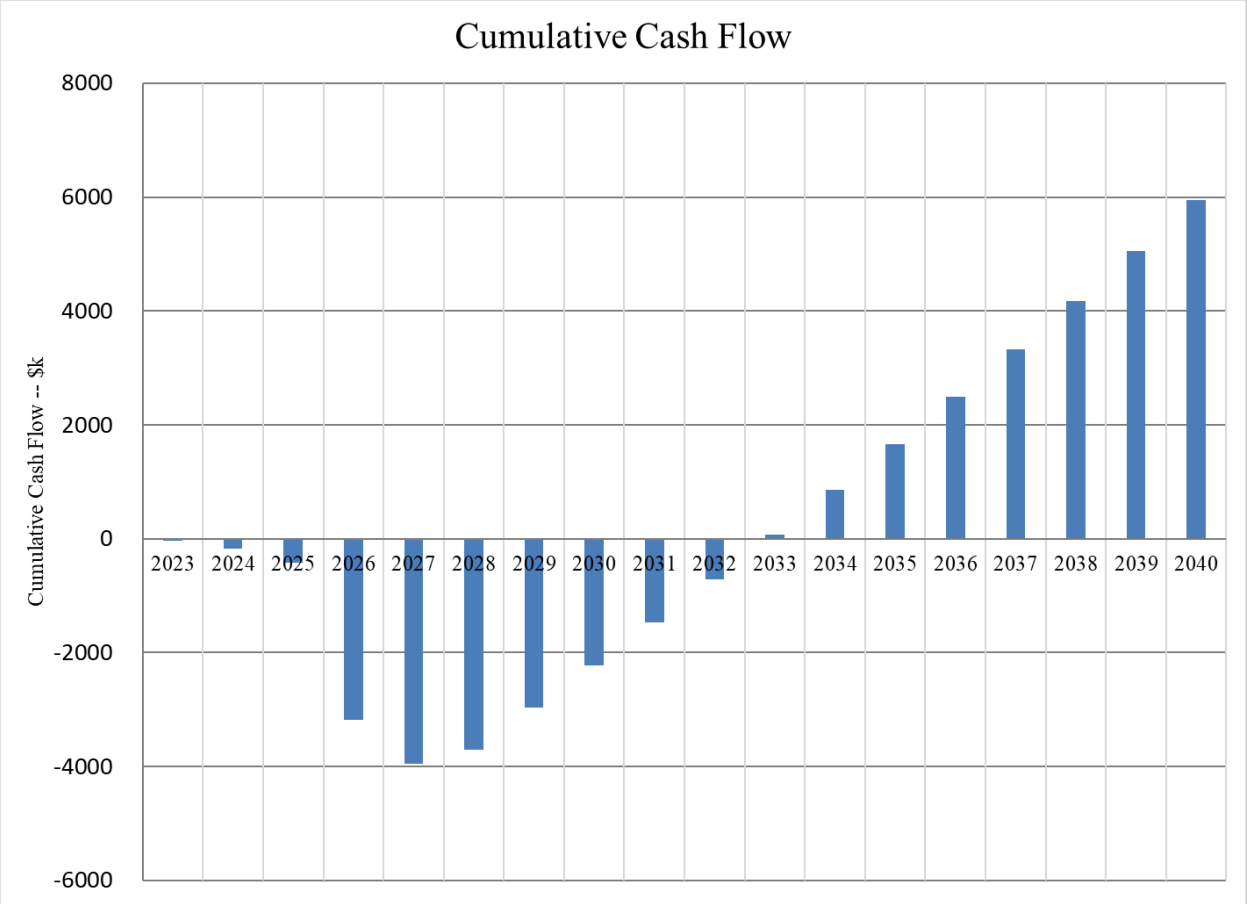


Figure A-6: Year-by-year cumulative cash flow chart for base case analysis from economics spreadsheet. *This chart displays year-by-year cumulative cash flows as calculated in the spreadsheet page displayed in Figure ? above.*

CASH FLOW ANALYSIS

ALL FIGURES ARE IN $k

USER INPUT
INCOME
OPERATING COST
CAPITAL COST
SOLVED BY MACRO / USER

Sensitivity Analysis can be performed by pressing the Sensitivity Analysis button. A dialog box allows the user to select various options. For the Sensitivity Analysis Macro, X variables should be cells set by the user (yellow or blue). Y variables should be chosen to be calculated values or values solved by the worksheet (aqua or blue). Selling Price can be an X variable or a Y variable.
The Set Selling Price macro will break down if an unfeasible target value is entered.

Macros created by Jeremiah Zartman.
Modified 6/15/04

COST OF CAPITAL =	4.5%		
TOTAL DEPRECIABLE CAPITAL COST =	$400	k	
SELLING PRICE =	$753,230.13	per Unit	
SALVAGE PERCENT=	0%		
SALVAGE VALUE=	0		

INFLATION =	1.9%	ANNUALLY (Applies to all costs)
INFLATION =	1.9%	ANNUALLY(Applies to selling price of product)
INCOME IS +, EXPENSES ARE -		

Total Replacement Cost Factor	0.0125 %
First year Replacement factor	0.03 %

	DESIGN	CONSTRUCTION		STARTUP	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	2023	2024	2025	2026	2027	2028	2029	2030	2031	2032	2033	2034	2035	2036	2037	2038	2039	2040
% OF CAPACITY	0%	0%	0%	50%	75%	90%	90%	90%	90%	90%	90%	90%	90%	90%	90%	90%	90%	90%
INCOME				11298	17270	21117	21519	21927	22344	22769	23201	23642	24091	24549	25015	25491	25975	26469
SALVAGE VALUE																		0
VARIABLE COST				-8065	-12327	-15074	-15360	-15652	-15949	-16253	-16561	-16876	-17197	-17523	-17856	-18196	-18541	-18894
FIXED COST				-4747	-4837	-4929	-5023	-5118	-5215	-5314	-5415	-5518	-5623	-5730	-5839	-5950	-6063	-6178
DEPRECIATION				-80	-130	-81	-53	-53	-26	-6	-5	-5	-6	-6	-6	-6	-6	-23
CAPITAL COST	-40	-132	-228	-12	-5	-5	-5	-5	-5	-5	-6	-6	-6	-6	-6	-6	-6	-6
LAND																		
WORKING CAPITAL			-10															13
ACTS RECEIVABLE	45			-1527	-807	-520	-54	-55	-56	-57	-58	-60	-61	-62	-63	-64	-65	-67
STARTUP COST			-12	-48														
GROSS INCOME			-12	-1641	-25	1034	1083	1104	1154	1196	1219	1242	1266	1290	1315	1340	1365	1374
INCOME TAX	24.0%		3	394	6	-248	-260	-265	-277	-287	-293	-298	-304	-310	-316	-321	-328	-330
DEPRECIATION				80	130	81	53	53	26	6	5	5	6	6	6	6	6	23
NET CASH FLOW	-40	-132	-247	-2706	-701	341	816	832	841	852	868	884	901	918	936	954	972	1008
CUMULATIVE CASH FLOW	-40	-172	-419	-3125	-3826	-3485	-2668	-1836	-996	-144	724	1609	2510	3428	4364	5317	6289	7297

NPV =	$3,286	k	(Cash flows at end of each period)
NPV =	$3,434	k	(Cash flows at beginning of each period)
GUESS =	12.5%		
AFTER - TAX REAL IRR =	15.0%		
TARGET=	15.0%		

NPV = 0 SET BY MACRO ($0.00)

DEPRECIATION RATES (HALF-YEAR CONVENTION)

YEAR1	20%
YEAR2	32%
YEAR3	19%
YEAR4	12%
YEAR5	12%
YEAR6	5%

ROI CALCULATION

Third year income	21,117	$k/yr
Third year variable cost	-15,074	
Third year fixed cost	-4,929	
Gross income	1,115	
Income Tax	-267	
Net Income	847	
Capital Investment (incl. Working Cap.)	3,196	$k/yr

ROI = 26.5%

PAYBACK PERIOD = 3.8 Years

Figure A-7: Resultant cash flow analysis from economics spreadsheet for 15% IRR. *This page of the economics spreadsheet contains all final inputs and calculated totals relevant to NPV, ROI, PBP, sale price of the CAR-T therapy, and year-by-year cash flows for a 15% IRR. These calculations build on inputs entered in Figures ? through ? above.*

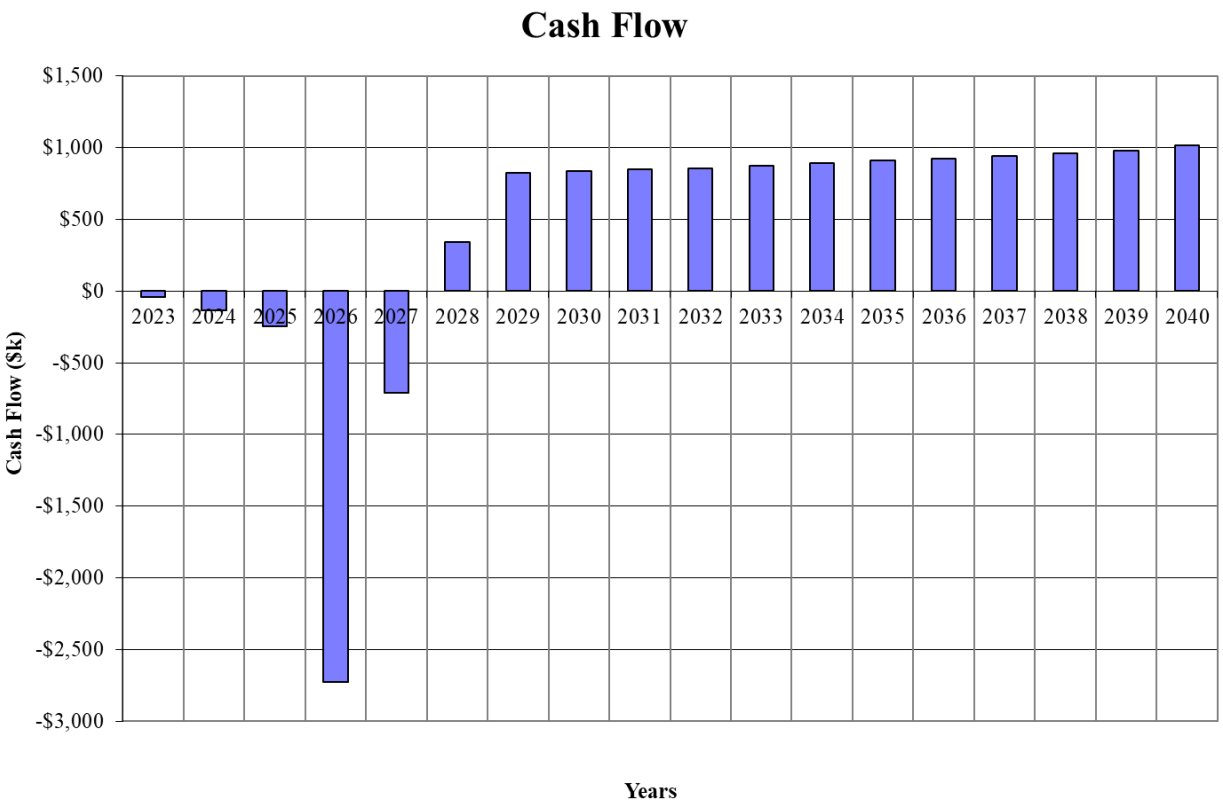


Figure A-8: Year-by-year net cash flow chart for base case analysis from economics spreadsheet. *This chart displays year-by-year net cash flows as calculated in the spreadsheet page displayed in Figure ? above.*

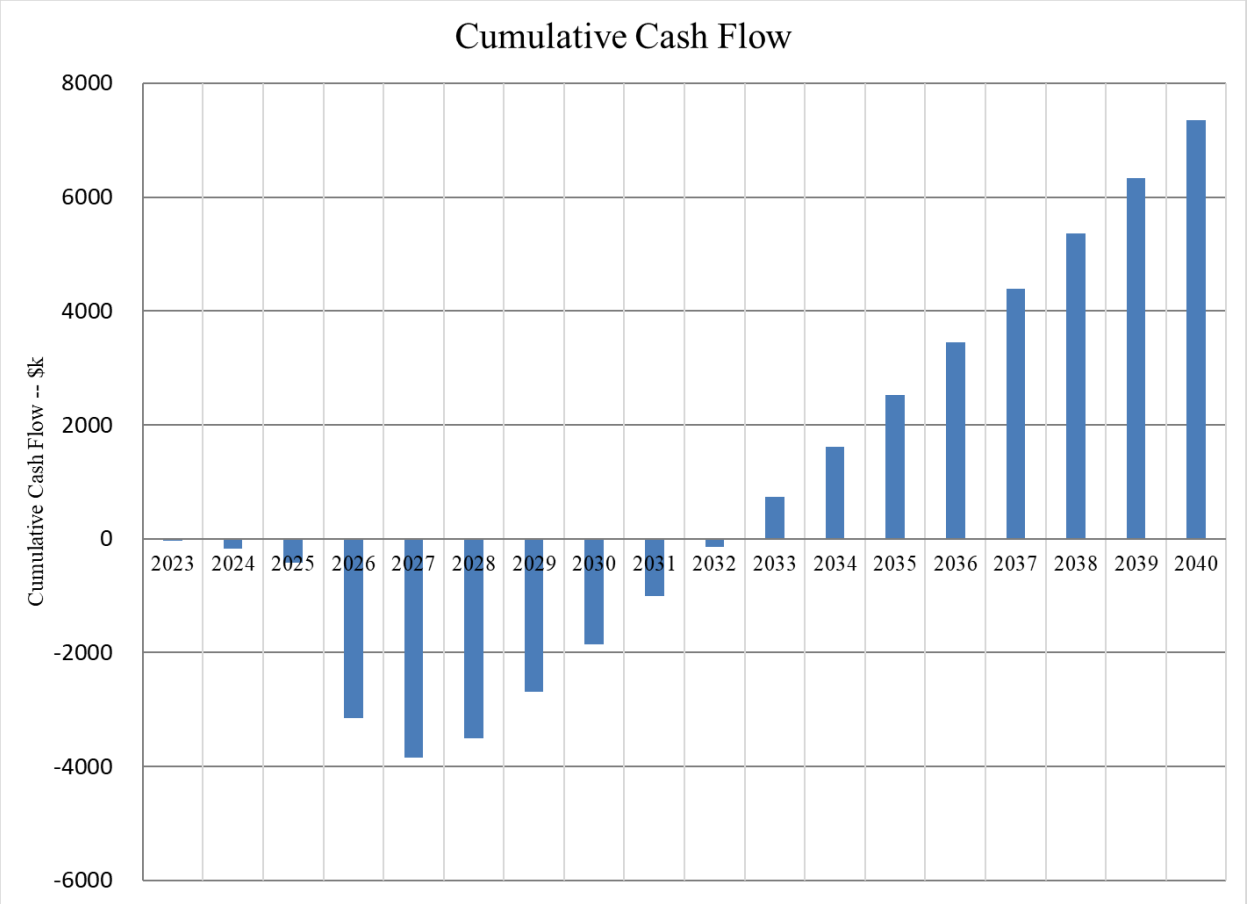


Figure A-9: Year-by-year cumulative cash flow chart for base case analysis from economics spreadsheet. *This chart displays year-by-year cumulative cash flows as calculated in the spreadsheet page displayed in Figure ? above.*

CASH FLOW ANALYSIS

ALL FIGURES ARE IN $k

USER INPUT
INCOME
OPERATING COST
CAPITAL COST
SOLVED BY MACRO / USER

Sensitivity Analysis can be performed by pressing the Sensitivity Analysis button. A dialog box allows the user to select various options. For the Sensitivity Analysis Macro, X variables should be cells set by the user (yellow or blue). Y variables should be chosen to be calculated values or values solved by the worksheet (aqua or blue). Selling Price can be an X variable or a Y variable.
The Set Selling Price macro will break down if an unfeasible target value is entered.

COST OF CAPITAL = 4.5%
TOTAL DEPRECIABLE CAPITAL COST = $400 k
SELLING PRICE = $761,869.98 per Unit
SALVAGE PERCENT= 0%
SALVAGE VALUE= 0

INFLATION = 1.9% ANNUALLY (Applies to all costs)
INFLATION = 1.9% ANNUALLY(Applies to selling price of product)
INCOME IS +, EXPENSES ARE -

Macros created by Jeremiah Zartman.
Modified 6/15/04

Total Replacement Cost Factor 0.0125 %
First year Replacement factor 0.03 %

	DESIGN 2023	CONSTRUCTION 2024	CONSTRUCTION 2025	STARTUP 2026	1 2027	2 2028	3 2029	4 2030	5 2031	6 2032	7 2033	8 2034	9 2035	10 2036	11 2037	12 2038	13 2039	14 2040
% OF CAPACITY	0%	0%	0%	50%	75%	90%	90%	90%	90%	90%	90%	90%	90%	90%	90%	90%	90%	90%
INCOME				11428	17468	21360	21765	22179	22600	23030	23467	23913	24368	24831	25302	25783	26273	26772
SALVAGE VALUE																		0
VARIABLE COST				-8065	-12327	-15074	-15360	-15652	-15949	-16253	-16561	-16876	-17197	-17523	-17856	-18196	-18541	-18894
FIXED COST				-4747	-4837	-4929	-5023	-5118	-5215	-5314	-5415	-5518	-5623	-5730	-5839	-5950	-6063	-6178
DEPRECIATION				-80	-130	-81	-53	-53	-26	-6	-5	-5	-6	-6	-6	-6	-6	-23
CAPITAL COST	-40	-132	-228	-12	-5	-5	-5	-5	-5	-5	-6	-6	-6	-6	-6	-6	-6	-6
LAND																		
WORKING CAPITAL			-10															13
ACTS RECEIVABLE	45			-1544	-816	-526	-55	-56	-57	-58	-59	-60	-61	-63	-64	-65	-66	-67
STARTUP COST			-12	-48														
GROSS INCOME			-12	-1512	173	1276	1330	1356	1410	1457	1485	1514	1542	1572	1602	1632	1663	1677
INCOME TAX	24.0%		3	363	-42	-306	-319	-325	-338	-350	-356	-363	-370	-377	-384	-392	-399	-403
DEPRECIATION				80	130	81	53	53	26	6	5	5	6	6	6	6	6	23
NET CASH FLOW	-40	-132	-247	-2625	-559	520	1003	1022	1035	1050	1069	1090	1111	1132	1153	1175	1197	1238
CUMULATIVE CASH FLOW	-40	-172	-419	-3044	-3604	-3084	-2081	-1058	-23	1026	2096	3185	4296	5427	6581	7756	8953	10190

NPV = $5,045 k (Cash flows at end of each period)
NPV = $5,272 k (Cash flows at beginning of each period)

GUESS = 12.5%
AFTER - TAX REAL IRR = 20.0%
TARGET= 20.0%
NPV = 0 SET BY MACRO ($0.00)

DEPRECIATION RATES (HALF-YEAR CONVENTION)

YEAR1	20%
YEAR2	32%
YEAR3	19%
YEAR4	12%
YEAR5	12%
YEAR6	5%

ROI CALCULATION

Third year income	21,360	$k/yr
Third year variable cost	-15,074	
Third year fixed cost	-4,929	
Gross income	1,357	
Income Tax	-326	
Net Income	1,031	
Capital Investment (incl. Working Cap.)	3,229	$k/yr

ROI = 31.9%

PAYBACK PERIOD = 3.1 Years

Figure A-10: Resultant cash flow analysis from economics spreadsheet for 20% IRR. *This page of the economics spreadsheet contains all final inputs and calculated totals relevant to NPV, ROI, PBP, sale price of the CAR-T therapy, and year-by-year cash flows for the base case 20% IRR. These calculations build on inputs entered in Figures ? through ? above.*

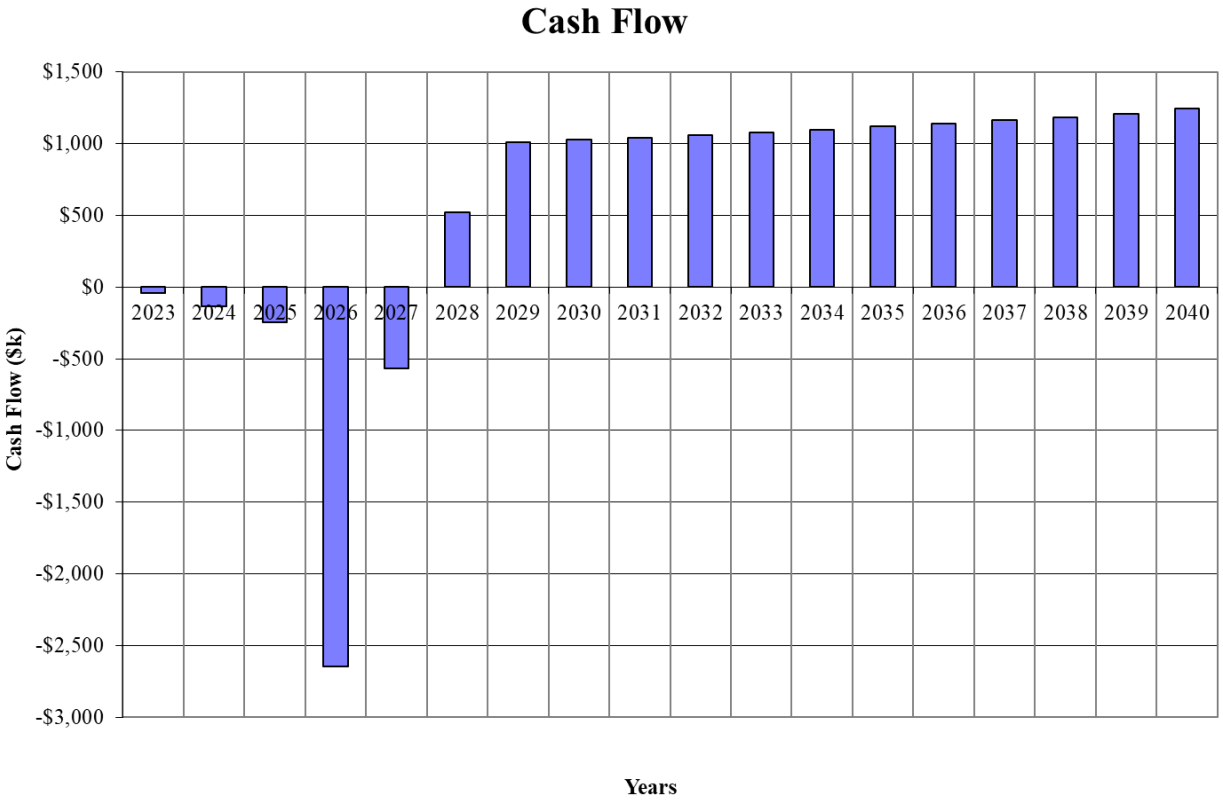


Figure A-11: Year-by-year net cash flow chart for base case analysis from economics spreadsheet. *This chart displays year-by-year net cash flows as calculated in the spreadsheet page displayed in Figure ? above.*

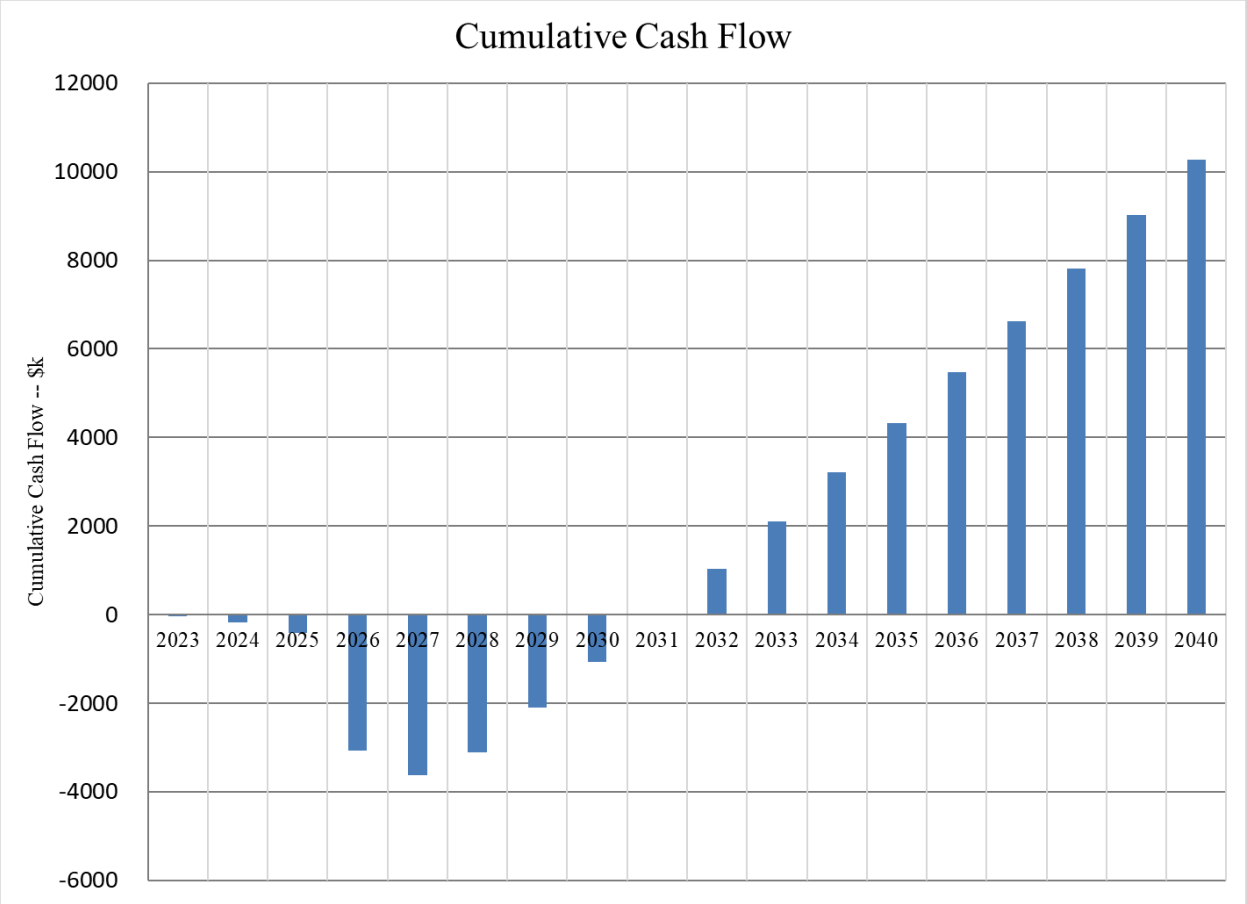


Figure A-12: Year-by-year cumulative cash flow chart for base case analysis from economics spreadsheet. *This chart displays year-by-year cumulative cash flows as calculated in the spreadsheet page displayed in Figure ? above.*

CASH FLOW ANALYSIS

ALL FIGURES ARE IN $k

USER INPUT
INCOME
OPERATING COST
CAPITAL COST
SOLVED BY MACRO / USER

Sensitivity Analysis can be performed by pressing the Sensitivity Analysis button. A dialog box allows the user to select various options. For the Sensitivity Analysis Macro, X variables should be cells set by the user (yellow or blue). Y variables should be chosen to be calculated values or values solved by the worksheet (aqua or blue). Selling Price can be an X variable or a Y variable.
The Set Selling Price macro will break down if an unfeasible target value is entered.

COST OF CAPITAL = 4.5%
TOTAL DEPRECIABLE CAPITAL COST = $400 k
SELLING PRICE = $770,814.82 per Unit
SALVAGE PERCENT= 0%
SALVAGE VALUE= 0

INFLATION = 1.9% ANNUALLY (Applies to all costs)
INFLATION = 1.9% ANNUALLY(Applies to selling price of product)
INCOME IS +, EXPENSES ARE -

Macros created by Jeremiah Zartman.
Modified 6/15/04

Total Replacement Cost Factor 0.0125 %
First year Replacement factor 0.03 %

	DESIGN 2023	CONSTRUCTION 2024	CONSTRUCTION 2025	STARTUP 2026	1 2027	2 2028	3 2029	4 2030	5 2031	6 2032	7 2033	8 2034	9 2035	10 2036	11 2037	12 2038	13 2039	14 2040
% OF CAPACITY	0%	0%	0%	50%	75%	90%	90%	90%	90%	90%	90%	90%	90%	90%	90%	90%	90%	90%
INCOME				11562	17673	21610	22021	22439	22866	23300	23743	24194	24654	25122	25599	26086	26581	27086
SALVAGE VALUE																		0
VARIABLE COST				-8065	-12327	-15074	-15360	-15652	-15949	-16253	-16561	-16876	-17197	-17523	-17856	-18196	-18541	-18894
FIXED COST				-4747	-4837	-4929	-5023	-5118	-5215	-5314	-5415	-5518	-5623	-5730	-5839	-5950	-6063	-6178
DEPRECIATION				-80	-130	-81	-53	-53	-26	-6	-5	-5	-6	-6	-6	-6	-6	-23
CAPITAL COST	-40	-132	-228	-12	-5	-5	-5	-5	-5	-5	-6	-6	-6	-6	-6	-6	-6	-6
LAND																		
WORKING CAPITAL			-10															13
ACTS RECEIVABLE	45			-1562	-826	-532	-55	-57	-58	-59	-60	-61	-62	-63	-65	-66	-67	-68
STARTUP COST			-12	-48														
GROSS INCOME			-12	-1378	378	1527	1585	1616	1675	1728	1761	1794	1828	1863	1899	1935	1971	1991
INCOME TAX	24.0%		3	331	-91	-366	-380	-388	-402	-415	-423	-431	-439	-447	-456	-464	-473	-478
DEPRECIATION				80	130	81	53	53	26	6	5	5	6	6	6	6	6	23
NET CASH FLOW	-40	-132	-247	-2541	-413	704	1197	1220	1236	1254	1278	1302	1327	1352	1378	1404	1431	1476
CUMULATIVE CASH FLOW	-40	-172	-419	-2961	-3374	-2670	-1473	-253	983	2237	3515	4818	6145	7498	8876	10280	11711	13187

NPV = $6,865 k (Cash flows at end of each period)
NPV = $7,174 k (Cash flows at beginning of each period)

GUESS = 12.5%
AFTER - TAX REAL IRR = 25.0%
TARGET= 25.0%
NPV = 0 SET BY MACRO ($0.00)

DEPRECIATION RATES (HALF-YEAR CONVENTION)

YEAR1	20%
YEAR2	32%
YEAR3	19%
YEAR4	12%
YEAR5	12%
YEAR6	5%

ROI CALCULATION

Third year income	21,610	$k/yr
Third year variable cost	-15,074	
Third year fixed cost	-4,929	
Gross income	1,608	
Income Tax	-386	
Net Income	1,222	
Capital Investment (incl. Working Cap.)	3,262	$k/yr

ROI = 37.4%
PAYBACK PERIOD = 2.7 Years

Figure A-13: Resultant cash flow analysis from economics spreadsheet for 25% IRR. *This page of the economics spreadsheet contains all final inputs and calculated totals relevant to NPV, ROI, PBP, sale price of the CAR-T therapy, and year-by-year cash flows for the base case 25% IRR. These calculations build on inputs entered in Figures ? through ? above.*

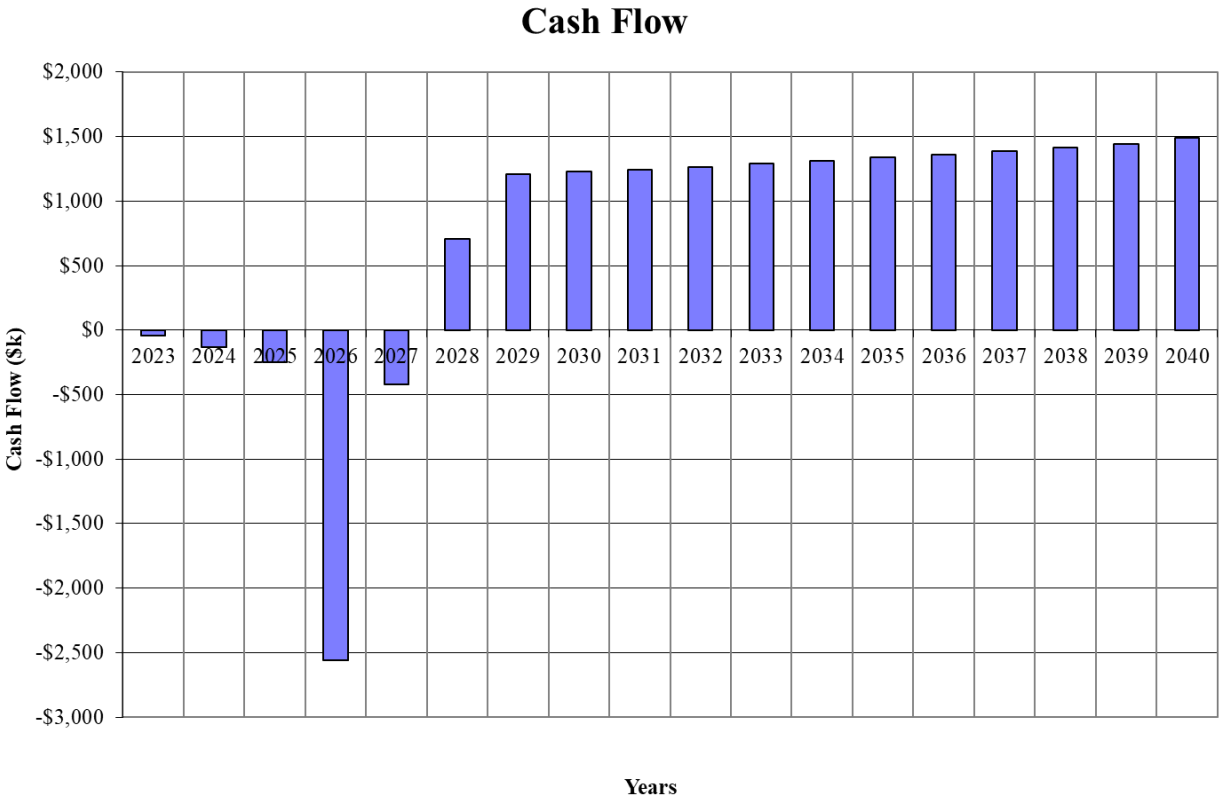


Figure A-14: Year-by-year net cash flow chart for base case analysis from economics spreadsheet. *This chart displays year-by-year net cash flows as calculated in the spreadsheet page displayed in Figure ? above.*

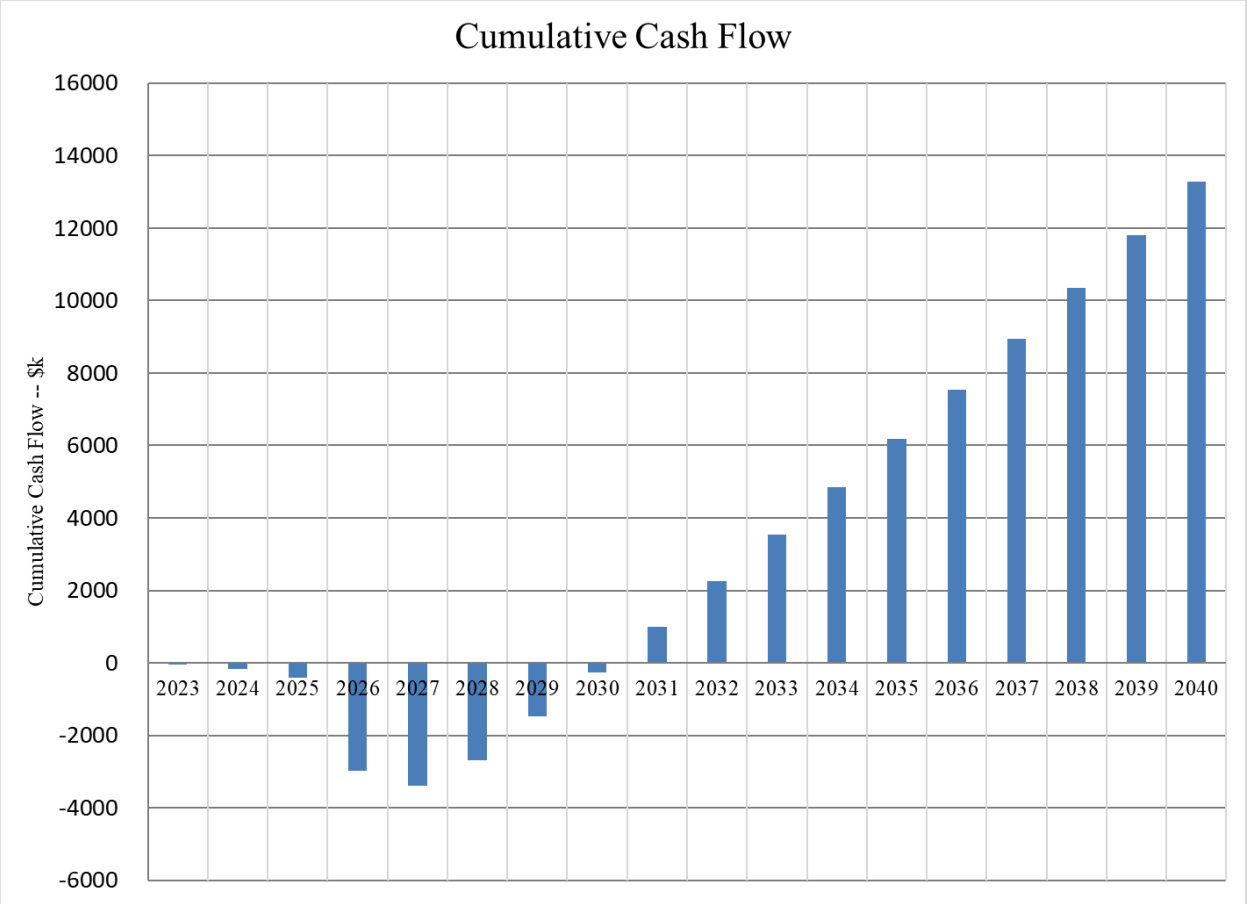


Figure A-15: Year-by-year cumulative cash flow chart for base case analysis from economics spreadsheet. *This chart displays year-by-year cumulative cash flows as calculated in the spreadsheet page displayed in Figure ? above.*

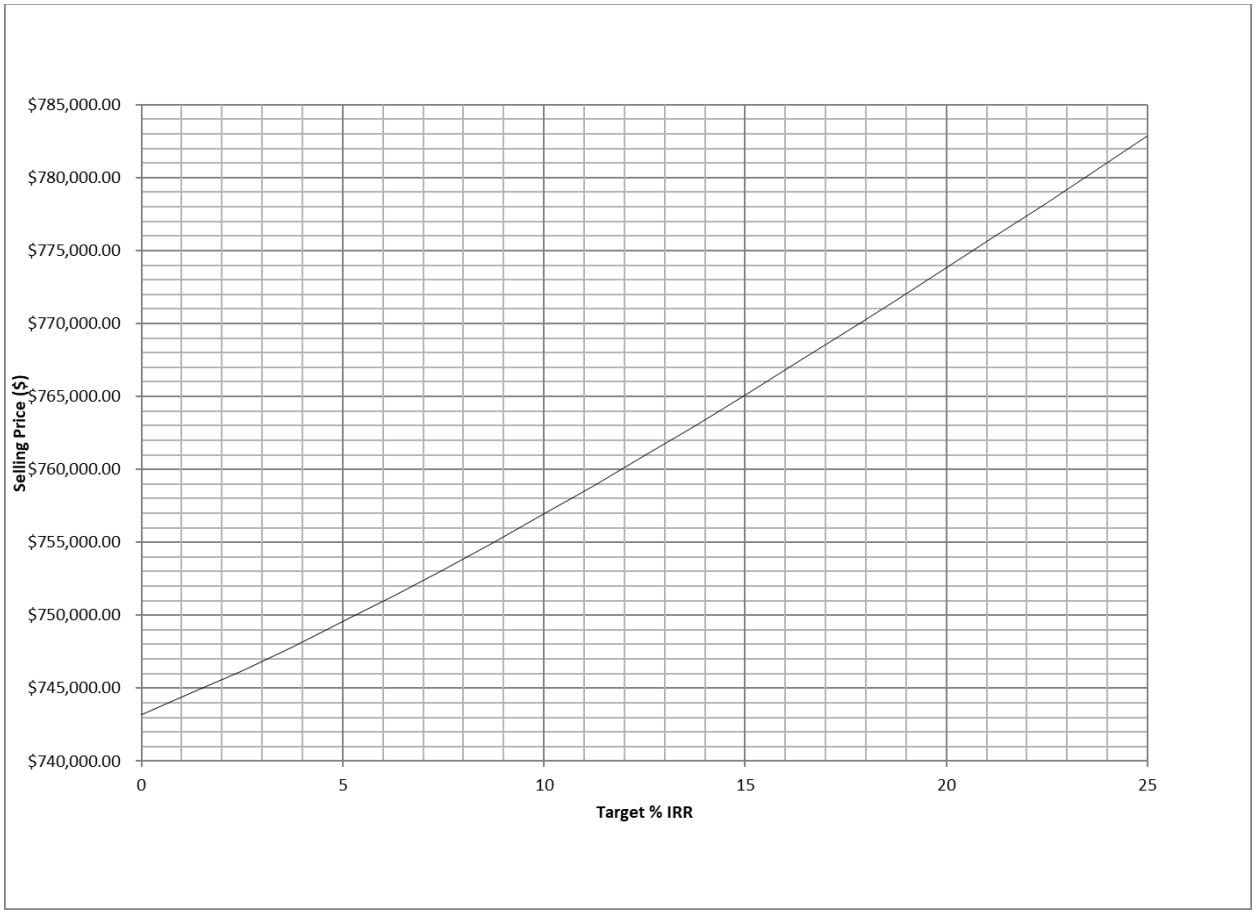


Figure A-16: Sensitivity analysis on selling price of the CAR-T therapy versus the target % IRR. *Using the economics spreadsheet, a sensitivity analysis was performed to determine the effects of a ±100% change in the base case 12.5% target IRR on the required sale price of the CAR-T therapy to meet that respective % IRR.*

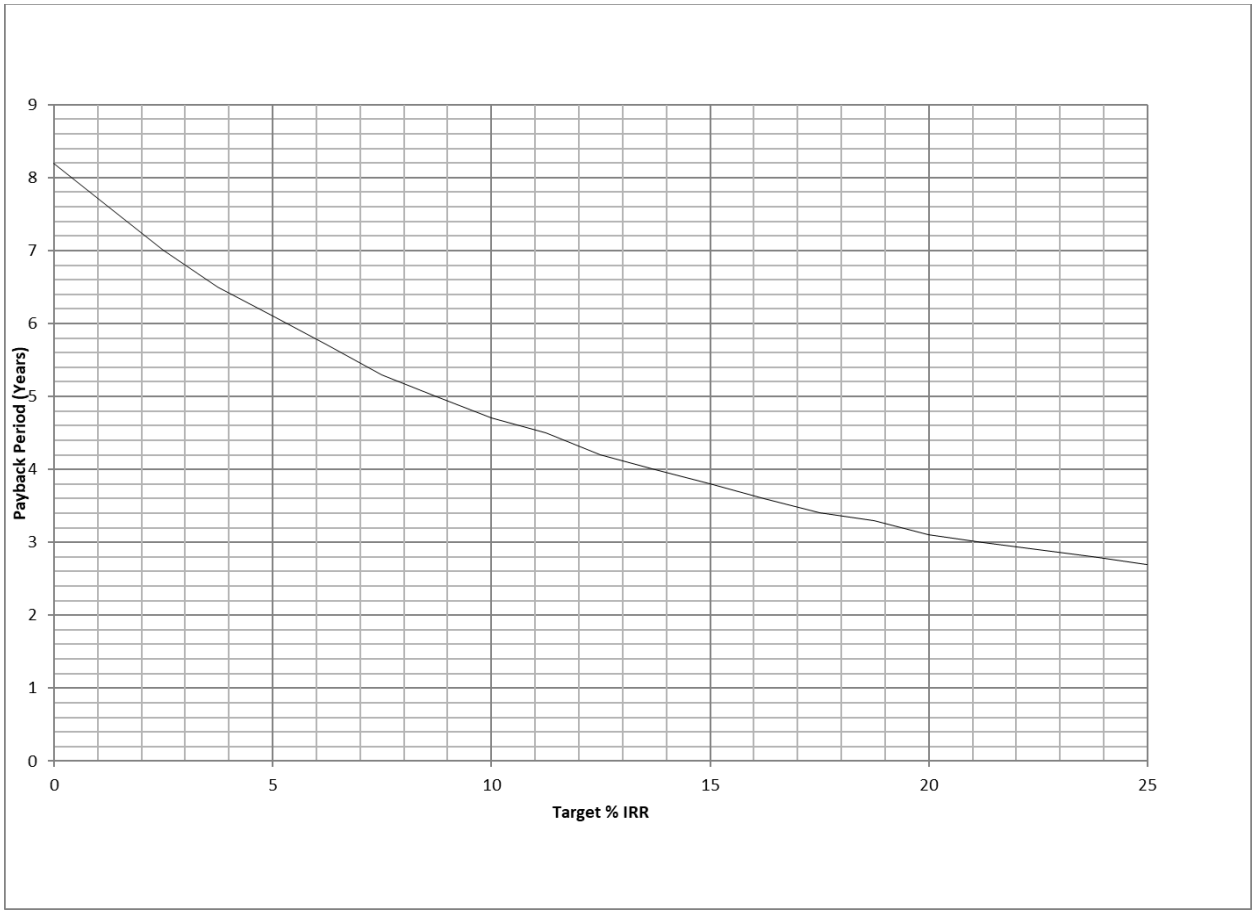


Figure A-17: Sensitivity analysis on payback period versus the target % IRR. *Using the economics spreadsheet, a sensitivity analysis was performed to determine the effects of a ±100% change in the base case 12.5% target IRR on the payback period.*

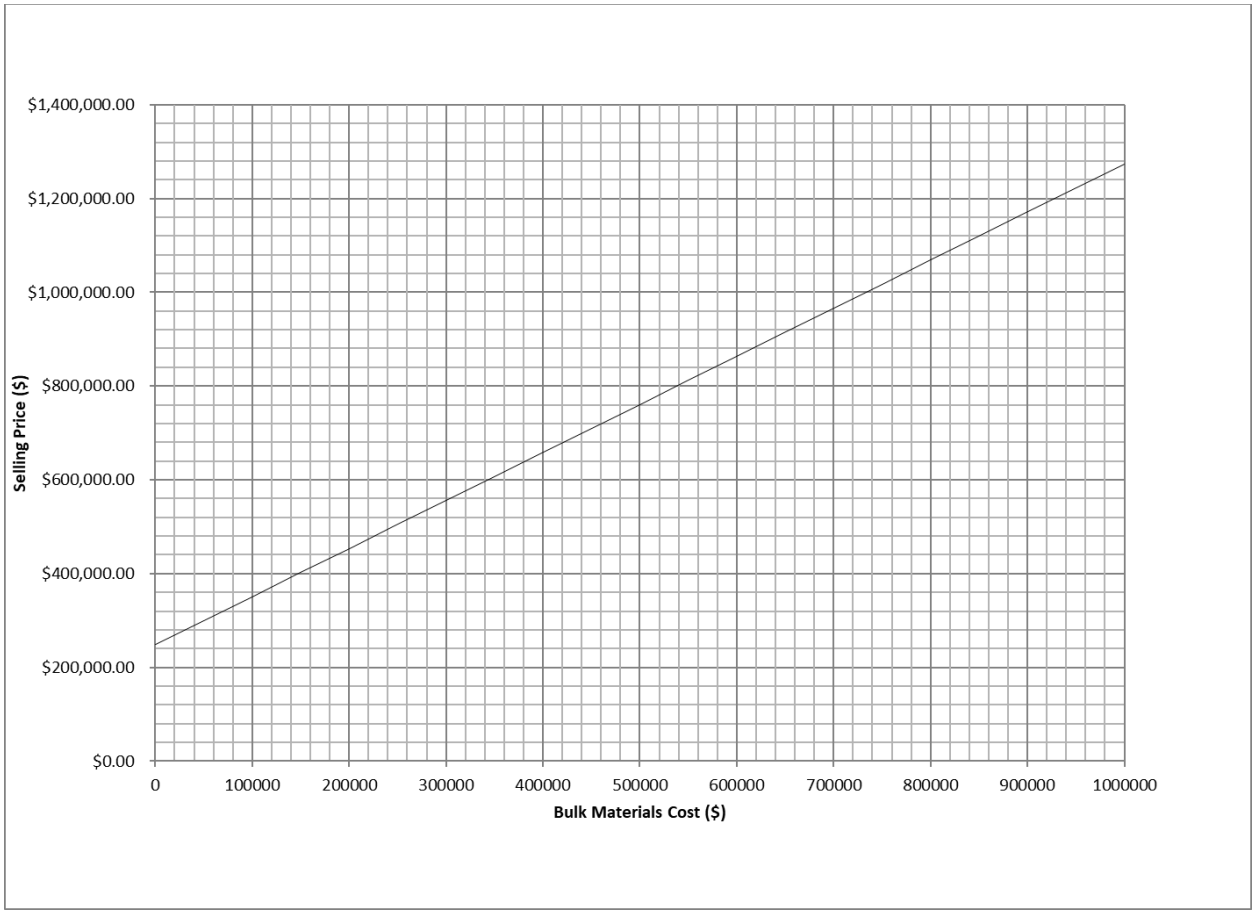


Figure A-18: Sensitivity analysis on selling price of the CAR-T therapy versus bulk material cost. *Using the economics spreadsheet, a sensitivity analysis was performed to determine the effects of a ±100% change in the assumed $500k bulk material cost on the required sale price of the CAR-T therapy to meet a 12.5% IRR.*

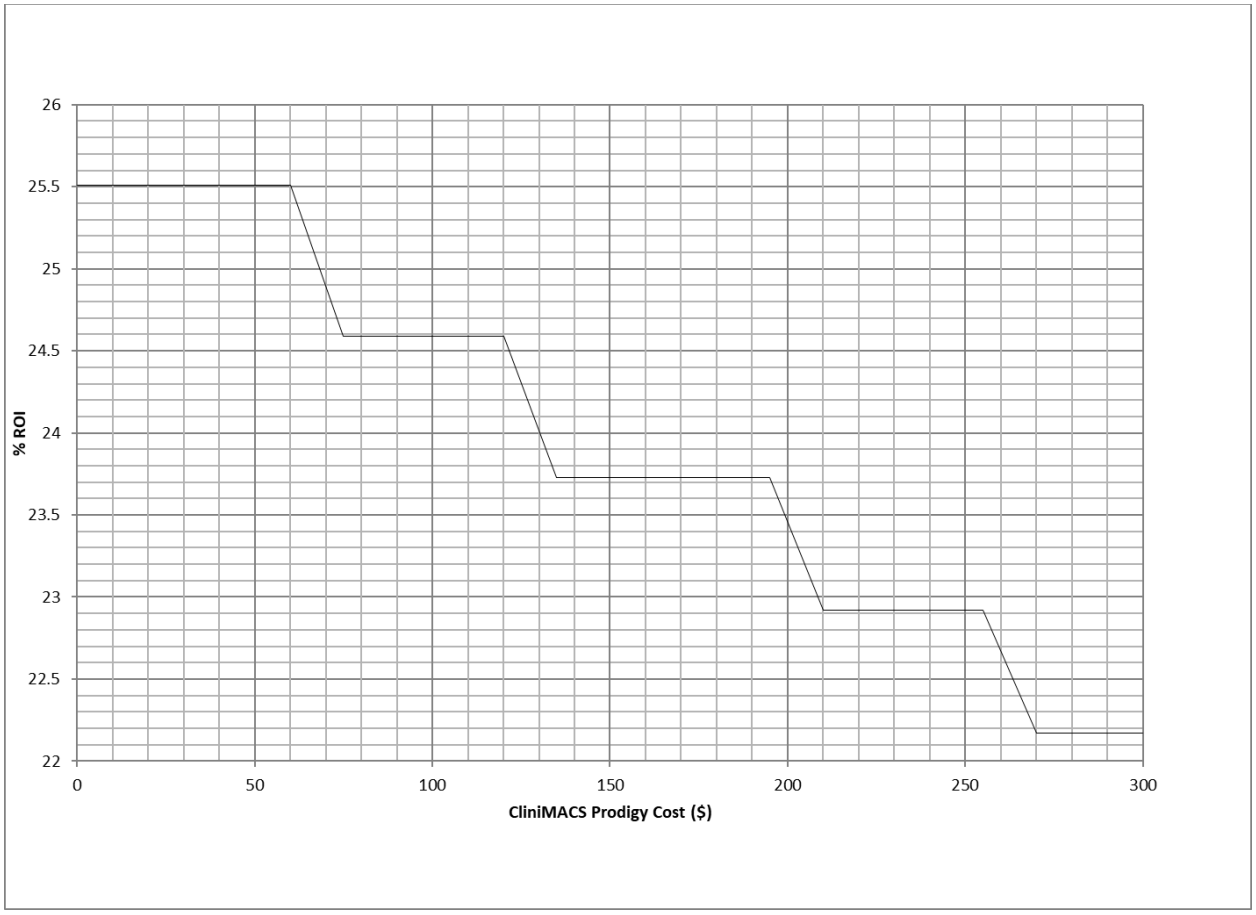


Figure A-19: Sensitivity analysis on the % ROI versus the CliniMACS Prodigy Cost. *Using the economics spreadsheet, a sensitivity analysis was performed to determine the effects of a ±100% change in the cost of the CliniMACS prodigy unit on the % ROI achieved with a base case 12.5% IRR.*

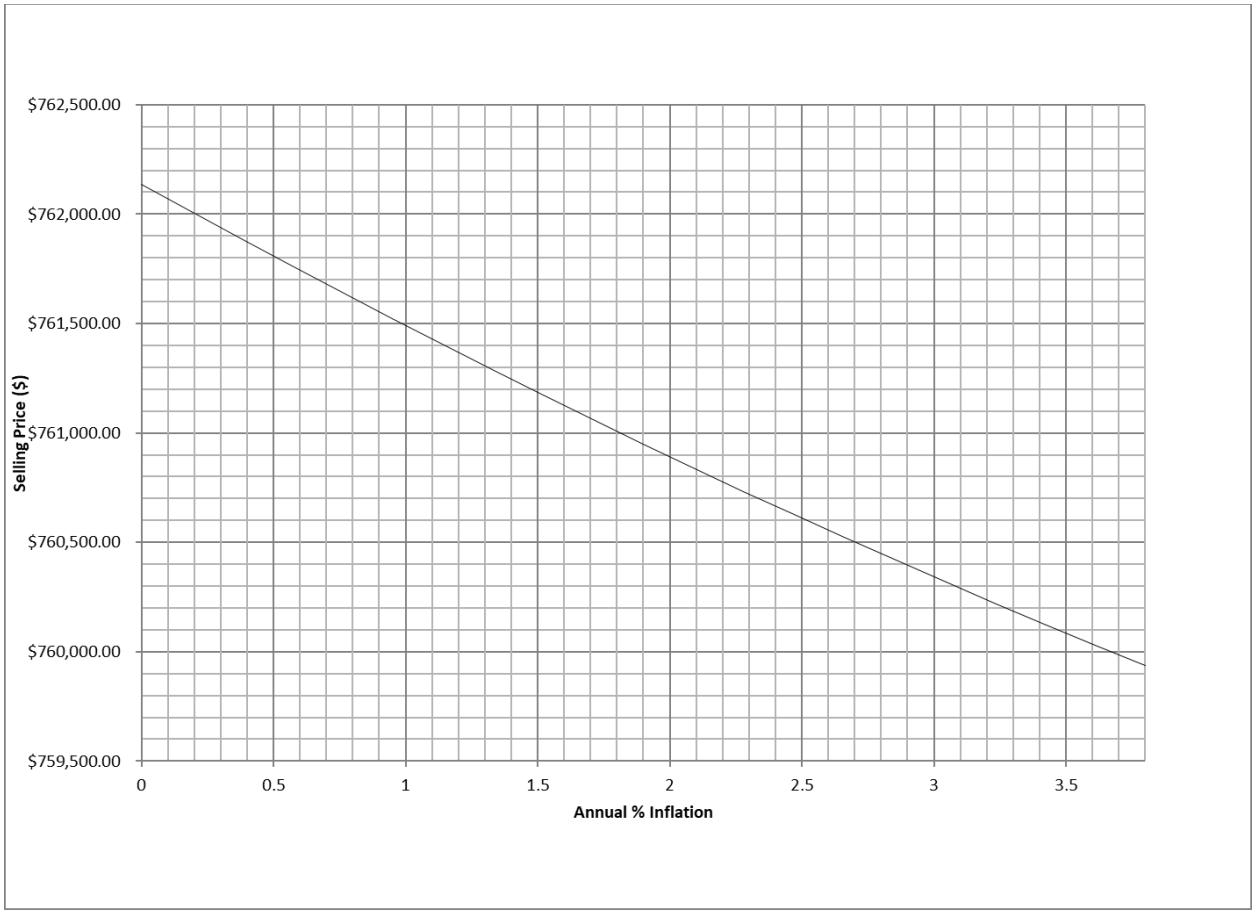


Figure A-20: Sensitivity analysis on selling price of the CAR-T therapy versus annual % inflation. *Using the economics spreadsheet, a sensitivity analysis was performed to determine the effects of a ±100% change in the annual % inflation on the required sale price of the CAR-T therapy using a base case 12.5% IRR.*

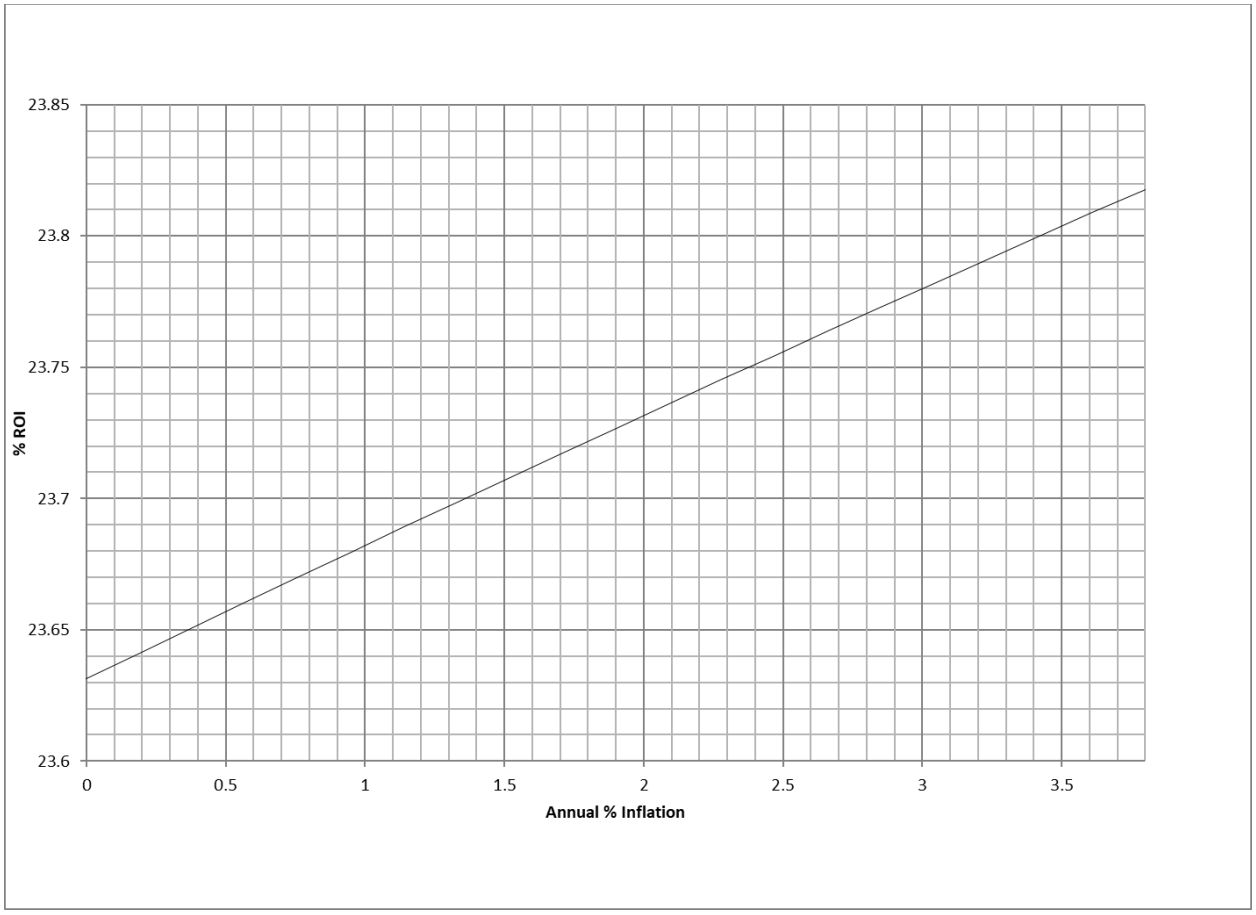


Figure A-21: Sensitivity analysis on the % ROI versus annual % inflation. *Using the economics spreadsheet, a sensitivity analysis was performed to determine the effects of a ±100% change in the annual % inflation on the % ROI for a base case 12.5% IRR.*

Appendix B: Gantt Chart

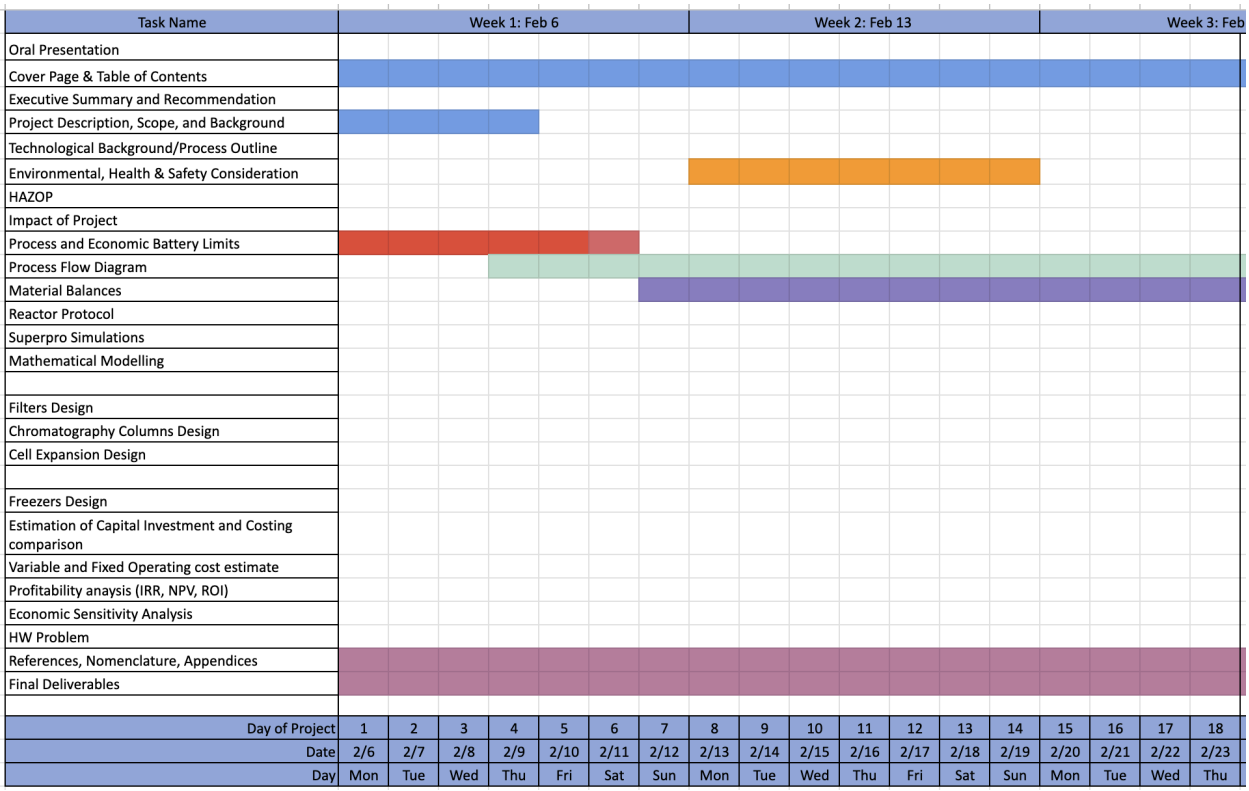


Figure B-1: Gantt Chart Weeks 1 and 2.

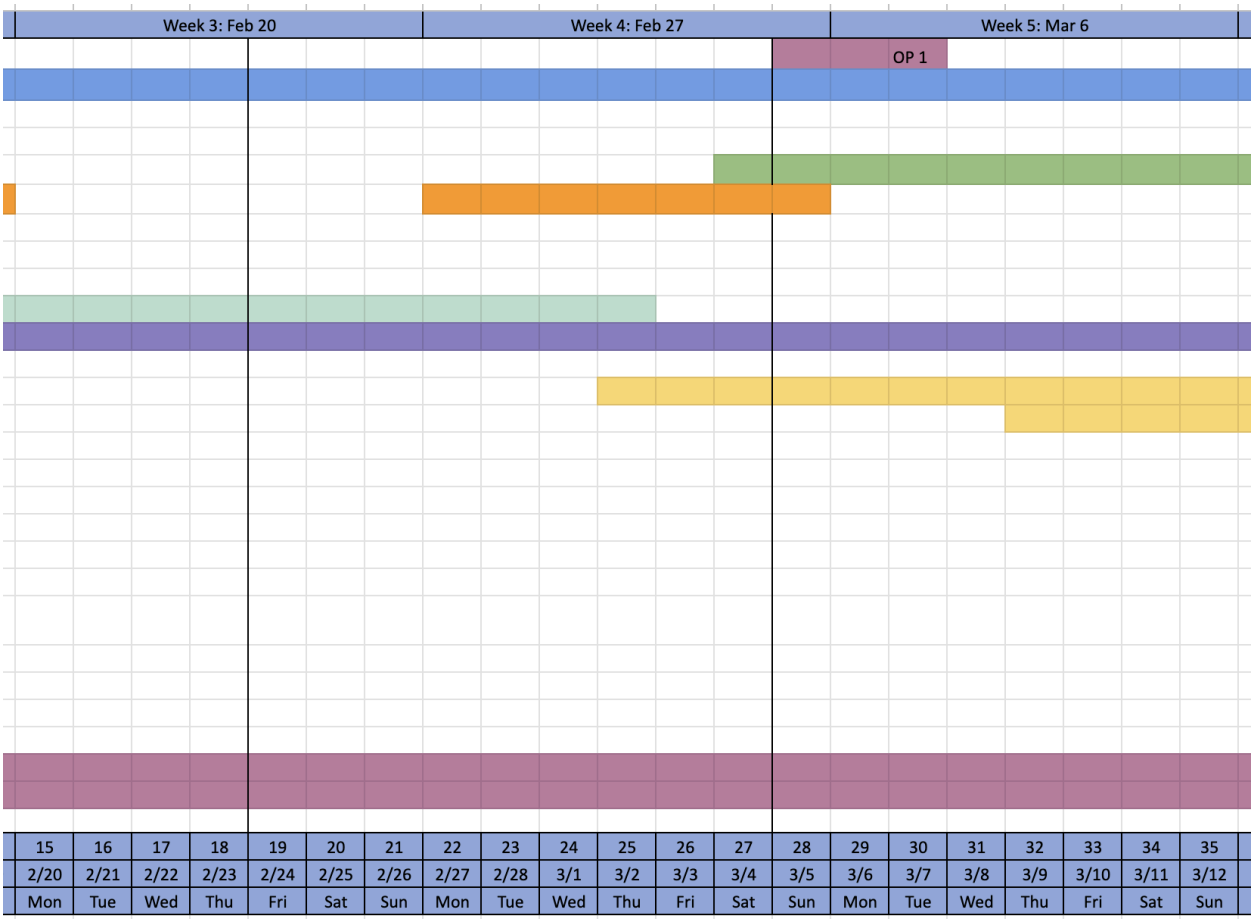


Figure B-2: Gantt Chart Weeks 3 - 5.

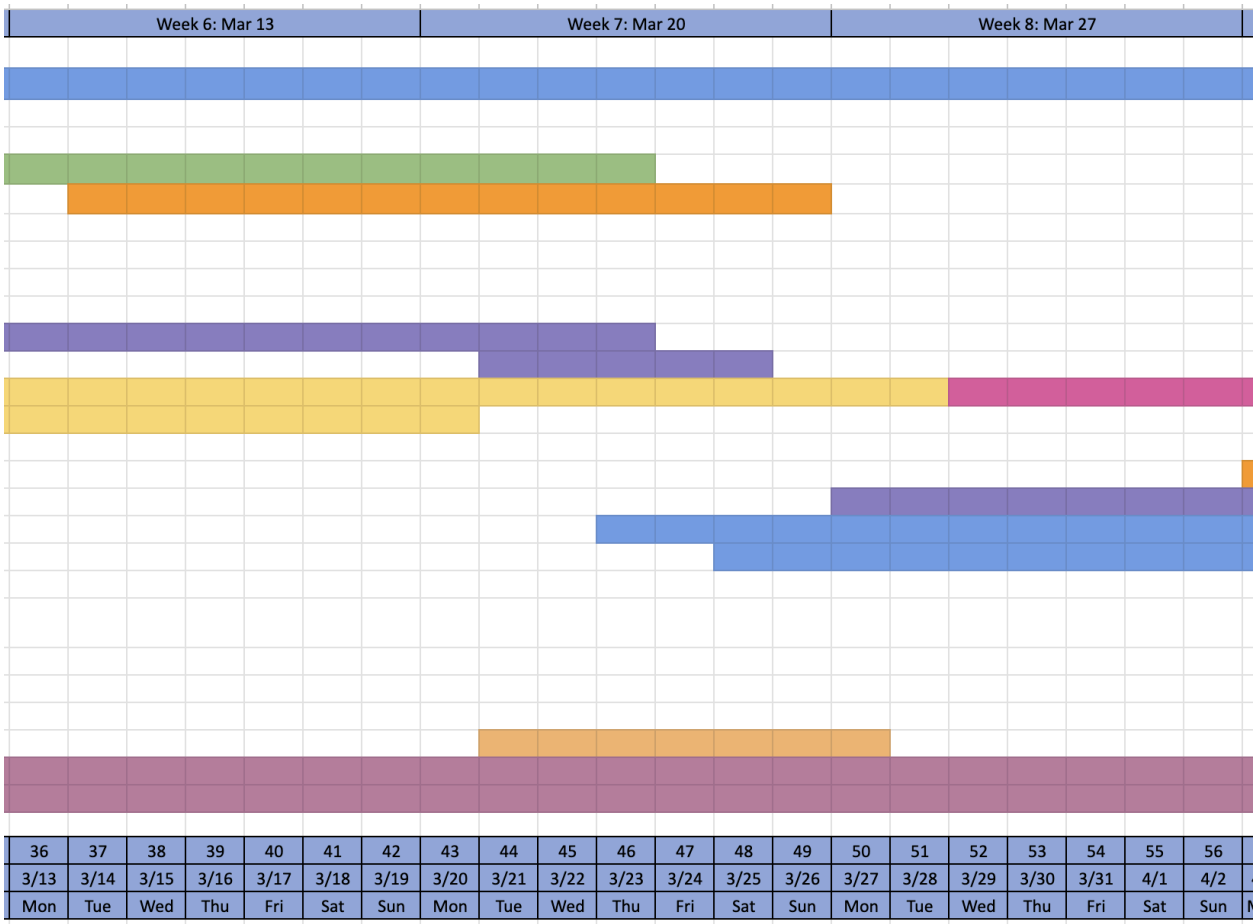


Figure B-3: Gantt Chart Weeks 6 - 8.

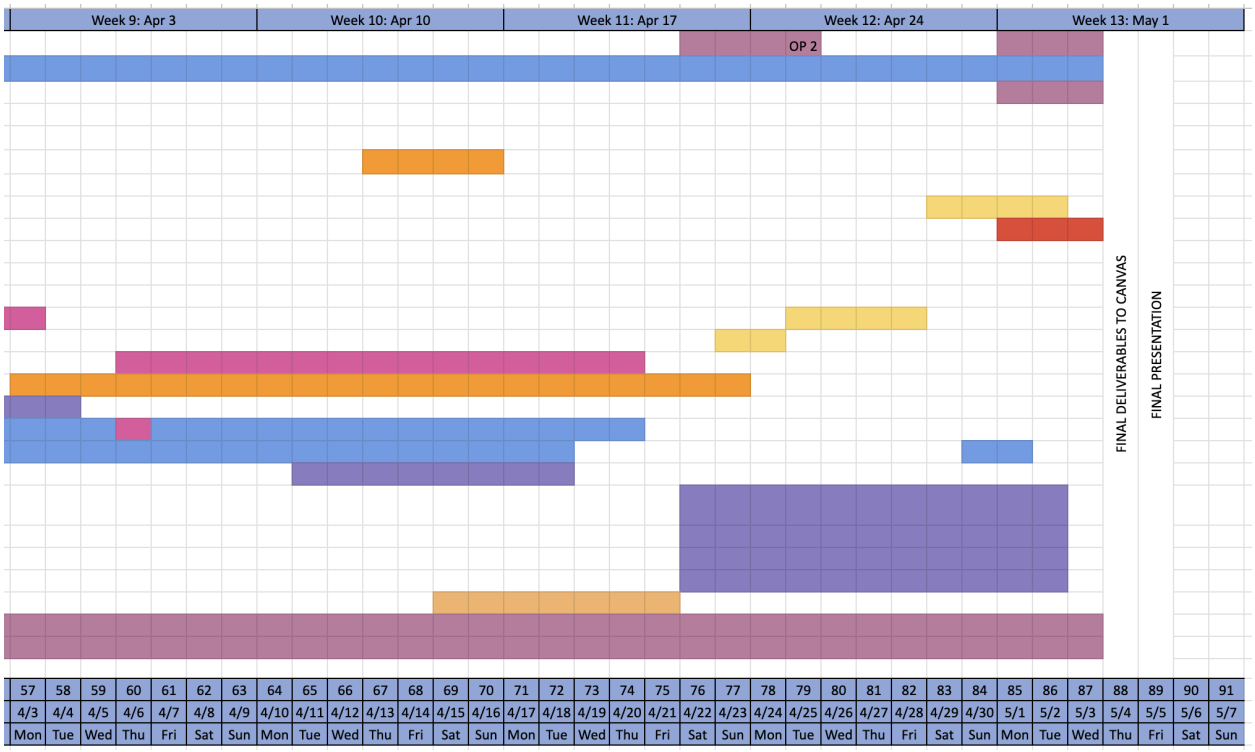


Figure B-4: Gantt Chart Week 9 - end of project.