# Seed Train Development for Vero Cells in the XDR-200

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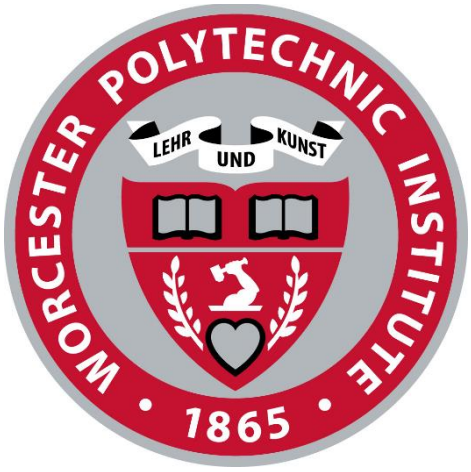


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# Authorship Page

All five group members contributed equaling to the writing of this report as well as every group member reviewed all sections before submission. The group jointly accepts the responsibility for the project and paper and will decline individual authorship.

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# Abstract

Bioreactors are used to produce trillions of cells for bioprocessing into useful biologics like vaccines and biopharmaceuticals. The cells required to sufficiently inoculate a bioreactor are produced using a seed train, the procedures taken to move from a frozen cell bank to a cell culture with a target density. One of the more useful cell types for vaccine production is the Vero cell, which requires a surface to adhere to in order to grow properly. The purpose of this project was twofold: to design and create a robust seed train process for scaling up to the XDR-200, and to evaluate the feasibility of microcarriers and single-use technology in the XDR-200. To accomplish this, the group utilized tissue culture flasks and spinner flasks to scale-up to the targeted cell density for inoculation of the XDR-200. Cell counts and media analysis indicated that the seed train was acceptable for scaling up and that microcarriers and single-use technology were feasible for Vero cell growth in the XDR-200 with future improvements like more efficient trypsinization of Vero cells in the XDR-200 to improve scalability.

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# Chapter 1: Introduction

The overarching goals expressed by GEHC can be broken down into two parts. The first is to determine the feasibility of using GEHC's XDR-200 bioreactor with microcarrier technology for vaccine production utilizing Vero cells. Before Vero cells can be introduced, the microcarriers must first be identified as usable within the bioreactor. Impeller tip speed and its effects on the microcarriers and their durability will be analyzed. The analysis will result in a protocol with the best possible parameters for an optimal method to produce cells at a large scale within the bioreactor using microcarrier beads that will result in minimal microcarrier destruction. After this, test trials will commence to ensure the microcarriers are viable under the mixing conditions. They must remain in-tact and suspended within the media without being exposed to too great a shear force. If this is accomplished, then Vero cells will be added and evaluated for cell viability. The data gathered from these 200 liter test runs will be compared with pre-existing data from a 50L bioreactor as a baseline.

The second is to explore the seed train process, looking for opportunities to scale up the culture more efficiently and to remove as much potential for error as possible. When culturing cells at such a large scale, any mistake or process failure becomes very costly. For instance, 200 L of media will cost around $6000, a 10 L wave bag costs $200, and a 200 L bag costs $9000. To avoid this, several different scale up processes will be tested and evaluated, and the most optimal method will be used to scale up to the XDR-200. Furthermore, bioprocess laboratories using similar equipment will be surveyed in attempt to uncover common points of failure in current processes. This data will serve as the basis for the final project goal of developing a design of a more efficient and robust process for Vero cell seed propagation.

Engineering a more efficient upscale process for adherent Vero cells serves to increase the vaccine production capability of the industry, ultimately increasing the number of patients who can be treated or vaccinated while simultaneously reducing the cost.

Vaccines play a vital role in US and global healthcare, with over 90% of the US population having received vaccinations for measles, mumps, rubella, and hepatitis B by the age of 17 (National Center for Health Statistics, 2015). Additionally, vaccines for tetanus, human papillomavirus, influenza, and numerous other viruses are available. Vaccines are also experiencing rising popularity in experimental treatment of cancer, as more and more data on immunizations for key cancer antigens is acquired (Rosenberg, 2004). This makes the production of vaccines an equally necessary and profitable industry. General Electric Healthcare (GEHC) Life Sciences, a unit of the American conglomerate dedicated to innovative medical technologies and patient care, has played a major role in pushing the field of vaccine production forward. However, with such a large demand for vaccines worldwide, a more productive and efficient upstream process is needed in order to mass produce cells at a large scale for high rate of vaccine production.

Perhaps their biggest contribution has been their work in the field of bioprocessing and the development of the cell culture seed train, which has benefited the field of health immensely over the years. They have provided equipment and procedures for medical companies around the globe so that they can produce their own cell culture seed train, such as the wave bioreactor and microcarrier beads, which will be discussed further into this report. A cell culture seed train is the process by which a scientist or organization moves from a small vial of a frozen stock of cells to billions and potentially trillions of cells, depending on the overall scale up of the seed train to ultimately be inoculated into a bioreactor. The products of these cells usually go through a

downstream process where they are processed through protein purification and can be used to produce vaccines, natural and recombinant proteins, and monoclonal antibodies. These cells can be used for a wide variety of purposes ranging from cell therapy, in which cells or the material they produce is used for a medical therapeutic treatment, to vaccine production, where the cells serve as hosts for viruses to replicate in. Vaccine production is one of the major goals of seed trains because of the large quantity of cells that are produced, in turn leading to larger quantities of viral antigen for vaccine production. One of the most commonly used cells for vaccine production is the Vero cell, an African green monkey kidney cell line that is immortalized, meaning it will continue to double without ever reaching senescence (Ammerman, 2009). This cell is anchorage-dependent, however, and therefore requires a surface to attach to in order to grow and function due to organ that these cells are derived from. Since these are kidney cells, they are immobilized and embedded in the connective tissue *in vivo*, which explains their anchorage dependence. Anchorage dependent cells are mainly used for virus production because of their ability to adapt to different substrate attachments. This characteristic allows the cells to attach to microcarrier beads. The ability to attach to microcarriers within media is vital for a successful adherent cell culture within a bioreactor, as microcarriers provide the surface area required for producing trillions of cells.

Bioreactors are a main component in the production of cells at a large scale. A bioreactor is a system that supports a biologically active environment. It is a vessel that carries out chemical processes and involves organisms or substances derived from organisms. It can also be used as a system to grow cells or tissues for a cell culture. Bioreactors are able to use microcarrier beads, which are very beneficial in the process of large scale production of cells. Microcarriers are a support matrix for cells to grow on inside a bioreactor. They are able to support the growth of

adherent cells on their surface, and provide a large production of adherent cells due to the ease of scale-up, the ability to precisely control cell growth conditions, a reduction in floor space and incubator volume needed for cell culture, and a reduction in technical labor. GEHC has pioneered single-use bioreactors and microcarrier beads in response to this requirement. Single-use bioreactors, like the XDR-200, allow precise control over the environment the cells grow in and ensure quick turnaround between cell growth stages by eliminating the need for extra cleaning and inspection. Microcarrier technologies include Cytodex 1 and Cytodex 3 beads. Cytodex 1 beads are suited for general purpose culture and established cell lines, as well as cultures of primary and diploid cell strains. Cytodex 3 beads are preferred for cells that can be difficult to culture, such as differentiated cell culture systems and cells with an epithelial-like anatomy. This is because Cytodex 3 beads are coated with a denatured collagen substrate, which has been shown to promote cell growth (GE Healthcare, 2006). They also require a maximum retention of viable cells at harvest, which permit adherent cells to grow in larger numbers in solution (GE Healthcare, 2006). The XDR bioreactor bag agitator has been modified to prevent and avoid microcarrier damage. Additionally, optimal operating conditions for cell culturing microcarriers in the XDR-200 can vary from cell line to cell line. Therefore, only limited information is available with respect to optimal conditions for this application.

The project/design phase is split up into two phases for the approach. The first phase is to study the vial to seed-train process and to discover common points of failure within. In the first term two separate seed train models will be tested and evaluated up to the 50L scale. These two seed trains will be analyzed and compared to one another to determine the viability of their cells, their growth rates, cost and ease of process. To accomplish this, samples will be collected from the 10L wave reactors to be counted, have their survival ratio determined, and their glucose

uptake rate analyzed. The cost, ease, time, amount of steps and ease of maintaining sterility of the process will also be weighted and considered for the final process design.

The second phase of the project will include the scaling up to the 200L XDR single use bioreactor. Finding the impeller tip speed and power input that is ideal for maximum cell growth is paramount to this stage. Once the cells have fully propagated and reached close to a maximum confluency of $1.76 \times 10^{10}$ cells through the 10L wave bioreactor, the cells will be placed into the 200L. Again, the viability of the cells, as well as the success of cell-to-cell transfer from bead to bead in the bioreactor will be assessed by adding more beads when the current beads in culture have become confluent. Bead-to-bead transfer will be determined by observing if the new beads have cells attached to them. This test will include taking samples of the beads daily and determine the number of microcarriers that are destroyed by the impeller, if any. The glucose uptake rate data from these samples will be compared to the 50L single-use bioreactor data to determine if improvements must be investigated. A higher glucose consumption rate will determine that the cells are metabolizing well and are growing and proliferating. If the 200L bioreactor runs are unsuccessful due to a low proliferation and growth rate of cells, contamination, cell death, or little to no attachment onto the microcarriers, suggestions will be made for improvements in either the bag, impeller, the cell environment controls or the microcarriers. A better solution will be determined based on observations from the procedure conducted.

This report will begin by discussing the background of the project based on material found in literature review to better understand the general problem encountered by many bioprocess facilities. The project will aim to address these problems, its objectives, as well as the project's goals. This chapter will discuss the functionality, applications and importance of

bioreactors and microbead technology used to scale up cells. It will also review current technologies and techniques used in the process of scaling up cells. The literature review will be followed by the project strategy, which will outline the ideas and procedure that will be taken to solve the problem addressed by General Electric Healthcare, which is the need for a more efficient and optimal technique to scale up cells from a 1.5 mL vial, into a 200 L bioreactor, specifically the XDR-200. Current customers of GEHC products in the bioprocessing fields have encountered numerous difficulties with the seed train process, and a goal of this project is to address these difficulties by implementing a more efficient protocol involving the upstream scaling up of cells. This chapter will discuss the strategy that will be taken in order to approach these complications, in addition to identifying solutions to the limitations of the bioreactor and microbead technology, such as the shear stress due to the impeller, foaming from addition of gases, and collision of microcarriers, in further detail. In addition, the industry, engineering, and regulatory standards will also be addressed.

The following chapter will delve into the design process that will be administered throughout the project. This section will discuss the objectives, constraints, functions, and specifications of the project design, beginning with requirements necessary in order to design a protocol that is able to optimally scale up cells into a large bioreactor with little to no cell death an absolutely no contamination. For this project in particular, it will require design aspects that will assist upscaling cells to a 200 L bioreactor while using microbead technology, while attempting to maintain cell growth, proliferation, and viability. This section will also describe the provided protocols, as well as the alternative methods that were developed throughout the project, that were tested in order to obtain the most optimal procedure for scaling up cells into a 200L bioreactor. The chapter will conclude with a discussion on the method or design that

resulted in the most efficient process to scale up the cells into the XDR-200. After discussing the final design, the ensuing chapter will discuss the results of the tests. Specifically the justification and verification of the method and design chosen by examining the results that are based on factors such as cell count or glucose uptake, as well as population doubling time. This chapter will be followed by the validation concerning the final design or method that has been decided upon. This consists of an explanation of how the objectives of the project were met and what has been accomplished by completing the project. In addition, various impacts will be discussed such as economical impacts, societal impacts, political ramifications, ethical concerns, health and safety issues, manufacturability, and sustainability.

Finally, the report will conclude with a discussion of the results of the project and how they accomplish the objectives. The results will also be compared with previous research and data used with bioreactors, and an explanation of the significance of the results. Additionally, limitations of the project will be discussed. This chapter will be followed by the conclusions and recommendations chapter, which will consist of a summary of the final results and how the objectives were met, in addition to recommendations on areas for further research.

# Chapter 2: Literature Review

## 2.1 Cell Culturing

Cell culture is a cornerstone of modern biological and medical research. The technique of growing cells in conditions favorable for replication, division, differentiation, or other desired cellular outcomes plays a major role in a wide range of applications. These applications include the use of cells as model systems for disease and cancer, toxicity tests, tissue and organ growth, drug screening, gene therapy, vaccine production, and many more (Shen, 2016). While the applications for cell culture are numerous, this project will be focusing on large scale cell culture for the purpose of later use in vaccine production.

The methods used for cell culture depend on the type of cell being utilized. There are two primary types of cell culture techniques used for production of larger numbers of cells: adherent cell culture and suspension cell culture. Adherent cell culture is the most common for cell culture, as cells primarily grow in contact with some kind of surface in any organism, typically other cells or a scaffold which might be made up of extracellular matrix. Suspension culture, on the other hand, is the result of cells having been adapted to no longer require a surface to adhere to (van der Pol, 1998).

## 2.2 Adherent Cell Culturing

Adherent cell culture is reliant on surfaces for cells to adhere to, and thus a proper surface to volume ratio must be achieved in the culture in order to maximize efficiency. The primary means of achieving this ratio is through the use of microcarriers suspended in a stirred-tank bioreactor (Kong, 1998). This technique relies on the use of patterned discs or spheres with

diameters in the range of ~200 μm that provide a floating point of attachment for cells. These microcarriers are hosted in a bioreactor. a giant stirred tank, which agitates the solution and assists in nutrient and gas exchange among the beads and solution. Other techniques utilized in adherent cell culture include roller bottles which rely on the motion of a bottle rolling to aid in gas and nutrient exchange and stacked plate systems which uses stacks of thin planes of treated material to house large numbers of cells (Zhang, 2000). Adherent cell culture allows for the use of most cells that have not entered senescence. The major advantage of adherent cell culture is this versatility in the type of cell produced. However, there are a number of disadvantages that factor into adherent cell culture.

The first is the surface area requirement, which directly limits the number of cells grown in culture. Microcarriers help circumvent this by providing additional surface area within a stirred-tank reactor, but there is a limit to the number of microcarriers that can utilized per unit volume. Another disadvantage is the requirement of a tissue-culture treated surface. While cells can still technically adhere to surfaces that have not been specially treated, studies have shown that pretreating the adherent surface with specific polymers can greatly increase cell attachment and viability (Yavin, 1974). Tissue culture vessels transitioned from pure glass, which had poor cell attachment rates, to polystyrene vessels. Polystyrene had one major drawback in its hydrophobicity, which made it difficult for cells to properly attach to the material. This led to the advent of synthetic polymer treated polystyrene, like poly-D-lysine coatings. These coatings maintained the excellent attachment properties of polystyrene but carried a positive charge, which can enhance cell attachment as well due to the slight negativity of cells (Ryan, 2008). Microcarriers are a specially prepared surface as well. They are typically comprised of cross-linked polymers like dextran that are saturated with media to induce swelling of the microcarrier

and provide nutrient and gas exchange from two directions, via the microcarrier and the surrounding media. A third major disadvantage of adherent cell technology is the necessity of dissociation from the adherent surface either mechanically or chemically. This dissociation is required for harvesting of the cells, whether it be for harvesting the final product of the cell culture or for moving the cells to a new host container. One of the most commons means of chemical dissociation is trypsinization, the use of the proteolytic enzyme trypsin to break down the proteins used by adherent cells to attach to surfaces. For adherent cell culture, one of the most commonly used types is the Vero cell. Vero cells will be the cell line utilized for completion of this project, due in part to their properties and value in vaccine production.

## 2.3 Suspension Cell Culturing

Suspension cultures bypass many of the disadvantages found in adherent cell cultures. Since cells are suspended in media, they do not need to be dissociated from a surface for harvest. The vessel for the cells does not need to be specially treated as in adherent culture, as once again cells are suspended in media. Finally, suspension cultures are limited only by the concentration of cells in media, due to required nutrients and gas. This constant limitation value of cells/unit volume makes it easy to scale-up to larger quantities of cells and media.

Suspension culture carries several disadvantages alongside it, however. The first is the requirement of adapting cells for suspension culture. There are protocols in existence for adapting adherent cells to suspension culture, but these protocols require several weeks to carry out and not all cells adapt successfully to suspension culture (Wu, 1990). This makes developing new suspension culture cells difficult and costly, in addition to the uncertainty of the process.

Whether or not the cells will properly adapt to suspension is the major question in this process. Adaptation to suspension requires a blend of the proper cell culture environment and media, and is entirely dependent upon the cells' ability to survive without an attachment. Toward this end, cells that survive initial suspension attempts are often grown further as the base of a successfully adapted suspension culture. The other major drawback of suspension culture is the necessity of agitation for proper gas exchange. This is typically a component of adherent culture as well, but it is always required in suspension cultures (Chu, 2001). Suspension culture is also typically carried out in a stirred-flask reactor, as these reactors provide the proper agitation for the culture and help to monitor levels of vital nutrients and gases like glucose and oxygen in the media.

Suspension culture is majorly advantageous for large-scale cell culture, as cells are easy to passage, follow specific growth curves that allow for accurate cell growth tracking, and do not require removal from an adherent surface through potentially traumatic enzymatic severing. The major reason that certain adherent cell lines are still utilized in large-scale cell culture is that they have not yet been successfully adapted to suspension culture. For example, Vero cells are a great platform for producing a wide range of viruses. Since the advantages of suspension culture over adherent culture are so significant, many groups are attempting to adapt Vero cells to suspension culture (Paillet, 2009). Aside from attempts to adapt new cell lines to suspension culture, there are a few cell lines that are widely used in industrial suspension cell culture. The three that are most commonly used are Chinese hamster ovary (CHO) cells and the murine myeloma lines, SP2/0 and NS0.

## 2.4 Vero Cells

In order to choose a cell line for virus cultivation, several factors must be examined. The most important criteria for selecting a cell line are the ability of the cell to propagate the virus in large amounts, the speed and efficiency with which the virus is expressed in the cell, and the suitability of the cell to a wide range of viruses (Rappuoli, 2006). For the purposes of this project, the Vero cell line will be used. The Vero cell line is derived from the kidney of an adult African green monkey, and has been around since 1962. The Vero cell is particularly susceptible to infection from a large range of viruses, which allows for easy intentional infection and harvesting of virus for vaccine production. Additional benefits of the Vero cell line are the ability to bank these cells, the well-understood characterization of the cells, the elimination of harvesting cells from animals and the ethics involved with that scenario, and the adaptability of the cells that allows for large batches to be grown in bioreactors (Sheets, 2000). When grown in industry, these cells are typically cultured in the presence of microcarriers, which have been designed to maximize the number of possible attached cells.

## 2.5 Cell Growth Techniques for Adherent Cells

Adherent bioprocess technology is a fast growing field in which many competing technologies are being developed and assessed. The three major classes currently in use are planar flask technologies, packed bed bioreactors and suspension microcarrier bioreactors. While each method has potential to produce hundreds of billions to trillions of cells per lot, they each have their own benefits and drawbacks.

Multi-layered flasks seen in **Figure 1a** have been the most commonly used and researched method of adherent cell culture for over 30 years. They are simply flasks which

attempt to maximize the surface area upon which the cells grow often taking cultures into the middle and late-stage development. They peak around 100-400 billion primary adult cells per lot with current planar technologies. The two ways planar technologies are able to increase their harvest size to this scale are by increasing the surface area available within the flasks and increasing possible cell density. (Rowley J, et al. 2012) Some of these stacked plate systems include Corning's CellStacks (Corning Inc. 2005) and Nunc Cell Factories (Thermo Fisher, 2016). While these technologies are very beneficial for scaling up large numbers of cells at a time due to their high surface area, they consist of some limitations. The biggest disadvantages of these technologies include the difficulty of obtaining samples from these flasks to ensure proper growth and cell conditions, media exchange within the flasks, and the high cost demand for utilizing these technologies.

Packed-bed bioreactors are reactors in which the cells are enclosed in a type of packed bed within a bioreactor. These packed beds can be hollow fibers, webbed fibers or different porous structures that are most commonly placed within the bioreactor chamber submerged in the media. Some notable advantages of packed bed systems are that they are capable of reaching high cell densities and the cells are not exposed to high shear stresses. Currently, packed-bed bioreactors can produce up to 4 trillion cells, which is comparable to fluidized-bed bioreactors, however this system uses suspension cells. This system works by having the cells suspended in an upward flow of medium within the bioreactor using an agitation pump. (Rowley J, et al. 2012) However, it can be difficult to keep the cells exposed to a homogenous mixture of nutrients due to how compact the cells and their scaffolding are, which can restrict the final density of the cell volume. It can also be difficult to strip the cells from their beds. The more densely packed the cells within the bioreactor, the more difficult it can become to remove the cells intact for

harvesting. Some of the current systems include the New Brunswick Celligen system (Eppendorf Inc. 2016) and the iCellis Bioreactor pictured in **Figure 1b** (Pall Life Sciences, 2015).

Lastly, the class of technology which will be studied here is suspension microcarrier bioreactors. These reactors work much like traditional suspension based reactors but use micron sized microcarrier beads for the cells to adhere to. This provides an increased surface area to volume ratio over the other methods, (e.g. 30 $cm^2$ in 1 mL using 5 grams of microcarriers) reducing the space needed for cultures. The beads surfaces and shapes can be tailored to the types of cells being cultured and modifications can be made to their surfaces to affect cell adhesion. The ability to control the cell environment is also improved over planar and packed-bed systems. The biggest drawback these technologies face is that shear stress from impeller-based systems could potentially tear the cells from their microcarriers or even destroy the beads themselves. Currently, a solution to this problem is the use of the wave bioreactor. Wave bioreactors produce little shear stress upon the cells due to a rocking motion with no impeller to produce shear stress on the microcarriers. A Wave bioreactor is pictured in **Figure 1c.** This is a potential means of circumventing harmful shear stress for cells. GE has demonstrated a cell density of $3x10^6$ cell/mL with their Cytodex beads within a 50L wave reactor. (GE Life Sciences, 2014) If these microcarrier cultures can be transferred into impeller suspension based bioreactors, they may also be able to produce quantities above the trillion cell count, suitable for more efficient production of therapeutics. (Rowley J, et al. 2012)

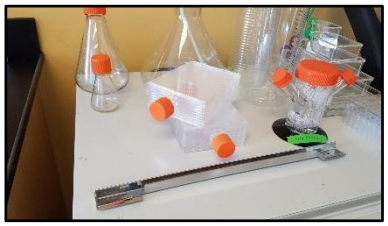
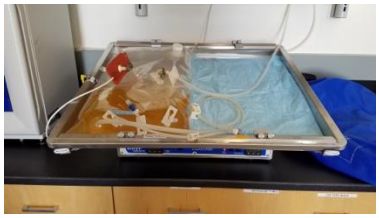
(a)  (b)  (c) 

*Figure 1: (a) Corning CellStack Culture Chambers, (b) iCELLis PBD Bioreactor, (c) GEHC Wave Bioreactor*

## 2.6 Microcarriers

Microcarriers have become a popular method for culturing adherent cells in industrial sizes. The main reason behind this is their higher surface area per volume compared to two-dimensional platforms like T-flasks. The microcarrier beads are spherical particles with a diameter ranging from 90-300 μm (Szczypka, 2014). This higher surface area per volume has been attractive since it lowers cost by increasing the cell yield in smaller volume reactors. In studies conducted by GE, microcarriers have reduced the cost of medium, serum, and labor costs by more than 50% (GE Healthcare file 18-1060-61 AG). The process by which cells attach, spread, and grow on the beads is dependent on the cell dissociation times, enzyme concentrations, incubation temperatures, media components, and microcarrier surface modifications like charged surfaces and cell-adhesion particles (Szczypka, 2014). Some examples of cell-adhesion particles that are incorporated in the microcarriers include collagen, laminin, and fibronectin that provide attachment sites for the cells (Szczypka, 2014).

## 2.7 Cytodex™ Microcarriers

The microcarrier products that GE makes for adherent cells are called Cytodex™ 1 and Cytodex™ 3. Cytodex™1 is the larger of the two and is a cross-linked dextran matrix bead that is suitable for

the production of viruses (GE Healthcare file 18-1060-61 AG). Cytodex™ 3 is a dextran matrix with a thin layer of denatured collagen on the surface to help with cells that may be hard to culture (GE Healthcare file 18-1060-61 AG). Cytodex™ 3 is also useful for harvesting cells as the collagen layer is easily digested by proteolytic enzymes (GE Healthcare file 18-1060-61 AG). A comparison of the physical characteristics of these microcarriers can be seen in **Table 1.** The beads used in the project are Cytodex™ 1 gamma-irradiated microcarriers from GE to allow for an easier preparatory process. Being gamma-irradiated means the microcarriers can be immediately swelled in cell culture media and only take one day to become ready for use. These microcarriers cut down time and labor costs by eliminating the process of sterilizing and swelling of the beads before they are used to culture cells (Gustaf).

*Table 1: Physical characteristics of CytodexTM microcarriers (GE Healthcare file 18-1060-61 AG).*

| | Cytodex 1 | Cytodex 3 |
|---|---|---|
| Density (g/mL) | 1.03 | 1.04 |
| Range of Diamter of beads *(µm) | 147-248 | 141-211 |
| Approximate area ($cm^2$/g of dry weight) | 4400 | 2700 |
| Approximate number of micro-carriers/g of dry with | 4.3*10^6 | 3.0*10^6 |
| Swelling factor (mL/g of dry weight) | 20 | 15 |

The Cytodex™ 1 beads are created by "substituting a cross-linked dextran matrix with positively charged DEAE (N, N-diethylaminoethyl) distributed throughout the matrix" (GE Healthcare file 18-1060-61 AG). These charged particles allow for optimal attachment to the beads and is

suitable for established cell lines such as Vero cells. A diagram of the microcarrier can be seen in **Figure 2**.

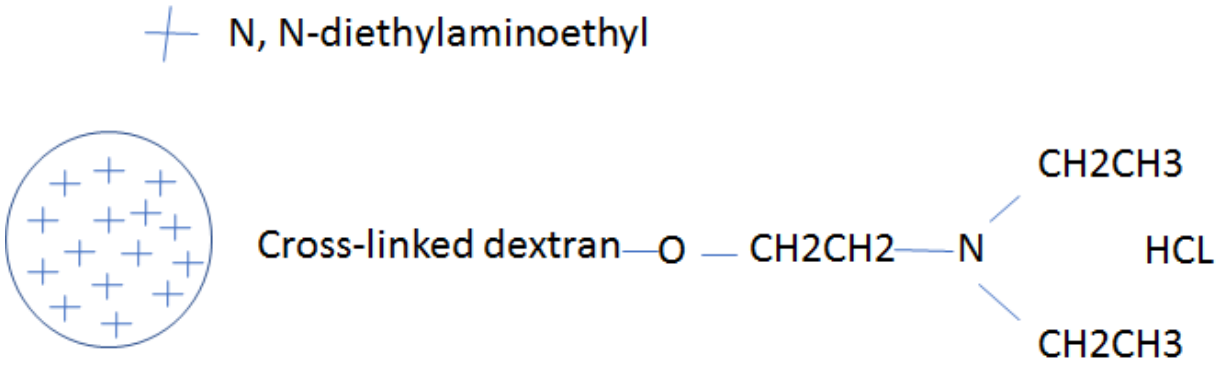



*Figure 2: CytodexTM 1 structure (GE Healthcare file 18-1060-61 AG).*

The Cytodex 1 beads also provide a useful tool for scaling up the cell culture to larger, industrial sized vessels. Microcarriers allow cells to be cultured at higher densities to be used for inoculation of larger vessels like stirred-tank bioreactors. This reduces the number of vessels needed to culture enough cells for inoculation of large production-scale bioreactors which eliminates cost and contamination (GE Healthcare file 18-1060-61 AG). Once microcarriers are used to inoculate larger vessels, the issue of scaling-up arises as different cell lines need to be scaled up differently. Some cell lines are able to undergo bead-bead transfer with Cytodex™ microcarriers which makes scaling-up easy by just adding culture volume and more beads (GE Healthcare file 18-1060-61 AG). The biggest issue comes from cell lines that can't transfer from bead-bead and need to be harvested and then attach to fresh beads that are introduced with increasing volume. The best way to harvest the cells from the beads is to trypsinize the beads and then to add more media and beads for the settled cells to attach to (GE Healthcare file 18-1060-

61 AG). The Vero cells that are used in the project have been proven to transfer from bead to bead with Cytodex™ microcarriers in a study done by Wang et al (Wang, 1999).

To determine the attachment efficiency of the beads when scaling-up, the Cytodex™ 1 beads can be extracted from the stirred tank reactor and nuclear stained using crystal-violet dye for microscopy. The beads are transparent which makes it easy to examine the attached cells under a microscope by a simple cell staining technique, like hematoxylin, to determine the attachment efficiency (GE Healthcare file 18-1060-61 AG).

Since the gamma-irradiated Cytodex™ 1 microcarriers are used in this project for scaling up to a 200L bioreactor, the parameters for optimal attachment must be investigated. The three main parameters that have been investigated for stirred-tank bioreactors with these beads are agitation rate (rpm), cell-to-microcarrier ratio (cell/μg), and FBS concentration in the media. In a study done by Souza et al, different agitation rates, cell-to-microcarrier ratios, and FBS concentrations were tested to see which parameters gave the best cell density (Souza, 2005). The run that gave the highest cell density output had an agitation rate of 40 rpm, 70 cells/μg of microcarriers, and 5% FBS concentration. This experiment showed that increasing cell-to-microcarrier ratio and FBS concentration together gave the highest cell density. Creating a higher cell density will allow for more cells to be infected, which ultimately yields more product for vaccine production.

## 2.8 Cell Culture Variables and Analytics

When preparing to use a bioreactor, it is important to prepare a model for the system, while also monitoring the cells within. It has been shown that multiple parameters (up to 35 in

some analyses) play into the success of a bioreactor run, such as cell growth rate, the rate of air introduction into the liquid culture (air sparge rate), dissolved oxygen (DO) and lactate concentration (Le, 2012). These variables all have a part in the bioreactor process, but some of these variables have a more significant impact on the cell culture than others. Optimal conditions may vary depending on the size of the bioreactor (Le, 2012). Some experiments start at a bench scale below a liter in working volume, and then move up to a full production stage of 12,000L. In this scale up process, parameters such as lactate concentration and growth rate are very indicative of whether the run will be successful. For example, at the 12,000L production scale, highly viable and efficient runs demonstrated that cells were actually consuming lactate. This proved to be valuable in early prediction of a run's eventual success. If lactate was being produced instead of consumed, the run was less likely to be as successful (Le, 2012). This shows how analyzing the cells may be used as an identifier should modifications be needed. Monitoring these parameters will be integral in ensuring the success of the project.

Another variable to take into account is maximum cell yield. Some bioreactors have a maximum density of cells (in cells/ml) that they can support. Some may range from $3 \times 10^6$ to $7 \times 10^6$ cells, as determined by many sampled runs (Tescione, 2014). One parameter that affects maximum cell density is maintaining correct dissolved oxygen levels. This becomes a challenge in larger reactors at the production scale (Xing, 2008). To allow for dissolved oxygen maintenance, spargers are used which introduce the oxygen into the reactor. However, due to current good manufacturing practice (cGMP) related constraints issued by the United States Food and Drug Administration (FDA) that must be followed, a certain type of sparger is largely used. The type of sparger that is primarily used is a pipe sparger, which is a pipe with holes drilled into it to allow for air escape. The other frequently used type of sparger is a frit sparger,

which utilizes a capillary tube attached to a porous frit to disperse tiny air bubbles into the culture. The pipe sparger is typically used instead of the frit sparger to allow for finer oxygen bubbles and better transfer (Xing, 2008). As this issue is met at the larger scale, different mixing models can be used to calculate the oxygen diffusion coefficient, along with dissolved carbon dioxide removal and mixing time. (Xing, 2008) This modeling allows for comparison with what is actually achieved after the run is completed, which can provide insight into how effectively the gases were actually dispersed in the cell culture.

To assess the cell culture within the reactor, some practices have become standard within the industry. For most users, it is common to evaluate the cell density and viability. This is completed by introducing a stain – Trypan Blue to a mixture of cells, and measuring the Trypan Blue exclusion. Trypan blue exclusion works through an absorption process of dead cells. If the cell membrane is not intact, then the dye will be absorbed. If the cell is alive, then the membrane will not include this dye (Strober, 2001) These cell mixture is then visually analyzed by a Cedex analyzer, or similar instrument. (Tescione, 2014) Likewise, other instruments are utilized to measure the lactate and glucose concentrations within the cell mixture. An example of an instrument for this is YSI 2700 enzymatic biosensor. (Tescione, 2014)

## 2.9 Current State of the Art

Currently, there are several competing technologies that show promise for scaling the culture of adherent mammalian cells. These include planar flask cultures, packed bed bioreactors and suspension microcarrier methods. However, few of these technologies have reached maturity, still requiring much more experimentation and improvement. Of these three major

classes of technology planar flasks are the simplest and most tested. Adherent cells have been grown in these flasks for over 30 years and the industry has experience with them. While planar flasks are a reliable method of culturing these cells, scaling up to lot sizes greater than $10^{12}$ cells becomes very labor intensive and requires very large volumes of incubator space. With more human labor involved, the possibility for error increases, increasing the cost of production further. Robotic instrumentation systems have been designed to perform a much of the physical manipulations of the flasks such as washing the cells and trypsinizing them. However, these systems are very expensive, often need to be custom built for the lab they are in and can be prone to error if something disrupts their process. (Rowley, 2012)

There is currently a need for more efficient methods to produce large quantities of mammalian cells on the order of $10^{12}$ (trillions) of cells. For the industry to be able to move forward with suspension microcarrier technologies, it must be proven that cultures can be grown at a cheaper cost than planar flasks and packed bed reactors, with equal or greater reliability and cell viability while adhering to all current good manufacturing processes (cGMPs). If this is proven to create a higher yield of cells in a robust process, then the industry will shift in this direction to be more efficient in their vaccine production operations.

## 2.10 Problem Areas

This project aims to develop a robust and efficient seed train process that is able to scale up cells into a 200 L single-use bioreactor utilizing microcarrier bead technology. This method is very advanced and contains some limitations within its procedure. Limitations of microcarrier bead technology that have been identified include the attachment of cells onto microcarrier

beads, and the destruction of the beads within the bioreactor due to a high amount of shear stress. The characteristics of microcarrier beads play important roles in how well cells are able to attach. Some important characteristics of microcarrier beads can include:

a. **Density:** The density of the microcarriers should be slightly greater than the culture medium to allow for an easier separation. The optimum microcarrier density should be around 1.03 g/ml in order to maintain an even suspension in culture. Beads of higher density will sink the bottom of the reactor towards the impeller, which will destroy the beads, and lighter beads will just float to the top. Cell attachments can slightly increase the density of the beads, so it is important to fabricate the beads at a lower density to take into account for cell attachment (Van Wezel et al, 1967).

b. **Diameter:** The microcarrier beads must have a sufficiently sized surface area that will allow for a maximum amount of cell attachment. According to GE a bead diameter of 100 to 200 μm has been found to be optimal (GE Healthcare file 18-1060-61 AG). Less than 50 – 70 μm has shown little to no cell growth. To ensure the microcarriers become confluent at the same time, there should not be a high variation in diameter between the microcarriers (Maroudas, 1973).

c. **Size distribution:** the size distribution of the beads needs to be small in order to ensure an even cell distribution on the beads following inoculation. Cell attachment favors larger beads because of the slower movement in culture

d. **Charge:** the surface charge of the beads should be around 2.0 ± 0.5 meq/g to support cell attachment. This characteristic is where researchers find the most issues. If the charge density is too low, the cell attachment will be insufficient, where less than 1 meq/g shows no cell growth. A very high charge density will have a toxic effect leading to limited or no cell growth within the

culture (Levine et al, 1977).

e. **Transparency:** The transparency of the beads is important because it allows for easy visualization of the cells under a microscope.

f. **Porosity:** Porosity should be low in order to prevent the reduction in concentration of added growth factors and serum via adsorption from the microcarrier beads (Moran, 1999).

g. **Surface binding material:** the strength and nature of cell attachment is highly dependent on the material used to create the surface of the microcarrier beads (Elsdale, 1972). Cells that are difficult to adhere prefer a collagen layer to attach to compared to the conventional dextran matrix (GE Healthcare file 18-1060-61 AG).

As described, there are many factors that go into the fabrication of microcarrier beads in order to optimize cell attachment.

One of the main problems encountered during the scale up process is the bead-to-bead transfer of cells when inoculating the bioreactor. Bead-to-bead transfer is the proliferation of cells from one bead moving to and beginning to populate another empty or partially-occupied bead, and typically involves some kind of forceful severance from the bead. Many methods have been tested for optimal transfer of cells, however a widely applicable method has not been devised yet. Some methods rely on cells detaching from the beads due to shear force from the impeller, or the presence of peptidases and proteolytic enzymes on the cell surface (Ryan et al, 1980). An alternative method for scaling up cells into bioreactors using microcarriers consists of enzymatic removal and resettling of harvested cells. Enzymatic methods include trypsinization, proteolysis, and metal chelation, which remove the factors involved in cell-substratum

attachment and cause cell detachment. The technique is used to remove cells off of glass or plastic surfaces when culturing cells, and can be used for cell-detachment off of glass-coated beads. Beads with a gelatin surface consist of special receptors and the linkage can be broken by the enzyme collagenase. The beads can dissolve easily, thus removing the beads from culture. It has proven to be more difficult to detach cells from microcarriers. Scanning electron microscope analysis has shown that is may be due to a greater cell to surface contact. Experiments have demonstrated a higher cell detachment rate when trypsinized under a high pH. A pH level of about 8.7 is able to reduce the surface charge of the microcarrier, therefore it can make cell detachment much easier (Varani et al, 1983).

Bead-to-bead transfer is viewed as an alternative to potentially traumatic methods for moving cells from one bead to another. In traditional bead-to-bead transfer, a fresh microcarriers and cell culture media are added to a small batch of highly confluent microcarriers. These new microcarriers settle on top of the confluent microcarriers and cells begin to bridge the gap between the two microcarriers. This method relies on intermittent agitation to allow for beads to settle and begin moving to the new microcarrier, with agitation serving to further disperse confluent and empty microcarriers amongst each other. Experiments utilizing this method of bead-to-bead transfer with Cytodex-3 and Vero cells demonstrated successful jumps in cell numbers and ended with a fully confluent microcarrier culture after four days of culturing (Wang, 1999).

The harvesting of vero cells from microcarriers depends on the surface characteristics of the beads. For Cytodex™ 1 microcarriers, the standard procedure for harvesting Vero cells uses the proteolytic enzyme, trypsin or dextranase (GE Healthcare file 18-1060-61 AG). To maximize

the harvesting efficiency, the media must be aspirated down to the settled bead slurry and then washed with a solution of EDTA-PBS multiple times (GE Healthcare file 18-1060-61 AG). This helps prevent the serum in the media from cancelling out the proteolytic enzymes effect on the detachment of the cells from the beads by diluting the solution. If the goal of the harvest is to collect the cells without damaging the beads, then trypsin should be used since it breaks the attachment proteins of the cells leaving the beads unharmed. For a more efficient harvest, dextranase can be used since it breaks down the dextran matrix of beads allowing the cells to be separated with a higher yield at the expense of the bead's structure (GE Healthcare file 18-1060-61 AG). The exposure time of the proteolytic enzyme to the culture should be between 8-12 minutes to prevent damage to the cells (GE Healthcare file 18-1060-61 AG).

Microcarrier beads allow for an agitated suspension that consists of a controlled environment, and under ideal conditions can consist of a homogeneous culture, which means that entire culture environment within the vessel is identical. In reality, this is not the case due to the agitation causing turbulence. All adherent cells, especially primary cells are sensitive to shear stress. Shear stress generated by large-scale cell culture devices, such as bioreactors, using microcarriers results in growth reduction, cell detachment, or cell death. Cells grown on microcarriers in an agitated culture system are exposed to shear stress and turbulences that can have a detrimental effect on cell growth and production. Specifically, shear stress can force cells to elongate and orient their major axis in the direction of media flow, as well as cause reorientation of the actin structures within the cell, resulting in an increased cell stiffness. This can have an influence on the ability of the cell to properly replicate, which is of the utmost importance in a biomanufacturing cell culture (Nerem, 1991).

## 2.11 Cell Growth Limitations

Along with cell attachment, there are other problems that arise with continued cell growth. The microcarrier concentration within the cell culture can be increased sufficiently high enough so that the surface area does not limit the growth of the cells. However, cell growth is sometimes limited due to the deterioration of the cellular environment. The culture can lack nutrients and oxygen supply, and can build up inhibitors within the culture. The precise factor that causes cells to stop growing in culture varies with the cell line, media, and growth conditions.

One factor that can affect the cell growth is the carbon energy source available, which can influence the cell's metabolic pathways. This can affect the energy state of the cell and the amount of waste that is produced, such as lactic acid and ammonia. A proper amount of glucose and glutamine need to be used in the culture for proper cell metabolism, as well as production of proteins (Eagle et al, 1958). Another factor that affects the growth of cells is the amino acid content, which aids in protein synthesis. Studies have shown that about 30% of amino acid uptake is not used for protein synthesis, which suggests that the amino acids are used for energy metabolism instead (Mizrahi et al, 1976). The rapid depletion of these amino acids, via energy metabolism, during cell growth can contribute to limited final cell yield in microcarrier cultures (Mizrahi et al, 1976). The growth of the cells in microcarrier cultures also depends on serums and hormones present in the culture. Typical cell cultures require 5 to 10% of some type of serum in the medium. This helps promote attachment of cells to the microcarrier surface by providing specific glycoproteins as well as promoting cell growth via growth factors. However, serums are chemically undefined and the hormonal and growth promoting components and vary between different types of serum, which can cause problems with the reproducibility of the

culture. Also, the addition of serum causes an increase in the overall protein content within the culture medium, which can increase the difficulty and cost of purification of the extracellular released products for vaccine production. Serum is also vulnerable to infection from viruses that are developed within the donor animal. Because of these limitations, attempts have been made to use reduced-serum or serum-free media to support cell growth in culture. Through recent experiments, it has been shown that a high serum concentration is often required at the early stages of culture to help provide the initial growth and proliferation to allow for efficient cell to bead attachment. However, the serum content does not have to be constant throughout the culture cycle. Therefore, the amount of serum can be reduced as the procedure goes on, without affecting the growth rates (Lambert et al, 1975). This will allow for a more robust method to optimize cell growth and minimize cost.

The most crucial parameter for cell culture is the oxygen content within the culture. In order to ensure oxygen doesn't become a limitation to cell growth, the oxygen transfer rate has to satisfy the oxygen utilization rate of the cells within the culture. Smaller cultures satisfy oxygen demand through the diffusion of oxygen from the headspace of the culture vessel. Previous experiments have demonstrated that cultures larger than 10 liters will need a larger demand of oxygen supply that diffusion from the headspace cannot satisfy on its own (Katinger et al, 1978). Different solutions have been experimented for this limitation, such as filling the headspace with oxygen instead of air so that the oxygen density is much higher per volume. Also oxygen can be supplied by diffusion through thin-walled silicone tubing that is introduced into the liquid medium (Fleischaker et al, 1981). When the oxygen content decreases in a large bioreactor, parameters are set to sparge in oxygen when it dips below a certain threshold which is usually around 40% dissolved oxygen.

A cell culture microenvironment can consist of inhibitors from the chemical degradation of the media, which limits the cell growth. These inhibitors include lactic acid and ammonia, and must be eliminated from the culture in order to maintain consistent cell growth (Zielk, 1976). Lactic acid is released into the culture medium as the product of anaerobic glycolysis from metabolizing glucose. Lactic acid significantly lowers the pH and can lead to many adverse effects including reduced cell growth, reduced cell viability, and reduced cell detachment from the microcarriers. The accumulation of lactic acid can be reduced by controlling the supply of glucose that is introduced to the culture, utilizing another carbohydrate source instead of glucose, such as fructose or galactose, or using a biotin to reduce lactate production (Pharmacia trade publication, 1981). The accumulation of ammonia in culture medium comes from the chemical decomposition of glutamine (Butler, 1984). This can be overcome by removing the spent media and replacing it with fresh supplemented media.

## 2.12 Shear stress

A critical issue of microcarriers in high-density cell culture within a bioreactor that leads to cell detachment is the shear stress caused by the agitation from the bioreactor, which is needed in order to create a homogeneous cell culture system. The agitation leads to the generation of turbulence, which exposes the microcarriers with cells attached to mechanical shear stress that results in apoptosis or cell death (Nienow, 2014). Beads can also collide with parts of the reactor, namely the impeller, causing additional damage to cells and even microcarriers (Cherry, 1988).

Most of the damage caused to cells is due to the shear stress from the agitation within the culture, especially when using solid microcarriers. Agitation at low levels is required to prevent sedimentation and aggregation of cells and microcarriers at high cell concentrations. However,

the maximum agitation is dependent on the culture support and cell line (Cherry, 1990). Previous experiments have shown that a shear stress of 0.26 $N/m^2$ had no effect on cell viability, but higher shear levels of 0.65 – 1.30 $N/m^2$ had some morphological changes and loss of viability when the cells were removed from the microcarriers. A shear stress level above 2.6 $N/m^2$ showed more than 75% cell detachment (Stathopolous, 1985).

## 2.13 Aeration

Aeration of large-scale cultures can only be performed efficiently through the injection of gas into the medium (sparging) for optimal gas transfer. However, the drawback to this method is the formation of foam, as microcarriers accumulate at the liquid-foam interface and can result in cell death. Foaming during the course of a bioprocess remains a major technological challenge. The foaming tendency of the cultivation induces a lot of direct effects, such as microbial cell stripping and contamination. Microspargers, which reduce bubbles, or tensioactive substances such as anti-foam, are used to prevent foam formation. Utilizing an anti-foam agent can reduce the foaming and bubbles, however these chemicals can also damage cells and cause cell death (Nienow, 2006). The addition of anti-foam modifies the properties of the medium, which leads to a toxic effect at the level of microbial metabolism and contamination of the downstream processing equipment (Delvigne, 2010).

## 2.14 Turbulence

Another problem that arises are the eddies that are generated from fluid turbulence, which directly interacts with the adherent cells and significantly damages them and results in more cell

debris and thus, a lower cell yield (Croughan et al, 1987). The eddies are formed from the fluid turbulence from excessive agitation from the impeller, in addition to the air turbulence when oxygen or carbon dioxide is pumped into the culture. In turbulent flow fields, short term hydrodynamic forces arise through the motion of turbulent eddies. The effect of turbulent eddies on the cells growing on the microcarriers can cause detachment. Cell death is observed when the average eddy length falls below two-thirds of the microcarrier diameter. Eddies decrease in size when the stirred speed in the bioreactor is increased. This decrease causes the eddies to directly apply shear stress on the beads and cells in different directions causing damage (Paul et al, 2004). If the eddies are larger, the microcarriers will move in the direction of the eddy, preventing shear damage.

## 2.15 Collision

Damage to microcarriers and cells are also caused by collisions within the vessel. Collisions can occur between the microcarriers themselves, the impeller, and the vessel walls. A higher number of microcarriers will result in more collisions within the bioreactor, which causes damage and results in cell death and decreased cell yield.

Microcarrier bead technology is very advanced and allows for the control the environment of large-scale cell culture while maintaining a homogeneous environment due to agitation. Specifically single use bioreactors can bring down the cost of large-scale cell culture using single use bags. They help reduce cleaning costs for equipment and do not require the same level of inspection that a reusable stainless steel reactor would to make sure it was prepared for the next run. Additionally, this lack of downtime for cleaning and preparation for another run allows for a much faster continual process, further reducing costs. However, there are some

drawbacks to the technology including detachment of cells from the microcarriers, as well as limitations to cell growth and cell death. A universal solution has not been determined for these drawbacks, and this project aims to identify a feasible method to upscale cells into a 200 L bioreactor utilizing microcarriers that will result in a maximum amount of cell proliferation and production.

## 2.16 Current Solutions and Advancements

One of the major goals of the project is to maximize cell proliferation. An obstacle that stands in the way of cell proliferation is the area available for cells to grow. Solutions have been developed for small-scale cultures, such as the utilization of roller bottles, multitarray stack units, and more recent developments such as hyperflasks. These devices have large surface areas to allow for a great amount of cell growth and proliferation. However, there are two major drawbacks to these methods. They have limited scalability, so they cannot be used for large cultures, and the culture conditions are not controlled with respect to pH or $pO_2$, which results in a continuous change of the physicochemical parameters over the culture duration (Castillo, et al, 2013). Thus, microcarrier beads, such as Cytodex 1, are used in bioreactors in order to accomplish successive scale up at a larger scale because of the large surface to volume ratio (Barret, 2009). Bioreactors, such as General Electric's Xcellerex XDR-200 used in this project, also have the advantage of containing a homogenous stirred suspension allowing for precise monitoring and controlling of environmental parameters, including pH, $pO_2$, and the concentration of media components, which results in a more optimal and reproducible cell culture process. In addition, cell samples can be taken and analyzed throughout the process (Van Wezel et al, 1967).

Foaming during the course of a bioprocess remains a major technological challenge. The

detection of foam is generally performed by using a resistivity probe located in the headspace of the bioreactor. The foam level is then detected by a contact probe, inducing the automatic delivery of the antifoam when foam is detected. The contact between the probe and the foam close the electrical circuit that supplies the antifoam delivery pump. However, this method does not take into account the dynamics of the foam formation, and can lead to the delivery of an inappropriate amount of antifoam. Research is currently being conducted that involves more sophisticated probes, which would be able to measure the conductivity at different levels of foam. The conductivity value could be used to measure the liquid content of the foam at any given point (Delvign, 2010).

High density (HD) cell banking offers substantial advantages in tie savings and simplification of the upstream process. This method provides the means to reduce the time required for culture inoculum expansion and scale-up by eliminating the need for multiple smaller scale shake flask-based operations. Tao et al. reported that using a HD cell bank containing 450 million viable cells/vial to directly inoculate a 20 L wave bag reduced the process time by 9 days since the previous steps of utilizing shake flasks were not taken. Productivity can be enhanced by 10% for a 50 day run and result in 5 to 6 batches of 100 million cells/mL every year (Wright et al, 2015).

Through the optimization of perfusion rate, rocking speed and aeration rate, the perfusion system is able to support peak cell densities of more than 20 x $10^6$ cells/mL in 5-mL CryoTube vials. The HD cell banks were able to be introduced straight into the Wave reactor (Tao et al, 2011). Heidemann et al. reported the success of freezing a cell bank in cry-bags used in blood cell banking at 100 million cells/mL. This method reduces the manual manipulation of shaker flasks under a laminar hood, which can make the cell culture vulnerable to contamination and

operation error. It also reduces the time needed for the culture by reducing the steps taken to reach a final working volume in the bioreactor (Wright et al, 2015).

Noninvasive methods for gathering data on small cell cultures, as in shake flasks and microtitre dishes, are also in demand. Typically the pH and and dissolved oxygen of the culture must be determined via withdrawing a sample from the culture container, which carries with it an inherent risk of contamination. One potential method for measuring dissolved oxygen in these containers noninvasively is through the use of fluorescence-based optical sensors. By utilizing small chemical optical oxygen sensors via the application of oxygen sensitive fluorophores, non-invasive measure of dissolved oxygen can be made possible (Schneider 2010).

When reviewing the biomanufacturing literature and interacting with current professionals, it is apparent that the field is attempting to move away from media components that may contain adventitious agents. These are agents that may disrupt the cell culture process via contributing to contamination (Furtak et al., 2015). One component of the cell culture media that may possess unwanted adventitious agents is serum. Serum can be derived from several animal sources, but is most commonly attributed to bovine roots. Instead of attempting to improve the diagnostics of the field and researching how to pinpoint the identification of malevolent viral and bacterial agents, one option is to halt the utilization of serum in the media. This would decrease the potential for any unidentified pathogens to contaminate the cell culture. However, when serum is removed from traditionally serum-infused cultures, difficulties may arise that lead to less successful cultures. Serum is often used to help supply the culture with proteins and growth factors that cells have access to in vivo. When this medium is taken out of the culture process, cells may not thrive as they did in the presence of serum. Not utilizing serum may also impact other cellular functions such as attachment to microcarriers (Genzel et al.,

2006). Serum free media may also contain detergents and low levels of $CaCl_2$ which negatively affect cell attachment (Genzel et al., 2006). To address this, the use of $CaCl_2$ may be used. This addition in serum-free media has shown improved cell attachment rates of up to 50% when compared with the traditional serum-supplemented media. Cell attachment was improved when using Cytodex I microcarriers, specifically. In addition, EDTA/PBS solution for cell harvesting instead of trypsin has also been shown to enhance cell attachment (Merten, 2000). These additions to cell culture media may be vital if serum is not utilized.

# Chapter 3: Project Strategy

## 3.1 Initial Client Statement

The first part of the initial client statement for this project was to "understand the earlier process of upstream bioprocess production (vial to seed train). GEHC wishes to understand customer challenges and potential opportunities for innovation to resolve these challenges." This included conducting client interviews with customers utilizing diverse seed train processes to better understand issues that they continually experienced. The second part was to "investigate the feasibility of XDR 200 and microcarrier cell culture technology for vaccine production utilizing Vero cells." There is a complete lack of data on using the XDR-200 to grow Vero cells on microcarrier beads, which is the driving force behind this project. Additionally, GEHC wanted the team to determine the optimal conditions for Vero cell growth in the XDR-200 and work to design a seed train that resulted in a larger population of viable Vero cells.

## 3.2 Design Requirements

### 3.2.1 Technical Requirements

The team developed a list of objectives, constraints, functions, and specifications that the final design must meet to be successful. In order to understand the earlier process of the upstream bioprocess production in addition to investigating the feasibility of the XDR 200 and microcarrier cell culture technology for vaccine production utilizing Vero cells, the team not only conducted multiple experimental runs, but also contacted customers of single-use technology to better understand the seed train process and the constraints they have come across

during the procedure. Obtaining customer pain points as well as running experiments helped develop design requirements for the seed train process. The design of the procedure has to be efficient with very little risk of contamination. The process must be able to allow for maximum cell growth and attachment to the microcarrier beads, as well as bead-to-bead transfer within the culture.

### 3.2.2 Objectives

The key goals of the project were determined from the need statement provided by the client. The goals allowed for an understanding of the objectives that are to be accomplished throughout the process, as well as the constraints of the design parameters.

The main goal of this project based on the client statement is to determine the feasibility of General Electric Health Care's XDR-200 Bioreactor in culturing viable Vero cells with microcarrier technology. The project will also develop an effective method for upscaling Vero cells into a 200 L bioreactor and producing the maximum amount of cells that can be used for vaccine production. This leads to the first objective, which is to determine the feasibility of using microcarrier bead technology in conjunction with single-use bioprocessing. This experiment will be conducted using and gathering data for the XDR-200 bioreactor. The data includes the speed at which the impeller will need to rotate in order to experience proper mixing, while applying minimum shear stress to the microcarrier beads that have cells attached. A proper impeller speed is required in order to acquire proper mixing and culturing. In addition, if the impeller speed is too high, it can crush the beads and destroy adherent cells. Therefore, along with obtaining data on agitation rate, the experiment will also measure bead-to-bead transfer in order determine the feasibility of using microcarrier bead technology with single-use technology. This will be done using visualization under a light microscope, as well as cell counting to determine if the cells are

proliferating and attaching to the microcarrier beads to prove that Vero cells can be grown on microcarrier beads in an efficient manner to produce a large mass of cells for vaccine production.

A second objective is to identify and maintain ideal cell conditions for proper cell growth and viability within the XDR-200 reactor. This includes the proper glucose balance, which should have a maximum of 5 grams per liter, and a decrease in glucose value will demonstrate cell growth and proliferation due to more cells uptaking glucose;  pH level, which should remain around a neutral pH between 7.1 and 7.4; temperature, which should stay steady around 37°C, and gas levels, which should maintain dissolved oxygen level close to 40%. Proper cell culture conditions play a major role in dictating the number of cells produced and their general health. By identifying the proper ranges and values through literature, speaking with experts in the industry, as well as experimentation for these influential factors, the team can maximize the number of cells produced.

The third objective is to scale up the culture of the cells more efficiently. When culturing cells, there is always a risk of contaminating the culture, leading to the destruction of potentially weeks worth of work, not to mention the financial loss. By minimizing the number of steps in the seed train and limiting human interaction and handling of the cells, potential risk is greatly cut. Additional attention must be paid to the means by which scaling occurs. Some process steps involve large financial commitments, like constructing a high-density seed back or purchasing multiple hyperflasks for culturing cells. A goal of this objective is to devise a more efficient and robust method for the seed train procedure in order to maximize cell viability within the bioreactor utilizing Vero cells for vaccine production. Figure 3 below illustrates the objectives of the project broken down into sub-objectives.

The final objective of the project is to better understand the pain points and constraints

that customers of single-use technology experience during their seed train process. The pain points would be relayed to General Electric so that they can better develop their products for retail. Obtaining pain points also helped provide various perceptions of how the industry approaches their seed train process, and provided better comprehension on the methodology that the group would proceed with during experimentation. An outline of the team's objectives can be seen in Figure 3 below.

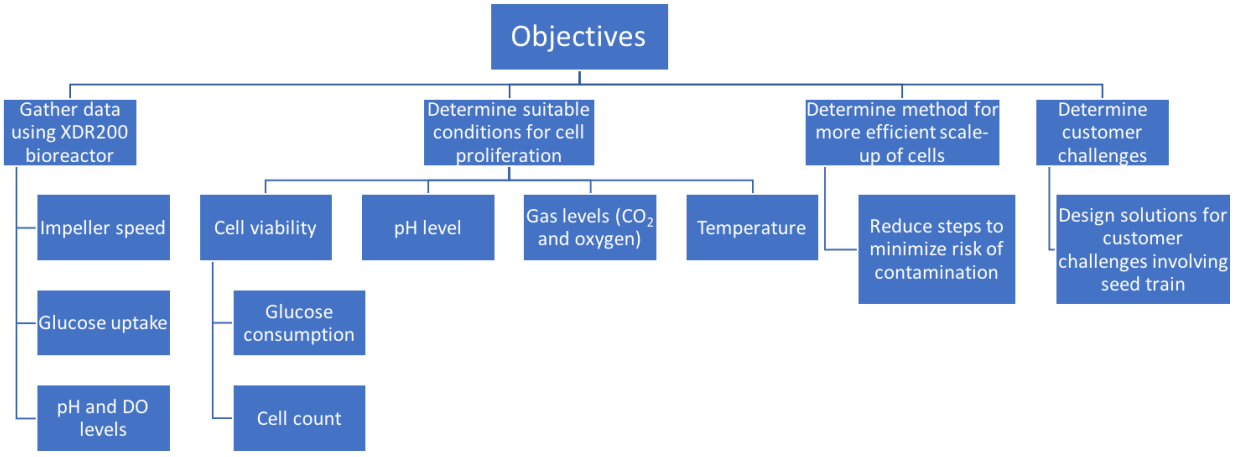



*Figure 3: Objectives of the Project*

### 3.2.3 Constraints

There are multiple constraints that need to be taken into consideration during the procedure. The biggest challenge consists of lack of time. The entire upscaling process from a 2 mL vial to a 200 L bioreactor can take at least a month, and that does not include the time it takes to receive materials. Careful planning will be required in order to ensure effective and efficient runs consisting of upscaling cells. The bioreactor redesign mandates a minimum of one month of

time in advance. The seed train into the bioreactor began on February 18th and the cell culture within the bioreactor was ended on April 7th. In addition to time, budget is also a constraint in the project, as each team member was provided $250.00, which totals to a total budget of $1250.00. In addition, the project is limited to mostly General Electric products, which reduces the amount of methods that can be utilized to find the best possible process to maximize cell viability. It would be costly to lose the progress of the production of a large sum of cells due to a mistake after many hours put into the procedure. The cell culture seed train process is expensive, especially concerning the purchase of material. The project requires purposeful runs to maximize the data gathered. This leads to the constraints involving the obtainment of project materials. The disposable materials such as bags and sensors are also costly to replace, so it restricts the amount of tests that can be run. Technical constraints must also be taken into account, like the maximum saturation of microcarrier beads in solution, which for Cytodex 1 is about 35 cells per bead; the limited surface area of these beads, approximately 4400 $cm^2/g$, and the limited amount of media in the reactor, which holds a maximum of 200 L.

### 3.2.4 Functions

First and foremost, the impeller tip within the reactor must not destroy the microcarriers. GE Healthcare has expressed issues with this dilemma in the past, and it is vital this does not occur. Microcarriers are the reason adherent cells can be grown in the bioreactor, as they provide a surface to attach to. If the impeller is decreasing the number of these beads, then surface area for the cells will be lost, along with the cells already attached to the beads. Secondly, bead to bead transfer must occur within the bioreactor. If cells are to grow and increase in number, then they must transfer from one bead to another. Third, the XDR-200L bioreactor must maintain consistent cell culture conditions throughout the course of the run. Cells are fragile and must not

undergo large changes in media composition, pH or temperature, as this can lead to altered growth rates (Wu, 2010). When adding in dissolved oxygen or raising the temperature/pH, it is imperative these types of actions do not result in changes that expose the cells to harmful levels. Lastly, cell viability and glucose uptake levels must be comparable to currently existing data for a 50L bioreactor. If this can not be achieved, then the goal of illustrating that the XDR-200L bioreactor can feasibly be used for vaccine production will not be met.

The second part of the project looks to create a more efficient seed train for the Vero cell line. This has been carried out within the industry of vaccine production for many years, but may be better optimized. A more robust method will be characterized by two main functions: minimize the risk of contamination and decrease the time it takes to move from frozen vials to the 10L Wave bioreactor. This will be achieved through the use of different flasks and altering how many cells are initially thawed from the seed bank.

### 3.2.5 Specifications

The specifications of the XDR-200L can be seen in the list below to ensure a proper cell culture includes maintaining a pH between 7.1 - 7.4. A mammalian cell culture, such as Vero cells, thrive best in a neutral environment, but some cells have been shown to function in slightly acidic environments (Microcarrier Cell Culture: Principles and Methods, GE, 1981). The culture also requires a carbon dioxide environment that maintains a 5% $CO_2$ ± 0.5% (GE, 1981), which helps regulate the pH of the environment, and can be controlled with a buffer solution such a HEPES, or a $CO_2$-bicarbonate based buffer solution. The pH of a culture is based off the balance between $CO_2$ and bicarbonate, so it is important to utilize proper levels of $CO_2$ when culturing in media that contains a buffer solution. The reactor will provide $CO_2$ to lower the pH, and provide a sodium bicarbonate to raise the pH. The cell culture also needs a 50% ± 10% dissolved oxygen

(DO) level to survive (McGlothlen & Asher, 2016). This is calibrated by using a DO probe within the reactor, that is set to 100% when the media is saturated with oxygen. Once the level drops below 40%, the controller will sparge air to provide more oxygen for the cells. The cell culture requires a media temperature of 37°C ± 1°C to keep the cells viable. The reactor control will pump cold water through the jacket if the temperature raises above the setpoint, and will pump hot water if the temperature falls below the setpoint (General Electric, 1981).

Specifications of the XDR-200 also require that the culture maintains a high cell viability in order to produce the maximum amount of cells possible for vaccine production. By taking in all factors mentioned in the previous paragraph, avoiding risk of contamination, and controlling the speed of the impeller in order to avoid the crushing of beads, the culture should maintain a cell viability greater than 90%. The XDR-200 must be able to hold $1.8 \times 10^{6}$ cells/mL, and a total of $3.6 \times 10^{12}$ cells. These are the baseline standard values that have been achieved by previous research. The group planned to upscale to this value within four weeks. In addition, the group planned to inoculate 100,000 – 300,000 cells/mL along with 4 grams/L of microcarrier beads.

These parameters must be held consistent within the bioreactor during upscaling of Vero cells. The last specification is impeller speed and power. If the impeller spins too rapidly, the cells will be put under harmful shear stress that could damage or outright kill the cells. On the other hand, if the impeller spins too slowly, there will be insufficient agitation, which will result in decreased gas and nutrient exchange, resulting in slowed cell growth or cell death. One of the objectives of this project is to determine the proper impeller settings for agitation.

In relation to the Vero cell seed train development, specifications are more difficult to impose. The current time frame for a scale up from frozen vial to working in the 200L bioreactor

takes about 17 days [GE Interview, 2016]. To be successful, this amount of time should be reduced. To test this, the group will experiment with different procedures to minimize the steps needed to reach the XDR. One method that can be utilized involves upscaling from T-300 flasks, to hyperflasks, into the wave bioreactor, and finally the XDR 200. This process eliminates the step of transferring between multiple T-flasks. Another method that can be utilized the use of spinner flasks instead of hyperflasks and the wave bioreactor. This method also reduces the steps of transfer between flasks by upscaling from T-flasks to 3 L spinners into the bioreactor. This will theoretically result in a reduction in the amount of time it takes to begin using the XDR-200. However, this method might result in a lower cell density compared to the hyperflask and wave bioreactor method.

## 3.3 Design Requirements (Standards)

Within the scope of the project, there are two focal points where the team needs to ensure standards are being followed. The first is in relation to cell culture and creation of procedures to upscale of Vero cells. The Code of Federal Regulations (CFR) Parts 210 and 211 mandate current good manufacturing practices (cGMPs) in relation to manufacturing of pharmaceuticals (Code Federal Regulations, n.d.). These cells will eventually be used for creation of vaccines, which fall under this jurisdiction. Subparts F: Production and Process Control and J: Records and Reports are the most notable. To be able to ensure product quality and reproducibility, procedures must be written explicitly. Also, records must be kept to check specific batches in case of recalls or investigation should occur. These cGMPs are being followed within the Biomanufacturing and Education Training Center (BETC), as procedures and notes are written with each lab entry. These are then compiled and added to a lab notebook to track the project's

progress.

Additionally, the second portion of the project focuses on the use of the XDR-200 bioreactor. When operating an instrument, it is imperative to follow the manufacturer's guidelines associated with that instrument to ensure proper use. GEHC has provided this manual, which entails working specifications, along with Standard Operating Procedures (SOPs). Should the team find it necessary to alter these, all steps will be approved through GEHC, thus certifying the actions. The instrument is intended for single use, but if contamination is to occur, then cleaning will comply with CFR 210 & 211 subpart J, where cleaning must be documented. (Code Federal Regulations, n.d.). These records will be kept along with the lab notebook. Also, if designs of the instrument or microcarriers are to be altered, then materials must meet specifications set by United States Pharmacopeia (USP) Class VI which regulates biocompatibility of materials. In terms of upstream biomanufacturing for vaccine production, ISO standards 9001: Quality Management Systems and 13485: Medical Devices - Quality Management Systems are used (ISO, 2016). Lastly, when models are created by CAD, they must be compliant with ISO 13567 and 16792: Technical Product Documentation (ISO, 2016). These outline specifications that design drawings must adhere to, which will be followed by defining all parts of drawings, and matching the criteria outlined by ISO.

## 3.4 Revised Client Statement

Further meetings with GEHC, WPI advisors, and specialists working at the Biomanufacturing Education and Training Center at WPI served to clarify the overarching goals of the project. One major goal that became apparent after meeting with GEHC was proving that the impeller used in the bioprocess was not damaging or crushing beads and therefore killing

cells. Previous data from a smaller bioreactor had indicated that this was a distinct possibility, so ensuring that no cells were lost due to bioreactor design was a major client goal. In addition, a greater focus on optimizing the early seed train process was stressed. The team had previously prioritized studying primarily the XDR-200 and its processes, but the importance of the entire upstream process was advocated by advisors. By minimizing the amount of time and resources spent in the early stages of cell growth before introduction into the bioreactor, the process as a whole would be simplified and shortened, leading to a lower risk of contamination or mechanical malfunction. GEHC also clarified that they would like to see the team submit any recommendations for improving the actual XDR-200 system or process, and put these recommendations into practice if time allowed it.

With these new objectives in mind, the client statement was expanded to encompass all of the goals of the project. The revised client statement is to optimize the earliest stage of the upstream bioprocess production by minimizing required steps, risks of contamination, and cost, to investigate the feasibility of the single use XDR-200 bioreactor in conjunction with microcarrier cell culture technology, especially the impeller, for growing Vero cells for vaccine production, and to recommend and implement design changes in the XDR-200 cell culture environment, impeller, and bioprocessor.

## 3.5 Project Management

The project approach can be broken down into six sections which are:

- Develop a Vero cell bank
    - High density and low density banks
    - Thawed from BETC Vero cells

- Development of a robust and efficient seed train process
    - Test several different methods for upscaling. One method included the utilization of hyperflasks and upscaling into the wave bioreactor before potentially inoculating the XDR at a high cell density. Another method was to use multiple T-flasks to upscale into 500 mL spinner flasks, and then upscaling into 3 L spinner flasks in order to condition the cells and the microcarriers in an environment that utilized a spinning impeller prior to inoculating the XDR 200.
    - Eliminate steps and minimize risk of contamination. First, the steps that were able to be eliminated had to be evaluated. Through literature review and discussion with experts in the industry, the team was able to conduct various methods in order to reduce steps in the early seed train process, which has the highest risk of contamination. Reduction of the transfer process from different flasks would be ideal to eliminate steps, which was tested through the use of hyperflasks and the Wave bioreactor.
- Analyze Bead-bead transfer
    - Analyze cell distribution over beads
    - Determine if impeller stop/trypsinization necessary
- Test the impeller and XDR-200 without cells
    - Particle analysis to gauge if beads harmed
- Test and sampling the vero cell culture in the XDR-200
    - Determine optimal ranges for pH, DO level, glucose supply, and agitation rate of the impeller for culture in the XDR-200
- Redesign the seed train or XDR-200 design if data fails and the experiment is not able to

obtain proper bead-to-bead transfer in addition to proliferation and high viability of cells.

- Troubleshoot encountered problems and propose solutions
- Submit potential designs to GE for manufacture

The sections can be seen in Figure 4 and a breakdown of the approach is illustrated.

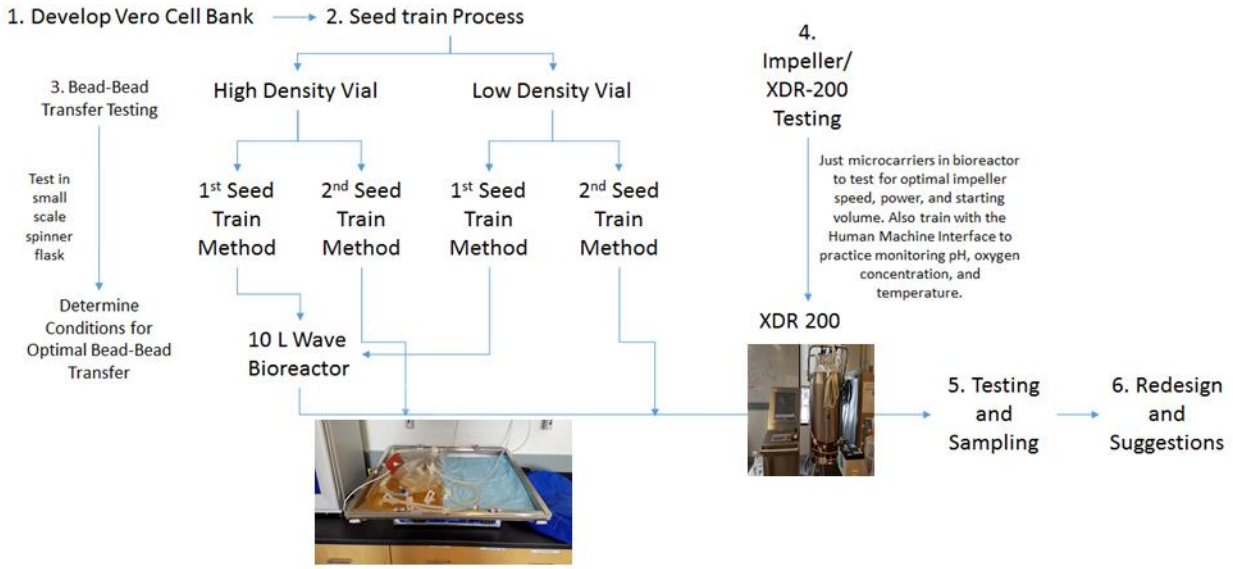



*Figure 4: Flowchart of Project Approach*

The first portion involves developing a Vero cell seed bank, seen in Step 1. This is created by culturing an initial vial, then refreezing cells for later use. This will help ensure variation in cell development is kept to a minimum, as the cells being used are all of the same cell line and population doubling level. Step 2 involves different methods for seed train development. Figure 5 shows two outlined methods for the seed train development process. Both methods will be carried out from frozen cell vials at high density (2mL vial with 20 million cells/mL) and low density (2mL vial with 10 million cells/mL). The low density vials are the conventional method used. The first method encompasses moving cells from a high density frozen vial to be plated on four T-185 T flasks. For the low density vial, the cells will be plated

on two T-185 T flasks. Each T-185 flask holds roughly 30 million cells at confluency. In four days the cells will be transferred from the T-185 T flasks to T-300 T flasks for four more days. The T-300 T flasks hold around 50 million cells at confluency. Then the cells will be seeded onto microcarrier beads in the 10L wave bioreactor at a 2 L working volume. Media and beads will be added until around 4 billion cells are present in the wave bioreactor with a working volume of 10 L. Then the cells will be transferred to the XDR-200 at a working volume of 40 L. The second method skips over the T-300 T flasks and wave bioreactor with the addition of hyperflasks. Each hyperflask can hold around 250 million cells which allows for the scale up to 4 billion cells for the XDR-200 with fewer steps. This method is able to reduce the steps in the early stages of cell culture by inoculating a high density of cells into the XDR without having to go through additional steps of trypsinization and the transfer into additional flasks. .

Step 3 is to analyze small scale bead to bead transfer to see if feasible for the XDR-200. This will occur through sampling microcarriers in a small scale reactor called a stir beaker. The steps include beginning with a confluent amount of microcarriers in culture, adding a known amount of new beads, and monitoring cell growth. If the population increases, it can be understood that cells are adhering to new beads.

Step 4, testing the impeller, will be conducted by carrying out test trials with just microcarriers in the XDR-200L reactor. Here, the team will slowly increase speeds and power input to see the effect on the microcarriers. This can be conducted visually through the use of a microscope after a sample is taken, or utilizing a particle analyzer to see if the beads have been broken into smaller pieces. After it is shown microcarriers can be used within the XDR-200, cells will be added and tested for viability in Step 5. Cell culture conditions such as temperature, pH, DO, and glucose uptake will be monitored as cell growth occurs within the bioreactor. If

need be, the seed train or XDR-200 design may need to be revisited in Step 6 if gathered data does not show feasibility of vaccine production.

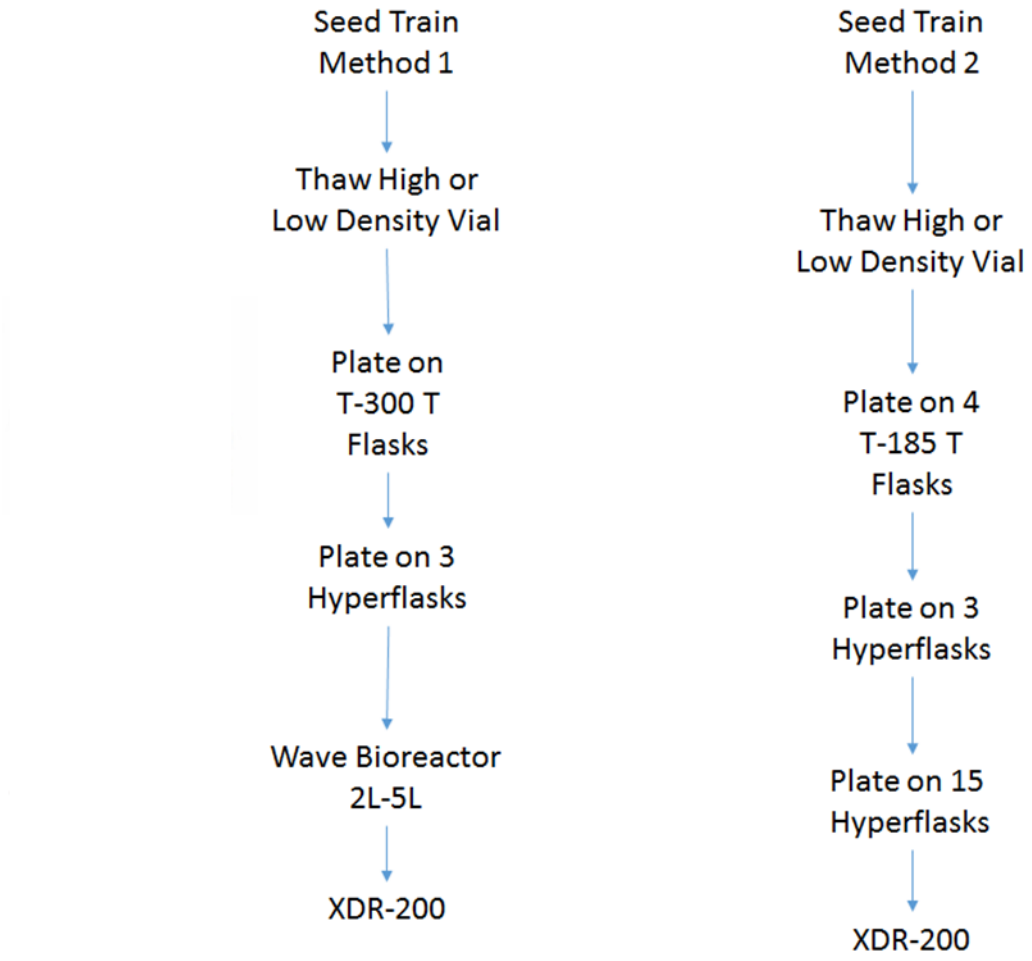



*Figure 5: Outlined Seed Train Methods*

The timeline for major milestones for each section of the project was plotted out in a Gantt chart that can be seen in Figure 6. The date for completion of the proposed tests was April 7th, 2017. The time constraint for each run of the XDR-200 is 35 days which includes the seed train process. Therefore seed train trials would be staggered to ensure more tests completed for the XDR-200.

| Objectives | 1/16 | 1/23 | 1/30 | 2/6 | 2/13 | 2/20 | 2/27 | 3/3 | 3/10 | 3/17 | 3/24 | 3/31 | 4/7 | 4/14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Wave Reactor Test 1 | | | | | | | | | | | | | | |
| 500mL Spinner Tests | | | | | | | | | | | | | | |
| Wave Reactor Test 2 | | | | | | | | | | | | | | |
| XDR-200 Final Run | | | | | | | | | | | | | | |
| Conclude Data Analysis | | | | | | | | | | | | | | |

*Figure 6: Gantt Chart of Experiment and Testing Timelines*

# Chapter 4: Design Process

## 4.1 Needs Analysis

The group developed a needs analysis based on the client statement and objectives of the project. In order to determine the actual requirements of the project, the group generated a pairwise comparison chart (PCC) to establish which parameters would be considered a "need" and which parameters would be considered a "want" for the project. A "need" is defined as a certain property that the final design must have. A "want" refers to properties that the group would like to have, but may not be possible due to possible constraints discussed in this section.

### 4.1.1 Needs and Wants

Based on the specifications, functions, and parameters of this project, the group developed the important functions for the final design. First of all, to meet the design baseline on what has already been tested, the XDR-200 must be able to hold $1.8 \times 10^8$ cells/mL, and a total of $3.6 \times 10^{13}$ cells. These are the baseline standard values that have been achieved by previous research. The group is looking at a time frame of under 4 weeks to upscale to this value. In addition, the group will need to inoculate 100,000 – 300,000 cells/mL along with 4 grams/L of microcarrier beads. Furthermore, it would be beneficial to utilize serum-free media, as this helps to eliminate the presence of unwanted adventitious agents (Furtak et al., 2015). Another cost effective parameter is to utilize the wave bioreactor before entering the XDR-200. This will minimize the amount of T-flasks used, and also reduces the risk of contamination during cell culturing since there is less transfer between different flasks. In addition, it would be beneficial to utilize bead-to-bead transfer when inoculating the cells into the bioreactor in order to avoid trypsinization and have more efficiency in the transfer of cells. Finally, ensuring that minimal

microcarriers are destroyed in the process is a major goal of both the team and GEHC. These parameters were compared against each other in the pairwise comparison chart (PCC, Table 2) below to determine the most important needs.

*Table 2: Pairwise Comparison Chart*

| Parameter | XDR-200 holds a total of $3.6x10^{13}$ cells | Under 4 weeks for 1 run | Serum-free media | Use of bead-to-bead transfer | No bead destruction | Use of wave bioreactor | Total |
|---|---|---|---|---|---|---|---|
| **XDR-200 holds a total of cells** | x | 1 | 1 | 0 | 1 | 1 | 4 |
| **Under 4 weeks for 1 run** | 0 | x | 0 | 0 | 0 | 1 | 1 |
| **Serum-free media** | 0 | 1 | x | 0 | 0 | 1 | 2 |
| **Use of bead-to-bead transfer** | 1 | 1 | 1 | x | 1 | 1 | 5 |
| **No bead destruction** | 1 | 1 | 1 | 0 | x | 1 | 4 |
| **Use of wave bioreactor** | 1 | 0 | 0 | 0 | 0 | x | 1 |

The most important needs of the project were determined by the PCC, where a 1 ranks a parameter that is more important, 0 is less important, and 0.5 is equally as important. Based on this method, it was decided that any parameter $\geq 4$ would be considered a need, and anything $< 4$ would be considered a want. These criteria established what was most important:

1. The use of bead-to-bead transfer

2. No bead destruction
3. The XDR-200 holds a total count of $3.6 \times 10^{13}$ cells

These three priorities are factors that will impact how efficient the use of the XDR is in growing Vero cells. Microcarriers will be the vessel cells are able to grow on, and throughout the culturing process, will be responsible for the continual growth of cells. As the beads come into contact with one another, cells are expected to spread and attach to other beads. A priority that pairs with this is the prevention of bead destruction. If beads are to be destroyed by the impeller, then this reduces the total amount of beads within the bioreactor, ultimately limiting how many cells may be grown. The final priority is to ensure that the XDR cultures $3.6 \times 10^{13}$ cells - as this total will be a benchmark of how successful the XDR-200 bioreactor culture is. This will not be able to happen if bead-to-bead transfer is limited or interrupted. If all three needs are able to be achieved, then the scale-up to the XDR-200 will be effective.

Behind the needs are what was determined to be wants - appearing from most desirable to least:

1. Use of Serum - free media
2. Use of Wave bioreactor
3. Under 4 week timeline for full culture

These needs were were deemed less important through the use of the PCC, but are still desired. Serum free media is notably less expensive and can reduce the total cost of the cell-scale up procedure. Before its viability is experimented with, however, the XDR must prove successful with bead-to-bead transfer and yielding a certain number of cells. Ultimately, the field of biomanufacturing is moving towards serum free media cultures and it would be desirable to meet this new practice. Additionally, utilizing the Wave bioreactor would also be advantageous. The

Wave bioreactor is a system that provides a reduced chance of contamination when compared with flasks. It also allows for easier sampling of the media. However, this step in the scale-up of Vero cells is not imperative to making the XDR-200 successful, as other methods may be employed. Lastly, creating a seed-train that can be completed in under 4 weeks would allow for quick turnover of a cell culture and allow for more scale-ups to occur. The shorter this timeline is, the more times it can be repeated which translates to more revenue for industry.

### 4.1.2 Physical Constraints

Due to various physical limitations, there were some parameters of the design that cannot be achieved. One of these constraints includes the volume limit of the XDR-200. The bioreactor has a 200 L limit, which restricts the amount of cells and microcarrier beads that can be inoculated into the bioreactor at one time. Another constraint is the surface area of the microcarrier beads in addition to the saturation level of the beads. The approximate surface area of the microcarriers that will be utilized, Cytodex 1, is about 4400 $cm^2/g$. These factors can be considered physical restrictions to the maximum amount of cells that can be attached to the microcarriers. Therefore, this reduces the number of cells that can be generated throughout the process. In terms of the microcarrier, they suffer from the physical constraint of having high shear sensitivity. Due to this, the amount of power put into the bioreactor must be limited. The company Sartorius' BIOSTAT single-use bioreactor uses a power output of 0.0223 W/L (De Wilde, Davy et al. 2009). This model would be used to set the impeller power output for the XDR at 0.892 W for the initial filling of the reactor and 3.35 W for the final fill of 150L. A high shear stress applied to the beads from the impeller can maim the beads, as well as the Vero cells attached to them.

## 4.2 Conceptual Designs and Feasibility Studies

A functions and means table was created by the design team to help facilitate brainstorming of different scaling up procedures. This also included XDR-200 design conditions which accomplish the functions needed for a successful project. The four functions listed in Table 3 were used to brainstorm different means to accomplish those functions. The means brainstormed for each function are detailed in Table 3.

*Table 3: Vero cell upscaling to XDR-200 functions and means*

| **Functions** | **Means** | | | | |
|---|---|---|---|---|---|
| **Bead-Bead Transfer** | Spinner flasks | XDR-200 | Wave bioreactor | | |
| **Prevent Crushing of microcarriers** | Adjust impeller tip speed/power output | Design mesh to prevent contact with impeller | Use of two impellers | | |
| **Seed train to n-1(step before XDR-200 inoculation)** | Hyperflasks | Tissue flasks | High/low density of cell bank | Wave bioreactor | Rolled membrane flask |
| **Optimal cell growth in XDR** | Starting volume | Density of microcarriers | Density of cells | Agitation rate | |

### 4.2.1 Feasibility Studies

This section will cover the methods which will be used to determine whether potential seed train designs and bioreactor parameters are capable of meeting the needs requirements.

#### 4.2.1.1 Bead-to-Bead Transfer

In order for the vero cells to efficiently grow within the XDR-200 bioreactor, it is essential for them to be able to spread to other microcarriers and gain room to grow. Once a microcarrier has achieved confluence, no new viable cells are produced on that microcarrier. The ability of the cells to transfer between beads is primarily affected as a function of agitation rate

and microcarrier density. Previous research has demonstrated that a shear stress between 0.65 - 1.35 $N/m^2$ produced morphological changes, loss of viability, and cell removal from the microcarrier beads. In addition, a shear stress value greater than 2.6 $N/m^2$ demonstrated more than 75% cell removal from the microcarrier beads (Otto-Wilhelm Merten 2015). Small scale tests in spinner flasks were performed to help determine an environment potentially most suitable for vero cell culture in the XDR-200.

#### 4.2.1.2 Spinner Flask Baseline Test (Constant Agitation)

The baseline test utilizes two 500 mL Corning spinner flasks to simulate an environment similar to the XDR-200 bioreactor. The baseline test parameters were determined from similar Millipore tests (McGlothlen, 2016) and GE's Wave Bioreactor manual. The flasks are filled to a 150 mL working volume of MEM 5% CFS with a microcarrier density of 4 g/L. They are inoculated with a vero cell density of $2.5x10^5$ cells/mL from T-300 flasks. Once inoculated the agitation rate is initially set to intermittently 50 rpm with a duty cycle of 55 minutes off 3 minutes on for the first 18 hours. Intermittent agitation is necessary to allow the vero cells to initially settle and attach to the microcarriers. The flask is then set to agitate at a constant rate for the remainder of the culture. Throughout the culture the microcarriers are sampled daily, visually inspected with phase shift microscopy and a media analysis is performed. These tests document the bead confluency percentage, track glucose uptake rate, lactate density, ammonia density, LDH density, L-glutamine density and glutamate. Once the microcarriers reach a confluency >75%, a final 150 mL (300 mL total) volume of conditioned bead slurry is added to the flasks. The flasks are then set to intermittently agitate with a 55 minute off 3 minute on duty cycle at 50 rpm for 18 hours. The agitation is again set to a constant 50 rpm for the remainder of the culture. The flasks are regularly sampled for the remainder of the culture. From this test we see that in

the small volume flasks the Vero cells are able to attach and grow on the newly added microcarriers.

One of our feasibility studies was to experiment bead-to-bead transfer within spinner flasks prior to inoculation of the XDR 200. Three 500 mL spinner flasks were inoculated at a $3.77*10^5$ cells/mL with 97.4% viability at a working volume of 300 mL and were observed over the course of 5 days. Illustrated below in Figures 7-9 are images showing cell attachment onto the microcarrier beads over the course of 5 days for each of the three spinner flasks.

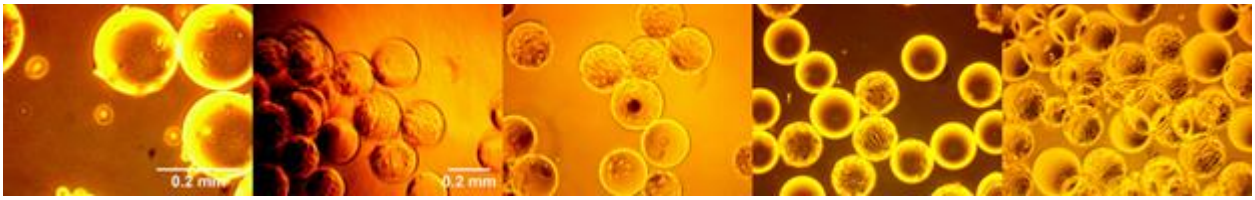



*Figure 7: Spinner flask 1 microscope images. From left to right is 3 hours post inoculation, day1, day 2, day 3, and day 4. Scale bars shown are 0.2 mm in length (Images 2-5 taken at the same magnification)*

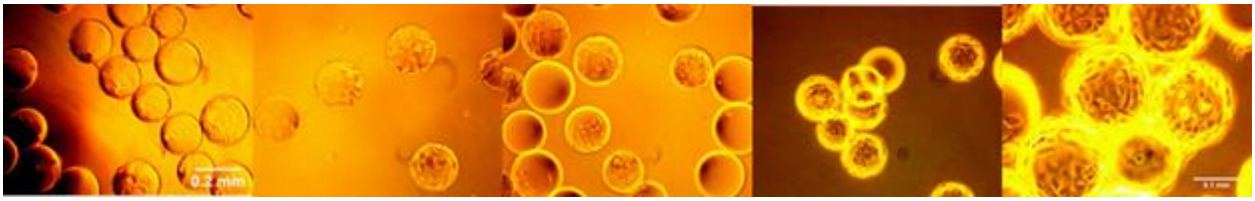



*Figure 8: Spinner flask 2 microscope images. From left to right is day1, day 2, day 3, day 4, and day 5. Scale bars shown are 0.2 mm in length (Images 1-4 taken at the same magnification)*


*Figure 9: Spinner flask 3 microscope images. From left to right is day1, day 2, day 3, day 4, and day 5. Scale bars shown are 0.2 mm in length on the left and 0.5 mm on the right (Images 1-4 taken at the same magnification)*

The first set of graphs pictured below in Figures 10-12 illustrate the glucose and lactate metabolite concentration for each of the three spinners. The initial low concentration of glucose was due to the utilization of unsupplemented media, and the spike in glucose occurred due to the supplementation of additional glucose on day 3. However, glucose concentration did decrease from 1 g/L to depletion, and then 4 g/L to 3.56 g/L in association with the increase up to 1.1 g/L in all three spinners, signifying slight cell growth and proliferation.

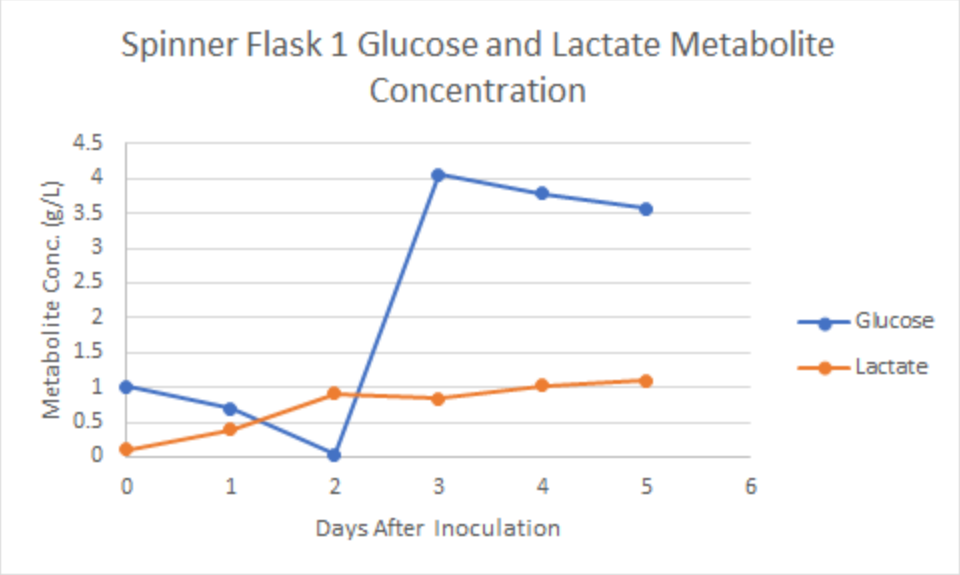



*Figure 10: Glucose and lactate metabolite concentration in spinner flask 1*

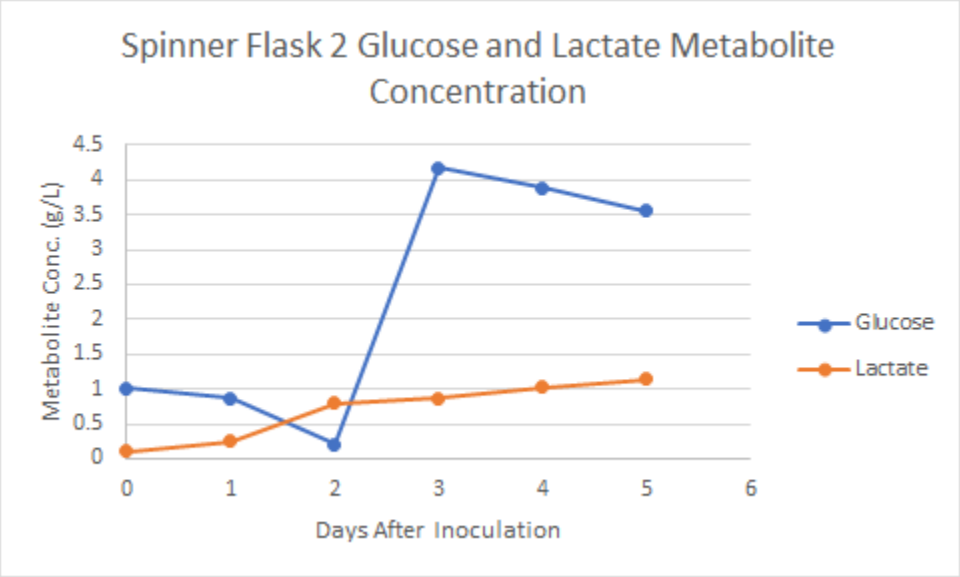



*Figure 11: Glucose and lactate metabolite concentration in spinner flask 2*

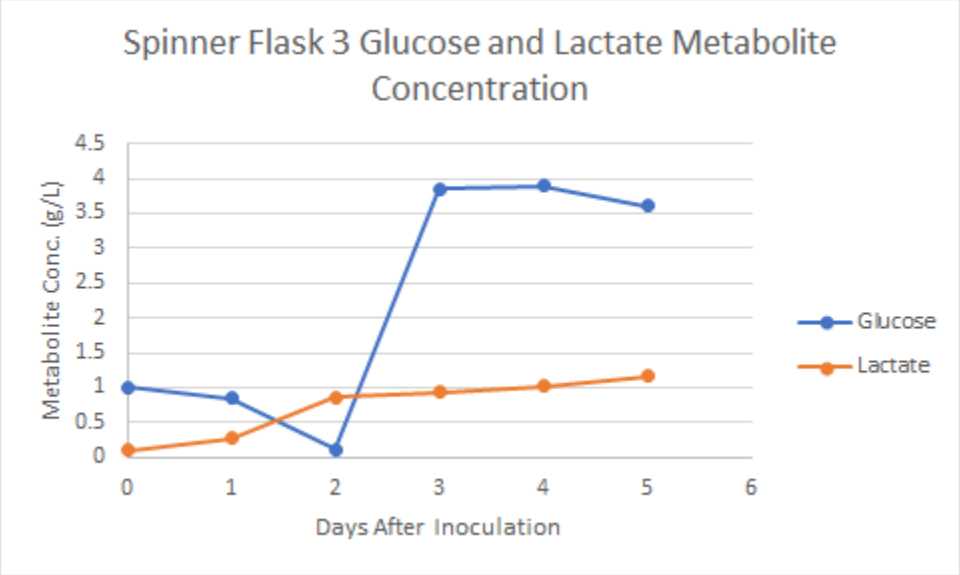



*Figure 12: Glucose and lactate metabolite concentration in spinner flask 3*

Figures 13-15 below illustrate the ammonium and glutamine metabolite concentration of each of the three spinners. Similar to glucose, glutamine was also supplemented on day 3. The decrease in glutamine and the increase in ammonium concentration signifies cell growth within each of the spinners. Spinner 3 resulted in an increase in glutamine on day 5.

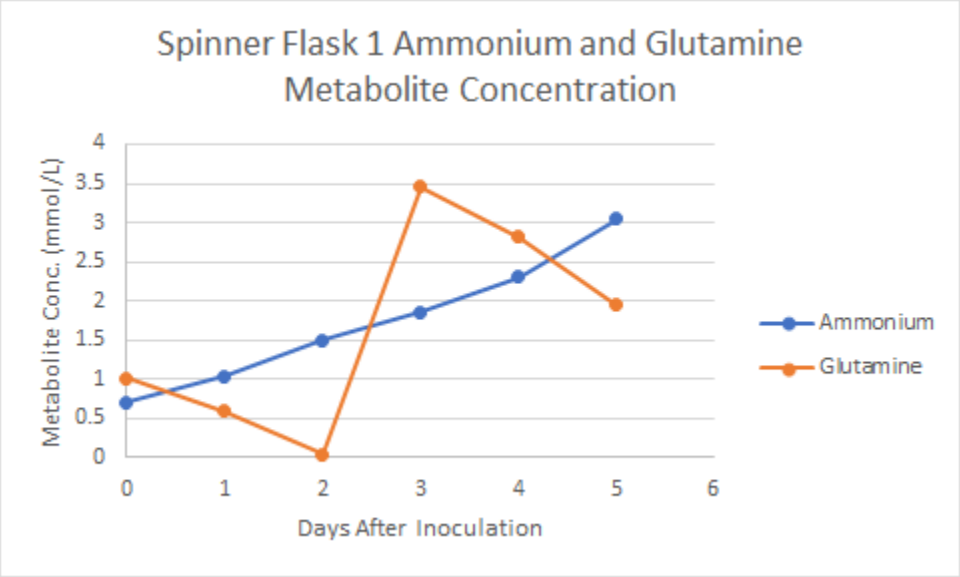



*Figure 13: Ammonium and glutamine metabolite concentrations in spinner flask 1*

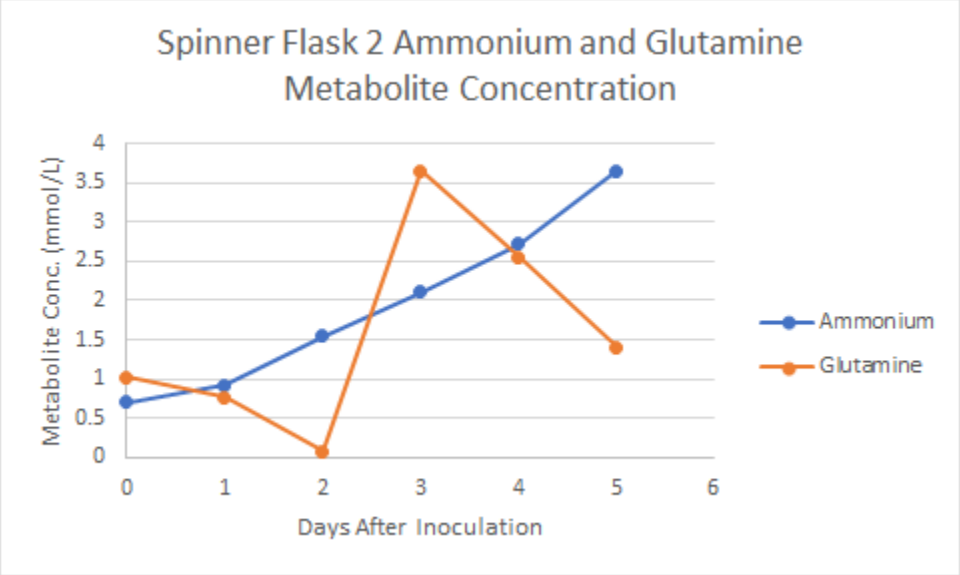



*Figure 14: Ammonium and glutamine metabolite concentrations in spinner flask 2*

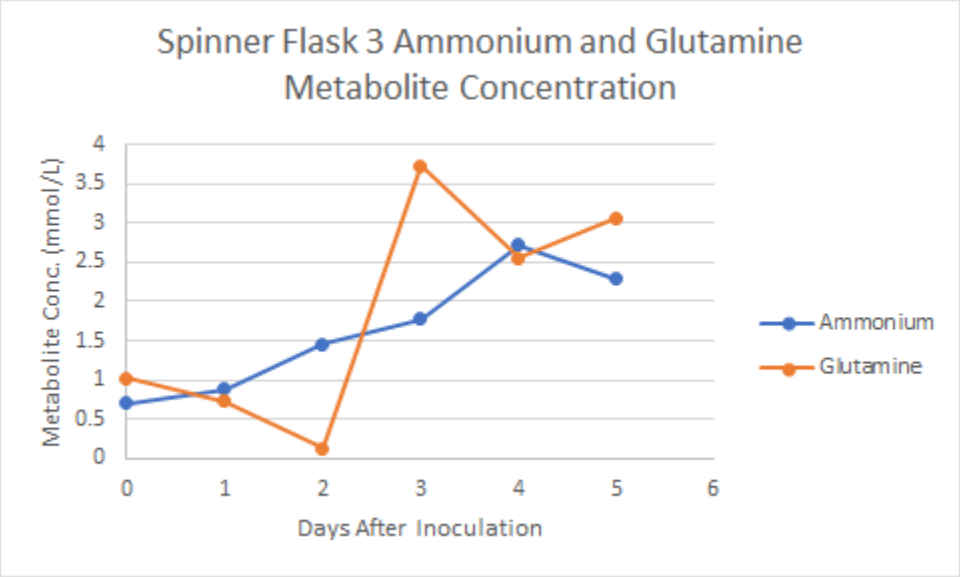



*Figure 15: Ammonium and glutamine metabolite concentrations in spinner flask 3*

The next set of figures, Figures 16-18, show the glutamate metabolite concentration. The increase in glutamate concentration shows cell growth due to the uptake of glutamine and release of glutamate. Spinner 2 demonstrates the most amount of cell growth, and spinner 3 has a decrease in glutamate on day 5, which is associated with the increase in glutamine.

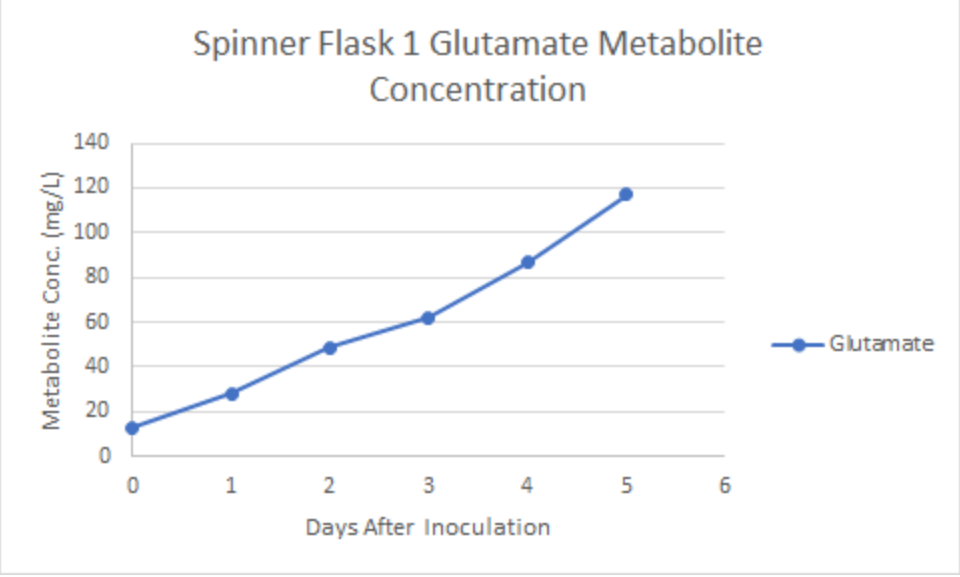



*Figure 16: Glutamate metabolite concentrations in spinner flask 1*

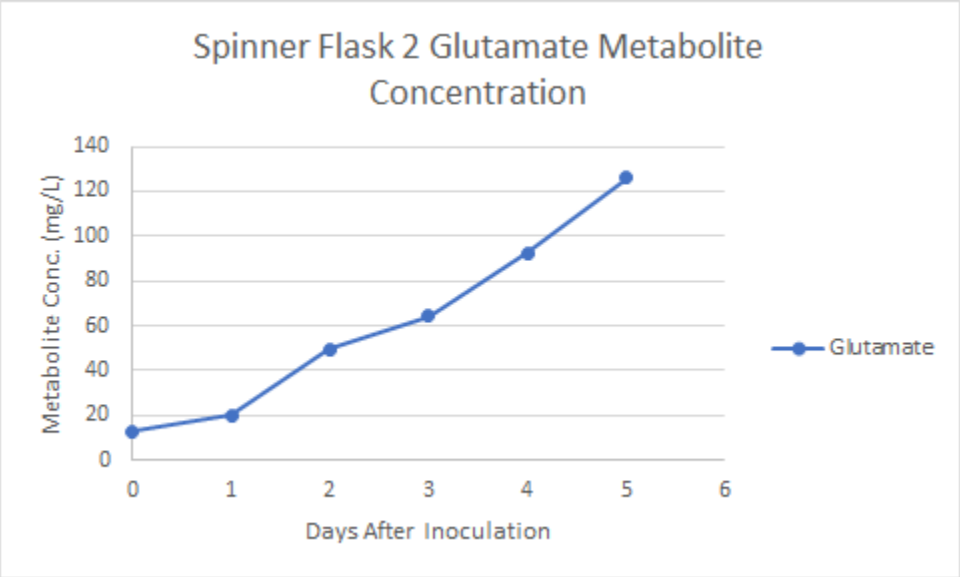



*Figure 17: Glutamate metabolite concentrations in spinner flask 2*

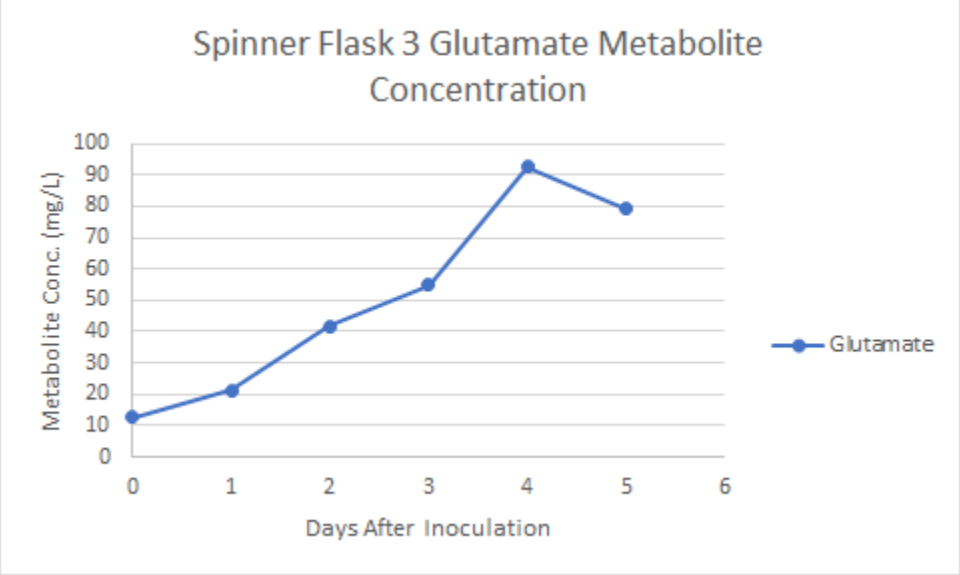



*Figure 18: Glutamate metabolite concentrations in spinner flask 3*

The next set of figures, Figures 19-21, illustrates the metabolite concentration of LDH in each of the spinner flasks. Spinner one had a steady increase in LDH, signifying little cell death over the first 4 days. However, there was a significant increase in LDH on day 5, which shows that there was considerable cell death on this day. Spinner 2 had a critical increase in LDH on day 3,

showing significant cell death on this day. Spinner 3 maintained steady LDH concentration, signifying there was little cell death throughout the 5-day span.

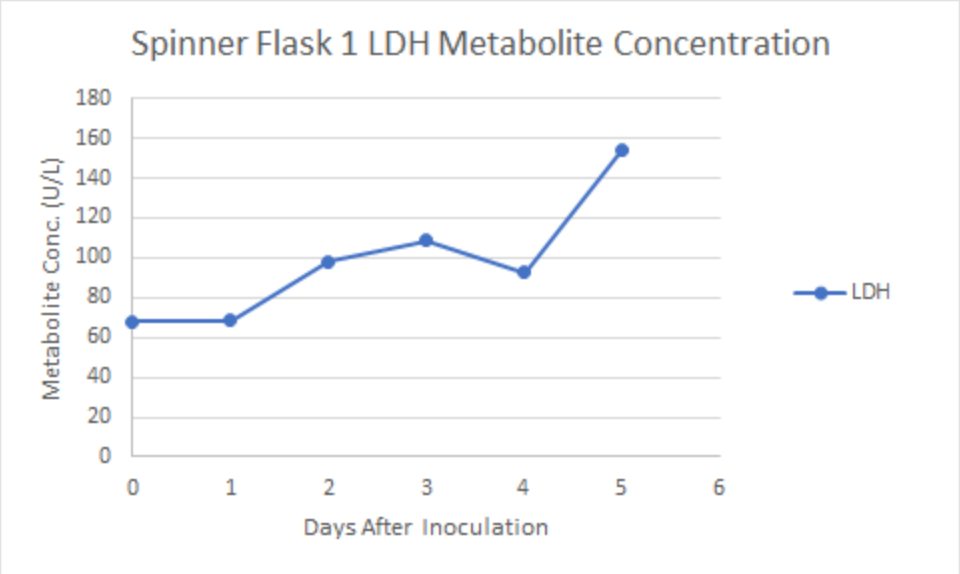



*Figure 19: LDH metabolite concentrations in spinner flask 1*

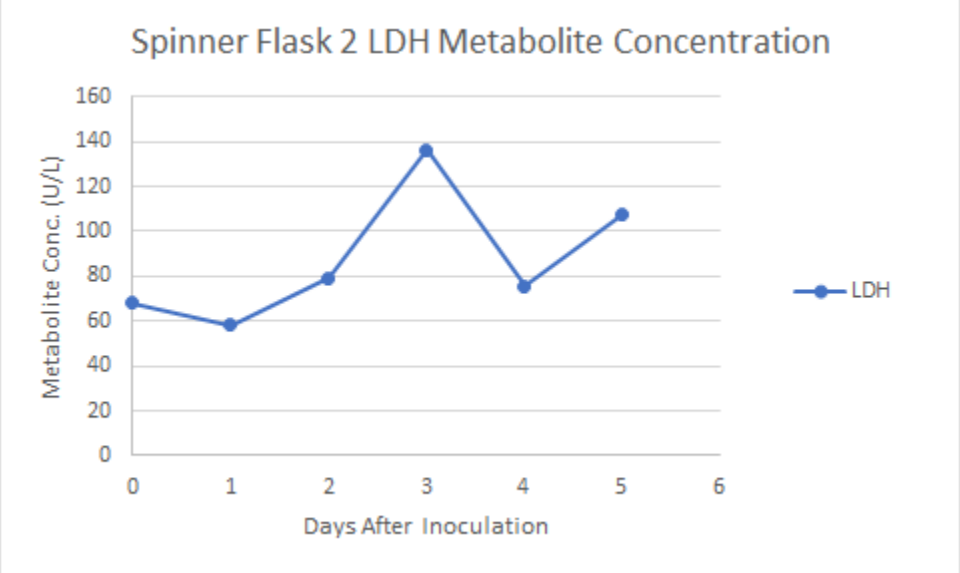



*Figure 20: LDH metabolite concentrations in spinner flask 2*

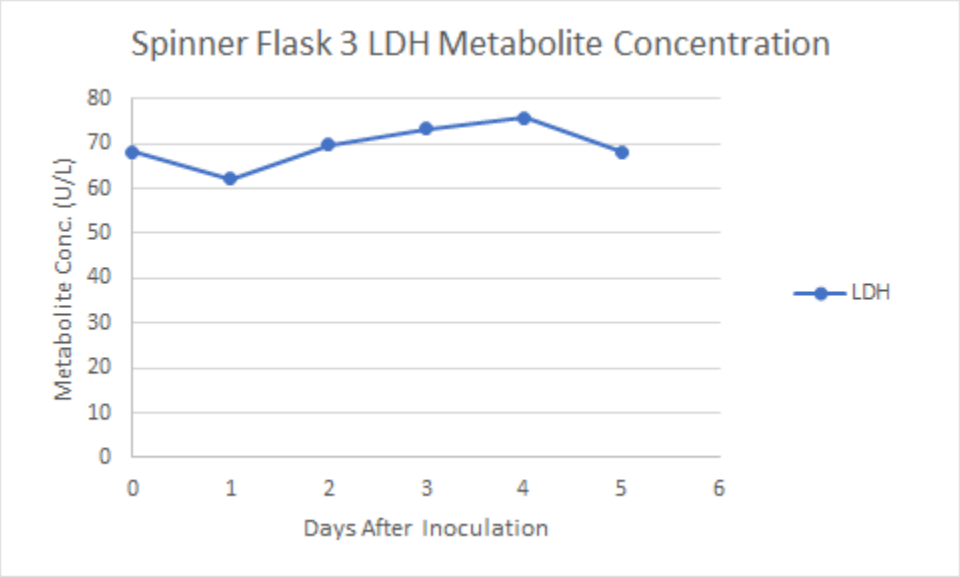



*Figure 21: LDH metabolite concentrations in spinner flask 3*

#### 4.2.1.3 Spinner Flask Test 2 (Intermittent Agitation)

This test is performed with all the same conditions as the spinner baseline test, however,. the cells are agitated with a duty cycle of 55 minutes off 3 minutes on at 50 rpm for the entirety of the culture. The 500 mL spinner flask is filled with 150 mL 4g/L 5% CFS MEM bead slurry and inoculated at a cell density of $2.5x10^5$ cells/mL.

#### 4.2.1.4 Wave Bioreactor Test

The GE Wave bioreactor is a proposed step in the seed train process by the group, therefore it is important to test for bead to bead transfer of cells in this step so it can be used for the scale-up to the XDR-200. The Wave bioreactor has a different agitation mechanism than the XDR-200 and spinner flasks because it uses a rocking motion with different angle parameters. The recommended agitation rate from the GE product guide of the Wave bioreactor for adherent cells is a 16 rpm rocking agitation at a 7 degree angle. These parameters will be tested with different values as well as using intermittent agitation to see which method facilitates bead to

bead transfer the best. To determine the feasibility of these methods, glucose uptake rates will be evaluated by a media analyzer, as well as cell counters to see if the cells are transferring from bead to bead. The results should show an increase in glucose uptake and an even confluency between sampled beads, which should be representative of the larger culture.

#### 4.2.1.5 Seed Train to n-1

In order to analyze cell culture within the XDR-200 bioreactor several seed train processes are tested. The preceding steps to XDR inoculation must be optimized to ensure meaningful data is collected from the 200L runs. In developing the seed train process, cost, ease of reproducibility, labor time, industry standards, sterility and cell growth consistency must be considered. Two initial seed train runs were developed from previous experiments conducted by GE (GE, 2014) and Millipore. (McGlothlen, 2016).

#### 4.2.1.6 Seed Train 1

Figure 22 below provides an overview of each step for the first seed train model with an estimated number of vero cells produced at confluency.

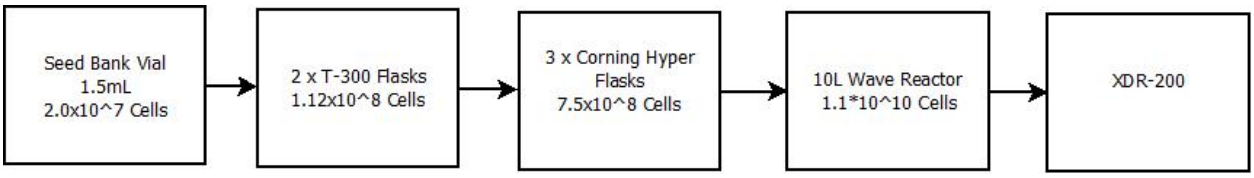



*Figure 22: A model of the first seed train*

The Wave Reactor seed train starts with a 1.5mL vial of twenty million cells. The cells are plated in 2 T-300 flasks and placed in a 37 degree incubator with DMEM 5% bovine fetal serum to be thawed for 96 hours or once confluent (whichever occurs first). The cells are then

trypsinized, suspended and transferred evenly into 3 Corning HyperFlasks. Each HyperFlask consists of 10 layers, which is the equivalent of 10 175 $cm^2$ flasks. They are then placed stored in a 37 degree incubator for 96 hours. The cells are again trypsinized, and moved to a 10L wave bioreactor with Cytodex 1 microcarriers. Here, they are regularly sampled and analyzed with a cell counter and glucose uptake analyzer to determine cell density and growth rate. The microcarriers are also visually inspected with a microscope to determine average confluency. The first seed train procedure that was run consisted of inoculating the 20 L Wave bioreactor at 10 L at a density of $1.75 * 10^5$ cells/mL at 99.6% cell viability with an average cell diameter of 20.5 micrometers. However, in addition to significant cell death, this seed train did not result in enough bead-to-bead transfer and cell proliferation to reach the XDR 200. The figures below, Figures 23, illustrate bead confluency of cells before inoculation, and post-inoculation at day 1, day 2, and day 5. As seen at day 5, there was very little attachment of cells to the beads, and the result was significant cell death.

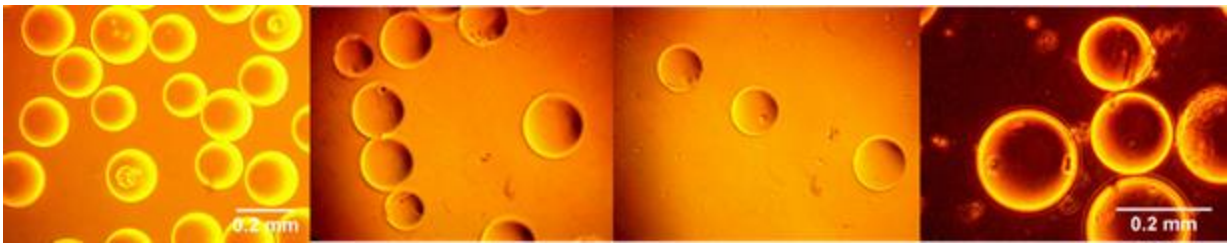



*Figure 23: Images of microcarriers in the first run of the Wave bioreactor. From left to right is before inoculation, day 1, day 2, and day 5. All scale bars shown are 0.2 mm in length (Images 1-3 taken at same magnification)*

The first graph, Figure 24, shows the pH level within the Wave throughout the 5-day culture period. The pH began to slightly decrease up to day 2 from 7.65 to 7.55, and suddenly increased

on day 5 to 7.85. The data indicates cell growth within the first 2 days, resulting in acidic byproducts from cell metabolism. However the increase in pH in the conclusion of the experiment indicates a slow rate in cell growth.

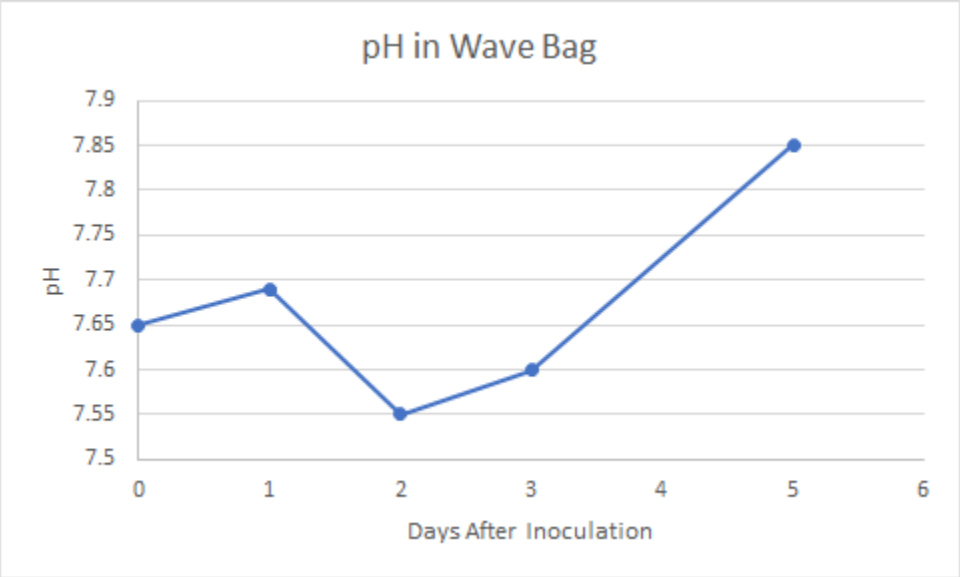



*Figure 24: pH in the first run of the Wave bioreactor*

The graphs below illustrate the results of the media analysis from the Wave bioreactor. The media analysis included glucose and lactate metabolite concentration, ammonium and glutamine metabolite concentration, glutamate metabolite concentration, and LDH metabolite concentration. The first graph (Figure 25) shows a decrease in glucose concentration from 1.04 g/L to 0.81 g/L, and an increase in lactate production to 0.21 g/L, signifying cell growth because the cells are consuming glucose and producing lactate as a byproduct. However, these changes are minor, as there was less than a 0.2 g/L concentration for both metabolites over the 5 day period, demonstrating minimal cell growth.

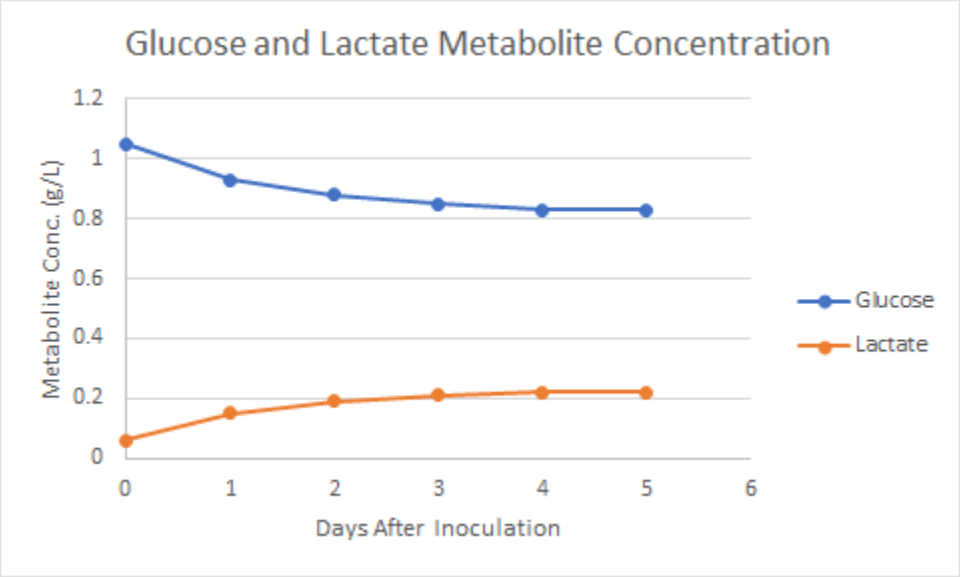



*Figure 25: Glucose and lactate metabolite concentration in the first run of the Wave bioreactor*

The following graph (Figure 26) illustrates an increase in ammonium concentration from 0.3 mmol/L to 1.41 mmol/L. The concentration of glutamine decreased from 1.6 mmol/L to 0.39 mmol/L over the 5-day culture period. The decrease in glutamine shows minor cell growth due to the uptake of glutamine to be used as an amino acid for energy use, and the increase in ammonium is a result of a toxic byproduct of glutamine use, which results in minimal cell growth as well as cell death due to the toxicity.

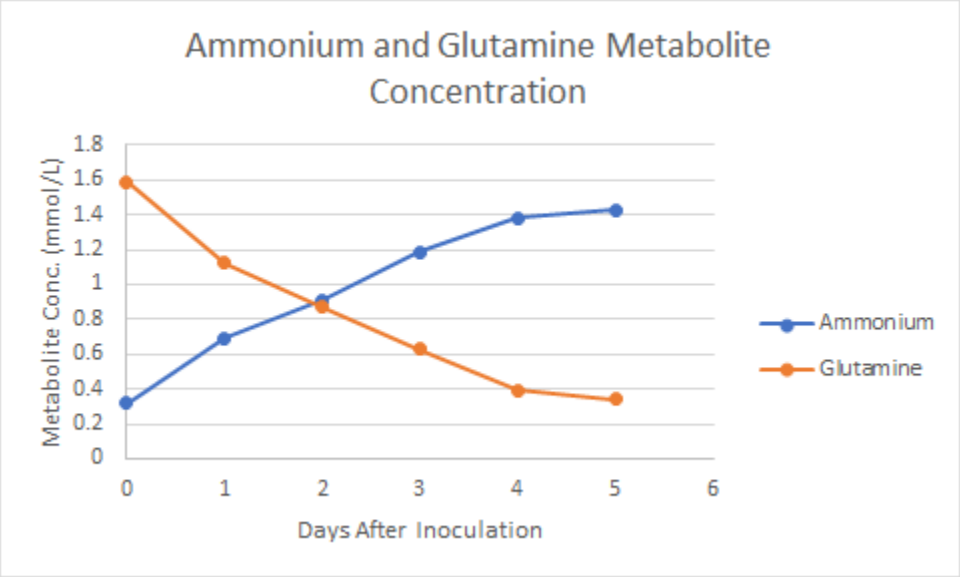



*Figure 26: Ammonium and Glutamine metabolite concentration in the first run of the Wave bioreactor*

The graph below (Figure 27) shows the glutamate metabolite concentration slightly increasing to 103 mg/L over the course of 5 days within the Wave bioreactor, signifying slight cell growth due to the production of glutamate from the metabolism of glutamine. The next figure, Figure 28, shows an increase in LDH metabolite concentration a maximum value of 250 U/L on day 4 which signifies cell death due to the release of this enzyme from cells when the cell membrane is compromised.

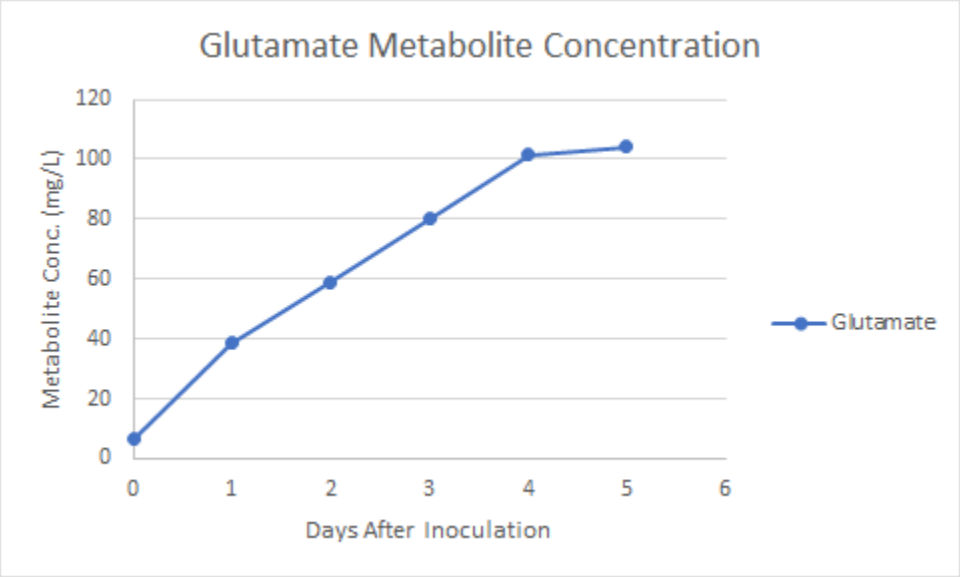



*Figure 27: Glutamate metabolite concentration in the first run of the Wave bioreactor*

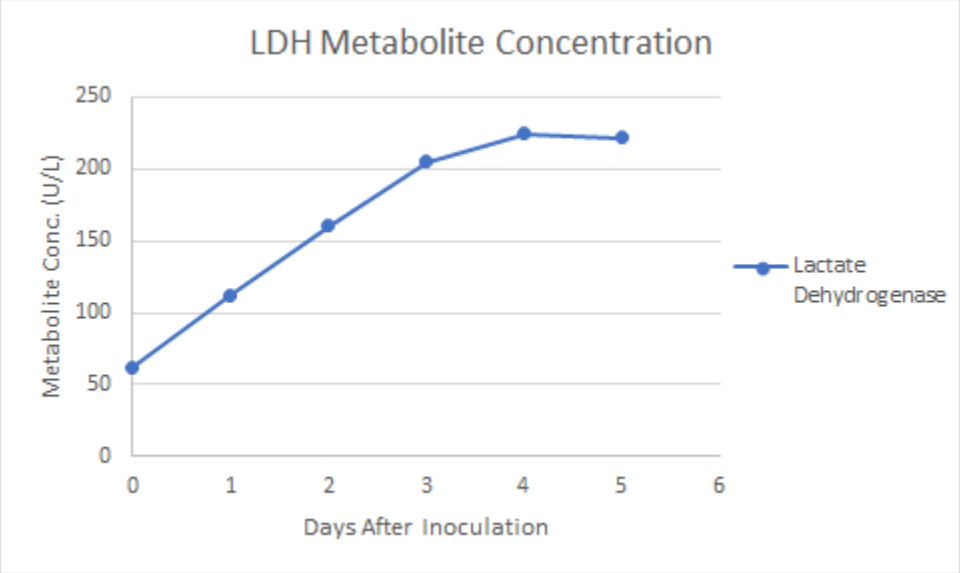



*Figure 28: LDH metabolite concentration in the first run of the Wave bioreactor*

#### 4.2.1.7 Seed Train 2

The flowchart below, Figure 29, provides an overview to the second seed train model. It is very similar to the first model, but uses 500 mL spinner flasks hyperflasks to inoculate the 10 L Wave bag.

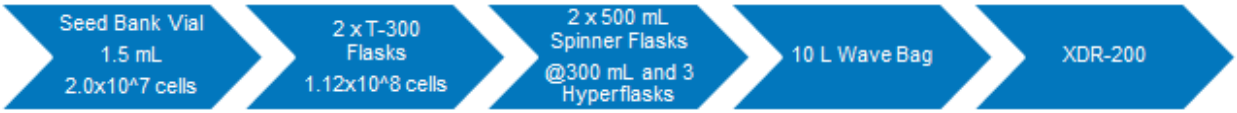



*Figure 29: Seed Train 2*

Seed Train 2 follows the same steps as Seed Train 1, described earlier, up to the 10L wave bioreactor. Instead of trypsinization and seeding in a 10 L wave bioreactor with Cytodex 1 microcarriers from only hyperflasks, cells are trypsinized and divided evenly into three hyperflasks and two 500 mL spinner flasks prior to inoculation of the Wave. Here, they will be incubated for 4 days. The cells will only be sampled in the spinner flasks daily whereas the hyperflasks will be sampled at the conclusion of this step due to a higher risk of contamination. The final feasibility test was a repeat of the first seed train utilizing the 20 L Wave bioreactor. The Wave bioreactor was inoculated at a density of $1.28 * 10^5$ cells/mL with a 99.1% cell viability at a working volume of 10 L. The images below in Figures 30 and 31 illustrate the progress of cell attachment to microcarrier beads over the course of 9 days. The image for day 0 shows viable cells floating around the empty microcarrier beads. By day 9, there was little attachment to be seen within the Wave bioreactor. It is important to note that on day 3, the microcarriers and cells from the 500 mL spinners were transferred to the Wave bioreactor to promote bead-to-bead transfer.

*Figure 30: Microscope images of the microcarriers in the second Wave bioreactor run. From left to right, hour 1, hour 2, hour 3, day 1. All scale bars shown are 0.2 mm in length (Images 1-3 taken at same magnification)*

*Figure 31: Microscope images of the microcarriers in the second Wave bioreactor run. From left to right, day 2, day 3, day3 (spinners added), day 6, and day 9. First scale bar shown is 0.2 mm in length and second is 0.5 mm (images 2-5 taken at same magnification)*

The first graph (Figure 32) shows the pH level within the Wave bioreactor throughout the course of 10 days. The pH began to slowly decrease as cells began to metabolise and release acidic byproducts such as ammonium, however when the additional beads and cells were added on day 3, the pH suddenly increased to an 7.76 before steadily decreasing back to a reasonable pH around 7.3.

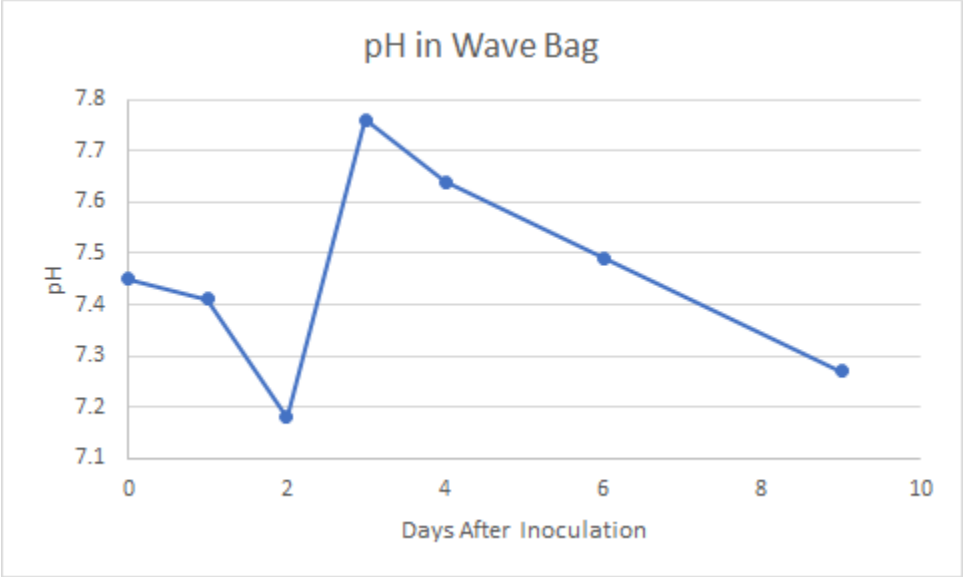



*Figure 32: pH in the second Wave bioreactor run*

The following graph (Figure 33) illustrates the glucose and lactate metabolite concentration within the Wave bioreactor over the course of 10 days. Glucose and lactate levels remained fairly steady, as glucose only decreased by a little more than 1 g/L from ~5 g/L to 3.6 g/L and lactate

increased by less than 0.5 g/L from 0 to ~0.4 g/L over the span of the experiment, demonstrating the cells were metabolizing at a very low rate and were not proliferating.

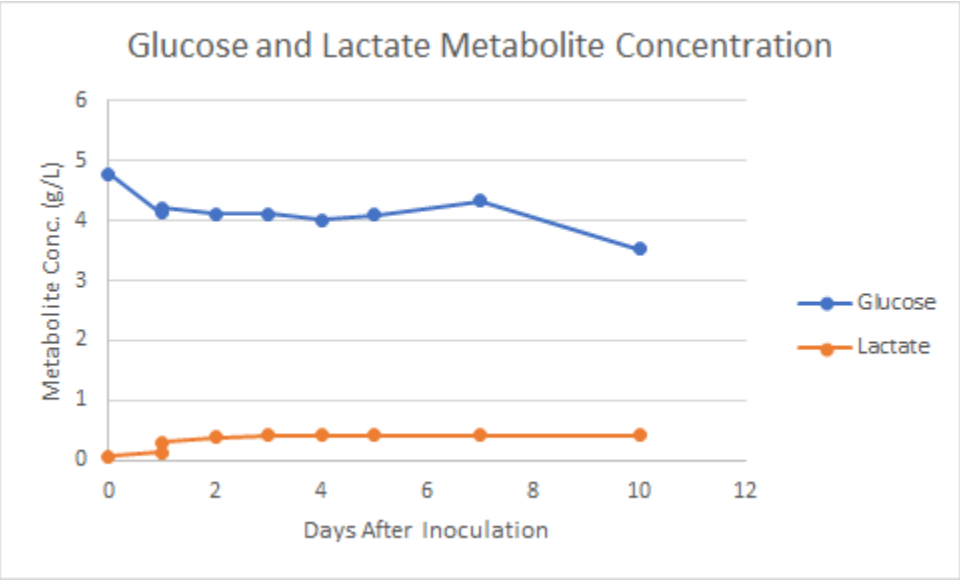



*Figure 33: Glucose and lactate metabolite concentration in the second run of the Wave bioreactor*

The next graph (Figure 34) illustrates the ammonium and glutamine metabolite concentration within the Wave bioreactor over the course of 10 days. Ammonium increased to ~4 mmol/L over the span of the experiment due to cell metabolism of glutamine. It is important to note that the Cedex media analyzer malfunctioned and was not able to provide the glutamine concentration after day 2. However, we can assume based on the ammonium concentration that the glutamine concentration had been slightly decreasing over the span of the experiment.

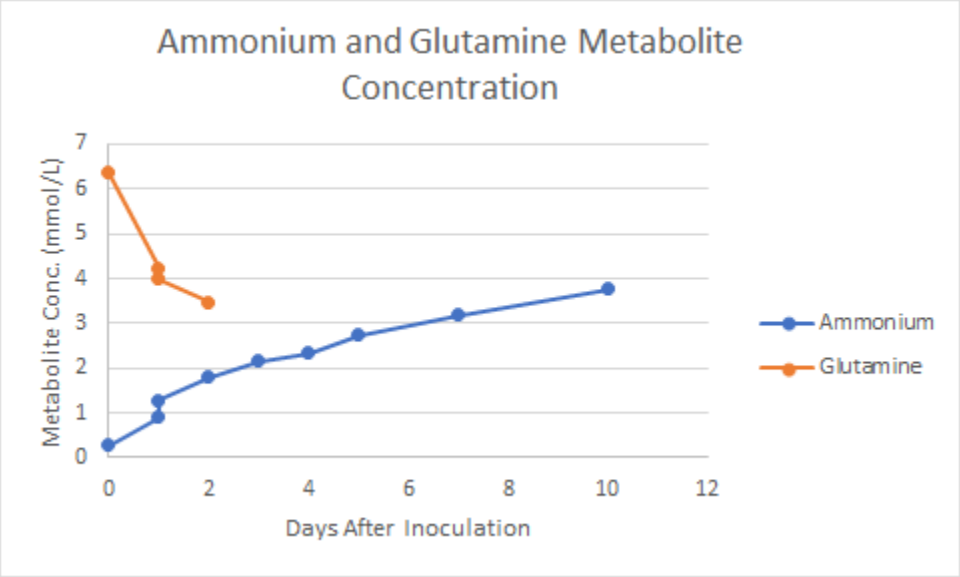



*Figure 34: Ammonium and Glutamine metabolite concentration in the second run of the Wave bioreactor*

The next figure (Figure 35) illustrates the glutamate metabolite concentration within the Wave bioreactor over the span of 10 days. Glutamate was shown to increase to ~250 mg/L, demonstrating that cells were metabolizing by consuming glutamine and producing glutamate as a byproduct of this metabolism.

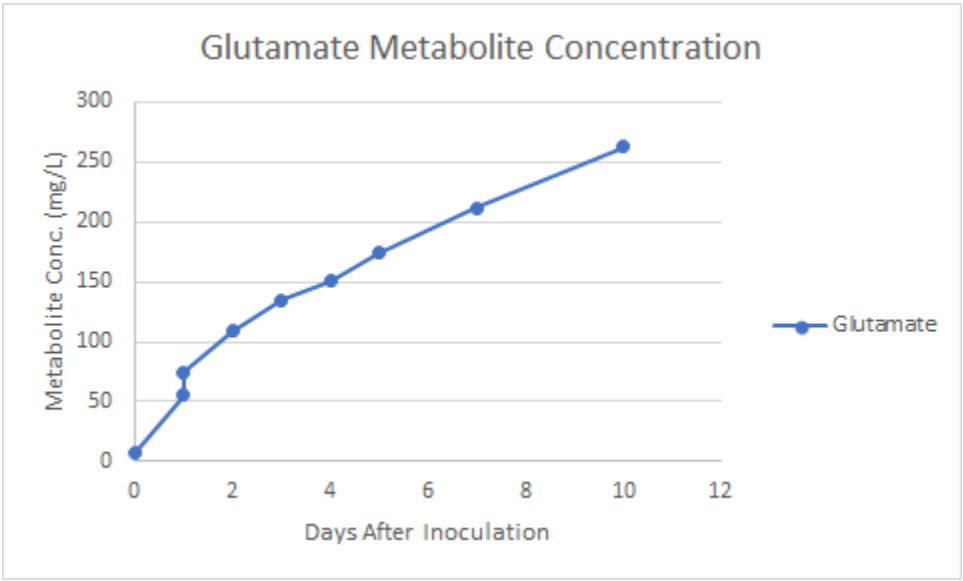



*Figure 35: Glutamate metabolite concentration in the second run of the Wave bioreactor*

The graph below (Figure 36) shows the LDH metabolite concentration within the Wave bioreactor over the course of 10 days. LDH had a sudden increase to 250 U/L on day 3, due to rapid cell death. LDH concentration than steadily decreased to 200 U/L after additional beads and cells were inoculated into the Wave bioreactor on day 3, demonstrating that the rate of cell death slowed down with the addition of cells.

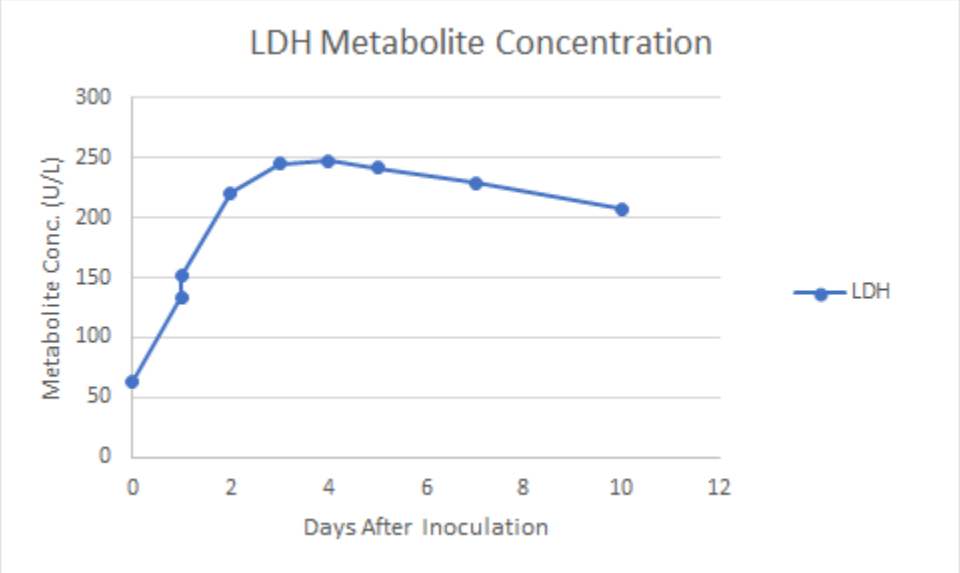



*Figure 36: LDH metabolite concentration in the second run of the Wave bioreactor*

#### 4.2.1.8 Cell Growth in XDR-200

One of the primary goals of this study is to determine the feasibility of Vero cell culture in the XDR-200 bioreactor. To accomplish this, tests were designed with varying starting volumes, microcarrier densities, and agitation rates. For each of the experiments cell growth and their environment are closely monitored and recorded.

## 4.3 Alternative Designs

The experiments conducted in this project were able to demonstrate that it is indeed

feasible to upscale a cell culture into a 200L bioreactor using microcarrier bead technology. The experiments were able to show cell attachment within the culture in addition to cell growth and proliferation based on the glucose consumption and lactate production, in addition to glutamine consumption and glutamate production. However, the group encountered obstacles throughout the experiment that hindered the efficiency of the upscaling process. First, the microbeads became crushed from the impellers within the culture vessels. This not only limits the surface area that cells can attach too, but the debris from the crushed beads would attack to the microcarriers that were intact, which takes up the area that the cells can attach to. Second, the group encountered issues involving the lack of attachment of cells to microcarrier in addition to cell viability. The cells were not able to obtain a homogeneous distribution across the microcarriers, and the lack of attachment lead to cell death, as shown by the ammonium production. This resulted in an acidic environment, as demonstrated by the decrease in pH within the culture. The group brainstormed alternative designs in order to find solutions to these obstacles.

### 4.3.1 Preventing Crushing of Microcarriers

To make sure that cells are growing properly, the surfaces that the cells attach to must stay intact and not undergo harmful shear forces. Preventing the loss of microcarriers is important for the XDR-200 performance because they create the adhering surface that are needed for the Vero cells to grow on. GE has spotted some issues with their impeller design in their 10L bioreactor, which showed beads being crushed under the impeller bearing. They have made changes by increasing the height of the bearing but have not tested it yet at a 200L level. The group will analyze samples of microcarriers within the culture and evaluating what percent of beads are physically damaged. If there are constantly deformed beads in abundance , then we

may assume they are damaged during the agitation of the culture. If damaging microcarriers is an issue, the group has brainstormed a few design modifications, like changing agitation rates and microcarrier density, that might be tested.

### 4.3.2 Mesh Design

One of the concepts brainstormed by the group was to include a mesh filter made of a sturdy, compatible material similar to the actual single-use bag above the impeller that prevents particles less than 0.1 mm in diameter to go through it, to prevent contact with the impeller or prevent the beads from being crushed by the eddy created by the impeller. The design can be seen in Figure 37. To test the feasibility of this, a CAD design would need to be approved by GE and then GE would fabricate the bag for testing. To test the effect of the filter in the system, the sensors would be used to see if the flow of nutrients and culture parameters is different among different parts of the bag. Also stress simulations of the filter from the power output of the impeller would have to be analyzed in SolidWorks to make sure the filter would not tear. The final feasibility test would be on the material of the filter and its effect on Vero cells.

One way the team has sought to ensure the viability of the microcarrier beads is to alter the design of the bioreactor. This concept focuses on creating a barrier to separate the impeller region and the microcarriers. The barrier will be a fine wire mesh that the beads will not be able to fit through.

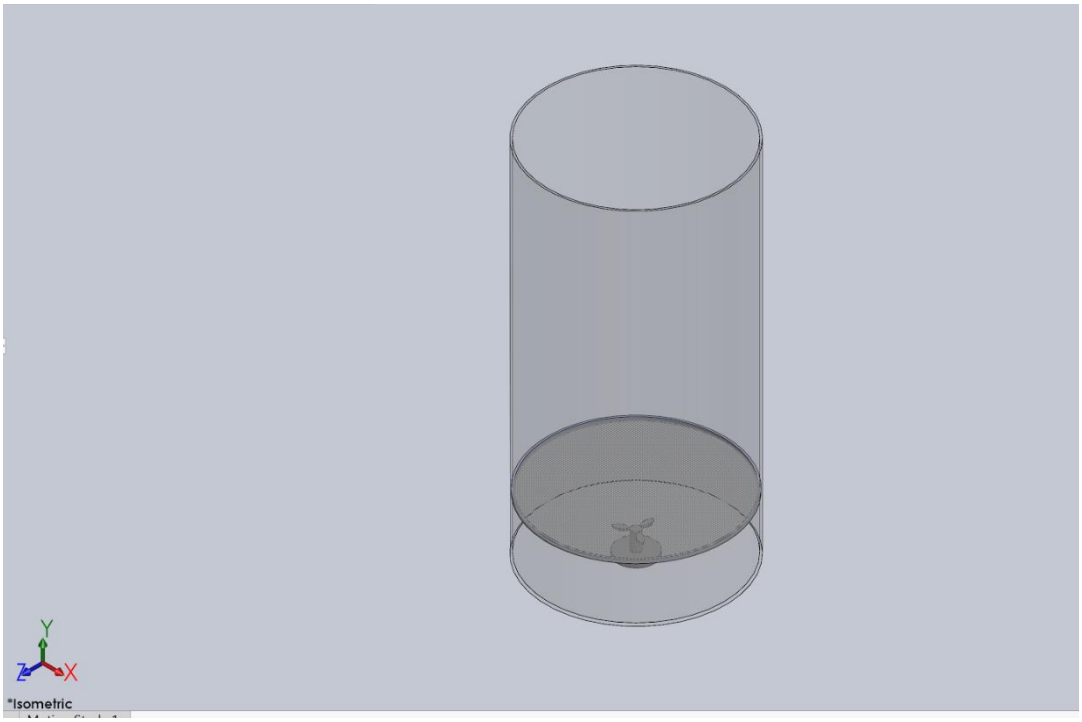



*Figure 37: Bioreactor Mesh Screen*

When culturing cells in the bioreactor, the impeller may create shear effects or come into physical contact with the microcarriers. The way the bioreactor is designed, the impeller is nested within the bag to which allows a single cell-scale up within the reactor. The impeller attaches magnetically to its base where the power is generated. For the separation to work, the mesh must also be designed within the bag, attached above the impeller. The team's original idea proposes a clearance of 20cm between the mesh screen and the impeller. The mesh will be made of a biocompatible, lightweight, semi-flexible polymer such as polypropylene. This material can also be siliconized to ensure that no cell attachment occurs - as is used with tissue culture flasks. The mesh is planned to be in the micron-scale for porosity, as the microcarriers are about 175 micrometers when swelled.

Limitations that may occur with this design focus on processability, bag attachment, and interrupting fluid flow of the bioreactor. To create a mesh this fine may be a challenge,

especially at large scale. It would also require incorporation into GEHC's production of the materials. Also, the process of clearing and manufacturing a new design may take too long to approve once the details are determined. Another issue is attaching the screen within the bag. The bags currently used within the bioreactor have no specific attachment point that can be readily used for this screen. To allow screen attachment, other modifications may be needed for the bag that could complicate the process. Lastly, with a fine screen in the micrometer range, this will introduce changes to the fluid flow of the system, which will need to be addressed. If the screen slows down the agitation and the culture becomes stagnant, then bead to bead transfer will not occur which then may induce cell death.

### 4.3.3 Dual Impeller Conceptual Design

Another conceptual design was the use of two impellers (one on top and one on the bottom) to maintain the suspension of cells between the impellers. The use of a dual-impeller system has been looked at previously, and shown that it is able to reduce the amount of shear within the suspension up to 59% (Buffo et al., 2016). A dual impeller design was found to generate larger eddies, which are less harmful to cells and their microcarriers. (Buffo et al., 2016) Ideally this would prevent the cells and beads from being crushed under the impellers as well as coming into contact with them. This works by providing a force from both ends of the bioreaction, which results in the beads to remain in suspension within the middle of the vessel, and keeps an even distribution of beads. The proposed design is depicted in Figure 38. To test the feasibility of this design, a Solidworks simulation must be run with the two impellers in the XDR-200 bag to see the shear forces created in the bag to determine if the beads or cells would subject to shear failure with this model.

Another way the team has set out to ensure the viability of microcarrier beads is to create

better mixing within the bioreactor bag. This may be accomplished by creating a more dynamic flow system with dual impellers stationed at the top and bottom of the single use bag.

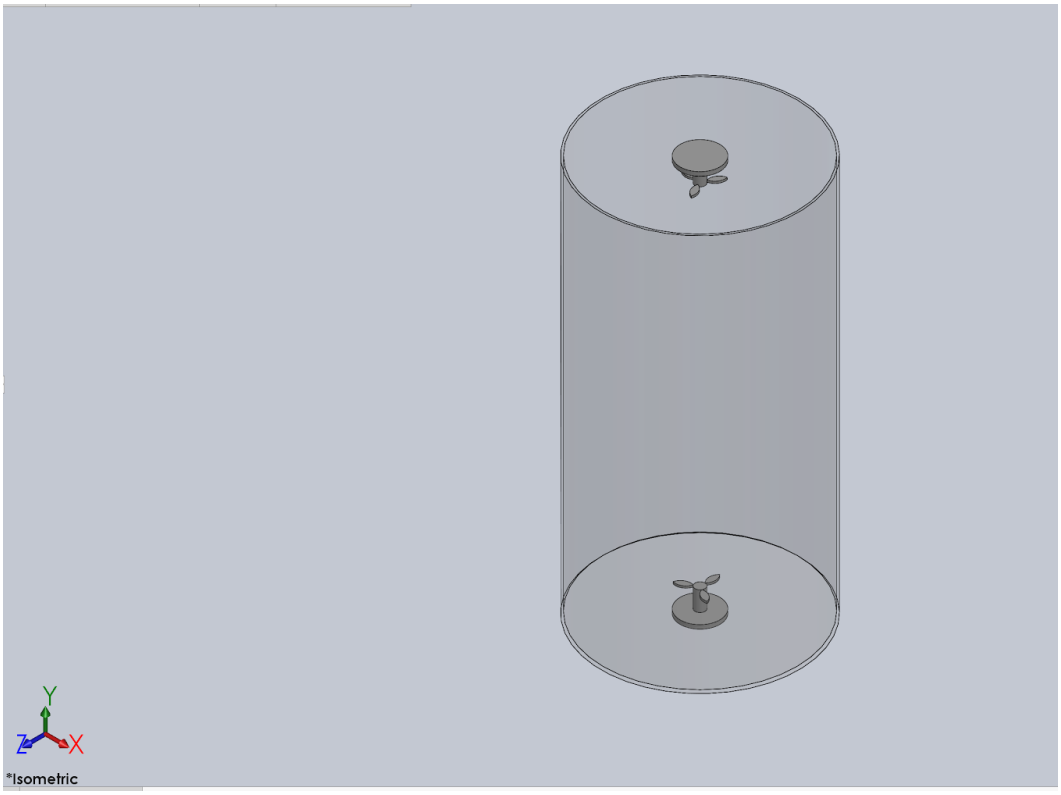



*Figure 38: Dual Impeller Single-Use Bag*

Due to anticipated difficulties that arise from the mesh screen design, an alternative to this may be to utilize two different impellers for cell culture suspension. This proposed concept will allow more even mixing within the reactor. With an impeller stationed only at the bottom, the flow gradient only pushes up from the bottom of the reactor. With flow being generated from the top and bottom, the flow gradient will keep the beads directed more towards the midline of the reactor, preventing beads from falling towards the impellers. This would have the aim of allowing microcarriers to stay away from the impellers. In addition to this, there would be the ability to reduce the shear force present in the original design (Buffo et al., 2016). The impeller will be made from the same material as the bottom impeller currently found in the XDR-200 -

high density polyethylene.

Difficulties that may arise from this design will be adapting the shell of the bioreactor to fit the upper impeller. With the current design, there is a station for the impeller to magnetically attach to at the bottom of the shell, while the top remains open. If there is to be an upper impeller, then the shell will need to be reworked to accommodate this. A power input station must be created for the impeller to attach to, which may be difficult for GEHC to incorporate into their system. Also, power input and fluid gradient will need to be recalculated with this change to the system.

### 4.3.4 Roller Membrane Flask

There have been papers outlining different flasks to be used for the scaling up of adherent cells, and a possible design that has been suggested but not created yet is the use of rolled membrane flasks. Rolled membrane flasks utilize coiled, spiraling scaffolds within the flask to support more anchorage-dependant cells. This method gives a significant increase in surface area compared to other flasks. This design gives 10 times the surface area per volume of a normal roller flask. This method could cut down on the flasks consumed in the seed train as well as minimized contamination. To test the feasibility of this design, cells would need to be seeded on the rolled membrane and then the bottle would be placed on a roller machine with media inside. The cell growth in the flask would be recorded each day to see if it is comparable in this flask to others used in industry.

One need to be addressed is the scale up of cells to reach the bioreactor. Before moving into the XDR-200 bioreactor, the culture must be around 4 billion cells. To facilitate reaching this number, different culture steps may be taken. One that may reduce space and increase yield is a roller membrane flask which is depicted below in Figure 39.

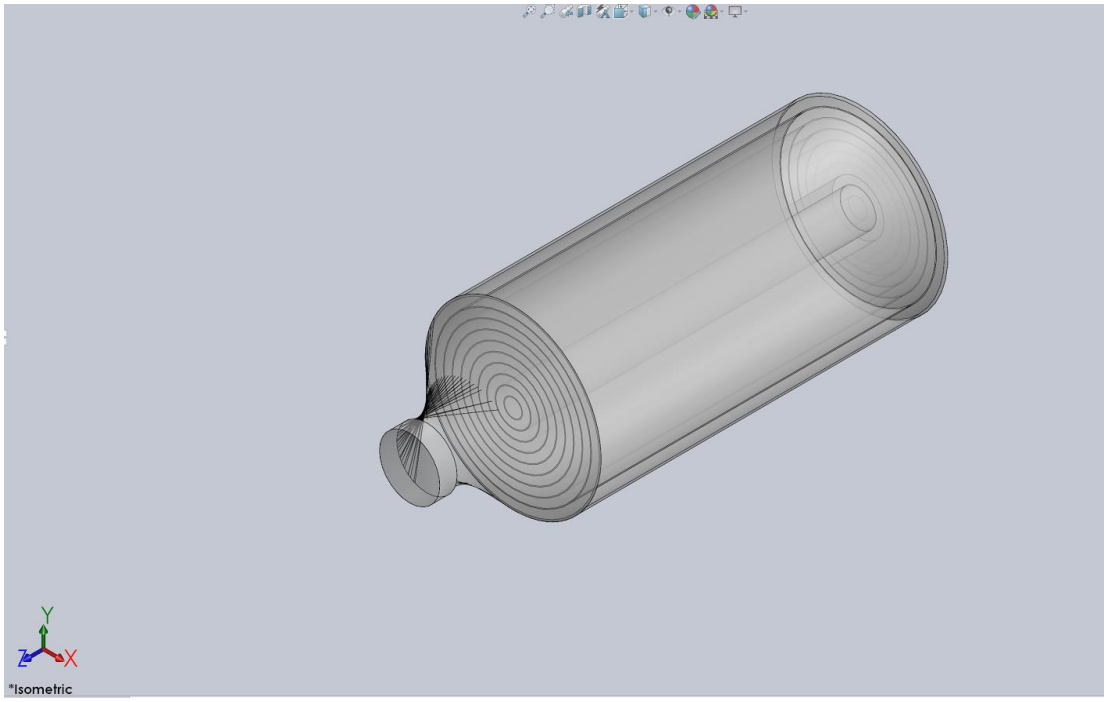



*Figure 39: Roller Membrane Flask*

As part of a current method to scale up cells, our team proposes the use of a hyper flask. This is a cell culture flask that employs the use of multiple layers, similar to stacking shelves. These flasks are cumbersome to deal with and introduce a risk for contamination when changing media or reseeding. To make the cell culture less cumbersome and utilize fewer flasks, a roller membrane flask may be of use. This flask works similar to a roller bottle that allows culture on the entire inside surface, as opposed to a T-flask. This is able to increase surface area to volume ratio and increase cell culture yield. The roller membrane flask utilizes this idea, but enhances it by spiralling the inside network. This will allow for increased cell attachment and fewer flasks to be seeded, reducing operations of the employee.

An expected difficulty to be overcome with this design is nutrient and gas exchange due to the increased surface area, which is comparable to microcarriers. Another problem that may occur is lack of resources to operate this design. It requires a special incubator or device to be placed in an incubator to ensure constant rolling occurs. This may take up too much space within

the incubators our team has available. Lastly, this design is not large-scale manufactured by any one company, so there may be issues with acquiring this flask. If the team was to design it, this make take too long to produce and slow down the progress of the project.

## 4.3.5 Alternative Seed Trains

### 4.3.5.1 Alternative XDR-200 Inoculation Density

We hypothesize that the XDR-200 inoculation cell density may be too low to allow for homogenous distribution of cells among the microcarriers. Therefore, it may be optimal to inoculate with a higher cell density. To accomplish this, the seed train must be changed to include a 20/10L wave bioreactor. The wave bioreactor holds approximately $1.7x10^8$ microcarriers which are capable of hosting $5.1x10^9$ cells at confluency. This provides the XDR-200 an inoculation cell density of $1.3x10^5$ cells/mL when short-filled at 40L. This process, depicted in Figure 40, also has the benefit of a reduced risk of contamination compared to conventional seed train methods because it requires less manipulation to inoculate the wave bioreactor and requires fewer steps to reach this reactor. This alternative process has few drawbacks other than a slight price increase and requiring culture to take place outside of final step in the seed train for 2 additional days which could cause a risk of contamination.

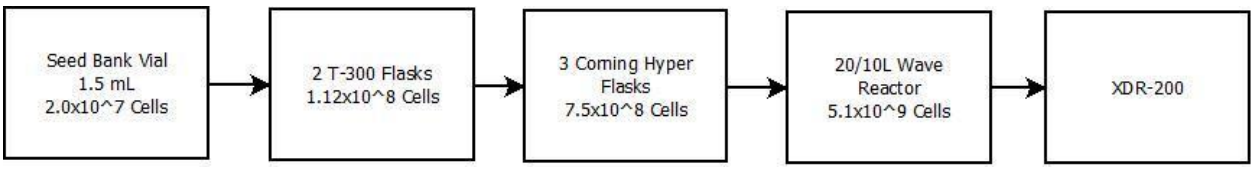



*Figure 40: Seed Train with 20L Wave Reactor*

### 4.3.5.2 High Density Seed Bank

If it is determined possible to thaw the banked cells directly onto microcarriers, initiating

the cell culture in a wave bioreactor from a higher cell density bank could reduce contamination risk, time and labor. The 20/10L wave bioreactor has a minimum working volume of 1000 mL, requiring 4 grams of beads ($1.7x10^7$ beads). Previous studies suggest the wave bioreactor needs between 3 to 10 cells per bead for inoculation. (GE, 2015) A cell bank of at least $5.1x10^8$ cells may be thawed into wave reactor at this initial volume and transferred from here into the XDR short-filled at 40L. The cost and number of steps in the seed train is reduced but the potential for cells to adhere to the microcarriers while thawing is currently unknown. This seed train can be seen in Figure 41.

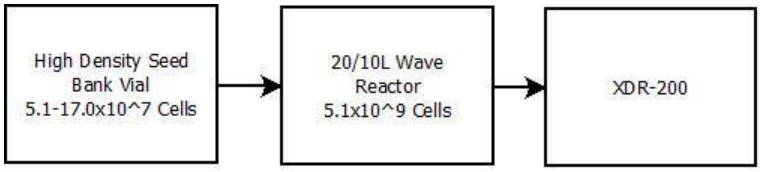



*Figure 41: Seed Train with High Density Seed Bank*

## 4.4 Final Design Selection

The final design chosen to scale up Vero cells to the XDR-200 incorporated T-300 culture flasks and various sized spinner flasks. The schematic of the seed train selected can be seen in Figure 42. This seed train proved to be the best choice after the conclusion of our feasibility studies. The previous designed seed trains had aspects that failed to grow Vero cells to the proper density to inoculate the XDR at 40 L. The feasibility study for the Wave bag reactor showed that the model used was not sufficient for Vero cell attachment as well as bead to bead transfer, which led to the elimination of that seed train. The feasibility study using the 500 mL spinner flasks showed a high confluency of cells as well as sufficient bead to bead transfer, which led to the selection of that seed train.


*Figure 42: Final Design of Seed Train to the XDR-200*

In terms of the final design selection for the XDR parameters, 3 g/L of bead volume showed sufficient growth for Vero cells at the 500 mL volume. Therefor 3 g/L for microcarrier density was used for the final design. Intermittent agitation and constant agitation were compared at the 500 mL volume and intermittent agitation for 24 hours showed the best results in terms of attachment leading to the selection of that method for the mixing parameter.

The alternative designs for the XDR bag were ruled out due to the limitation of time needed to approve the design as well as implement. After all of the alternative designs were compared and evaluated through the feasibility studies as well as time constraints, the final design using the seed train in Figure 42 along with the parameters shown above proved to be the best option for the final design.

# Chapter 5: Final Design Verification

## 5.1 First Seed train

The final experiment's microcarrier culture began with inoculating three 500 mL spinner flasks at a density of 4.05 * $10^5$ cells/mL at a working volume of 300 mL for a span of 4 days. The images of the microcarrier beads and cell attachment are shown below in Figure 43. As shown, there was immediate cell attachment onto the microcarrier beads after day 1, and by day 4, ~80% of the microcarrier beads were confluent with cells.

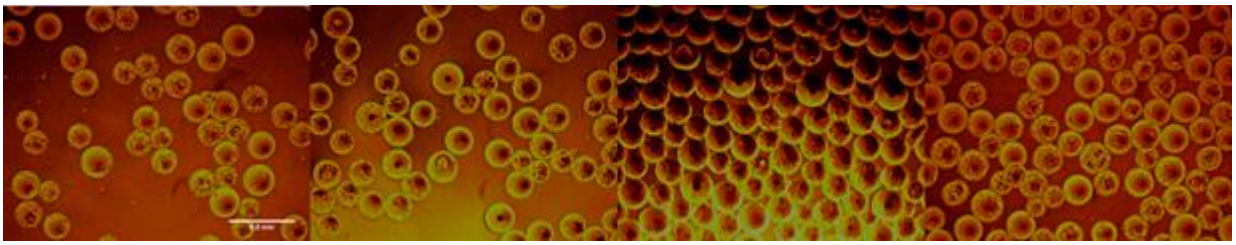


*Figure 43: Microscope images of the microcarriers in 500 mL spinner flasks. From left to right, day 1, day 2, day 3, and day 4. Scale bar shown is 0.5 mm in length (All images taken at same magnification)*

The first graph (Figure 44) shows the glucose and lactate metabolite concentration within the spinner flasks over the course of the procedure. Glucose was shown to steadily decrease from 5 g/L to 3.7 g/L, while lactate was shown to increase to 1 g/L. This data demonstrates that cells were proliferating by metabolizing the available glucose and releasing lactate as a byproduct.

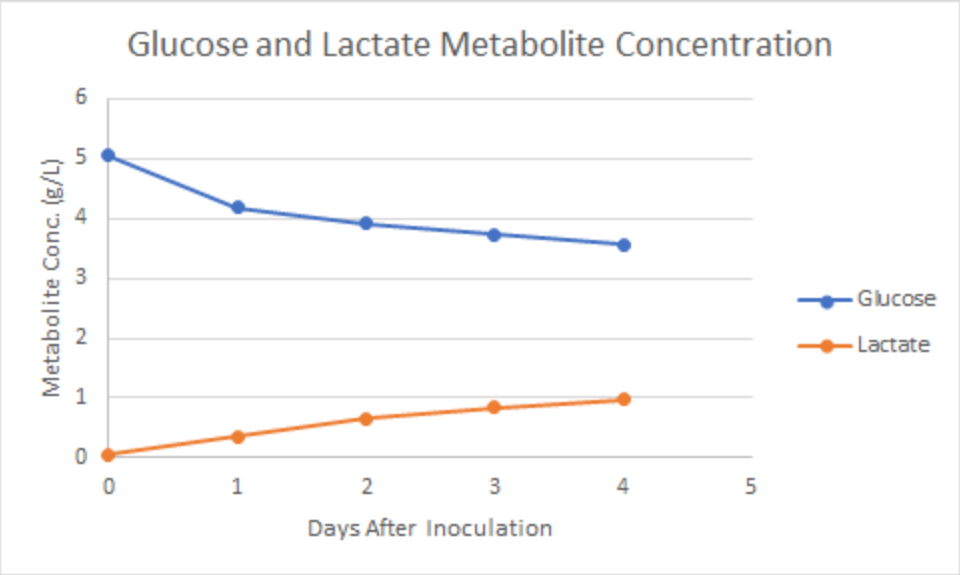



*Figure 44: Glucose and lactate metabolite concentration in the 500 mL spinner flasks*

The following graph (Figure 45) shows the ammonium and glutamine metabolite concentration within the spinner flasks over the course of 4 days. Glutamine was shown to decrease from 4.4 mmol/L to 2.3 mmol/L and ammonium was shown to increase from 0.5 mmol/L to 3 mmol/L. This demonstrates cells proliferating by metabolizing glutamine as energy from amino acids and producing ammonium as a byproduct of this metabolism.

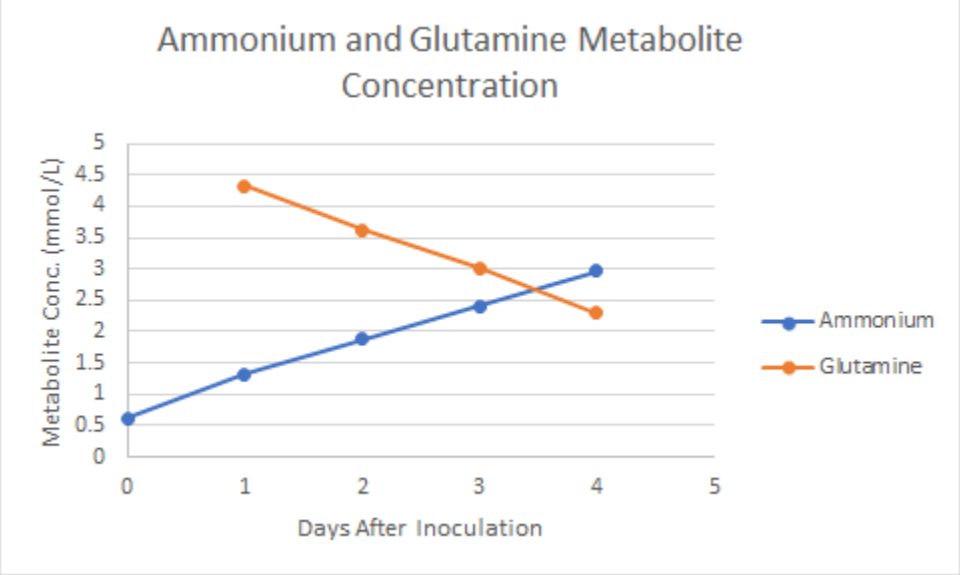



*Figure 45: Ammonium and glutamine metabolite concentration in the 500 mL spinner flasks*

The following graph (Figure 46) shows the glutamate metabolite concentration within the spinner flasks over the course of 4 days. The glutamate concentration was shown to increase to 115 mg/L over the span of the experiment, showing cell metabolism of glutamine and the release of glutamate as a byproduct in the media.

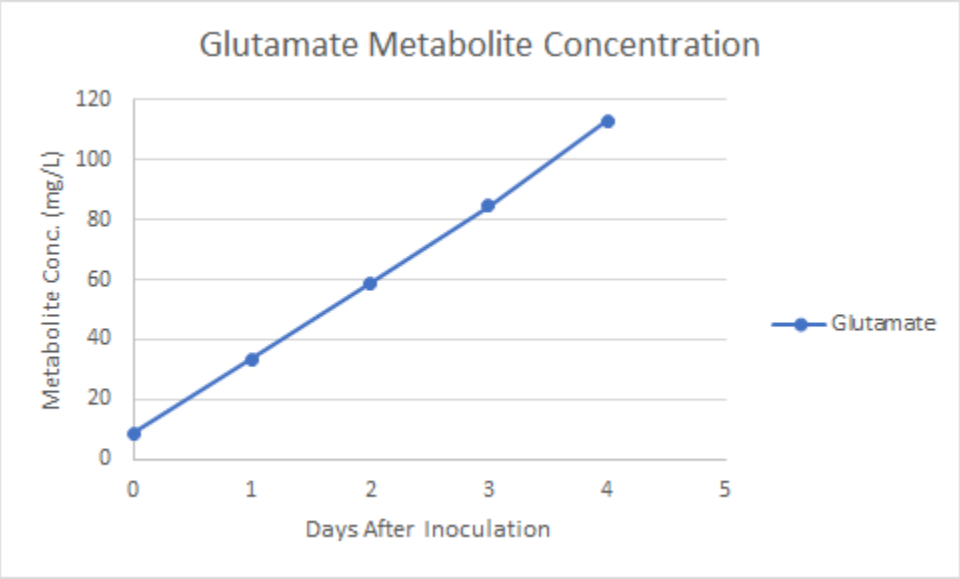



*Figure 46: Glutamate metabolite concentration in the 500 mL spinner flasks*

The following graph (Figure 47) shows the LDH metabolite concentration within the 500 mL spinners over the course of the experiment. LDH concentration was shown to increase from 60 U/L to a maximum of 110 U/L on day 3, and then dropped to 105 U/L on the final day of the experiment. The steady increase in LDH shows a slow rate of cell death within the 500 mL spinners.

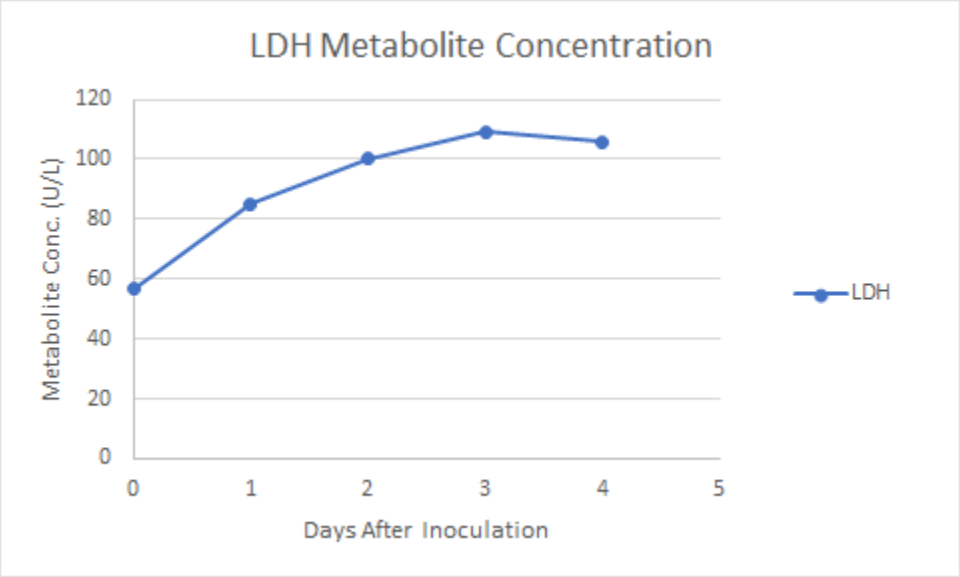



*Figure 47: LDH metabolite concentration in the 500 mL spinner flasks*

## 5.2 3L Spinner Flask at 1L fill

Following the culturing within the 500 mL spinners, the microcarrier beads and the cells within these spinners were transferred into 3 L spinners at a working volume of 1 L. The following images, Figures 48 and 49, illustrate the cell attachment onto the microcarrier beads within the 3 L spinners over the course of 22 days. Day 12 began to show an increase in debris due to the crushing of the microcarriers under the impeller within the 3 L spinners. By the final day of the experiment, ~70% of the microcarrier beads were confluent with cells.

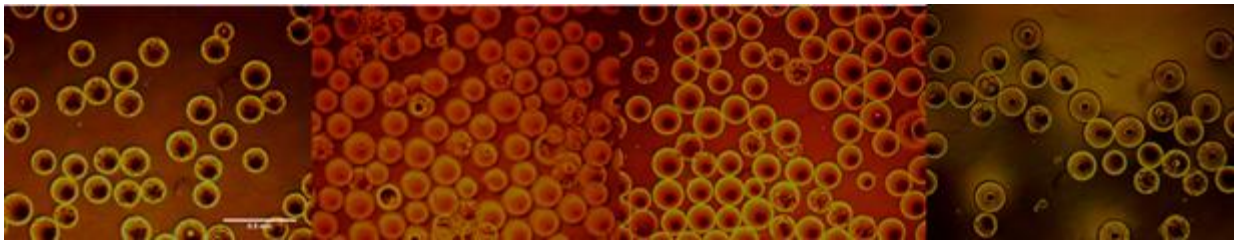


*Figure 48: Microscope images of the microcarriers in the 3 L spinner flasks. From left to right, day 1, day 3, day 5, and day 8. Scale bar shown is 0.5 mm in length (All images taken at same magnification)*

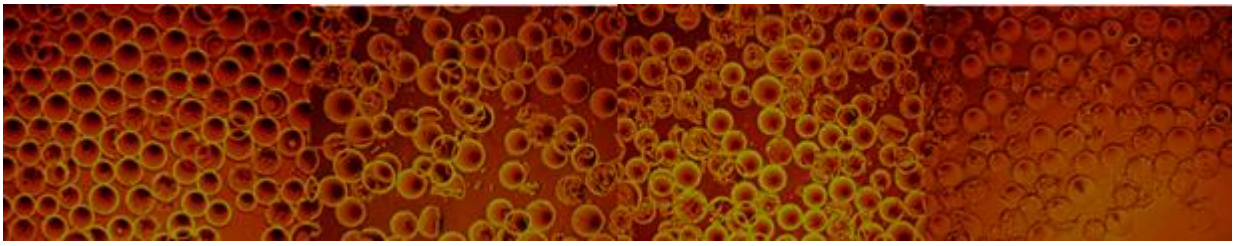


*Figure 49: Microscope images of the microcarriers in the 3 L spinner flasks. From left to right, day 9, day 12, day 19, and day 22. Scale bar shown is 0.5 mm in length (All images taken at same magnification)*

The first graph (Figure 50) shows the pH level within the 3 L spinner flasks over the course of the experiment. The pH was shown to drastically fluctuate throughout the experiment, reaching a maximum value of 7.5 on day 1 and a minimum value of 6.5 on day 13. The pH began to drop as glucose was being consumed and cells began to die, creating an acidic environment. However, when glucose was supplemented on day 11, this resulted in normalization of pH since cells began to thrive again and release $CO_2$ into the culture, thus raising the pH over time. The pH than continued to drop as the supplemented glucose was being consumed over the final days of the culture period. The experiment concluded with a final pH of 6.71, slightly below the optimal range.

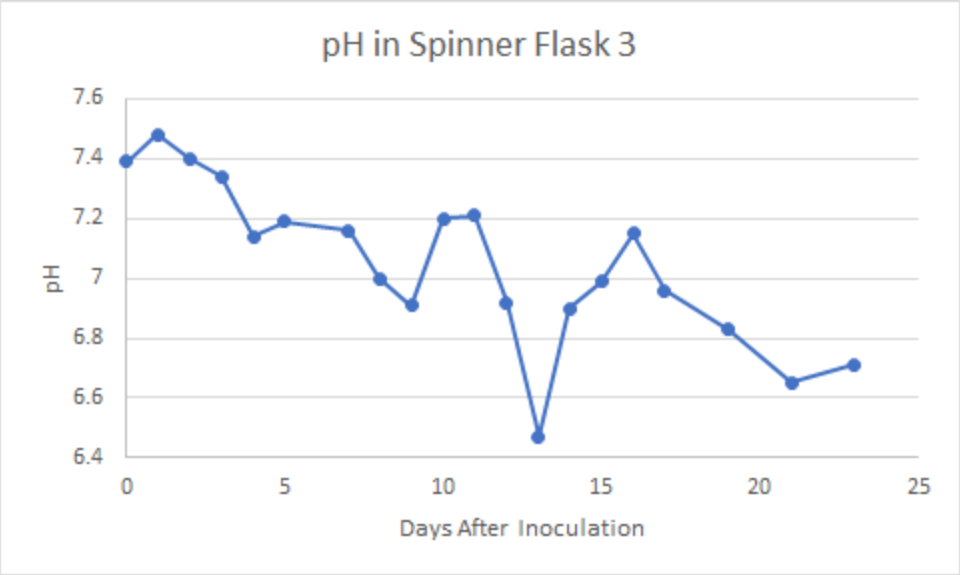



*Figure 50: 3 L spinner pH analysis*

The following graph (Figure 51) presents the glucose and lactate metabolite concentration within spinner 3 of the 3 L spinner over the course of the experiment. Glucose was shown to decrease from 5 g/L to 1.5 g/L, while lactate increased to 2.05 g/L over the experiment. Glucose was supplemented on day 11 and day 17, which is shown by the slight increase in glucose and slight decrease in lactate in the following days. The decrease in glucose and the increase in lactate demonstrates cell growth and proliferation due to metabolism of glucose and production of lactate.

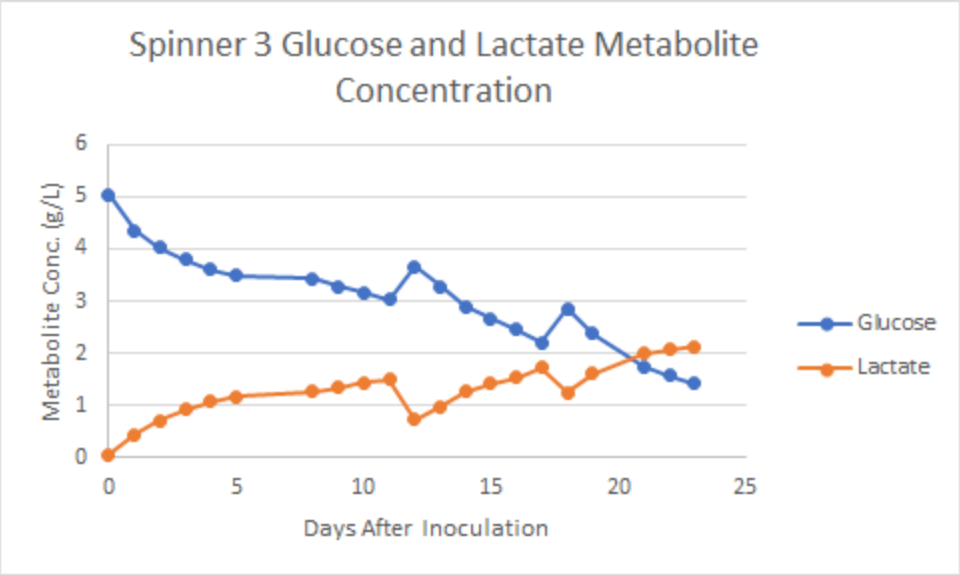



*Figure 51: Glucose and lactate metabolite concentration in the 3 L spinner flasks*

The following graph (Figure 52) shows the ammonium and glutamine metabolite concentration within Spinner 3 of the 3 L spinner flasks over the course of the experiment. Glutamine began at 4 mmol/L and concluded with a final concentration of 2.1 mmol/L, while ammonium increased to a maximum value of 5.8 mmol/L. Glutamine was supplemented on days 11 and 17, which is shown by the sudden increase in glutamine concentration on these days. The decrease in glutamine and increase in ammonium over the course of the experiment signifies the proliferation of cells due to metabolism of glutamine and production of ammonium within the culture.

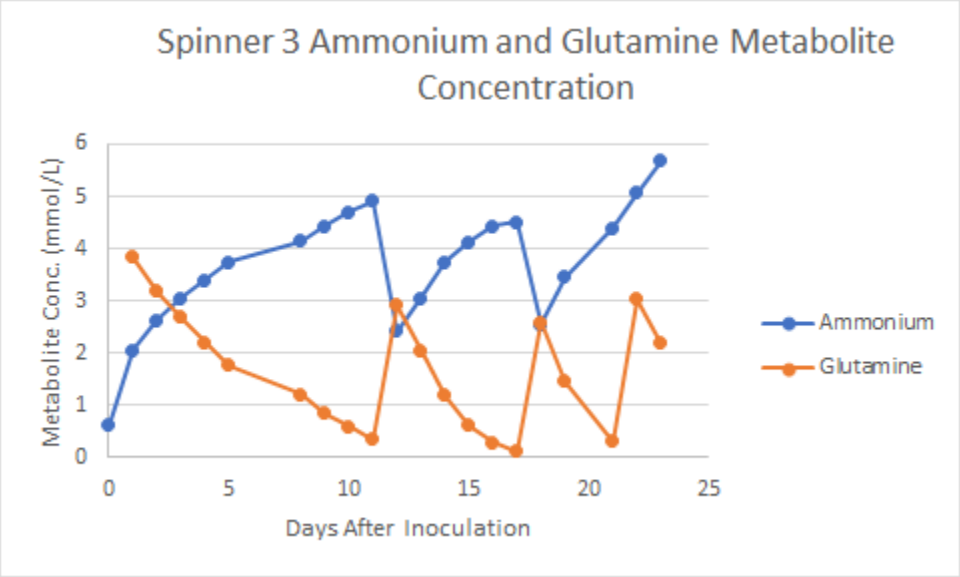



*Figure 52: Ammonium and Glutamine metabolite concentration in the 3 L spinner flasks*

The following graph (Figure 53) shows the glutamate concentration within Spinner 3 of the 3 L spinners over the course of the experiment. Glutamate increased to 350 mg/L by the conclusion of the experiment, signifying cell proliferation due to the metabolism of glutamine into glutamate. Glutamine was supplemented on days 11 and 17, which is shown by the decrease in production of glutamate on these days.

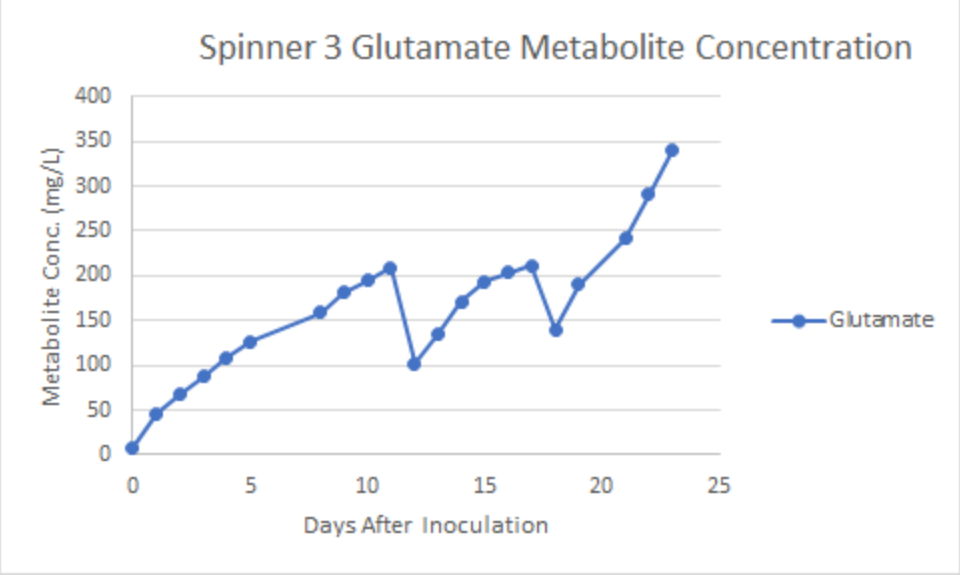



*Figure 53: Glutamate metabolite concentration in the 3 L spinner flasks*

The following graph (Figure 54) presents the LDH metabolite concentration within Spinner 3 of the 3 L spinner flasks over the course of the experiment. The LDH concentration reached a maximum concentration of 395 U/L on day 21, signifying the most amount of cell death.

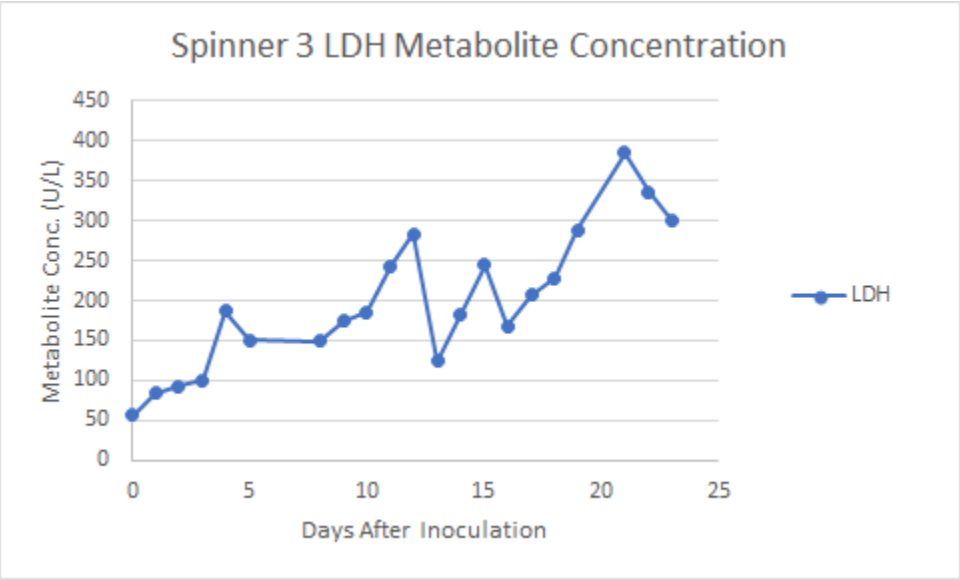



*Figure 54: LDH metabolite concentration in the 3 L spinner flasks*

Contamination was found in Spinner 1 on day 14 of the experiment, and was replaced by an additional spinner, Spinner 4, on day 18. Cells were thawed into 7 T-300s and 4 T-185s and upscaled into the 3 L spinner with no 500 mL spinner in the n-1 stage. This spinner had its own experiment that spread over the course of 5 days and concluded at the same time as the other two spinners on day 23. The images below (Figure 55) show the cell attachment onto the microcarrier beads throughout the span of 5 days. Cells showed immediate attachment after day 1. The image from day 5 showed about 90% of the microcarrier beads confluent with cells.

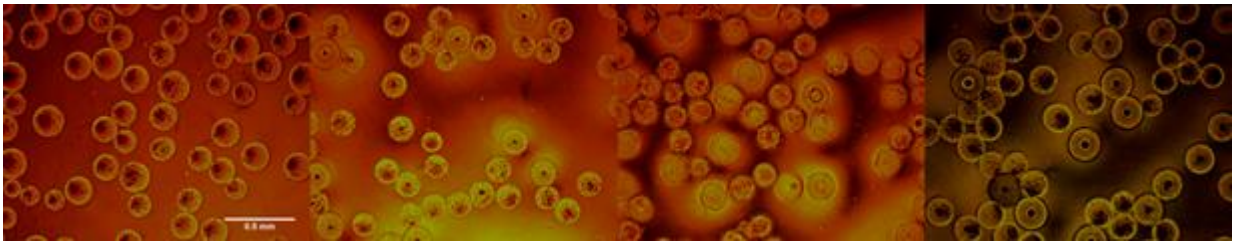


*Figure 55: Microscope images of the microcarriers in the 3 L spinner flask 4. From left to right, day 1, day 3, day 4, and day 5. Scale bar shown is 0.5 mm in length (All images taken at same magnification)*

The following graph (Figure 56) represents the pH level in Spinner 4 over the course of 5 days. The pH is shown to drop from 7.3 to 6.2 in the span of the experiment. The sudden drop in pH may be due to the high rate of cell proliferation, which results in a large amount of ammonium production and acidity within the media.

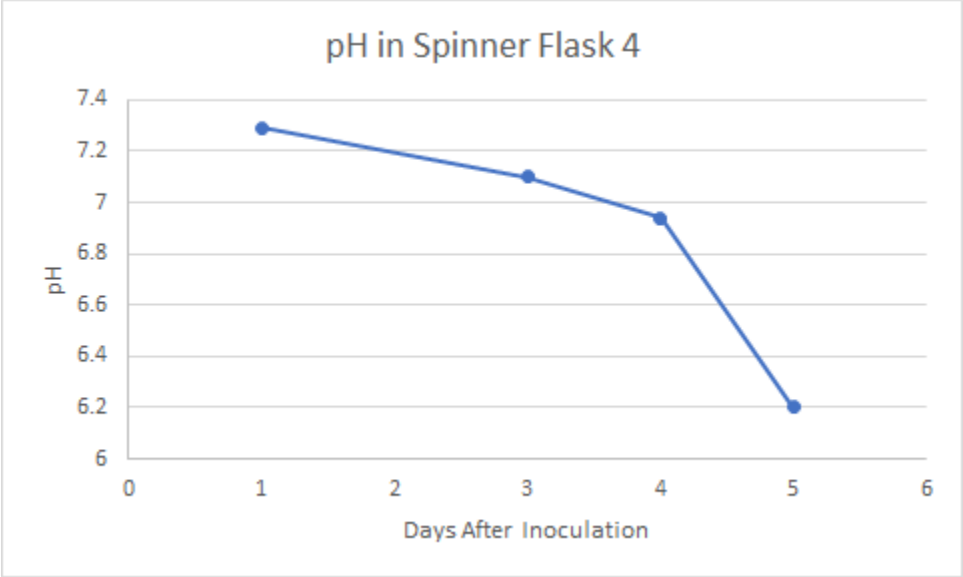



*Figure 56: 3 L spinner flask 4 pH analysis*

The following graph (Figure 57) shows the glucose and lactate metabolite concentration in Spinner 4 over the span of 5 days. The glucose concentration decreased from 5 g/L to 1.25 g/L while the lactate steadily increased to 1.5 g/L over the span of 5 days. This data demonstrates the metabolism of glucose into lactate, and therefore cell growth and proliferation within the spinner.

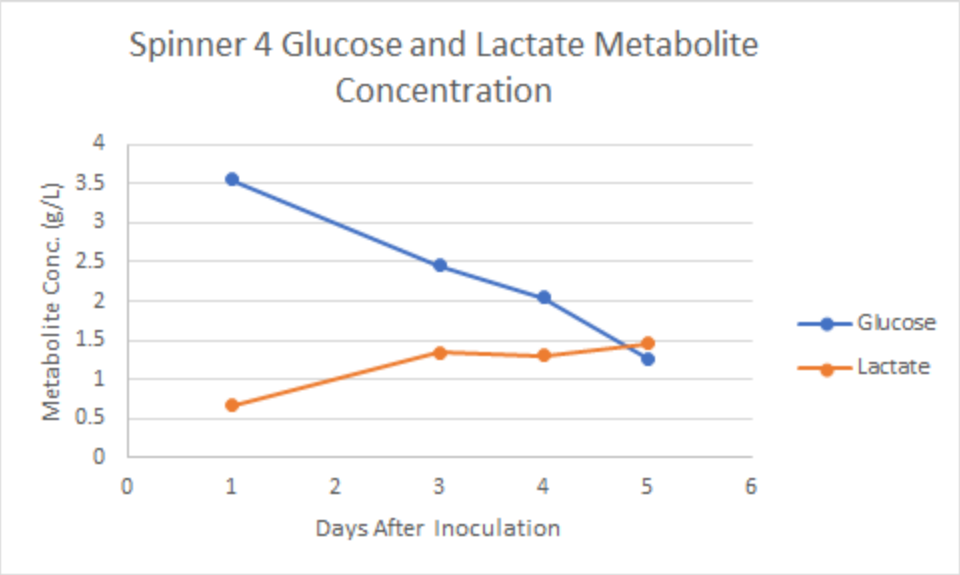



*Figure 57: Glucose and lactate metabolite concentration in the 3 L spinner flask 4*

The following graph (Figure 58) displays the ammonium and glutamine metabolite concentration within Spinner 4 throughout 5 days. Ammonium was shown to steadily increase over the course of 5 days up to 5.6 mmol/L and glutamine decreased from 4 mmol/L to 1.8 mmol/L. The spinner was supplemented with glutamine on day 3 because of complete depletion of glutamine. This could have been due to a high rate of cell proliferation, which results in a large amount of glutamine consumption.

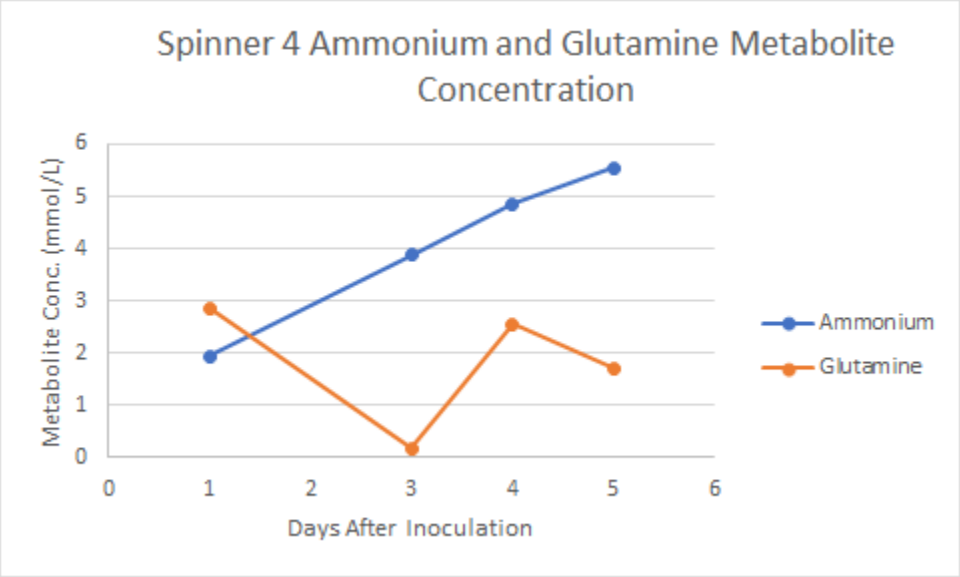



*Figure 58: Ammonium and Glutamine metabolite concentration in the 3 L spinner flask 4*

The following graph (Figure 59) presents the glutamate metabolite concentration within Spinner 4 throughout the span of 5 days. The glutamate concentration was shown to increase from 51 mg/L to 220 mg/L throughout the 5 days, demonstrating cell growth and proliferation due to glutamine metabolization.

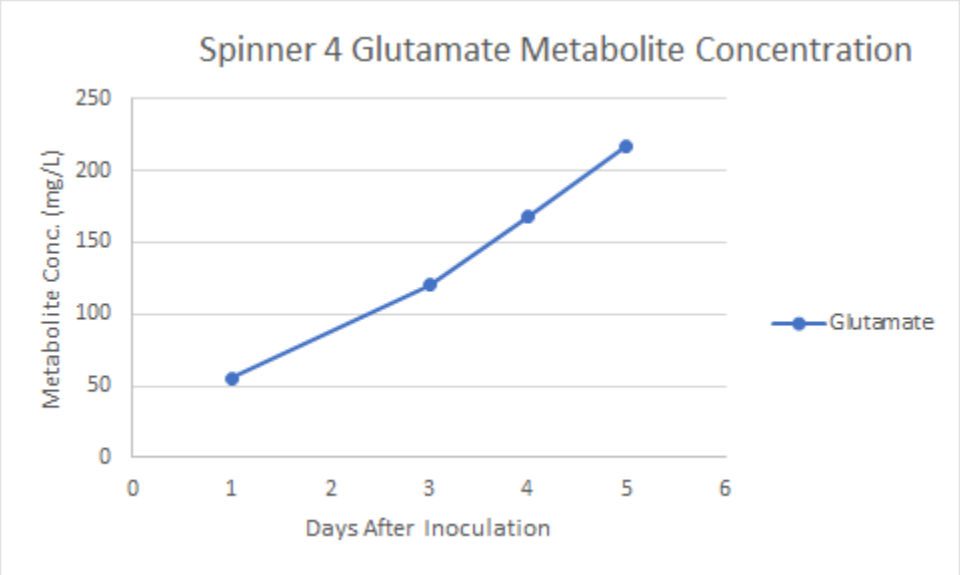



*Figure 59: Glutamate metabolite concentration in the 3 L spinner flask 4*

The following graph (Figure 60) presents the LDH metabolite concentration within Spinner 4 over the course of 5 days. The LDH concentration was shown to increase to 230 U/L. The steady increase demonstrates a slow rate of cell death within the spinner flask.

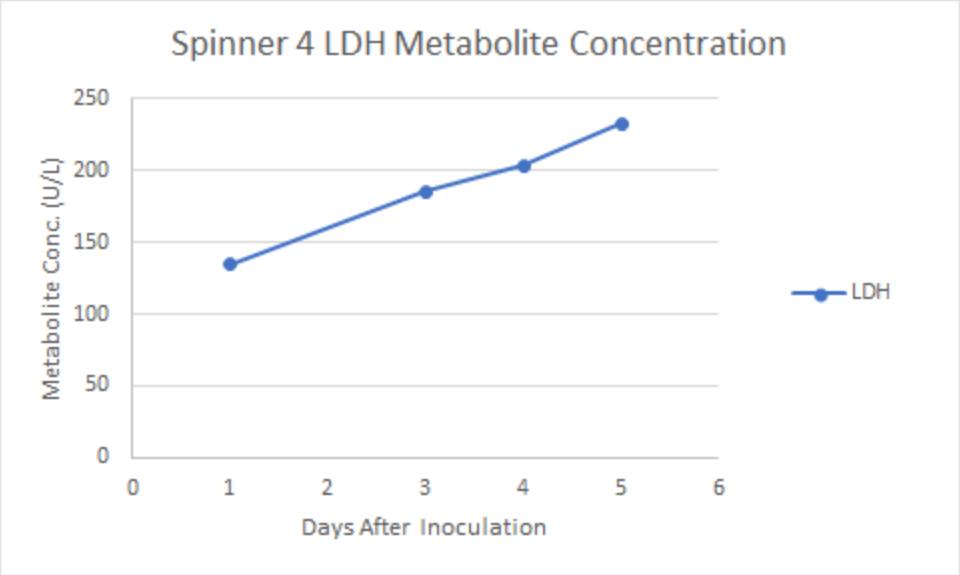



*Figure 60: LDH metabolite concentration in the 3 L spinner flask 4*

## 5.3 Redistributed cells among 3 spinners at 1.5L fill

Following the 23 days of culture within the three 3 L spinner flasks at a 1 L working volume, the cells were trypsinized within the spinners and evenly distributed among the three 3 L spinners at a working volume of 1.5 L for a 3 day culture period. This was the final step prior to inoculating the XDR 200. The cell attachment onto the microcarrier beads are illustrated in Figure 61 below. The image of day 3 demonstrates ~90% of beads confluent with cells, in addition to a high amount of debris due to the crushing of microcarriers from the impeller within the spinner flask.

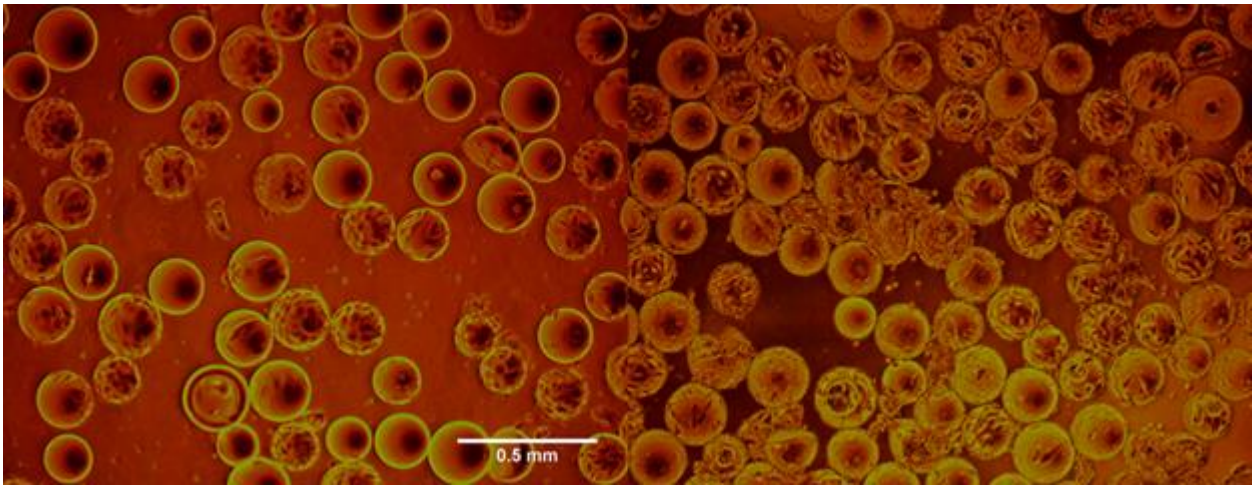



*Figure 61: Microscope images of the microcarriers in the redistributed 3 L spinner flask (flask 2). From left to right, day 1, and day 3. Scale bar shown is 0.5 mm in length (All images taken at same magnification)*

The following graph (Figure 62) presents the pH level within Spinner 2 of the 3 L spinners throughout 5 days. The pH decreased from 7.1 to 6.83 over the span of this experiment, which demonstrates cell growth due to the release of acidic byproducts from metabolism of glucose and glutamine.

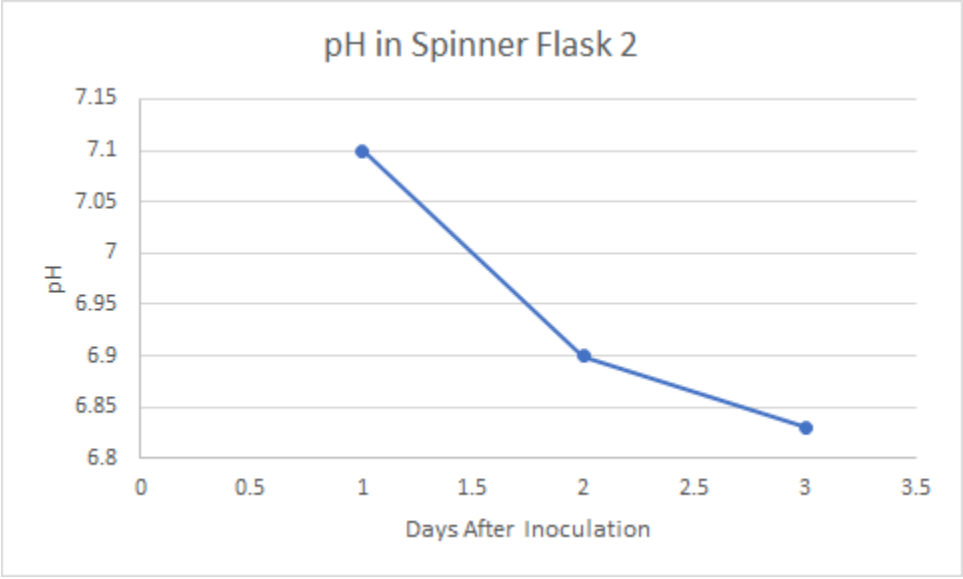



*Figure 62: pH in redistributed spinner flask 2*

The following graph (Figure 63) shows the glucose and lactate metabolite concentration within Spinner 2 of the 3 L spinners over the course of 3 days. Glucose was shown to steadily decrease from 3.1 g/L to 1.56 g/L, while lactate increased from 0.5 g/L to 0.8 g/L over the span of this experiment. The data demonstrates steady growth of cells due to the metabolism of glucose into lactate.

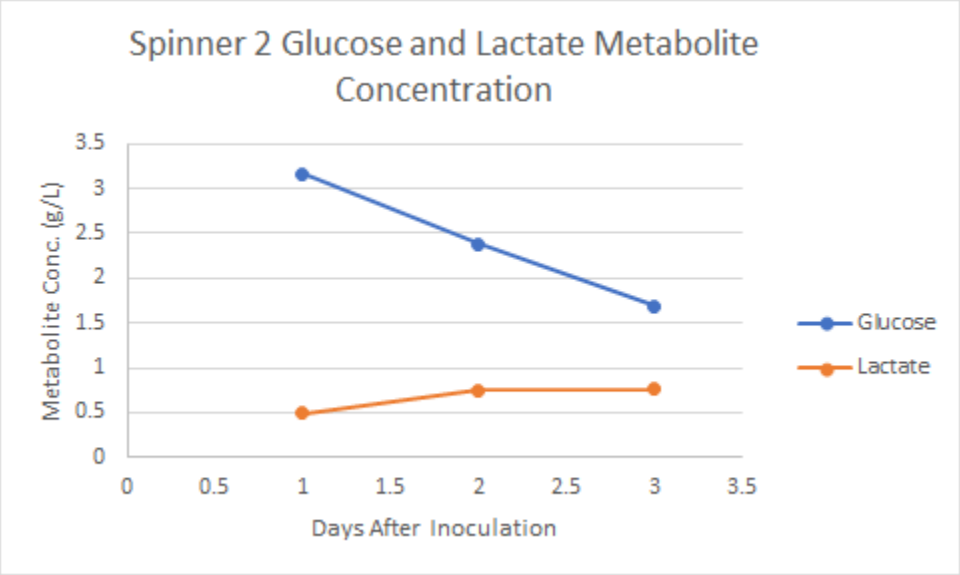



*Figure 63: Glucose and lactate metabolite concentration in the redistributed 3 L spinner flask 2*

The following graph (Figure 64) shows the ammonium and glutamine metabolite concentration within Spinner 2 of the 3 L spinners throughout the span of 3 days. Glutamine was shown to drop from a concentration of 3.5 mmol/L to 1.9 mmol/L while ammonium increased up to 2 mmol/L. This data indicates the metabolism of glutamine by the cells for energy, therefore ammonium is created as a byproduct. The minor change in these concentrations also show that the growth is at a slow rate.

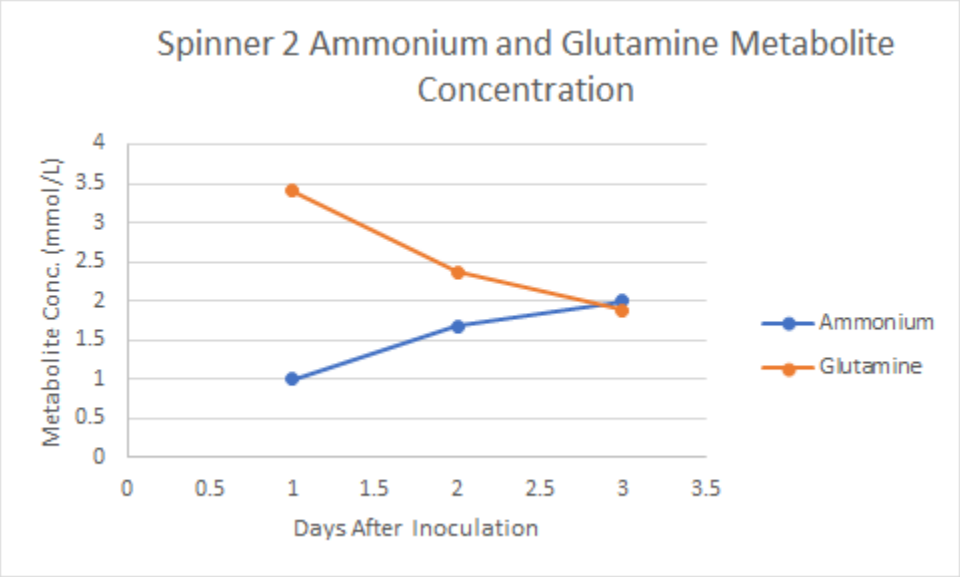



*Figure 64: Ammonium and glutamine metabolite concentration in the redistributed 3 L spinner flask 2*

The following graph (Figure 65) displays the glutamate metabolite concentration within Spinner 4 of the 3 L spinners throughout 3 days. The glutamate concentration was shown to increase from 50 mg/L to 150 mg/L, which indicates metabolism of glutamine and therefore, cell growth and proliferation.

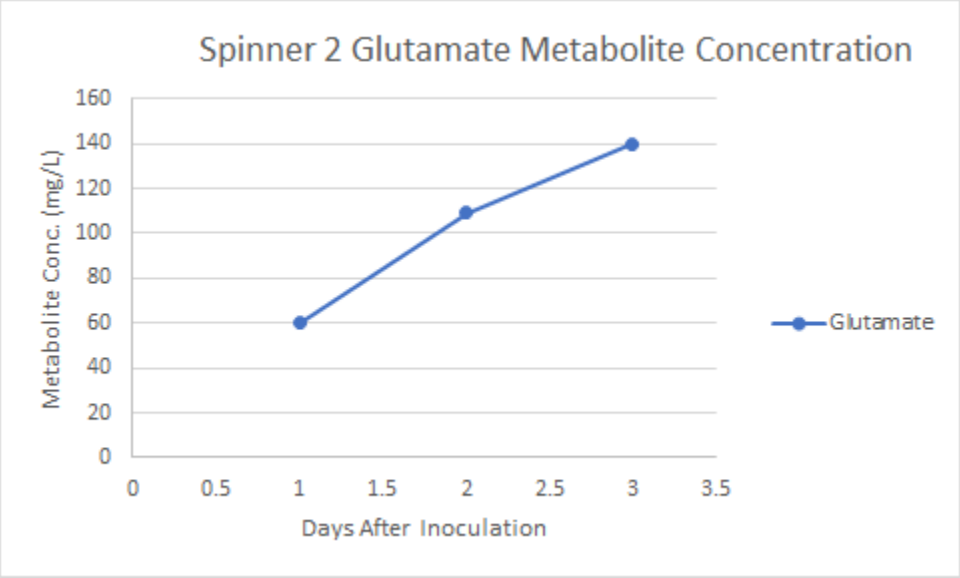



*Figure 65: Glutamate metabolite concentration in the redistributed 3 L spinner flask 2*

The following graph (Figure 66) represents the LDH metabolite concentration within Spinner 2 of the 3 L spinners throughout the culture period of 3 days. LDH levels showed very little cell death between days 1 and 2, as the LDH concentration remained at 150 U/L. However, LDH concentration increased to 355 U/L the following day, indicating an increase in cell death.

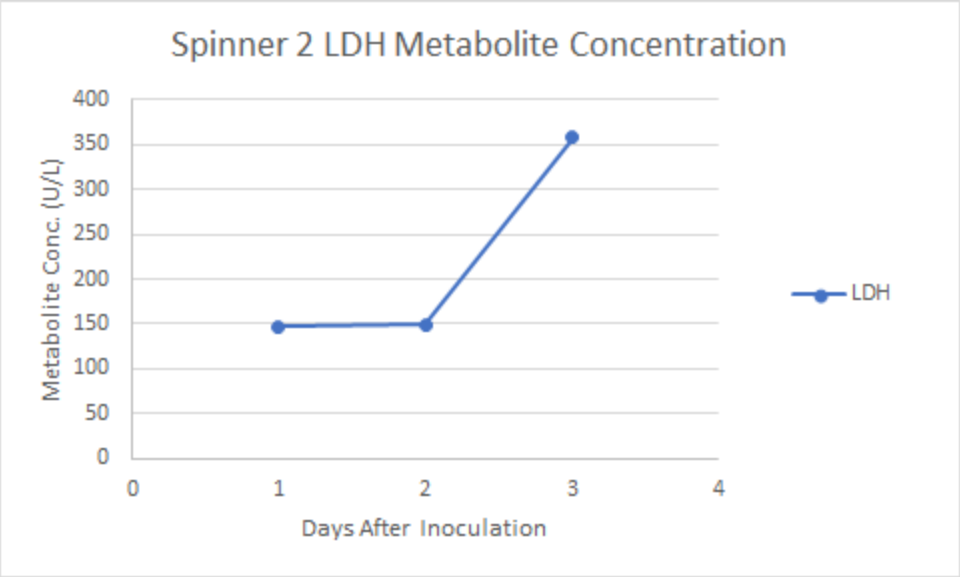



*Figure 66: LDH metabolite concentration in the redistributed 3 L spinner flask 2*

## 5.4 XDR-200 Run

Following the 3 day culture period within the three 3 L spinners at a 1.5 L working volume, the cells were once again trypsinized and the inoculum was transferred into the XDR at a 52 L working volume for a culture period of 7 days. The cell attachment to microcarrier beads is displayed in the images below in Figures 67 and 68. The image shown in day 4 indicates the accumulation of debris within the culture, due to the destruction of microcarrier beads and cell death. The image in day 5 presents what is believed to be a deformed microcarrier bead. By the conclusion of this experiment, ~60% of the microcarrier beads were confluent with cells within the culture, however some microcarrier beads had debris attached to them.

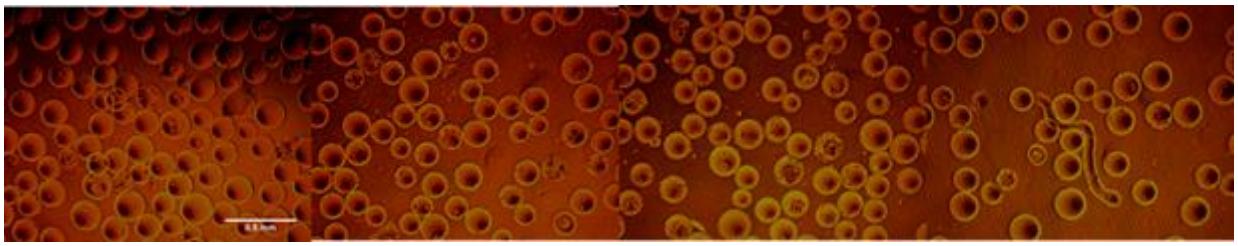


*Figure 67: Microscope images of the microcarriers in the XDR- 200 at a 52 L volume. From left to right, day 1, day 3, day 4, and day 5 (bead deformation). Scale bar shown is 0.5 mm in length*

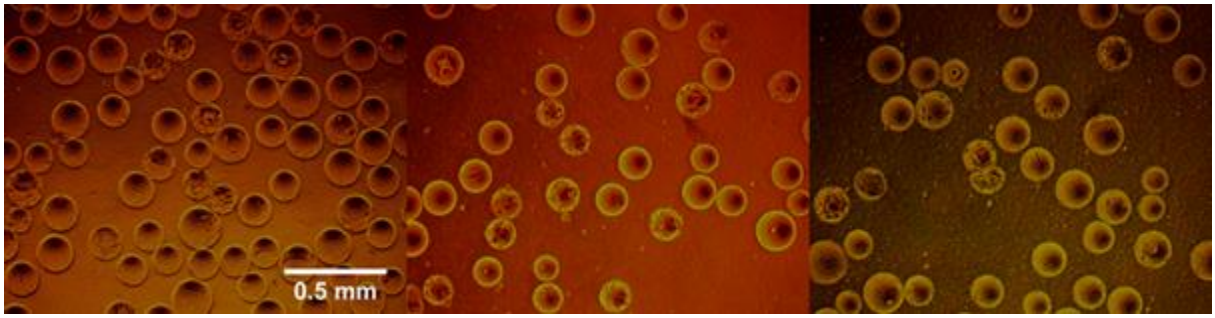



*Figure 68: Microscope images of the microcarriers in the XDR- 200 at a 52 L volume. From left to right, day 5, day 6, and day 7. Scale bar shown is 0.5 mm in length (All images taken at same magnification)*

Following the 7 day culture period at 52 L within the XDR 200, the cells were trypsinized within the XDR 200 by draining the culture media to 19 L, washed twice with 20 L of PBS, and trypsinized with 6.5 L of trypsin. The resulting final volume within the XDR 200 was 150 L. This cell culture period lasted for 7 days. The following images (Figures 69 and 70) display the cell attachment onto the microcarrier beads throughout the 7 day culture period. The images indicates very little cell attachment to the microcarrier beads, in addition to a high amount of debris, suggesting the trypsinization process was not successful.

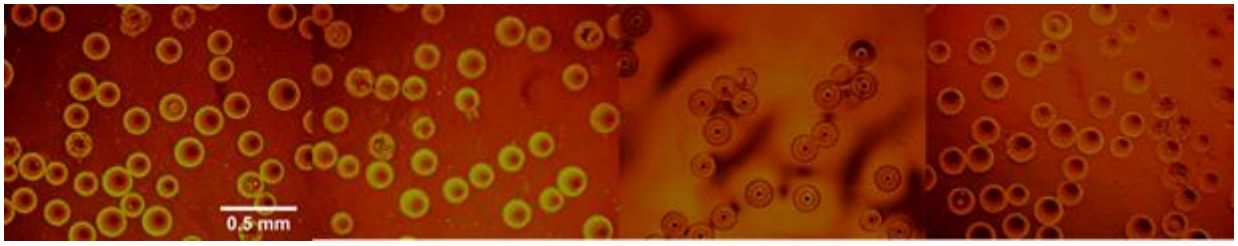



*Figure 69: Microscope images of the microcarriers in the XDR- 200 at a 150 L volume. From left to right, day 1, day 2, day 3, and day 4. Scale bar shown is 0.5 mm in length (All images taken at same magnification)*

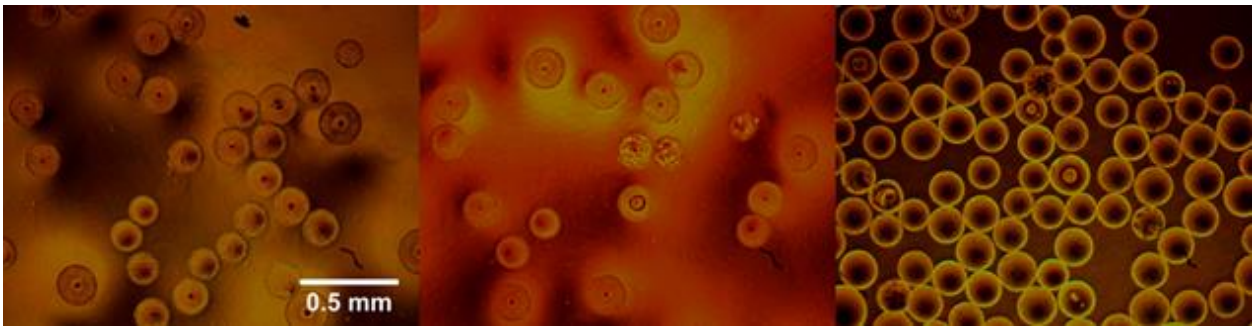



*Figure 70: Microscope images of the microcarriers in the XDR- 200 at a 150 L volume. From left to right, day 5, day 6, and day 7. Scale bar shown is 0.5 mm in length (All images taken at same magnification)*

The following set of graphs (Figures 71 and 72) displays the cell counts within the XDR 200 over the 14-day culture period. Graph (a) shows the first 7-day period in the XDR 200 with a working volume of 52 L. The actual cell growth was found to be much lower than the expected calculated cell growth. Graph (b) shows the second 7-day period in the XDR 200 with a working volume of 150 L. This data demonstrates very little cell growth throughout the cell culture, and found to be significantly lower than the expected calculated cell growth.

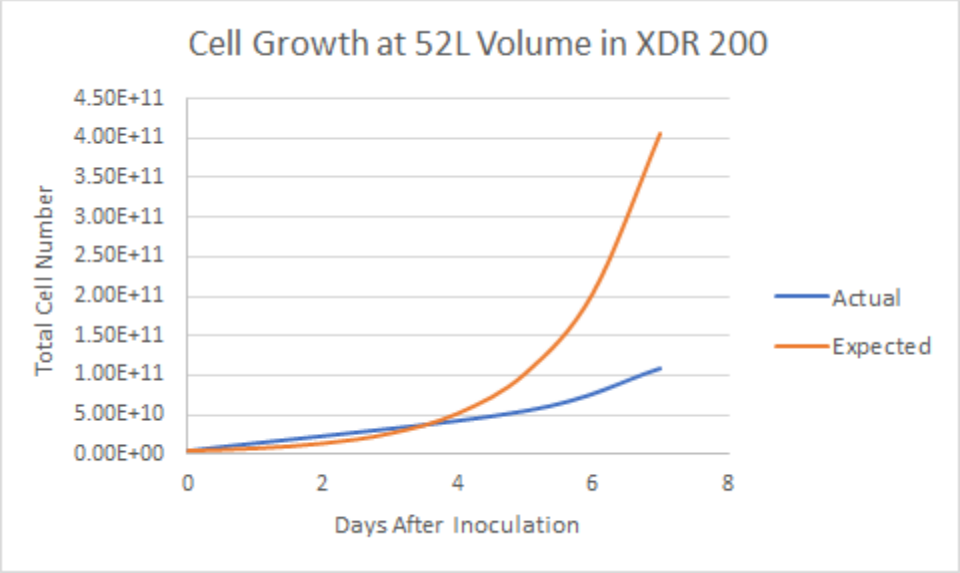



*Figure 71: Actual cell count vs expected cell count in the XDR-200 at the 52 L volume*

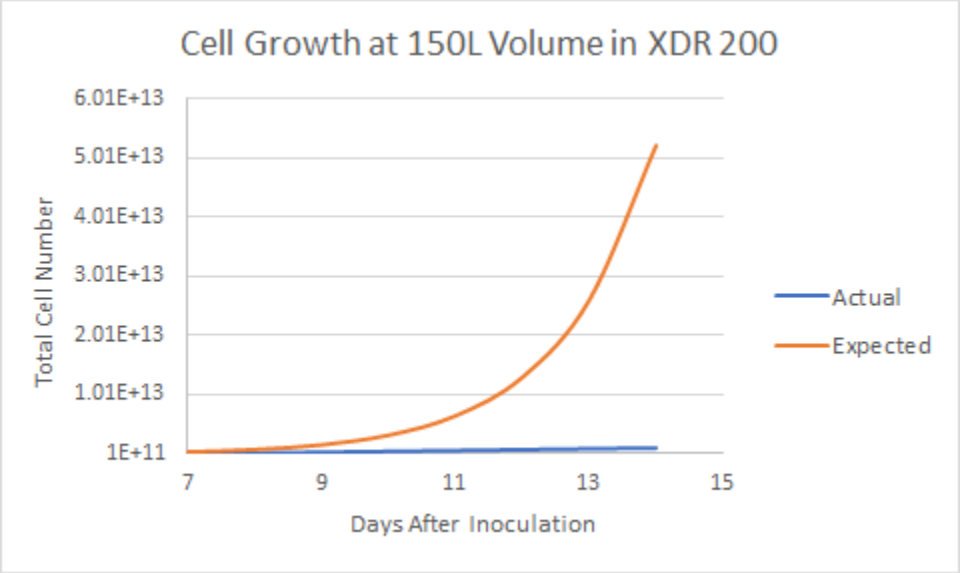



*Figure 72: Actual cell count vs expected cell count in the XDR-200 at the 150 L volume*

The following graph (Figure 73) displays the glucose and lactate metabolite concentration within the XDR 200 over the 14-day culture period. Glucose was supplemented on day 8 during the process of trypsinization and increasing the working volume to 150 L. Within the first 7 days, the glucose concentration decreased from 4.2 g/L to 1.1 g/L. The second 7-day culture period shows

a decrease in glucose concentration from 4 g/L to 2.4 g/L. LDH remained fairly steady throughout the 14-day culture period, and had very little change in concentration. This data indicates a very small rate of cell growth and proliferation, especially during the second 7-day period where there was minor glucose consumption and minor LDH release.

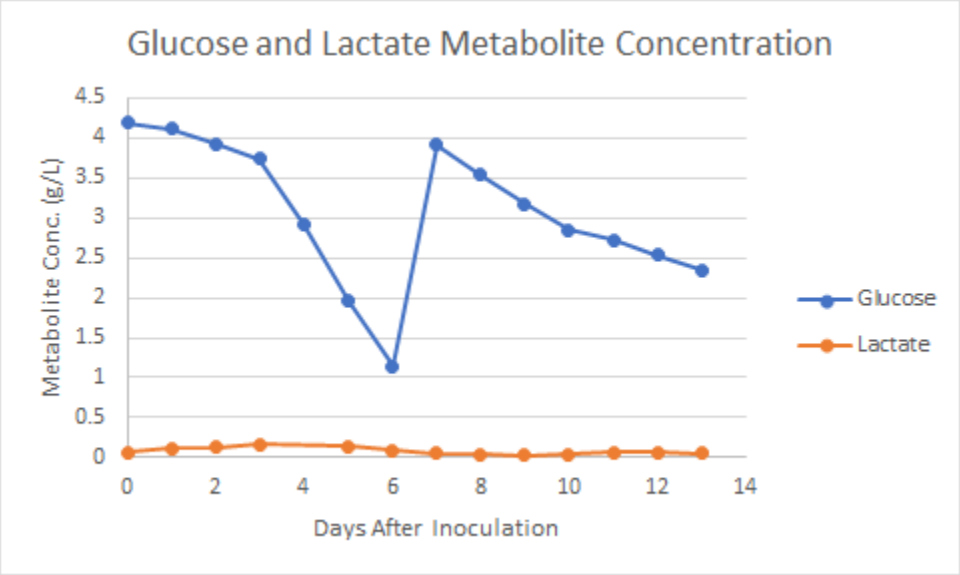



*Figure 73: Glucose and lactate metabolite concentration in the XDR-200 at a 52 L volume*

The ammonium and glutamine metabolite concentration over the 14-day culture period is shown in Figure 74 below. Throughout the first 7-day culture period at 52 L within the XDR 200, the ammonium increased up to 2.1 mmol/L and the glutamine decreased from 5.9 mmol/L to 3.1 mmol/L. This data indicates slight cell growth and proliferation over the first 7-day period. Glutamine was supplemented on day 8 when the cells were trypsinized and the working volume was increased to 150 L. Glutamine decreased from 4.5 mmol/L to 2.9 mmol/L, while the ammonium concentration increased from 1 mmol/L to 2 mmol/L over the second 7-day period at 150 L working volume. This data indicates very minimal cell growth and proliferation due to the minimal change in concentration of glutamine and ammonium. This data suggests that the cells

are not consuming glutamine for energy, and therefore little ammonium is produced as a byproduct.

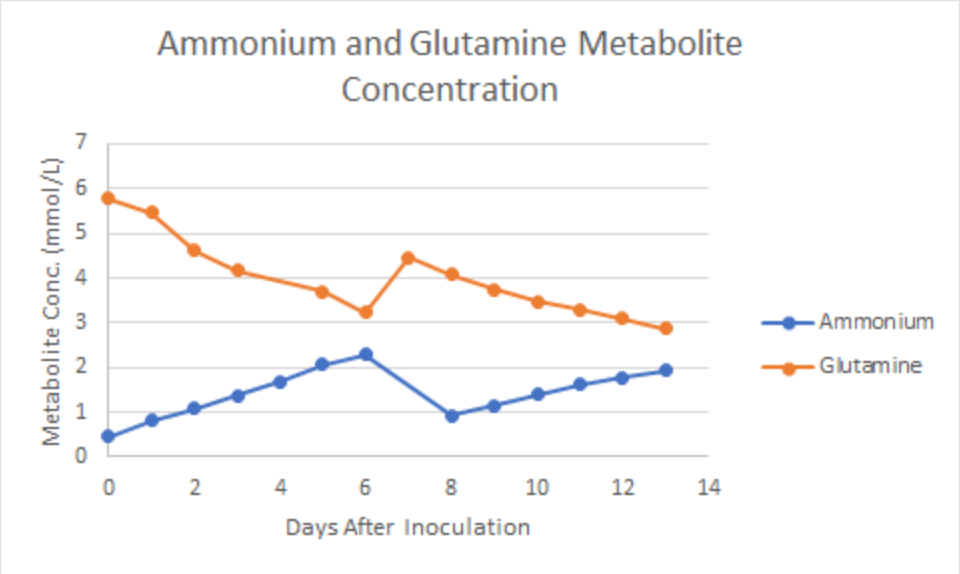



*Figure 74: Ammonium and Glutamine metabolite concentration in the XDR-200 at a 52 L volume*

The following graph (Figure 75) shows the glutamate metabolite concentration within the XDR 200 over the 14-day culture period. The first 7-day culture period had an increase in glutamate concentration from 10 mg/L to 90 mg/L. The second 7-day culture period at a working volume at 150 L within the after trypsinization and supplementation of glutamine within the XDR 200 had a slight increase from 10 mg/L to 51 mg/L over the 7-day culture period. This growth is significantly lower than the first 7-day culture period at a 52 L working volume. This is due to the minimal uptake of glutamine, which results in little release of glutamate as a byproduct.

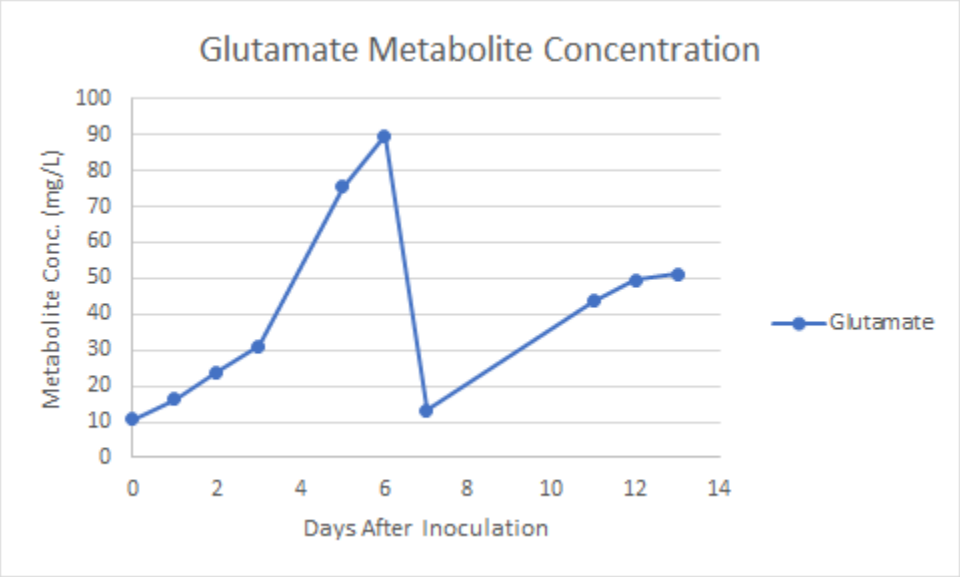



*Figure 75: Glutamate metabolite concentration in the XDR-200 at a 52 L volume*

The following graph (Figure 76) displays the LDH metabolite concentration over the 14-day culture period within the XDR-200. The first 7 days at a working volume of 52 L shows an increase of LDH from 59 U/L to 107 U/L, indicating an increase in cell death within the culture. The second 7-day culture period within the bioreactor at a 150 L working volume displays a constant average around 60 U/L with little LDH production, indicating little cell death compared to the first 7-day period at a 52 L working volume.

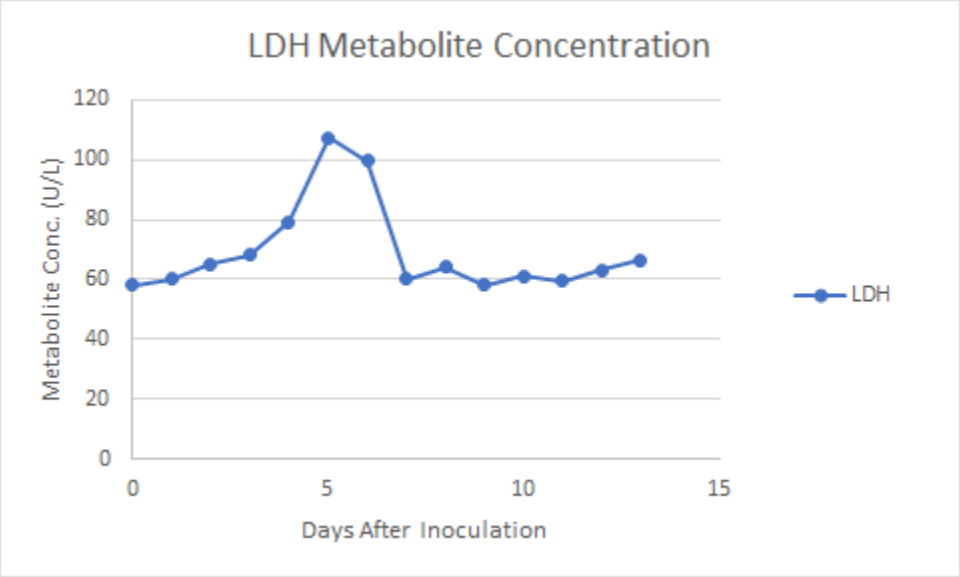



*Figure 76: LDH metabolite concentration in the XDR-200 at a 52 L volume*

# Chapter 6: Final Design and Validation

The purpose of this project ultimately was to develop a seed train methodology which would support the culture of adherent mammalian Vero cells on microcarriers in a 200 L XDR bioreactor. Through customer interviews, alternative designs and verification experiments the most effective seed train designs and cell culture methods were used to design the final cell culture. From this final cell culture the team was able to propose further changes to bioreactor and seed train design which could better fulfill the following project objectives outlined in Chapter 3.

1. Scale the culture from a frozen seed bank to a large enough inoculation density for the XDR.
2. Determine feasibility of using the XDR-200 for Vero Cell culture while utilizing Cytodex microcarriers for bead to bead transfer.
3. Ensure the XDR-200 maintains ideal cell conditions for cell growth and viability.

The following sections describe how the final experimental design was performed while discussing how it was able to meet the project objectives.

## 6.1 Seed Train Design

The flowchart seen in Figure 77 below visualizes the major steps in the final cell culture design.


*Figure 77: Final design outline*

### 6.1.1 Thaw Cells

A 1.5 mL vial of approximately $20x10^6$ Vero cells were thawed onto 2 Celltreat T-300 cell culture plates in accordance with SOP 01. The cells were submerged in 40 mL 5% CFS GE MEM media and placed in 5% CO2 incubator at 37 C. The cell growth was observed through phase shift microscopy over 48-72 hours. Once high confluency was achieved, (>80% of culture plate covered by Vero Cells) the cells were trypsinized, resuspended in media and counted with a CEDEX cell counter. Figure 78 below provides an example of a highly confluent T-300 flask. A cell count of $60\text{-}80x10^6$ cells should be resuspended to proceed.

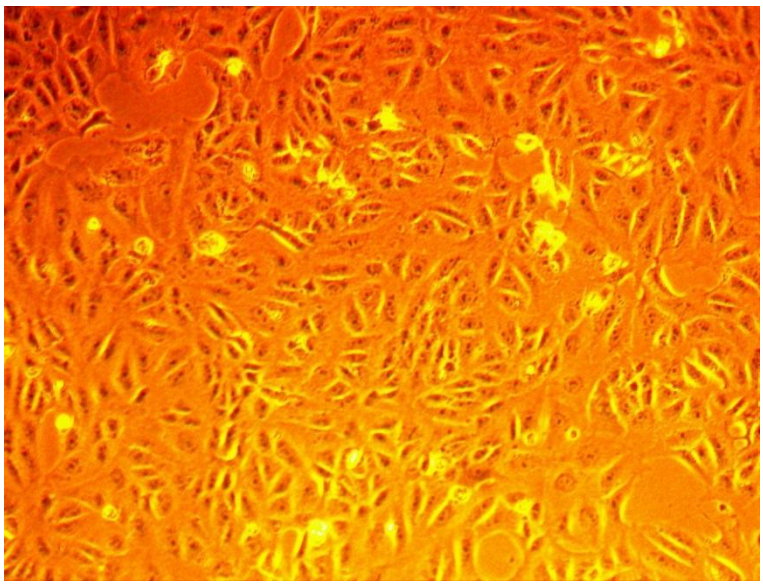


*Figure 78: Cells in T-300 flask 48 hours after inoculation.*

### 6.1.2 T-300 Culture Plates

The suspended cells were equally divided into 6 Celltreat T-300 cell culture plates in the same conditions as the previous step. Once high confluency was achieved the cells were again trypsinized, resuspended and counted. A cell count of 1.8-2.4x$10^8$ cells should be achieved to proceed.

### 6.1.3 500 mL Spinner Flasks

3 x 500 mL Corning disposable spinner flasks were filled to a maximum 300 mL working volume of bead slurry at least 24 hours before the T-300 plates reached confluency. The bead slurry consisted of 5% CFS GE MEM media, 3g/L Cytodex 1 Gamma microcarriers, and was supplemented to 4-5g/L glucose and 4 mmol L-glutamine. The pre-filled spinner flasks were placed into a 5% CO2 incubator at 37 C onto spinner plates set to 40 RPM for 24 hours. This allows for the media to warm and the Cytodex microcarriers to condition and expand. Before inoculation a sample is taken to observe homogenous microcarrier diameter seen below in Figure 79.

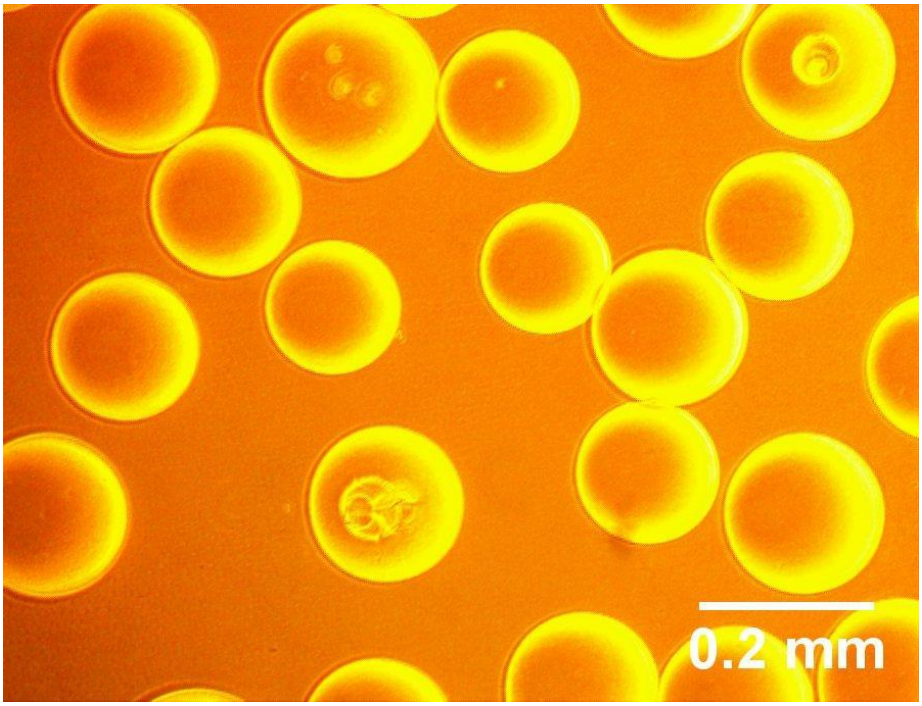



*Figure 79: Cytodex microcarriers after 18 hours of being conditioned.*

Once bead conditioning was determined the suspended cells taken from the T-300 flasks were pipetted evenly into the 3 spinner flasks. A baseline media metabolite analysis was performed with a Cedex Bio Analyzer to determine initial levels of glucose, lactate, lactate dehydrogenase, ammonia, glutamate, L-glutamine and pH. These tests provide markers for:

1. Cell growth - glucose, glutamate and L-glutamine
2. Cell death - lactate dehydrogenase
3. Media conditions - ammonia

The spinner flasks were placed in an incubator and set to intermittently agitate for 18-24 hours with a duty cycle of 3 minutes on 55 minutes off at 50 RPM. Once complete the agitation was set to continuously spin at 50 RPM. The cells were sampled daily. Each sample had 1.5 mL taken for media analysis in the Cedex Bio Analyzer. To prevent the microcarriers from clogging the analyzer the sample was spun in a centrifuge at 1400 RPM for 2 minutes to separate the

microcarriers from the supernatant. 1-2 mL was sampled daily and viewed under phase shift microscopy to determine overall bead confluency pictured in Figure 80 below. <5 mL was sampled and placed in a conical tube to measure pH of the media.

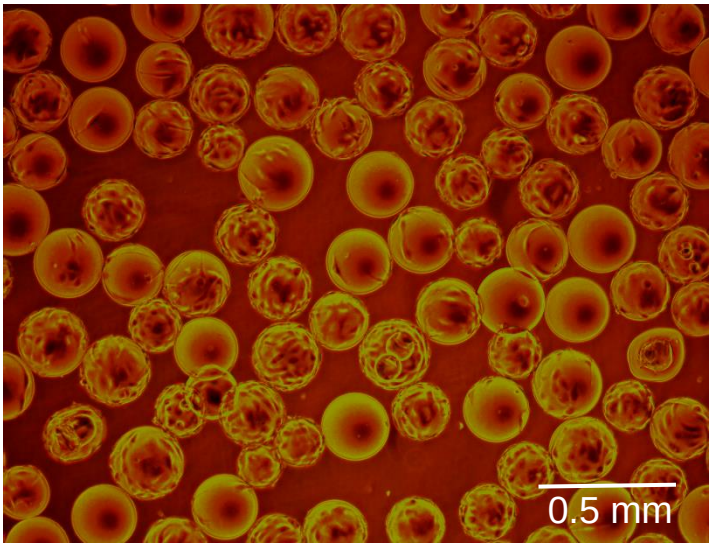



*Figure 80: 500 mL Spinner bead confluency check 96 hours after inoculation*

Once high confluency, >75%, was reached. The flasks were placed into a biosafety cabinet where the beads were allowed to settle for 5-10 minutes. Approximately 150 mL of metabolized media was pipetted out of the flasks. The cells were then pumped into 3x 3 L disposable corning spinner flasks at a working volume of 1 L. To transfer the cells ¼" sterile bio-welded c-flex tubing was connected between each spinner flask. The 500 mL flasks were then pressurized with <5 psi air filtered through a 0.22 um filter. This method was used over pipetting because it reduces physical manipulation within the flasks, which could risk contamination. Air pressure was used rather than a peristaltic action pump to reduce crushing of microcarriers.

### 6.1.4 3L Spinner Flasks

24 hours before transferring the cells from the 500 mL to the 3 L flasks, 850 mL of bead slurry was prepared and conditioned in each 3 L spinner flask. The stock 5% CFS GE MEM

media was supplemented to 5 g/L glucose and 4 mmol L-glutamine. 3 g/L microcarriers were weighed in a biosafety cabinet and poured into each flask containing the media. The spinner flasks were set to constantly agitate at 40 rpm in a 5% CO2 incubator at 37 C for 24 hours.

The contents from each 500 mL spinner flask was then pumped into each 3 L spinner flask. A new baseline media analysis was performed to track glucose consumption and the media metabolites described earlier in this section. The flasks were set to spin intermittently for the first 18-24 hours at 40 RPM with a duty cycle of 3 minutes 55 minutes off to allow for more physical bead-bead interaction. The agitation was then set to a constant 30 RPM.

The cells were sampled daily using the same methods described for sampling the 500 mL spinner flasks. After 96-120 hours it was observed that cell confluency and bead coverage had not been increasing, pictured below in Figure 81. Glucose uptake rate remained constant, indicating that no new cells were being produced. From this, the team determined that bead-to-bead transfer of cells without trypsinization was not a viable method for Vero cell culture at larger scales than demonstrated in the spinner flask tests performed in section 4.

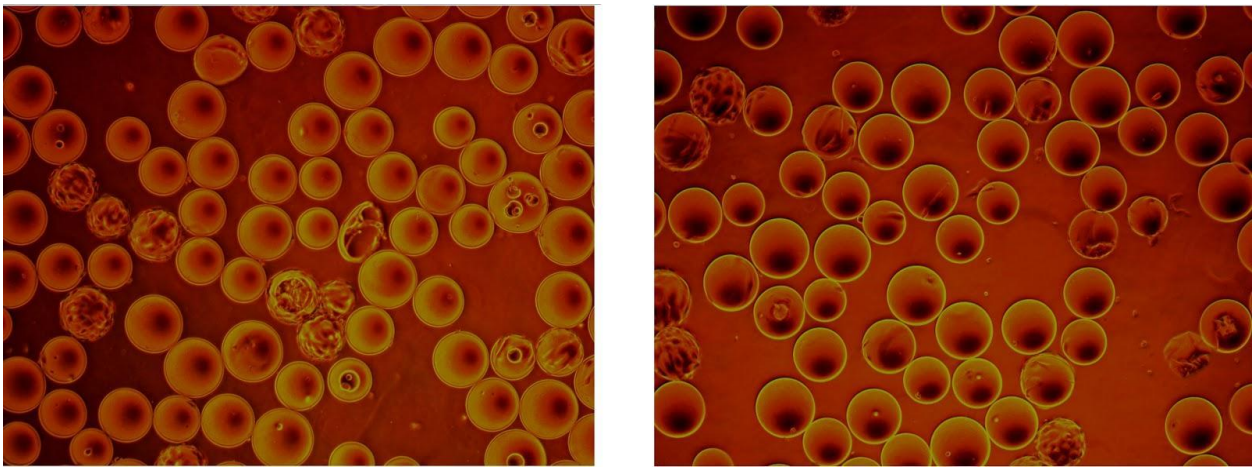


*Figure 81: Cell confluency 3L spinners - day 1 (left) vs. day 5 (right)*

In order to resume cell culture 12 x T-300 culture plates were inoculated with thawed

cells using the methods described earlier in section 6. These cells were to be ultimately harvested and distributed evenly among each spinner flask. Before adding the cells, excess metabolized media was pipetted off each flask and replaced. The harvested cells from the T-300 flasks were distributed evenly between each spinner flask. The spinners were set to agitate intermittently for 24 hours, before resuming constant agitation. Within 96 hours the newly added cells had distributed themselves and improved bead confluency to cover approximately 40%, visible in Figure 82. At this time cell growth had peaked, confluency remained stagnant and glucose consumption remained constant.

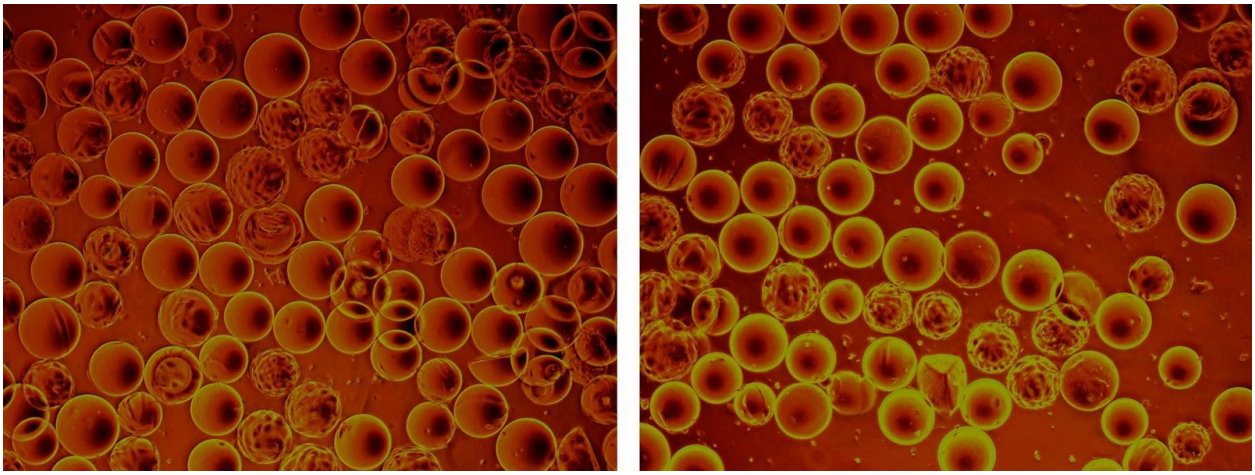


*Figure 82: Cell confluency 3L spinners 96 hours after cell addition*

To achieve the maximum working volume of 3 L in the spinner flasks and an appropriate inoculation density for the XDR-200, the cells were trypsinized from the beads before adding additional bead slurry. The spinner flasks were placed in a biosafety cabinet for 5-10 minutes to allow the microcarriers to settle. The supernatant was removed via pipetting. 500 mL of trypsin was added to each flask. The flasks were then placed in an incubator with agitation set to 50 RPM for 15 minutes to allow the cells detach from the microcarriers. 2 L of supplemented bead slurry, 4.5 g/L beads, prepared the day before was pumped into each flask. Additionally,

approximately 9.0 x $10^7$ cells, pre-cultured on T-flasks were added to each flask, because of the low confluency observed before the addition.

A baseline media analysis was performed and the flasks were set in an incubator with intermittent agitation for 24 hours, with a duty cycle of 3 minutes on 55 minutes off at 30 RPM. The spinner flasks were then set to constant agitation and samples were taken daily. The cells successfully distributed themselves among the beads, reaching >75% confluency within 5 days as seen below. The cells were ready to be added to the XDR-200. A manual cell count was performed using a crystal violet nuclei stain on a hemocytometer in accordance with SOP 02. Images of the 3 L spinner at day 1 and day 5 can be seen in Figure 83.

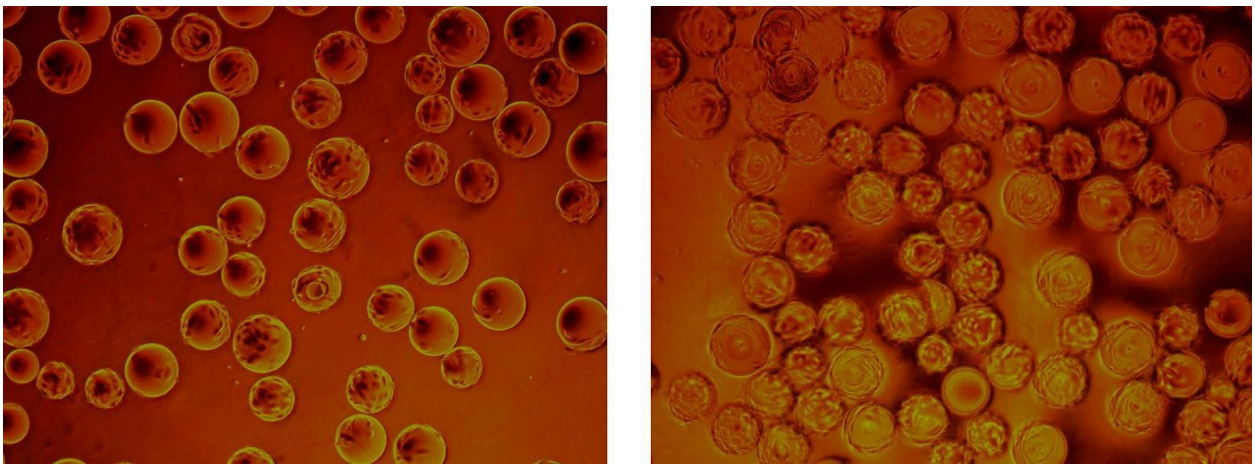


*Figure 83: 3 L spinner final volume: day 1 (left) day 5 (right)*

### 6.1.5 XDR-200

45 L of supplemented bead slurry, 3g/L beads, was pumped into the XDR-200 reactor 48 hours before inoculation using sterile biowelded c-flex tubing. The agitation rate was set to 45 RPM, temperature to 37 C, pH to 7.1 and DO to 40%. Before inoculation, a sample was taken visually with phase shift microscopy to ensure the microcarriers were conditioned and not being destroyed by the reactors impeller.

The contents from each 3 L flask was pumped into the XDR-200 through biowelded ¼" c-flex tubing by pressurizing the flasks. A baseline media analysis was performed. The XDR was set to agitate intermittently for 12 hours with a duty cycle of 55 minutes off 3 on at 25 RPM. The Agitation was then set to a constant 45 RPM and the cells were sampled daily. After 6 days little improvement in confluency was observed and glucose uptake rate remained constant (pictured below in Figure 84).

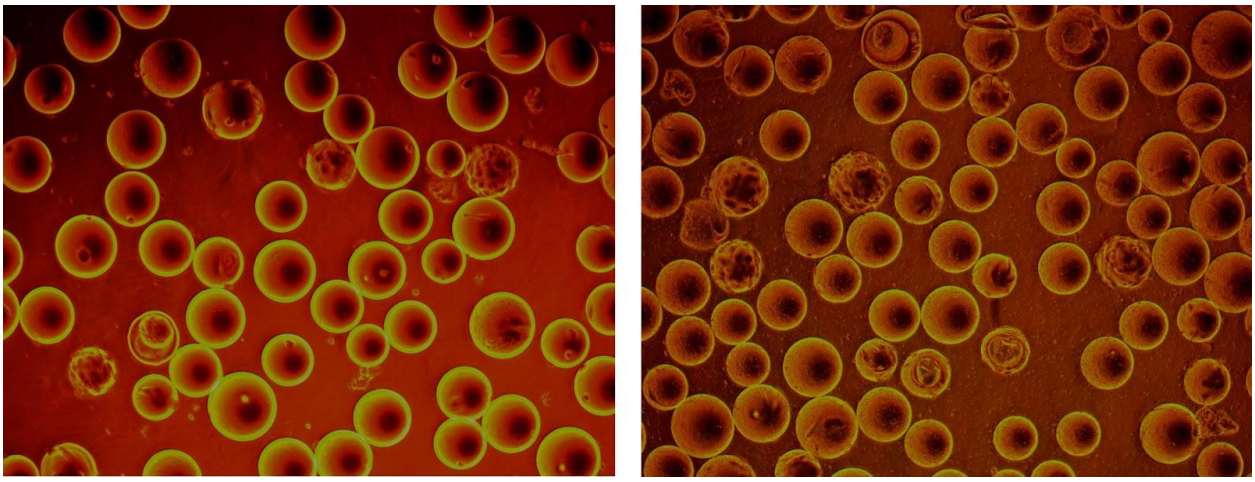


*Figure 84: XDR-200 confluency: day 1 (left) vs. day 5 (right)*

The cell coverage did not reach the levels the team was hoping for (>75%) before adding the final volume of bead slurry to the reactor. However, due to time constraints the team decided to trypsinize and resuspend the cells we had while adding the final volume of bead slurry. 24 hours before the final volume was achieved, 25 L of bead slurry was prepared in a in a flexboy bag, 5% CFS GE MEM, with 35 g of microcarriers 5g/L glucose and 4 mmol L-Glutamine. The bag was placed on the wave platform over night set at 37 C to warm and condition the beads.

Before adding the final volume of bead slurry to the XDR-200 the cells needed to be trypsinized. A 50 L waste bag was biowelded onto one of the XDR kleenpak ports. Agitation was turned off and the beads were allowed to settle. Once settled as much media was drained off the top into the

waste bag as possible. The XDR was drained down to 20 L. In attempt to wash as much serum containing media as possible 25 L of PBS was pumped into the XDR through a sterile biowelded port and drained off into the waste bag twice. 6 L of trypsin was pumped into the XDR bag. The agitation was set to spin at 45 RPM for 15 minutes in attempt to have the cells detach from the microcarriers. The 25 L of supplemented bead slurry was added to the XDR, and an addition 100 L of 5% CFS GE MEM media was added slowly over the next 24 hours. The XDR was set to intermittently spin at 25 RPM intermittently with a duty cycle of 55 minutes off 3 minutes on for the first 24 hours. A baseline media analysis test was performed and the reactor was then set to spin constantly at 45 RPM.

Samples were taken daily, monitoring cell confluency, media metabolites and reactor conditions. Over the next 5 days, it was observed that no improvement in cell confluency was achieved and glucose consumption rate did not significantly increase. The cells did not effectively transfer between microcarriers. This was likely the case for two reasons. One, The cell density was not high enough to proceed to the final in the XDR. Two, the final trypsinization process did not effectively remove the cells we had off the beads. This is because the team was not able to drain and wash the initial volume of serum containing media from the culture effectively. Too much media was left within the reactor when the trypsin was added. The serum from the media inactivated the trypsin before the cells were able to detach. Therefore, no bead-to-bead transfer occurred and only <2% of microcarriers contained cells.

## 6.2 Economics

Bioprocessing plays a major role in the production of a wide range of products, including vaccines, cell and protein therapeutics, and cells for biological research (Lim, 2010). Vero cells

play an especially large role in the production of vaccines, due to their susceptibility to a broad range of viruses. The aim of this project was to evaluate the seed train process and the feasibility of using microcarriers and single-use technology in the XDR-200 for Vero cell production. The purpose behind the project is ultimately streamlining and optimizing Vero cell production to obtain maximum cell yields with minimal time, effort, and resources. In optimizing the production of these cells, the production of larger amounts of viral antigen for vaccine creation is made possible. This results in a greater profit margin for the company producing the vaccine, as well as a direct reduction in the cost of the vaccine as the cost of production drops. Additionally, the supply of the vaccine would also increase. This would make it less expensive for the general populace to vaccinate themselves as well as allowing charitable organizations and companies to donate more vaccines.

## 6.3 Environmental Impact

One of the most important considerations of single-use technology is the impact on the environment. One study was conducted regarding the environmental impact of a traditional biopharmaceutical manufacturing facility that utilized reusable stainless steel equipment in comparison to the environmental impact of a facility using disposable, single-use technology. The results of this study found that at a 3 x 2000 L scale in the production of monoclonal antibodies, the disposable facility used significantly less water (87%), energy (30%), and space (38%) (Sinclair, 2008). This remarkable decrease in water usage and energy greatly reduces the carbon footprint of the manufacturing facility. Combined with the convenience and ease-of-use that comes alongside disposable technology, it becomes extremely appealing for industry. However, there are negative environmental impacts for disposable technology. The primary means of disposing of the disposables, including the single-use bags and the microcarriers, is

incineration, which can release chemicals into the air after the product has been burned (Pietrzykowski, 2011). Although there is a need to better address the disposal of single-use technologies, the environmental benefits for the technology currently outpace the drawbacks.

## 6.4 Societal Influences

The societal influence of the product of this project essentially ties back into economics. In reducing the cost of vaccines, it becomes easier for families and individuals to afford to vaccinate themselves. As a result, protection from dangerous, painful diseases like mumps, measles, tetanus, and even the seasonal flu can be extended to a larger portion of the population. This should also contribute to better disease safety for the entirety of the populace through the principles of herd/community immunity. With more and more of the population properly vaccinated, the odds of an infectious outbreak occurring are greatly reduced (Fine, 2011). Finally, reduced vaccine cost and increased supply should lead to a better rate of conversion of financial donations into actual vaccine supplies for charitable outreach, allowing for better vaccination coverage in impoverished areas.

## 6.5 Political Ramifications

The political ramifications of increased vaccine production and reduced vaccine cost are tricky to predict. Less expensive and more readily available vaccines could have an impact on the global market, for example. As more and more people became immune to certain diseases, the demand for treatment or medication for those diseases would likely drop, resulting in pharmaceutical companies selling less product depending on the targeted diseases. Similarly, drops in mortality from proper vaccination might lead to longer life spans, which could impact social security programs. Additionally, there might be a political shift from generations of people

living longer and continuing to influence the direction of politics. It is unlikely, however, that vaccine production would significantly affect the culture of other countries, beyond improving life expectancy.

## 6.6 Ethical Concerns

The discovery of the vaccination process was one of the most important scientific advancements in human history. For example, it is estimated that measles vaccinations have saved over 17.1 million lives between the years 2000 and 2014 (Krisberg, 2016). Vaccinations play a major role in public health and safety, preventing individuals from contracting a multitude of diseases while simultaneously working to prevent a disease outbreak or epidemic from occurring. There have been some claims of a causal relationship between vaccines like the MMR vaccine and autism, but in-depthy studies and reviews have rejected any meaningful link between vaccination and autism (Immunization Safety Review Company, 2004). Regarding the Vero cell production, there are a couple of ethical considerations to take into account. The first is the purpose behind the production of large numbers of Vero cells for research, namely what that research is attempting to discover or produce. It is important to maintain a humanitarian focus in research. The second is the use of animal products in media used to grow Vero cells, such as serum. Not only must customers be properly informed of the use of these products, but scientists should ensure that they are obtained humanely from animals.

## 6.7 Health and Safety Issues

Health and safety go hand-in-hand with vaccination, as mentioned previously. Aside from a disproven theoretical claim that vaccines might cause autism, vaccines have been proven to be extremely safe, with serious adverse events occurring infrequently (Maglione, 2014). They

actively work to shield individuals from harmful diseases and provide communal protection from rapid disease outbreaks. Beyond the safety of the end product, the manufacturing processes for both the production of large numbers of cells and their infection and harvesting for viral antigen are extremely safe, given the scientists carrying out the process follow safety protocols and wear the correct personal protective equipment. By wearing nitrile gloves, a laboratory coat, proper laboratory attire like long pants and closed-toe shoes, and using a biosafety cabinet to handle the product, the risk of contaminating the product and personally contacting the materials is minimized. Aside from manual handling of the cell culture during the early stage of development, every other step of the process uses welded tubing to transfer materials, further isolating the cell culture and maintaining user safety.

## 6.8 Manufacturability

This experiment would be moderately challenging to reproduce in its entirety. The materials and general procedures the group utilized over the course of the project are straightforward, but several of the steps of the process were nonoptimal and replicating them would not be advisable per the group's data. For example, the general seed train process is easily reproducible, from laying down a Vero cell seed bank to plating T-300 tissue culture flasks to trypsinizing and moving into the 500 L spinner flasks and then into the 3 L spinner flasks. After that, however, the cells were kept in the 3 L spinner flasks for more than three weeks, which the group would recommend avoiding. Additionally, spinner flask 1 was contaminated during the course of this culture, leading to the seeding of a new spinner flask, spinner flask 4. The eventual trypsinization of these flasks and pooling together to redistribute the live cells from spinner flask 4 along with the stagnant cells from spinner flasks 2 and 3 would not be a normal step in a seed train established by the group. Moving from the spinner flasks into the XDR-200 was also

straightforward, as well as the trypsinization and additional filling of the XDR-200 from the original 52 L short-fill to the 150 L final volume. The major issues regarding reproducibility of experiments involving cell culture are the potential differences amongst cells between experiments and the possibility for human error, both of which could easily alter the results of an experiment aiming to reproduce this project.

## 6.9 Sustainability

Sustainability in terms of energy does not apply to the scope of the project tackled by the team. The only possible connection between the project and sustainable energy results from the fact that electricity was used for refrigerators, incubators, water baths, stir plates, and bioreactors for the project. The source of the electricity used in similar work in the future is dependent upon the location of cell manufacture and the energy policies of the party producing the Vero cells. The methods the group utilized to scale up the seed train to the XDR-200 ultimately took longer than a normal seed train typically would, as the culture spent much longer in the 3 L spinner flasks than the group had planned. The seed train the group designed would not have taken any longer than a typical process if it had been flawlessly executed. In fact, using a high density seed bank as the group had considered would have greatly reduced the amount of time in culture. Similarly, the amount of resources utilized would also have been equivalent to a standard seed train had the planned seed train been flawlessly executed. In fact, both the amount of time and resources required would have been reduced if a high-density cell bank been established and utilized.

# Chapter 7: Discussion

Throughout the duration of the project, the team strived to create a robust and effective cell expansion process that would concluded with successful growth in the XDR-200 bioreactor. In working towards this, the following objectives were created to measure the team's success:

- Reduce chances for contamination
- Yield high cell counts
- Establish good bead-bead transfer
- Propagate cells in minimum time possible
- Prevent microcarrier destruction

While not all experiments coincided with these aforementioned criteria, these objectives helped shape our design and ultimately led to a seed train and XDR-200 scale up that was moderately successful in showing Vero cell growth on microcarriers with single-use technology.

## 7.1 First Seed Train

Beginning the experimentation of varying cell expansion methods, the first started with thawing a frozen vial of $20x10^6$ Vero cells and concluded with the inoculation of a 20L Wave bioreactor. In this experiment, pH, media and visual samples were analyzed, which depict a result that was not expected when beginning our work. When first inoculating the Wave reactor with the Vero cells, it was expected that cells would easily attach to the microcarriers and become settled within the first three hours. There was difficulty in achieving this and is corroborated through the pH values, along with metabolite concentrations. As the culture

progressed and was sampled for another 5 days, slowly decreasing glucose and slowly increasing lactate (Figure 25) occurred due to many cells not attaching and growing. With this, pH initially seemed stable, but increased as cells died. With a stagnating culture, there was little point in continuing the experiment, as most cells were not alive, indicative of rising LDH which peaked on day 4 of the cell culture (Figure 28).

When the experiment began, it was assumed the platform for the Wave reactor would be stable and hold a constant temperature to incubate the cells. However, this was learned to not be the case, as the heating element of the platform was not functional unless the platform was rocking. This led to difficulties in settling times, along with keeping temperatures constant at 37 degrees Celsius. While the rocker platform was held still, it stopped along an angle causing the contents of the cell culture to be pooled to one side. This may have affected the cells by not allowing even distribution or easy access to the microcarriers. While this was occurring, uneven temperatures compiled on top, dropping to values near 30 degrees. This combination proved to be difficult for the cells to thrive in, as there was very little attachment to beads. Another factor to look at would be the cell density the Wave reactor was inoculated at ($1.75*10^5$ cells/mL), but literature has shown successful cell cultures starting at a similar density (Trabelsi et al, 2006). This points to the previously discussed factors of inadequate settling times and fluctuating temperatures to be the believed cause in a failed cell culture within the Wave reactor.

Additionally, a second run within the Wave reactor was made. The Wave reactor was inoculated at a lower density at $1.28*10^5$ cells/mL. This is a bit lower compared to other literature values, and may have contributed to the unsuccessful culture. A second run was made with the aim of keeping a warmer average temperature. Regardless, the data showed what was concluded in the first run – cells were not able to attach within the Wave reactor initially.

Throughout the first three hours, viable cells were to be seen within the samples, but did not attach. To keep the heating platform on to achieve a warmer temperature, the cells were not allowed to settle as much and underwent increased agitation. Comparatively to the first run, this may have helped, as a bit more bead attachment was observed after the first two days. This is also indicated by the decrease in pH from 7.45 to below 7.2 (Figure 32), along with the initial drop in glucose concentration (Figure 33).

However, by day 3 of the cell culture, slowing glucose consumption and rapidly increasing LDH values (Figures 33 & 36) indicated the same fate as before. To address this, previously cultured cells already attached to microcarriers within spinner flasks were added to the Wave reactor. This may be the cause for the large increase in pH as an entirely separate culture was introduced. Due to the circumstances of the experiment and in trying to promote cell growth within the Wave reactor, cell counts were not conducted. Therefore, we do not know the cell density that was introduced to the Wave. Despite the new addition of already attached cells to microcarriers, glucose and lactate concentration changed slightly, indicating the cells may have been surviving if they were already attached, but not proliferating or attaching to new beads.

During this experiment, it was assumed that cells on covered beads would come into contact with uncovered beads and may transfer this way. Ultimately, this was shown to be very difficult and does not prove successful when aiming to expand a Vero cell culture. Some literature has shown not using trypsin as a means of cell expansion, but with different cell types, a stirred tank reactor, and varying microcarrier density (Leber et al, 2017). With several different experiment parameters, it is difficult to say why bead-bead transfer did not occur within the Wave reactor. This may point towards bead density being an underlying factor in bead-bead

transfer without trypsin, as a separate spinner culture experiment did not show adequate bead-bead transfer with a much lower bead density than used in the literature. Ultimately, both Wave reactor runs were able to show that different settling/agitation protocols must be employed at a constant, warmer temperature to achieve a successful cell culture.

## 7.2 500ml Spinner Flask

While testing varying parameters that may affect cell growth, such as seeding density and agitation rates, three different spinner flasks were inoculated and cultured for a period of six days. The breakdown was as follows:

- Spinner Flask 1 = High Density ($7.16 \times 10^5$ cells/ml)
- Spinner Flask 2 = Constant Agitation ($3.77 \times 10^5$ cells/ml)
- Spinner Flask 3 = Intermittent Agitation ($3.77 \times 10^5$ cells/ml)

During all three cultures, it was realized the media used was running low on supplements such as glucose and glutamine (Figures 44 & 45), which were added in mid culture. After concluding these three different cell cultures, the spinner flasks contents were moved into the Wave reactor during the second attempt at Seed Train 1 with the aim of preventing a failed Wave reactor run. This led to not completing final cell counts, but the group was still able to gather insights from these experiments.

The first of the spinner flasks was seeded at a higher density than the other two, with the main parameter of inoculation density being isolated to learn of its effects on cell culture growth. Spinner flasks two and three were compared through means of varying agitation patterns to learn if additional settling at later stages in the cell culture would affect cell growth. After concluding the six-day culture, all three spinner profiles appeared to look very similar in regards to their

metabolite analysis. Among all three spinners, glucose and glutamine dropped fairly quickly within the first two days, nearly plummeting to 0 g/L (Figures 10 -15). While the consumption was expected, the low concentration within the media was not. Having little glucose/glutamine for the cells to feed on may have adverse effects on cell proliferation, as these supplements are part of their food source. To amend this, both metabolites were additionally supplemented on day 3. We believe this may have slowed cell metabolism and growth, as the rate of glucose/glutamine consumption slowed after day 3 in all spinners. This is corroborated by a general increase in LDH levels starting at the day 2 marker for the spinners (Figures 19 – 21).

In lieu of the slowed cell growth, all three cultures were able to produce viable cells and confluent microcarriers as can be seen by the visual phase shift microscopy images. While final cell counts were not conducted due to movement of cells to the Wave reactor, the group still made inferences as to the effects of altered parameters of inoculation density and agitation rate. It was not expected to see much variation among the different agitation profiles, but increased inoculation density was proposed to raise the cell density. According to the metabolite analysis, there were no statistically significant differences, indicating similar growth and proliferation pattern. From visual checks, the group felt that the high-density cell culture performed better initially, as more microcarriers were confluent quicker. This may simply be attributed to an increased number of cells initially, and depleted nutrient sources slowed their growth. The group was hopeful of the higher inoculation density, and feels it may still be impactful under correct nutrient conditions.

In regards to the varied parameters, our cell cultures experiments were not able to show any numerical differences between each other. Aside from this, the group was able to confirm previous methods of microcarrier cell culture in spinner flasks. All three spinners proved

successful in bead attachment via Vero cells. This proved valuable in moving forward with Vero cell cultures for the final XDR-200 run, as a baseline was set to carry out future expansion methods in spinner cultures.

## 7.3 XDR-200 Final Run

After concluding the feasibility studies and looking at the effects of altered spinner flask cultures, a final seed train was employed to reach the XDR. The microcarrier cultures began with three 500mL spinner cultures similar to the previous experiment. After following a hunch that was not confirmed, the group inoculated at a higher cell density of $4.05*10^5$ cells/mL. This appeared to be successful as the visual samples showed quick cell attachment and increasing confluency as the culture progressed for four days. Metabolite analysis showed that the culture was behaving similar to before with an initial decrease in glucose, but appeared to slow after two days. As visual samples were continually evaluated, microcarrier confluency was increasing. There was also a much lower concentration of LDH as compared to the Wave reactor, showing cells were not staying suspended and dying. This was expected to occur given the previous success of the 500mL spinner flask experiments.

After expanding the cells in the 500mL spinner flasks, the microcarriers were transferred to a 3L spinner at a 1L fill. Despite previous experiments, the group was hopeful of bead-to-bead transfer occurring via microcarriers coming into contact with one another. Throughout the first week of the culture, visual samples showed many empty beads due to the increased amount of microcarriers in the larger fill volume. It is believed that the beads from the 500ml culture stayed confluent and did not continue proliferating onto the new beads within the 3L spinner. This is backed up by the slow decrease of glucose (Figure 51) over a ten-day period, as the cells that

were alive continued to live, but rapid growth and proliferation did not appear to occur.

As the culture stagnated, one spinner was found to have contamination which was noted at the beginning of the seed train. Due to a faulty weld between two tubing ends, the media being transferred into the spinner may have come into contact with the air and caused a delayed contamination. To rectify the lost cells, a new 3L spinner was inoculated at $3.95 \times 10^5$ cells/ml at a 1L fill. This resulted in a successful cell attachment to the microcarriers as seen in the 500mL spinner flasks which was not expected due to the other stagnating cultures within the 3L spinners. This illustrated to the group that the problem had not been with the spinner flasks, but had to do with when the cells were originally introduced to microcarriers. If the cells were able to distribute among the microcarriers, then they were able to attach and grow well. The attachment and growth was corroborated by the rapid drop in glucose and glutamine (Figures 51 and 52), which was also able to explain decreasing pH (Figure 50). As cells metabolize, they create ammonium, contributing to the acidity of the media.

As this new spinner showed improved signs of vero cell growth, the other two cultures continued to stagnate as we let them sit to try to increase our total cell count. The 3L spinners were to be used to inoculate the XDR-200 in a final scale up, so the cell density still needed to increase. As the microcarriers within Spinner 4 became confluent, the cells needed more room to grow. In response to previously failed bead-to-bead transfer and input from advisors at GE Healthcare, Spinner 4 contents along with Spinner 2 and 3 were all trypsinized and evenly redistributed among the 3L spinners at a fill volume of 1.5L. Visual samples here showed much cell debris as was expected, along with many confluent microcarriers. Due to the cultures being held for over 3 weeks, many cells had died, and microcarriers had been damaged due to a heavier impeller within the 3L spinners. Despite this, the beads were well covered after three days within

the spinners. Glucose and Glutamine decreased faster than the initial Spinner 2 profiles, showing increased cell proliferation. This can be attributed to the newly distributed cells with more room to grow - they were not crowded on already confluent microcarriers. This showed that trypsinization was necessary for successful bead-to-bead transfer and would be needed in the larger scale culture at the XDR-200 level. Finally, this is also shown through the slow increase of LDH within the newly distributed cells, as there was not a large increase of cell death in this 1.5L fill volume.

After cultivation of the cells and based on previous success of the trypsinization process, cells were again trypsinized within the spinner flasks before being transferred to the XDR-200 at a working volume of 52L. Originally, microcarrier attachment was seen within the XDR-200, however, the beads that had cells seemed to gather more cells than desired, leading to quick confluency being reached on beads that had cells. Analyzing glucose profiles (Figure 73) show that slow cell growth occurred within the first three days, followed by an increase in consumption. There may have been a slight lag period that was overcome, allowing cells to better grow on the microcarriers that were not already confluent. This however did not reach what was expected within this working volume for a cell count, showing that different agitation methods may be needed to better distribute the cells among the microcarriers.

Additionally, upon scale up to the 150L working volume, issues were encountered during the washing and trypsinization phase. Due to placement of the outlet port and restricted shell of the bioreactor, not all media was drained from the 52L culture stage. This led to extra time being spent which held the cells at a lower temperature than desired. The PBS and Trypsin introduced to the reactor were held at room temperature which dropped the reactor temperature to roughly 30 degrees Celsius. This colder shock could be related to the slow growth seen after the scale up.

Also, due to media still being present during trypsinization, this could have nullified the effects of trypsin, thus not stripping cells from beads. As seen in previous experiments, if the cells are not distributed amongst the microcarriers upon scale-up, cell proliferation and growth is nearly halted. To be able to efficiently grow cells within the bioreactor with microcarriers, the draining port may need to be altered.

## 7.4 Limitations

Throughout the entirety of the project, there were several limitations experienced by the group. First and foremost was the design specifications of the Wave bioreactor rocker. While inoculating this bioreactor, it was discovered that the heating platform needed to be rocking in order to heat. Without the incubating hood to accompany this model, only certain agitation/settling patterns could be achieved. Additionally, temperatures could not be kept high enough to for proper incubation. This affected the experiments within the Wave bioreactor and limited the seed train development to exclude this platform. Also, with cell expansion reaching a larger scale than traditional T-Flasks, each run took a few weeks' time to prepare and complete, limiting how many attempts were made to the XDR-200. Once moving into the XDR-200, the non-commercial model being used had a few design alterations that affected how much media could be drained. This impacted the trypsinization and cell distribution process. Lastly, the team was not well-versed in cell counting procedures at the beginning of this project, potentially leading to approximate cell counts. However, with repeated trials, the group improved upon this skill. Despite the limitations encountered during the project, the team was able to express how to achieve bead-to-bead transfer, and conduct a semi-successful culture within the XDR-200.

# Chapter 8: Conclusions and Recommendations

The results of the project differed from the results that the group expected at the outset of this project. Changes in the timeline of the project, the means by which the group would scale up the cell culture, and in the availability of the group and the project's advisors all had pronounced effects on the final results and conclusions derived from the project.

The final seed train executed by the group differed majorly from the seed train proposed at the beginning of the project. The group's two unsuccessful attempts at using the Wave bioreactor to scale up to the XDR-200 failed due to temperature, pH, and settling issues, and forced the group to search for a viable alternative to reach the XDR-200. The group settled on using 3 L spinner flasks due to the ease of sampling and agitation adjustment, as well as previous success with 500 mL spinner flasks. The cell cultures were successful in the tissue culture flasks and in the 500 mL spinner flasks, but ultimately stagnated in the 3 L spinner flasks and failed to demonstrate adequate bead-to-bead transfer. The team hypothesizes that trypsinization is required for sufficient cell spread and resettling on new beads. One of these original three 3 L spinner flasks later became contaminated, which forced the team to seed a new 3 L spinner flask directly from trypsinized tissue culture flasks. This new 3 L spinner flask demonstrated a thriving cell culture and excellent bead distribution, which further emphasized the importance of trypsinization. The team decided to attempt to use this thriving culture to support the other two stagnant 3 L spinner flasks and trypsinized and redistributed amongst the three flasks.

These flasks reached a sufficient level of confluency for inoculation, but only contained a final working volume of 1.5 L each for a total of 4.5 L, half of what the team had planned to inoculate the XDR-200 with originally. The team made the decision to inoculate the XDR-200 with this 4.5 L of inoculum in addition to cells separately cultured in tissue culture flasks.

The inoculation of the XDR-200 was successful, although the starting cell density was lower than expected and the XDR-200 bag was filled to 52 L instead of 40 L due to issues with probe placement. The XDR-200 culture demonstrated excellent growth for the cell density that it began with, although the total percentage of confluent beads was much lower than the group's target. Once again, time constraints forced the team to act and scale up to the 150 L with a smaller cell density than expected. This scale up step was where the team ran into its major issues with the XDR-200, as well as limitations of the project in general. The media and PBS utilized during the rinse were room temperature due to lack of incubator space, and the cell culture spent several hours in this cool media, which likely lagged the cells if it did not outright kill some of them. In addition, the group was unable to completely drain the supernatant media from the XDR-200, leading to an ineffective trypsinization process. After the 150 L fill of the XDR-200, the culture appeared to stop growing, demonstrating very little glucose consumption and visually appearing to have failed to spread cells from bead to bead during trypsinization. The culture remained stagnant in terms of growth until the end of the week.

The group discovered a lot about the basic seed train process as well as operating inside of a bioreactor. The major takeaway from this project was the importance of trypsinization in allowing cells to be removed from a fully confluent bead and used to populate an empty bead. Bead-to-bead transfer may work for small cultures, but it takes too long and is generally unreliable in large-scale cell cultures. Another key takeaway was the effectiveness of intermittent agitation in allowing cells to settle and attach to beads before shifting them routinely to allow for better distribution. The optimal settings for this intermittent agitation require further data collection. Finally, the influence of starting cell density on a successful culture was made readily apparent. If the group had not been operating on a strict timeline, efforts to continue to increase

cell density before progressing with the seed train would have been made. This project emphasizes how potentially useful a high density seed bank that could allow for immediate inoculation of larger culture vessels would be.

Moving forward, there are a number of points that the group would address if further experimentation were conducted. These points include general recommendations for changes in materials, methodology, and process and device design, as well as topics of interest that the group would further investigate given the required time and materials.

The first point the group would address would be the starting materials utilized during the course of the experiment, beginning with the microcarrier beads. This experiment utilized gamma-irradiated Cytodex 1 beads to test bead-to-bead transfer. However, literature has shown that Cytodex 3, with its collagenase surface, has demonstrated effectiveness with bead-to-bead transfer because of their superior properties for harvesting and redistribution of cells within culture. When cells are harvested from Cytodex 3 microcarriers, the collagen surface layer is digested by proteolytic enzymes like collagenase, which allows for the harvest of a larger percentage of attached cells while maintaining cell viability and membrane integrity. These properties might potentially prove to be superior to those of the Cytodex 1 beads utilized by the group, and experimentation with them would provide useful data. It is also important to note that these beads would then be destroyed, which would require the addition of new beads. In terms of media, it is better to use DMEM than MEM for adherent cell cultures. DMEM contains a higher concentration of amino acids and vitamins, as well as additional supplementary components that are vital for cell growth. DMEM also contains a higher concentration of glucose, eliminating the need for additional supplementation of glucose during the course of the experiments. Additionally, higher percentages of serum within the media were found to be more effective,

although whether the higher cell densities and better attachment would justify the increased cost for serum is currently unknown.

The next point the group would address would be modification of the seed train process during the upscaling of cells. The first point is the most important issue concerning the seed train process, and it involves the use of trypsinization when working with bead-to-bead transfer. The group highly recommends trypsinizing the beads when using microcarrier beads for cell cultivation. Based on the experiments conducted in this project, it was found that bead-to-bead transfer via settling periods was very unreliable by itself and was ultimately unsuccessful. However, when the cells were trypsinized in the T-flasks and inoculated directly into the 3 L spinner (as was the case for spinner flask 4), it resulted in successful cell attachment onto the microcarrier beads. The group hypothesized that if trypsinization of the 300 mL cultures in the smaller spinner flasks was conducted when scaling, it would have resulted in more successful cell attachment and cell growth on the microcarrier beads in the 3 L spinner flasks.

The next point the group would bring up concerns the swelling and conditioning of the microcarrier beads. The group recommends avoiding the use of magnetic stir bars when conditioning microcarrier beads. During the experiment, a number of microcarrier beads were crushed during conditioning in a glass bottle with a magnetic stir bar. The resulting debris attached onto other microcarrier beads within the culture, which prevented cells from attaching to the affected beads.

The final recommendation in the seed train process concerns the use of the Wave bioreactor when upscaling cells with microcarrier beads. The older Wave model that the team had access to was not able to maintain temperature when the system was turned off. During intermittent agitation, when the bioreactor was turned off for long periods of time, the

temperature would drastically drop, which resulted in unfavorable conditions for cell growth and even cell death. Another issue with the older model was that the settling angle, the angle of the platform at rest, was not 0°. This resulted in all of the beads settling at one end of the bag instead of being evenly distributed across the wave bag at rest. Finally, the Wave bioreactor should have the ability to better monitor and control pH. The pH in the culture was unstable throughout the experiment and the team was unable to monitor pH without constantly withdrawing samples. The Wave bioreactor should be able to maintain ideal pH, or at least indicate when pH needs to be adjusted, throughout the culture period via an electronic control.

The next set of recommendations the group would address concerns the design of the XDR-200 bioreactor. The most critical issue that the group encountered was during the process of trypsinizing the cells within the bioreactor prior to increasing the working volume. The XDR-200 should have a better system for removal of supernatant that will allow for drainage of the spent media while avoiding losing microcarrier beads. During the experiment, it was difficult to drain the media from the bioreactor because of the placement of the drainage port. It was located too high up on the bag to drain the media from the bottom of the bioreactor. A possible solution might be placing the drainage port at the bottom of the bioreactor and including a filter with pore sizes that are small enough to prevent microcarrier beads from draining through the port, although clogging this port might be an issue. Another solution might be to lower the tubing belt line. One potential height would be at 15 L, which would allow for further drainage of the reactor. This would be especially useful for 200L cultures, as the final bead volume for a 200 L culture with a microcarrier concentration of 3 g/L would be 12 L. A height this low might prove problematic for cultures with larger microcarrier concentrations, however, as a 200 L culture at 5 g/L bead concentration would have a final bead volume of around 20 L. Lowering the drainage

port would help during the trypsinization process by reducing the amount of media in the culture, which would reduce the amount of PBS needed to dilute the media, in addition to requiring less trypsin to remove cells from the microcarriers. Reducing the amount of trypsin required would be especially useful, as trypsin is a significant cost in the process.

The next issue concerning the XDR-200 design is the placement of the pH and dissolved oxygen probes. The group recommends placing these probes at the 40 L mark with a lower belt line to allow for a short-fill at 40 L. During the experiment, the group had to short-fill to 52 L in order for the media to reach the pH and DO probe for analysis. Also, it is difficult to access the lower belt line without disturbing the probes with the current placement of the probes.

Finally, another issue encountered with the XDR-200 design was during the insertion of the microcarrier beads into the XDR-200. The group encountered clogging of the C-flex$^{TM}$ tubing when transferring the microcarrier beads into the XDR-200. The microcarrier beads mixed with the liquid media, which resulted in clumping within the tubing and led to a blockage. It required several hours of careful application of vacuum and airflow to move the bead slurry through the tubing. A solution to this issue would be to have the quick connects for the tubing located at the top of the XDR-200 bag so that the media would not interact with the microcarriers that are being pumped into the bioreactor until they were actually in the reactor. The only drawback for this solution might be beads accumulating in folds in the walls of the bioreactor bag, although the number of stuck beads would likely be trivial and would be reintroduced to the media when a complete fill of the bioreactor was conducted.

Further research should also be conducted on different agitation and settling rates for the microcarrier beads and cells during intermittent agitation. An ideal agitation and settling rate would result in better cell distribution and attachment in the culture. Additional studies on

methods for better cell distribution within a cell culture containing microcarrier beads would also provide valuable information for successfully scaling up an adherent cell culture using microcarriers.

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# Appendix A:

Standard Operating Procedure (SOP) 1 - Thawing Cell Bank into Tissue Culture Flask

| WPI BETC | MASTER BATCH RECORD | Document Number WPI-0074 | REV 1 |
|---|---|---|---|
| Title<br>VERO 76 GE MQP T-FLASK - BIOREACTOR | | Batch Number: Flask 1 Rev | |

**1.0 References**

**BETC SOP-001; Operation and Cleaning of the Beckman Coulter Allegra 6 Bench Top Centrifuge**
**BETC SOP-002; Operation and Cleaning of Class II Biosafety Cabinets at BETC**
**BETC SOP-006; Operation and Cleaning of the Lab-Therm LT-X CO2 Incubator**
**BETC SOP-007; Operation and Cleaning of the VWR CO2 Incubator model 2375**
**BETC SOP-028; Solution Transfers and Filtrations**
**BETC SOP-041; Gowning Procedure for BETC Laboratory and Biosafety cabinets**
**BETC SOP-055; Operation of the Waterbath**
**BETC SOP-056; Cell Counting**

**2.0 Operator Signature Log**

| Print Name | Signature | Initials | Date |
|---|---|---|---|
| Nick Dalton | Nick Dalton | ND | 1/31/17 |
| | | | |
| | | | |
| | | | |
| | | | |
| | | | |
| | | | |

| WPI BETC | MASTER BATCH RECORD | Document Number WPI-0074 | REV 1 |
|---|---|---|---|
| Title **VERO 76 GE MQP T-FLASK - BIOREACTOR** | | Batch Number: Flask 1 Run | |

### 3.0 Bill of Materials

| Material | Qty Required |
|---|---|
| VERO 76 MCB Vial | 1 |
| 300cm² tissue culture flasks with vented caps, sterile | 2 |
| Corning Hyper flasks | 4 |
| 15/50 mL Conical centrifuge tube(s) | As Required |
| MEM / 5% CBS growth Medium | As Required |
| Cytodex 1 gamma microcarriers | As Required |
| 0.25% Trypsin, 0.53mm EDTA | As Required |
| Microfuge Tubes | As Required |
| 1 mL Sterile Pipette | As Required |
| 2 mL Sterile Pipette | As Required |
| 10 mL Sterile Pipette | As Required |
| 25 mL Sterile Pipette | As Required |
| 50 mL Sterile Pipette | As Required |
| 70% Isopropyl Alcohol | As Required |
| Clean Room Wipes | As Required |
| | |
| | |

| WPI BETC | MASTER BATCH RECORD | Document Number WPI-0074 | REV 1 |
|---|---|---|---|
| Title VERO 76 GE MQP T-FLASK - BIOREACTOR | | Batch Number: | |

## 4.0 Equipment List

| Description | |
|---|---|
| $CO_2$ Incubator | Micro-pipettes |
| Biological Safety Cabinet (BSC) | Pipette Aide |
| Water Bath | Peristaltic Pump |
| Centrifuge | Timer or Stopwatch |
| Microscope | Cedex Cell Counter |
| 20/50 Wave Bioreactor | XDR 200 disposable Bioreactor |

## 5.0 Operating Parameters

| Condition | Target | Operating Range (IPC) |
|---|---|---|
| Incubator Temperature Setpoint | 37.0 °C | 36 – 38 °C |
| Incubator$CO_2$ Setpoint | 5 % | 3 – 7 % $CO_2$ |
| Water bath Temperature | 37.0 °C | 36 – 38 °C |
| Centrifuge Speed | 800 rpm | 750 – 850 rpm |
| Centrifuge Time | 5 min | 4 – 6 min |
| Centrifuge Temperature | RT (room temp) | RT |

| WPI BETC | MASTER BATCH RECORD | Document Number WPI-0074 | REV 1 |
|---|---|---|---|
| Title VERO 76 GE MQP T-FLASK - BIOREACTOR | | Batch Number: Flask1 Run | |

## 6.0 Flask Operations

**Manufacturing Instructions**

**NOTES:**

- In the event that any operating parameter or performance parameter is not within range contact a supervisor.
- All technicians are to be properly gowned according to BETC SOP-041.
- All aseptic procedures are to be conducted in Biological Safety Cabinets.
- The steps listed in these directions are in the order of normal occurrence, however to facilitate manufacturing processes, some steps may be performed out of sequence. This is to facilitate optimal set-up times. The acquired data must be entered at the time of performance.
- All actual data is to be entered at the time of execution.

| Instructions | Data Required | Actual Data | Performed By / Date | Verified By / Date |
|---|---|---|---|---|
| **Pre- Thaw** | | | | |
| 6.1. Ensure incubator is set to 37.0 °C and is 5 % $CO_2$ according to BETC SOP-006 or BETC SOP-007. | Incubator ID #: | | DW 1/31/17 | |
| | Incubator CO2% Setpoint Entered: | 5.0 % (X%) | | |
| | Incubator Temp Setpoint Entered: | 36.8 °C (XX.X °C) | | |
| 6.2. Obtain MEM/ 5% CBS growth media from the 4°C refrigerator. Sanitize/Spray with 70% IPA and place in the BSC. | MEM/CBS medium Batch #: | | | |
| | MEM/CBS medium Exp. Date: | | | |
| 6.3. Ensure the incubator is 36.0 - 38.0 °C and place the Medium into the incubator for ≥ 60 minutes. * MEM w/ CBS = A GE Media = B | Incubator Temperature: | 36.8 °C (36.0 - 38.0 °C) | DW 1/31/17 | |
| | Time Medium Placed in Incubator: | A: 9:38 AM B: 9:42 AM | | |

| WPI BETC | MASTER BATCH RECORD | Document Number WPI-0074 | REV 1 |
|---|---|---|---|
| Title **VERO 76 GE MQP T-FLASK - BIOREACTOR** | | Batch Number: Flask 1 Run | |

| Instructions | | Data Required | Actual Data | Performed By / Date | Verified By / Date |
|---|---|---|---|---|---|
| 6.4. | Clean the Biological Safety Cabinet (BSC) according to BETC SOP-002. | BSC ID #: 1040 | 1040 | EK 1/31/17 | |
| | | BSC Certification Due Date: | 17/Jun/16 | | |
| | | Magnahelic Reading: | .6 | | |
| 6.5. | Obtain MEM/ 5% CBS growth media from the incubator. Sanitize/Spray with 70% IPA and place in the BSC. | Time removed from Incubator #: 11:25 | 1125 | EK 1/31/17 | |
| 6.6. | Spray all equipment, to be used in the BSC, with 70% IPA and place in BSC.<br>**NOTE:** Allow all equipment to dry prior to use. | N/A | N/A | ZAM 1/31/17 | |
| 6.7. | Aseptically transfer 170 ± 1mL of MEM growth medium with 5% CBS into two (2) sterile 300cm² tissue culture flask. | Amount Transferred: | 170 mL | EK 1/31/17 | |
| 6.8. | Aseptically transfer 10 mL of MEM growth medium with 5% CBS into two (2) sterile 15mL conical tubes. | Amount Transferred: | 10 mL | EK 1/31/17 | |
| 6.9. | Label the Flasks and conical tubes with "MEM/5% CBS, initials and date". | Flask/Tubes Labeled: | (Yes) or No | ZM 1/31/17 | |
| 6.10. | Ensure the incubator is 36.0 - 38.0 °C and place the Flask and conical tubes into the incubator for ≥ 10 minutes. | Incubator Temperature: | 36.6 °C (36.0 - 38.0 °C) | ZM 1/31/17 | |
| | | Time Medium Placed in Incubator: | 1139 | | |
| **Vial Thaw Day 0** | | | | | |
| 6.11. | Clean, fill and set the Waterbath to 37 °C according to BETC SOP-055. | N/A | N/A | EK 1/31/17 | |
| 6.12. | Clean the Biological Safety Cabinet (BSC) according to BETC SOP-002. | BSC ID #: 1040 | 1040 | EK 1/31/17 | |
| | | BSC Certification Due Date: | 17/Jun/16 | | |

| WPI BETC | MASTER BATCH RECORD | Document Number WPI-0074 | REV 1 |
|---|---|---|---|
| Title **VERO 76 GE MQP T-FLASK - BIOREACTOR** | | Batch Number: Flask 1 Run | |

| Instructions | Data Required | Actual Data | Performed By / Date | Verified By / Date |
|---|---|---|---|---|
| | Magnehelic Reading : | .6 | EK 1/31/17 | |
| 6.13. Obtain one vial of the VERO 76 Master Cell bank and transport to the inoculation area. | Batch #: | WPI-GEMQP-MCB01 | ZAM 1/31/17 | |
| | Vial #: | 12 | | |
| 6.14. Verify the Waterbath temperature is at 37 ± 1.0 °C and thaw the vial for ~ 90 seconds using a stop watch.<br>When thaw is complete, spray vial(s) with 70% IPA and place in the BSC. | Time Thaw Start: | 1149 | ZAM 1/31/17 | |
| | Time Thaw End: | 1151 | | |
| 6.15. Remove the conical tubes and flasks containing media from the incubator spray with 70% IPA and place in the BSC.<br>Calculate the media warming time. | Time Removed from Incubator: | CT 1150 | EK 1/31/17 | |
| | Time Placed into Incubator ( **From Step # 6.10**): | 1139 | | |
| | Total Medium Warming Time: | 11 min Hrs (≥ 30 Min.) | | |
| 6.16. Spray all equipment, to be used in the BSC, with 70% IPA and place in BSC<br>**NOTE:** Allow all equipment to dry prior to use. | N/A | N/A | ZAM 1/31/17 | |
| 6.17. Aseptically transfer the MCB vial using a 2 mL pipette into one (1) of the 15 mL conical tubes with warmed media.<br>**NOTE:** Ensure no ice crystals are visible within the vial. If ice crystals are visible, warm the vial in hand until thoroughly dissolved. | Volume Transferred from the Vial into 15mL conical tube: | 2 mL | EK 1/31/17 | |

| WPI BETC | MASTER BATCH RECORD | Document Number WPI-0074 | REV 1 |
|---|---|---|---|
| Title **VERO 76 GE MQP T-FLASK - BIOREACTOR** | | Batch Number: Flask 1 Run | |

| Instructions | Data Required | Actual Data | Performed By / Date | Verified By / Date |
|---|---|---|---|---|
| 6.18. Centrifuge the cell broth for 5 minutes at 800rpm per BETC SOP-001 | RPM Setpoint entered: | 800RPM | ZAM 1/31/17 | |
| | Centrifuge start time: | 1157 | | |
| | Centrifuge End time: | 1202 | | |
| | Total centrifugation time: | 5 min | | |
| 6.19. In BSC decant the supernatant into an appropriately sized sterile container. | N/A | N/A | EK 1/31/17 | |
| 6.20. Aseptically re-suspend the cell pellet with 10mL of MEM/5% CBS and pipette until suspension is fluid. (No visual cell clusters). Once The cell pellet is re-suspended, pipette 5mL of the thaw contents into each of the sterile 300cm² T-Flask. | Volume Transferred to each T-Flask: *Small volume spilled | a - 5mL mL<br>b - 4.5mL | EK 1/31/17 | |
| | Inoculation Time/Date: | 1/31/17 12:17PM | | |
| 6.21. Label the flask with the Batch #, Flask # 1 a / b, date and initials. | T- Flask Labeled: | (Yes) or No | ZAM 1/31/17 | |
| 6.22. Ensure that incubator temperature is 36.0 - 38.0 °C and $CO_2$ % is 5 ± 2 %, and then place the T- flask into the incubator. | Incubator Temperature: | 36.8 °C (36.0 - 38.0 °C) | ZAM 1/31/17 | |
| | Incubator $CO_2$ %: | 3.5 % (3 - 7 %) | | |
| | Time/Date T- Flask Placed into Incubator: | 1218 1/31/17 | | |
| 6.23. Monitor cells daily or every other day. When cells reach a target ≥ 90% confluent monolayer, proceed to " Expansion to Hyper Flask" steps. | Time Date confluency met: | | | |

| WPI BETC | MASTER BATCH RECORD | Document Number WPI-0074 | REV 1 |
|---|---|---|---|
| Title: VERO 76 GE MQP T-FLASK - BIOREACTOR | | Batch Number: Flask 1 Run Flask ~~2~~ 3 1, 4 | |

| Instructions | Data Required | Actual Data | Performed By / Date | Verified By / Date |
|---|---|---|---|---|
| 7.7. Warm Trypsin, 450mL Media and PBS | ~~Fla~~ | 10:40 | 6 Feb 17 CAH | |
| 7.8. Pour out four flasks ID them here | Flasks ~~1~~, 2, 3, ~~4~~ | | | |
| 7.9. Wash Flasks w/ 20mL PBS ea, | 2 and 3 | 13:03 | CAH | |
| 7.10. Pour out PBS | | 13:05 | CAH | |
| 7.11. Add 15 mL 0.25% Trypsin | ~~Flas~~ | | | |
| 7.12. Rat in incubator record time | Flask 4: 13:06 – 13:~~14~~<br>Flask 2: 13:09 | Flask A only has 9mL Tryp | | |
| 7.13. Take out incubator | Flask 4 13:14 | | | |
| 7.14. Observe dissociation | | | | |
| 7.15. Neutralize trypsin w/ 25 mL Media | Flask 4: 13:17<br>Flask 1 | | | |
| 7.16. Pour into collection bottle | | 13:28 | CAH | |
| 7.17. ~~Sample~~ Break up clumps | | 13:30 | CAH | |
| 7.18. Sample ~~1~~ 1.5 mL from inoculate | | 13:31 | CAH | |
| 7.19. Cell Density? want $1 \times 10^{+5}$ cells/mL x2 | $1.36 \times 10^{6}$ cells/mL<br>99.1% viable @ 26mL | | N/S | |

| WPI BETC | MASTER BATCH RECORD | Document Number WPI-0074 | REV 1 |
|---|---|---|---|
| Title VERO 76 GE MQP T-FLASK - BIOREACTOR | | Batch Number: Flask 1 Run Flask 1, 4 | |

**8.0 Comments Log**

| Comment # | Step # | Comment | Initials / Date |
|---|---|---|---|
| 7.20 | | ~~Put into~~ Inoculate and label 1 spinner @ 14:05 | WF / 2/6/17 |
| 7.21 | | 3 minutes on, 30 minutes off 50 rpm 12-18hrs is spinner flask profile for 1 | CAH / 2/6/17 16:05 |
| | | | |
| | | | |
| | | | |
| | | | |
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| | | | |

**Microcarrier / Nuclei Stain Counting Technique**

Patrick Guertin Xcellerex, Inc.

[For Stain Solution Formulation]

Citric Acid 10 Grams per Liter
Crystal Violet 5mL per Liter
WFI or Milli-Q water

[If making 200mL of Stain Solution]

200mL of WFI
2 Grams of Citric Acid
1mL of Crystal Violet

Sampling Procedure

1. Sample 5mL from mixed flask or spinner and transfer into 15mL conical tube and allow beads to settle. Use Sharpie marker to mark off the meniscus reached by total volume.
2. Pipette to obtain supernatant for Nova Bio Profile analysis or other application.
3. Wash settled pellet by removing the supernatant and adding at least 2 times the bead volume of DPBS (i.e. Bead vol. 0.5mL add ≥1.0 mL of DPBS) and let beads settle.
4. Aspirate or pipette to remove the DPBS Supernatant.
5. Add approx 1.5mL Stain buffer (as referred above) then mix well by pipette and/or vortex. Let stand for 2 minutes.
6. Add an amount of DPBS to bring sample back to original sample volume. (i.e. if 2mL in tube add 3mL of DPBS to obtain 5mL original sample volume). Ensure that the meniscus of the total volume reaches the premarked line.
7. Pull supernatant sample for hemocytometer count and dilute 1:5 w/ DPBS if necessary.