ABSTRACT

IJEOMAH, BRENDA IJEOMA. Adeno-Associated Virus Production in Serum-Free Suspension Adapted HEK293 Cells for Gene Therapy. (Under the direction of Dr. Balaji Rao).

Adeno-associated virus (AAV) is one of the most commonly used vectors for gene therapy applications. With increasing demand for the manufacture of large quantities of pure and potent recombinant AAVs (rAAVs) for clinical testing, many developments have been made, such as new serotypes and scalable methods for production. This study highlights an easily scalable process for the production of AAV-2 in suspension and serum-free Human Embryo Kidney (HEK) 293 cells by triple transient transfection, adapted from an adherent, serum dependent HEK293 cell line. First, the adherent HEK293 cells obtained from a qualified cell bank repository, ATCC, was adapted to a serum-free suspension medium in shake flasks. To demonstrate scalability the suspension cells were scaled up to 5L WAVE bag bioreactors and transfected for large scale AAV-2 production. The AAV-2 production in the suspension adapted cells was compared to the adherent cell line production as a baseline. The suspension HEK293 cells generated a vector genome titer of 2.37E+02 vg/cell compared to the adherent cell line that generated a vector genome titer of 1.19E+02 vg/cell. The results show that the AAV genome production from the suspension HEK293 cells is greater than the vector genome produced with the adherent HEK293 cells, with increased ease of scalability.

Adeno-associated Virus Production in Serum-Free Suspension Adapted HEK293 Cells for Gene Therapy

by
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BIOGRAPHY

Brenda Ijeomah was born in Kaduna, Nigeria and currently lives in Raleigh, North Carolina. She is the second child of Ikechukwu and Paulina Ijeomah. She has two siblings, an older brother and a younger sister. In 2009, Brenda moved to the United Stated of America in pursuit of a better education.

In 2012, she enrolled in North Carolina State University as a Chemical and Biomolecular Engineering student where she had the opportunity to pursue a minor in Biotechnology. The Biotechnology courses at North Carolina State University sparked her interest in the field. Brenda graduated with her Bachelors of Science degree in May 2016 and was gainfully employed at Biogen shortly after as an entry-level Cell Culture Engineer.

In the fall of 2017, Brenda re-enrolled in North Carolina State University to pursue a higher degree in Biomanufacturing, where she became involved in a gene therapy research project that shaped the basis of this thesis. She is currently a Process Engineer at Pfizer, working on several gene therapy projects.

Outside of all academic endeavors, Brenda enjoys traveling around the world and experiencing different cultures, cooking, and reading. She also enjoys spending time with family and volunteering at dog shelters in the Raleigh-Durham area.

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CHAPTER 1

Introduction

The ability for genes to be altered and rapidly repaired has been made possible since the discovery of DNA as the biomolecule of genetic inheritance. The development of nucleic acid sequencing and the potential to perform complete human genome sequencing has allowed for the identification of genes that result in particular diseases (Naso et al.). Monogenic diseases, caused by single gene mutations, could be treated at the molecular level if mutant genes could be repaired, or by altering the expression of overactive and underactive genes (Mueller and Flotte). This concept has been the basis of gene therapy for the last couple of decades.

Gene therapy is the experimental treatment involving the introduction of genetic material into a patient's cells to treat or prevent diseases. It involves the replacement of mutated genes that cause specific diseases with a healthy copy of the gene and the inactivation of improperly functional mutated genes. New genes must be delivered to the patient's cells using a carrier or vector, since a gene cannot be directly inserted into the cell. Vector systems used in gene therapy include viral and non-viral vectors. There are numerous viral and non-viral vectors that could be used for gene delivery, each with their specific advantages and limitations.

Gene Delivery Systems

The efficacy and safety of gene transfer determines the effectiveness of gene therapy. In order to protect the genetic material in the extracellular environment from degrading, the vector system used for gene delivery must be used (Liu and Kirn). A vector is defined as a vehicle for the delivery of genes into target cells and their nucleus. Under ideal conditions, an exemplary vector should be able to accommodate foreign genes, deliver the gene of interest to a specific cell, and have high levels and duration of transgenic expression to rectify the defect while

maintaining non-immunogenicity (Mali). The two types of vectors used for gene transfer include viral vectors for gene transduction and non-viral vectors for gene transfection. However, in the scope of gene therapy, each vector type has its limitations and advantages.

Viral Vectors

The use of naturally occurring agents, such as viruses, which have evolved to deliver DNA or RNA for replication into a host cell have been used extensively (Naso et al.). Recombinant viruses have demonstrated promising and efficient *in vitro and in vivo* gene transfer. Depending on the target genes, numerous viral agents could be used for the purpose of gene therapy. However, viral vectors also possess some undesired properties, such as the tendency to cause cancer due to their immunogenic profiles. Several types of viruses such as retroviruses, lentiviruses, herpes viruses, adenovirus, and adeno-associated viruses (AAVs) have been used and proposed for preclinical studies (Liu and Kirn). These viruses each have their advantages and limitations and they are used in best suited applications. For example, retroviruses integrate permanently into the genome of infected cells, however in order to be transduced, they require mitotic cell division. Lentiviruses offer efficient and stable gene transfer, however there are potential problems with insertional mutagenesis. Herpes simplex viruses are capable of delivering exogeneous DNA in large amounts, but cytotoxicity remains an issue. Adenoviruses are able to deliver genes to both dividing and nondividing cells efficiently, but *in vivo* gene expression is limited due to immune elimination of infected cells. AAVs are also capable of infecting dividing and nondividing cells, but its DNA capacity is limited (Naso et al.). A detailed analysis of these viral vectors and their applications is discussed further in this section, with emphasis on AAVs as the viral vector for gene therapy.

Retroviruses

Retroviruses are small RNA viruses that include DNA intermediates in their replication cycle. They were among the first vectors constructed for gene therapy with the basis being the removal of genes required for virus replication and replacing them with therapeutic genes. Genes such as *gag* (capsid), *env* (envelope glycoprotein), or *pol* (reverse transcriptase) are deleted and a therapeutic gene of less than 10 kb is inserted. The deletion of these genes causes defective viral replication, therefore viral particles can be produced only when the vector is transfected into a specialized cell line known as "packaging cell line" (PCL) (Nafe et al.). The vector containing the therapeutic gene can transfect PCLs even while producing defective retroviruses, which can deliver the transgene but cannot initiate infection. The vector contains the sequences needed in *cis* for viral replication, packaging, and integration of RNA, along with the therapeutic gene, while the PCL carries the *trans*-acting genes needed for viral replication. The separation of *cis* components into the vector and *trans* components in the PCL is the premise behind the development of viral vector systems. Viral particles cannot be replicated and produced by recombinant viral vectors after the transduction of target cells because they do not code for the *trans* genes needed for replication. To eliminate the risk of generating replication-competent retroviruses due to recombination between PCLs and retroviruses, the *trans* genes are divided into separate chromosomal locations in the PCL genome (Gardlík et al.)

High tissue specificity of targeted retrovirus gene transfer can be achieved by fusing a portion of the envelope glycoprotein gene to the ligand of a tissue-specific receptor. Retroviral vectors are derived from the Moloney murine leukemia virus and they are able to maintain their viral sequences during cell division, which is advantageous in the treatment of chronic and

inherited diseases. However, over-expression of the therapeutic gene could lead to mutagenesis and toxicity (Scott-Taylor et al.).

Other limitations of retroviral vectors include low vector titer of approximately 1E+07 infection particles per ml, low transfection efficiencies, inability of non-dividing post-mitotic cell transduction due to infection of only proliferating cells, and particle instability making concentration difficult. This makes retroviral vectors conducive for *ex vivo* gene therapy applications since they cannot infect non-dividing cells.

Lentiviruses

Lentiviruses are a subtype of retroviruses that have the ability to infect both actively dividing and non-dividing cells and their vectors have been constructed to overcome the limitations of retroviruses. The most common type of lentiviruses is the human immunodeficiency virus type 1 (HIV-1). In the Lentivirus system the env gene has been replaced with genes from other viral genomes such as the Ebola virus, vesicular stomatitis virus, etc. in vector PCL. Lentiviral vectors have 6 out of 9 genes deleted, except the *gag*, *pol*, and *env* genes, and are further modified by deleting the terminal signal sequences. This transfer system makes long-term expression and efficient transfer possible without eliciting an inflammatory response (Quinonez and Sutton). Transgene expressions are higher when infecting lympho-hematopoietic cells and central nervous systems than in muscle and liver cells.

In its original form, lentivirus is a highly virulent virus, so it poses major safety concerns. There is a slim chance for HIV-1 wild-type mutation to occur after transduction, so efforts have been made to minimize vector mutations such as the removal of accessory genes and proteins from the viral vector genome. The deletion of these genes to reduce the potential for pathogenic vector mutation poses no adverse effects to the lentivirus vector function(Kim et al.).

There are three generations of the lentiviral particles, based on the packaging plasmid used to produce the lentivector. The first lentiviral generation uses a packaging plasmid to provide all the gag and pol sequences, the accessory genes (vif, vpr, vpu, and nef), and viral regulatory genes (tat and rev) required (Escors and Breckpot). In the second generation, the four accessory genes are removed without any negative impact in regards to vector yield and infection efficacy. Removal of these accessory genes also improves the safety of the lentivirus vector because all virulence factors are devoid by removal of replication-competent lentivirus. The third generation includes an improved packaging system, which consists of a split-genome system for packaging to improve the safety of the viral vector. In the split-genome packaging system, a separate plasmid is used to express the viral regulatory rev gene and a strong promoter that is tat-independent replaces the 5' long terminal repeat on the transfer vector (Dull et al.)

Table 1.1: Viral vector genome size (Ortiz et al.)

Vector	Packaging Capacity
Adeno-associated virus	**4.7 kb**
Adenovirus	**7-10 kb**
Herpes simplex virus	**25 kb**
Lentivirus	**8 kb**
Retrovirus	**8 kb**

Herpes Simplex Viruses

The herpes simplex virus (HSV) is a highly infectious neurotropic DNA virus that is a suitable vector for the introduction of foreign genetic material to cells. It is a double stranded DNA virus consisting of a trilaminar lipid envelope with 10 embedded viral glycoproteins for receptor-mediated cellular entry.

Herpes simplex virus type-1 (HSV 1) are used as gene therapy vectors and can be prepared in one of two ways. The gene of interest is introduced into the plasmid containing an HSV packaging signal and origin of replication by the amplicon system. The plasmid construct is then delivered into cells by transfection, followed by infection with a helper HSV virus, which results in amplicon packaged viral particles. Alternatively, the gene of interest can be inserted directly into the HSV genome by cloning the gene, flanked by specific HSV viral sequences, into a plasmid vector. Then transfecting the plasmid into the cells along with the HSV DNA, where recombination between the plasmid vector viral sequences and the corresponding virus genome sequence occur (Latchman). Reporter genes, such as green fluorescent protein (GFP) or β-galactosidase can be used to differentiate recombinant and non-recombinant viruses. Due to the HSV pathogenicity, the goal in vector construction is to inactivate any pathogenic viral genes by either removing the genes needed for growth in non-dividing cells, or by deleting immediate early genes needed for replication and encapsidation (Cotter and Robertson).

The greatest advantage of using HSV as a vector is their large genome capacity for the insertion of foreign genes. HSV has one of the largest genomes among viral vectors and can accommodate approximately 25 kb of foreign genes with the deletion of other genes without any significant effect on viral replication.

Adenoviruses

These linear, double stranded DNA viruses with a genome size ranging from 26 kb to 45 kb, and a diameter of 70 nm, encode 50 viral polypeptides. Adenoviruses are non-enveloped viruses with an icosahedral conformation, designed to overcome retroviral vector limitations for gene transfer such as low vector titer and inability to transduce non-dividing cells. Recombinant adenoviruses can accommodate up to 8.5 kb of foreign DNA, which is sufficient enough for most therapeutic genes. Although they are able to deliver large transgenes, their DNA is unable to integrate into the host genome, so it resides in the host nucleus (Gardlík et al.).

There are more than 50 different adenovirus serotypes, however adenovirus type 5 (Ad5) is the most commonly used serotype for gene therapy since it can be easily manipulated and obtained at concentrations of 1E+10 to 1E+11 viral particles per mL in *in vitro* experiments. Ad5 has a genome of approximately 36 kb and the DNA is flanked by inverted terminal repeats (ITRs) on both ends. The ITRs serve as a self-primer, which promote primase-independent DNA synthesis and host genome integration. The adenovirus genetic element also consists of a packaging signal required for proper packaging of viral transcripts. The viral transcripts are classified as early or late transcription units. The early units (E1, E2, E3, and E4) express non-structural proteins that regulate the functions of viral DNA replication, while the late units (L1, L2, L3, L4, and L5) encode for the Ad virion structural components (Lee et al.).

The advantages of adenoviruses include the ability to efficiently infect both dividing and non-dividing cells, making gene delivery with high degree of differentiation easier. Adenoviral vectors are complex, stable, and have a broad range of cell specificity. Since the viral DNA cannot be integrated into host genome, transient expression is used to express the foreign delivered genes. A disadvantage of adenoviral vectors is an inflammatory response mediated by

B- and T- cells from the activation of immune cells, affecting the health of the patient and gene delivery efficiency. Another disadvantage is the short-term expression of adenoviruses, limited transduction of cells, immunogenicity, and transient expression of foreign genes (Lusky et al.).

Adeno-associated viruses

Adeno-associated virus (AAV) is a widely used gene therapy vector. AAV is a small virus of approximately 25 nm from the *Parvoiridae* family, composed of a non-enveloped capsid protein shell containing a linear single-stranded DNA genome of approximately 4.7 kb (Balakrishnan and Jayandharan). The single stranded genome of AAV contains *Rep* (Replication), *Cap* (Capsid), and *aap* (Assembly) genes, which result in at least nine gene products through the use of differential splicing, alternative transition start-sites, and three promoters. The *Rep* gene is required for viral genome replication and packaging, while the *Cap* gene forms the capsid shell protecting the viral genome. The *Rep* region encodes for four proteins, Rep78, Rep 68, Rep 52, and Rep 40, designated according to their molecular mass. The *Cap* gene encodes three structural proteins, VP1, VP2, and VP3, which share the same reading frame. These capsid proteins form the outer capsid shell and provide protection for the viral genome. The *aap* gene provides the scaffolding function for capsid assembly by encoding the assembly-activating protein (AAP) overlapping the *cap* gene alternatively (Naso et al.). The AAV genes are flanked by two palindromic inverted terminal repeats (ITRs), which form T-shaped structures and are the only *cis* elements required for AAV replication, packaging, integration, and rescue. As seen in Figure 1.1, the four proteins in the *Rep* region and the three proteins in the *Cap* region share the same reading frame.

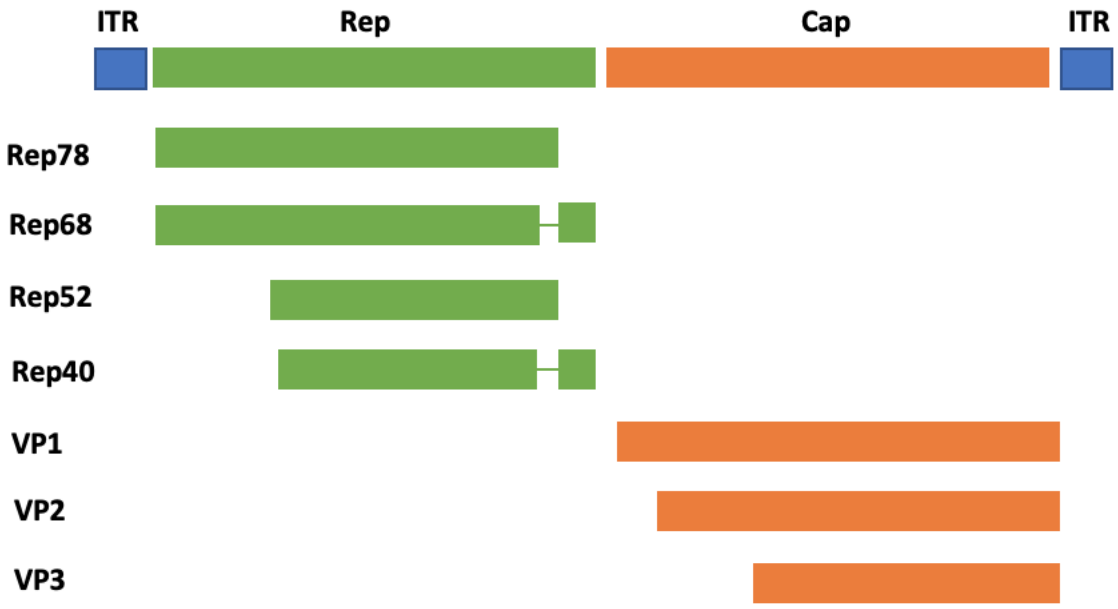


Figure 1.1: Schematic of AAV Genome ((Samulski and Muzyczka), Cell Biolabs, INC AAV-2 Helper Free Packaging System VPK-402 Product Manual)

There are 12 AAV serotypes and 108 isolates that have been identified and classified to date. Recombinant AAV (rAAV) is an engineered nanoparticle that lacks wild-type viral DNA and it is used to cross the cell membrane for the delivery of DNA cargo into the cell's nucleus (Gao et al.). In rAAVs, the ITR-flanked transgenes can form episomes, circular concatemers, in transduced cells' nucleus. Due to the inability of recombinant episomal DNA to integrate into host genome, the DNA would become gradually diluted as the cell undergoes constant replication, resulting in the loss of transgene expression (Choi et al.).

AAV-2 was the first serotype to be isolated, characterized, and fully sequenced. Due to the vast knowledge of its biology, AAV-2 has been historically adopted as a gene therapy vector. AAV production is versatile, allowing for the generation of hybrid AAV vectors composed of the same AAV-2 ITR-flanked transgenes with any available AAV capsids. AAV vectors have unique transduction abilities, such as cell tropism and transgene expression kinetics, due to the capsid interactions with target cells (Colella et al.). Table 1.2 indicates the optimal AAV

serotypes used for specific organ transduction, with AAV-2 targeting the central nervous system, kidney, and eyes.

Table 1.2: Tropism of AAV serotypes indicating optimal serotype for particular organ transduction (Srivastava)

Tissue	Optimal AAV Serotype
Central Nervous System	1, 2, 4, 5, 8, 9
Heart	1, 8, 9
Kidney	2
Liver	7, 8, 9
Lung	4, 5, 6, 9
Pancreas	8
Photoreceptor Cells	2, 5, 8
Retinal Pigment Epithelium	1, 2, 4, 5, 8
Skeletal Muscle	1, 6, 7, 8, 9

Adeno-associated virus is beneficial for gene therapy due to its long-term gene expression, inability to replicate without a helper virus, lack of wild-type infection pathogenicity and weak immune response. Despite their benefits, using recombinant AAV for human gene therapy has its shortcomings (Grieger et al.). One of the challenges is scaling up the manufacturing process in compliance with current good manufacturing practices (cGMP) to yield sufficient quantities of purified vectors to meet the growing clinical need.

Table 1.3: Summary of the advantages and disadvantages of gene therapy viral vectors

VIRAL VECTORS	CHARACTERISTICS	ADVANTAGES	DISADVANTAGES
RETROVIRUS	Single-strand RNA; Allows 8 kb exogenous DNA	Stable transgene expression; Easy to design; Integration into cell DNA	Small capacity for transgene insert; Infects only dividing cells; Insertional mutagenesis (integration)
LENTIVIRUS	RNA virus; HIV-1 derivative	Infects dividing and nondividing cells	Insertional mutagenesis (integration)
HERPES SIMPLEX VIRUS	Double-stranded DNA; Allows 30 kb exogenous DNA	Large capacity for transgene insert; Ability to infect non-dividing cells; Availability of anti-herpetic drugs	Immunogenicity
ADENOVIRUS	Double-stranded DNA; 36 kb genome	Large capacity of transgene insert; Biologically safe	High immunogenicity
ADENO-ASSOCIATED VIRUS	Single-stranded DNA that requires helper virus; allows 5 kb of exogeneous DNA	Stable transgene expression; Infects only dividing cells	Small capacity for transgene insert

Non-viral vectors

Non-viral delivery systems for gene transfer provide a simple and safer alternative to the viral counterpart. With non-viral vectors, therapeutic genes can be inserted into the plasmid directly, and then the plasmid can be introduced into cells by direct injection or a variety of ways. They were developed to achieve prolonged gene expression, virtually unlimited insert capacity, high efficiency, and low toxicity. Non-viral delivery systems possess low host immunogenicity with relatively cheap and simple production. The low immunogenicity of these

vectors allows for possible re-dosing since it is impossible for viral recombination to occur that would result in a competent virus (Lundstrom and Boulikas).

Particle-mediated gene transfer, such as "the gene gun" is another method for gene transfer where DNA is bound to miniature gold particles, which are shot into the cells under high speed and pressure to pass the cell membrane. Another non-viral gene transfer strategy is gene-activated matrix, where cDNA is loaded on a scaffold of porous biomaterial and packed directly into a wound, where the transfer of the gene into endogenous cells occurs (Macklin et al.).

Liposomes and Nanoparticles

Liposomes can be used to carry genetic material in tissue culture in order to increase DNA uptake. Cationic liposomes are amphiphilic molecules comprised of a lipid bilayer membrane that interact with negatively charged DNA, thus protecting it from circulating endonucleases. When DNA is incubated with liposomes, the resulting structure is a lipoplex. A cationic lipoplex induces cellular uptake by binding to the negatively charged cell membrane, although the uptake rate is low (Tong et al.). There are no replication risks in liposome-mediated gene transfer. Pegylated liposomes are known to localize to solid cancers and delivering radio sensitizing agents to tumor tissue, thus improving the therapeutic ratio of chemotherapy (Gardlik et al.).

Other artificial technologies, like nanoparticles, have been used to encapsulate the nucleic acid and deliver them through the cell membrane, while protecting the DNA and RNA from degradation (J. Chen et al.). Nanoparticles can be lipoplexes, polymers, or ceramic-based and they can accumulate in tumors due to the enhanced permeability and retention effect when used as vectors (Chisholm et al.).

Naked DNA

Naked DNA or plasmid DNA consists of a ring of extrachromosomal DNA found in bacteria. The therapeutic gene can be inserted into the plasmid using recombinant DNA techniques and expressed when plasmids contain promoters and polyadenylation sequences. Plasmid DNA is easy to manufacture and can contain large exogeneous inserts. They are highly stable during storage and are safer than viral vectors with respect to genomic integration and immunogenic responses. Although naked DNA is considered safe, it has low transfection efficiencies compared to viral vectors (Hendrie and Russell).

Table 1.4: Summary of the advantages and disadvantages of non-viral vectors for gene therapy

NON-VIRAL VECTORS	ADVANTAGES	DISADVANTAGES
LIPOSOMES AND NANOPARTICLES	Large gene capacity; Nanoparticles can accumulate into tumors due to enhanced permeability and retention (EPR) effect	Inflammatory toxicity and low transfection efficiencies; Requires intraperitoneal transfer
NAKED DNA	Easy engineering and low immunogenicity	Low transfection efficiencies; Rapid clearance

Although there are numerous viral and non-viral agents, each with their unique attributes that could be used in the application of gene therapy, their advantages and disadvantages make them more or less suitable for specific profiles. The disadvantages of some viral vectors, such as their immunogenicity and predisposition to cause cancer, have limited their use in clinical applications. Other non-viral technologies have exhibited unwanted safety signals that require further understanding. Adeno-associated virus remains the most actively used gene therapy vectors and the AAV-2 serotype will the primary focus for the purpose of this research development due to its broad cell tropism.

Clinical Manufacturing

A living system is required to produce adeno-associated virus for gene therapy. Despite the copious amounts of research on AAV production, current methods of manufacturing remain expensive. The most robust production of AAV occur in adherent cells grown in cell stacks or roller bottles, capable of achieving titers of approximately 1E+05 genome copies per cell or 1E+14 genome copies per liter. However, to support the growing manufacturing needs, scalability of adherent processes remains a limitation (Naso et al.).

Transient Transfection Platforms

Currently, the industry relies on HEK293 cells, transfected with two or three plasmids, as a platform for production of AAV. One plasmid contains the gene of interest, one carries the AAV *rep/cap* genes, and the other constitutes of helper genes. The AAV Helper-Free System by Cell Biolabs depicted in Figure 1.2 promotes the production of AAV virions without using a helper virus, thus promoting a safe and convenient gene delivery system. For this to be possible, most of the adenovirus gene products, such as E2A, E4, and VA RNA gene, required for AAV production are supplied with a pHelper plasmid. The *rep* and *cap* genes are removed from the ITR-flanked viral vector and supplied in *trans* on the pAAV-RC plasmid. This allows for the gene of interest to be inserted in the viral genome. Figure 1.2 depicts the triple transfection schematic for the AAV Helper-Free System for AAV production.

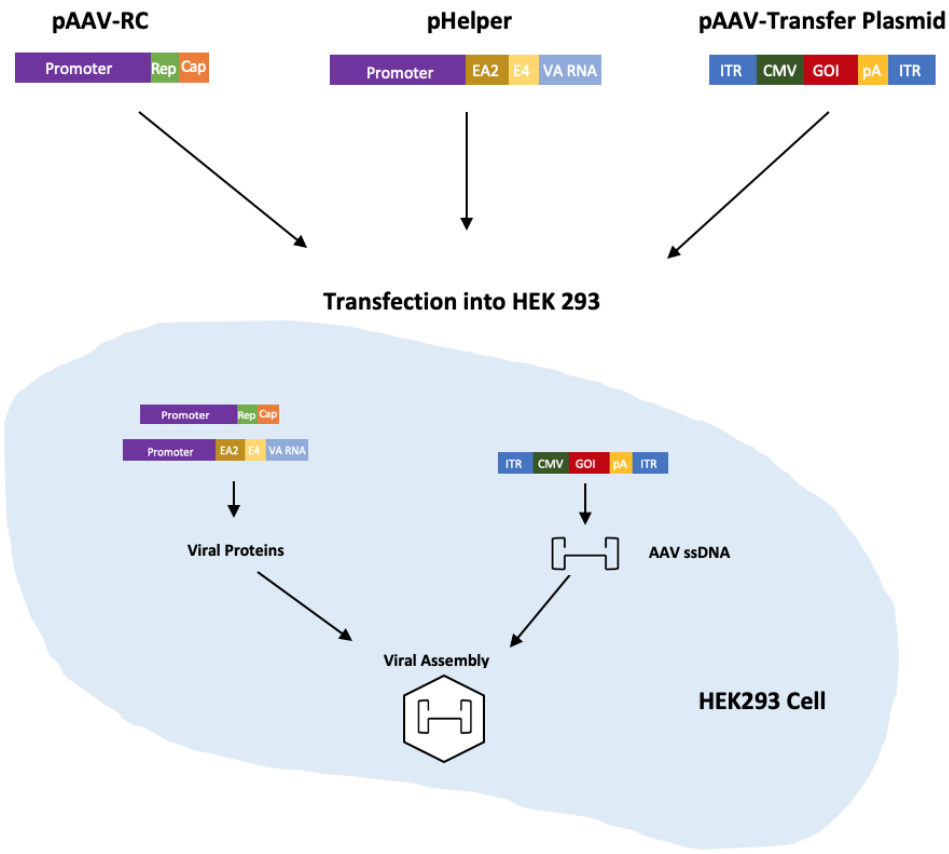


Figure 1.2: AAV Helper-free system (Cell Biolabs, INC AAV-2 Helper Free Packaging System VPK-402 Product Manual)

Producer Cell Lines

Genetic components for the manufacturing of AAV have been integrated into the genomes of insect and mammalian producer cell lines. Most helper genes required for the production of AAV cannot be stably transfected, except the E1a and E1b adenoviral genes (Naso et al.). The E1a and E1b genes supply the helper functions necessary for AAV production, and they induce the expression of the AAV *rep* gene causing toxicity in insect and mammalian cells. To overcome this, mammalian cell lines have been developed by either co-infecting baby hamster kidney cells with replication-defective herpes simplex viruses to encode the ITR-flanked transgene and *rep/cap* genes or by utilizing a stable producer HeLa cell line to carry the ITR-flanked transgene and *rep/cap* genes. The helper genes are provided by the infection of

adenovirus particles after co-transfection of the AAV packaging plasmids and rAAV vector (Xiao et al.).

Sf9 insect cell lines combined with baculovirus infection has also been used to produce large quantities of AAV. To do this, two or three particles of baculovirus are used to infect Sf9 cells to initiate AAV production. In this system, one virus contains the *rep* gene, another contains the *cap* gene, and the third baculovirus particle carries the ITR-flanked transgene. If the SF9 cells are engineered to contain the ITR-flanked transgene, then only two baculovirus particles are required to initiate AAV production. The SF9 system has proven to produce high levels of AAV, approximately 1E+05 genome copied per cell or 1E+15 genome copies per liter, with the ability to be easily manipulated and high-density growth. While the Sf9 system is becoming increasingly popular for AAV manufacturing, there are concerns regarding post-translational modifications and baculoviral instability (Naso et al.) (Mietzsch et al.).

HEK 293 cells are the preferred host cells used in the gene therapy industry because they are highly transfectable, with the ability to support high levels of viral production. HEK293 host cells supply the remaining adenoviral genes and stably express the E1 adenovirus gene. HEK293 cells are commonly maintained in adherent cultures using T-flasks, roller bottles or cell factories in laboratory-scale and clinical production. The use of adherent systems poses challenges in terms of scalability due to the manipulation of large numbers of vessels, availability of manufacturing space, and increased risk of contamination due to open handling during the aseptic process. The adherent method is also labor-intensive and time-consuming. One way to reduce risk during clinical operations is to use large bioreactors with cells floating freely in suspension (van der Loo and Wright).

The industry relies on HEK293 cells, transfected with two or three plasmids as a platform for AAV production. These cells are maintained in adherent cultures, which pose challenges in terms of scalability. In order to meet the growing clinical needs while remaining cGMP compliant, suspension cultures are necessary. This project reports the development of a serum-free suspension HEK293 cell line for the production of AAV-2 that matches the production of AAV-2 in adherent serum-dependent HEK293 cells.

CHAPTER 2

Materials and Methods

Adaptation of HEK293 cells to suspension from an adherent HEK293 master bank

To begin generating a scalable high-titer process, an adherent HEK293 cell line growing in fetal bovine serum (FBS)-containing medium was obtained from a cell bank repository (ATCC). The process of adapting adherent HEK293 cells to suspension HEK293 cells occurred in two phases; weaning the cells off serum while remaining adherent, then adapting to serum free suspension media. The first step in the process was thawing a vial of ATCC CRL-1573 293 Embryonic Kidney Human cells in ATCC-Formulated Eagle's Minimum Essential Medium (Part #: 04729) supplemented with 10% Gibco fetal bovine serum (FBS) (Part #: 10099-141) and cultured in Corning tissue culture treated T-75 T-flasks for several days to allow cells to recover from cryopreservation. The cells were passaged in Dulbecco's Modified Eagle Medium (DMEM) (Part #: 30-2002) every three to four days over a four-week period, while gradually reducing the amount of FBS from 10% to 5% to 2.5% every couple of passages. The adherent cells were incubated in a Forma Series II Water Jacketed CO_2 Incubator at 5% CO_2, 37°C, and 80% humidity and they were passaged at 20,000 cells per cm^2 at approximately 90% confluency. The cells in 2.5% FBS were passaged into HyClone Hycell TransFx-H serum-free suspension media (Ref #: SH30939.02) and grown in Corning Erlenmeyer baffled shake flasks while their growth rates and viabilities were monitored. Once adapted, the suspension cells were expanded and frozen down to create a master cell bank (MCB) to be used later in this investigation. The cells were grown and maintained using Infors Multitron Shaker Incubators at 37°C, 80% humidity, 5% CO_2, and 125 rpm. The cells were passaged at 0.5E+06 viable cells per mL and maintained at cell densities between 2.5E+06 viable cells per mL and 3.5E+06 viable cells per

mL. Cells densities and viabilities were counted with a Vi-CELL XR (Beckman Coulter) and imaged on an EVOS M5000 (Invitrogen).

Triple Transfection of HEK293 suspension cells

Cell density and viability were determined on the day of transfection using a Beckman Coulter ViCell XR Cell Counter. The suspension cells were grown in HyCellTransfx-H media and diluted down to 1E+06 viable cells per mL in a 125 mL Corning Erlenmeyer baffled shake flask with a working volume of 28.5 mL. A transfection cocktail was prepared by mixing the following reagents in order: plasmid DNA, FreeStyle F17 Expression medium (Thermo Fisher Scientific Part #: A13835), and Polyethyleneimine (PEI) Max (MW 40,000),. The ratio of the Cell Biolabs plasmids used were 1:1:1 molar ratio of pAAV-RC2:pHelper:pAAV-GFP and 1.0 μg/mL total DNA. Transfection grade PEI Max was used at a volumetric ratio of 2:1 PEI to DNA. FreeStyle F17 media was added to bring the transfection cocktail volume to 5% of the final flask volume. The transfection cocktail was mixed thoroughly by inversion and allowed to incubate for 10 minutes at room temperature. The cocktail was pipetted into the flasks and returned back to the incubator. Three hours after transfection the flasks were supplemented with HyClone CDM4HEK293 media (Thermo Fisher Scientific Part #: SH3085802) at 10% of the final flask volume in order to stop the transfection and prevent any PEI-related cell aggregation. Experiments were done in 30 mL volumes to optimize the process and cells were harvested 72 hours after transfection.

Triple transfection of HEK293 adherent cells

The adherent cells were transfected using the following method. Twenty-four hours prior to transfection, T-25 flasks were inoculated with 126,000 cells per cm^2 and placed in a Forma Series II Water Jacketed CO_2 Incubator at 5% CO_2, 37°C, and 80% humidity. On the day of transfection, the cells in one of the T-25 flasks were trypisnized and counted on a Vicell to determine the amounts of plasmids required for transfection. The plasmids for the adherent transfections were calculated on a per 1E+06 cells basis, so obtaining the cell density prior to transfection was essential. The transfection cocktail was prepared by aliquoting 1.4 mL of Opti-MEM media (Thermo Fisher Scientific Part #: 31985-070) in a sterile Falcon 15 mL conical tube. The plasmid DNA was added into the conical tube containing the Opti-MEM media, followed by PEI Max and the mixture was shaken vigorously. The solution was incubated at room temperature for 15 minutes. Next, 2.8 mL of DMEM supplemented with 2% FBS was added to the cocktail. The T-25 flasks were removed from the incubator and the spent media was aspirated out and replaced with approximately 4.5mL transfection cocktail (final volume dependent of the plasmid molar ratios and PEI to DNA ratio). The flasks were returned to the incubator for 24 hours. Twenty-four hours after transfection, the media was exchanged with DMEM media without FBS and allowed to incubate for an additional 48 hours. On the day of harvest, the flasks were observed under the EVOS M5000 imaging system to observe GFP expression. To detach the cells, the flasks were tapped gently and observed under the microscope to confirm complete detachment. The cells were counted using the Vicell cell counter.

Suspension HEK293 cells lysis and harvest

Cells were lysed 72 hours post-transfection by adding Sodium Deoxycholate to a final concentration of 1.0%. They were incubated at 37°C for 120 minutes. During the development process, 800 µL aliquots of the transfected cell culture were lysed in microcentrifuge tubes while shaking at 1800 rpm on an Eppendorf MixMate shaker. Then Novagen Benzonase (Part # 70664-3) and Magnesium Chloride, with a final concentration of 50 units per mL and 2mM, respectively, were added and incubated for 30 minutes. Post-lysis, the cells were centrifuged at 14,000 rpm for 10 minutes on an Eppendorf Centrifuge 5418 benchtop centrifuge. The supernatant was collected and stored at -80°C.

Determining rAAV titer from cell lysate using qPCR and ELISA

Two microliters of the cell lysate were placed in a 96-well round bottom PCR plate for DNase treatment. Eighteen microliters of DNase reaction mixture were added to the PCR plate containing the cell lysate. The DNase reaction mixture composed of two microliters of 10x DNase I buffer (Invitrogen Part #: AM8170G), one microliter of Deoxyribonuclease I – 137 U/run (Invitrogen Part #: 18047-019), and 15 microliters of nuclease-free water per reaction. The plate was sealed, vortexted, and spun down at 2,400 x g for 1 minute. The DNase samples were incubated at 37°C for 1 hour to initiate the DNase treatment, 95°C for 20 minutes to inactivate the DNase enzyme, and then held at 4°C. Twenty microliters of Proteinase K reaction solution were added to each well of the PCR-plate. The Proteinase K reaction solution was made by mixing 19 microliters of 2X Proteinase K buffer (Thermo Fisher Scientific Prototype Part #: RD090518) and one microliter of Proteinase K (Invitrogen Part #: 100005393). The plate was sealed, vortexed, and spun down at 2,400 x g for 1 minutes. The plate was incubated at 60°C for 1 hour to initiate the proteinase treatment, 95°C for 10 minutes to inactivate the Proteinase K

enzyme and held at 4°C. A 10 microliter aliquot of the Proteinase K digestion sample was diluted in 90 microliters of nuclease free water to be analyzed by qPCR. A bulk qPCR reaction mixture was prepared by mixing 2x qPCR Expression Super Mix with premixed ROX (Thermo Fisher Scientific), AAV-GFP-1 probe, GFP specific primers (F/R), and nuclease free water. The AAV qPCR protocol uses the probe 5′-FAM-CACTCCCTCTCTGCGCGCTCG-TAMRA-3′, the forward ITR primer: 5′-GGAACCCCTAGTGATGGAGTT-3′, and the reverse primer: 5′-CGGCCTCAGTGAGCGA-3′. A standard curve was prepared by diluting one microliter of 58 ng of linearized pAAV-GFP in 99 microliters of ddH_2O to result in 1 x 10^8 DNA copies per microliter. Ten microliters of the 10^8 sample was diluted in 90 microliters of ddH_2O to result in 1 x 10^7 DNA copies per microliters. Serial dilutions were made in this manner until 1 x 10^1 DNA copies per microliters was achieved.

Sixteen microliters of the qPCR reaction mixture were mixed with four microliters of the eight-standard curve samples and the diluted cell samples in a 96-well PCR plate, the plate was sealed, vortexed and spun down at 2,400 x g for 1 minute. The samples were incubated at 50°C for 2 minutes (Uracil-DNA Glycosylase (UDG) incubation), denatured at 95°C for 2 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The PCR plates were analyzed on an Applied Biosystems 7500 Fast Real-Time PCR System. An American Research Products AAV-2 Titration ELISA (Part #: PRATV) assay kit was used for the quantitation of AAV-2 serotype particles in the cell culture lysate.

CHAPTER 3

Process Development

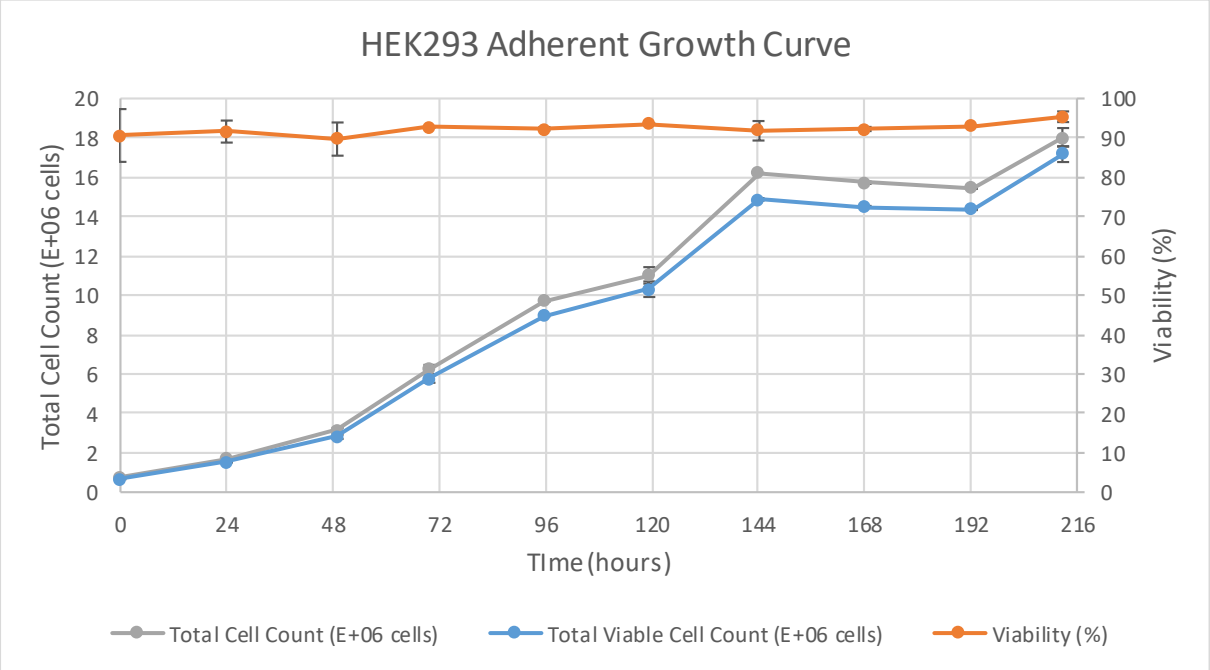
In some AAV manufacturing platforms, the genetic components necessary for AAV production are integrated into the genome of mammalian or insect cell lines for clinical use. With many developments being made on AAV as a gene therapy vector, efforts have been put forth to develop a scalable manufacturing process that can generate highly pure and potent AAV vectors at high titers. One such scalable methods is utilizing the transfection capabilities of HEK293 cells, which is relatively simple and efficient (Grieger et al.).

Development of a suspension HEK293 cell line

The goal of this project was to develop an HEK293 cell line that grows well in suspension culture in animal-component free (ACF) medium and produced equal or better titers of AAV-2 when compared to the original adherent HEK293 cell line. The adherent HEK293 cells were retrieved from a qualified master cell bank from ATCC, expanded and frozen down to develop a master cell bank. A growth curve for the serum dependent adherent cells was performed to set a baseline for cell growth.

The adherent cell growth curve was performed by inoculating 18 T-25 T-flasks at 20,000 cells/cm^2 to take daily cell counts and metabolite data of three flasks per day. The cells were detached by first aspirating out the spent media and washing the cells with 5 mL of phosphate buffered saline (PBS) and then aspirating that out. Next, 2.5 mL of TrypLE Select (Thermo Fisher Scientific Part #: 12563011) was added to the flask and allowed to incubate over the cell surface for 3 minutes, then an equal volume of DMEM media supplemented with 10% FBS was added to completely detach the cells from the flask surface. A total of 5 mL of cell culture was present in the flask prior to taking a cell count. The cell density data reported in Figure 3.1 is a

total cell count of the entire 5 mL working volume.



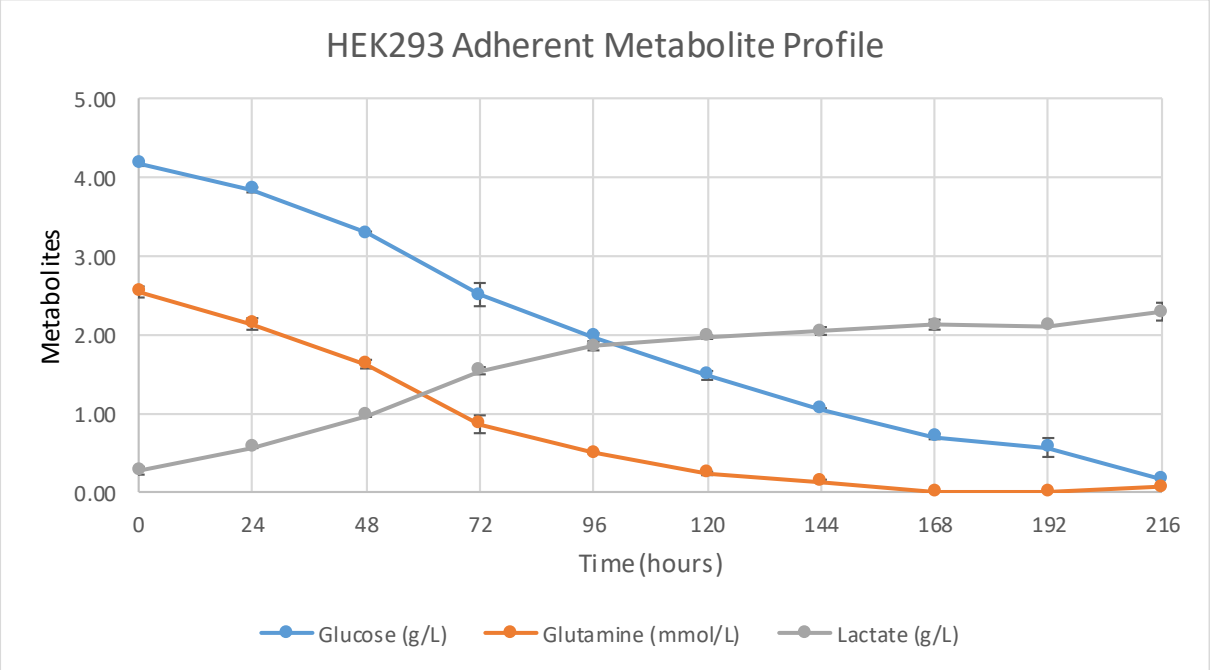


Figure 3.1: Growth curve and metabolite profile for adherent cell culture in T-25 flask. Error bars represent the standard deviation of triplicate biological replicates.

The growth curve shows the cells in exponential growth phase from days 0-4 and the cells enter stationary/death phase after day 6. There appears to be a second surge of growth at day 9, however due to a limited number of flasks it could not be confirmed if the cells had in fact reached stationary growth. The average specific growth rate in exponential growth phase was 0.025 hr^{-1} and the average doubling time was approximately 31 hours. The cell density reached a peak of 14.88E+06 total viable cells on day 6, however the final total viable cell count on day 9 was 17.18E+06 cells.

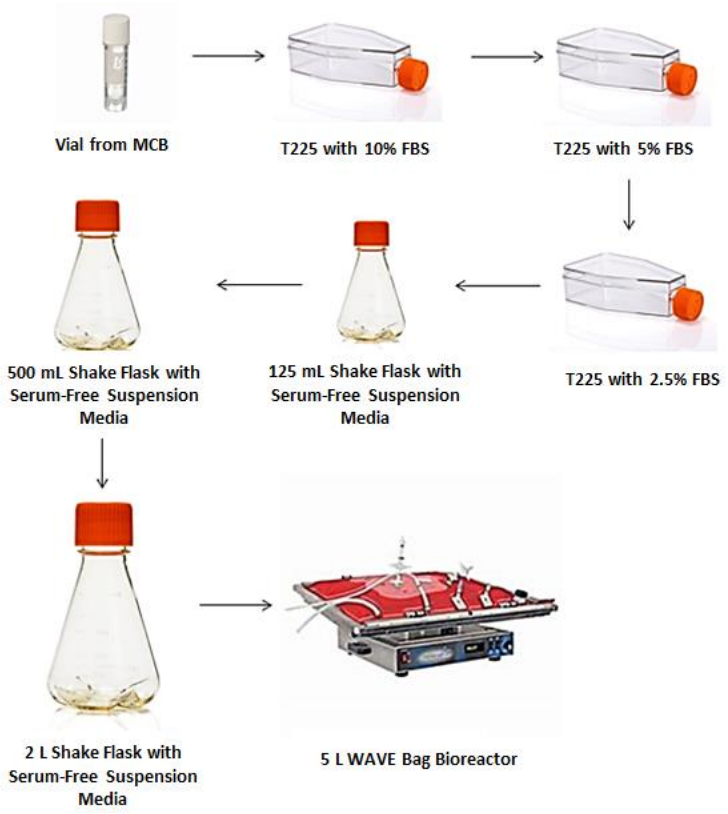
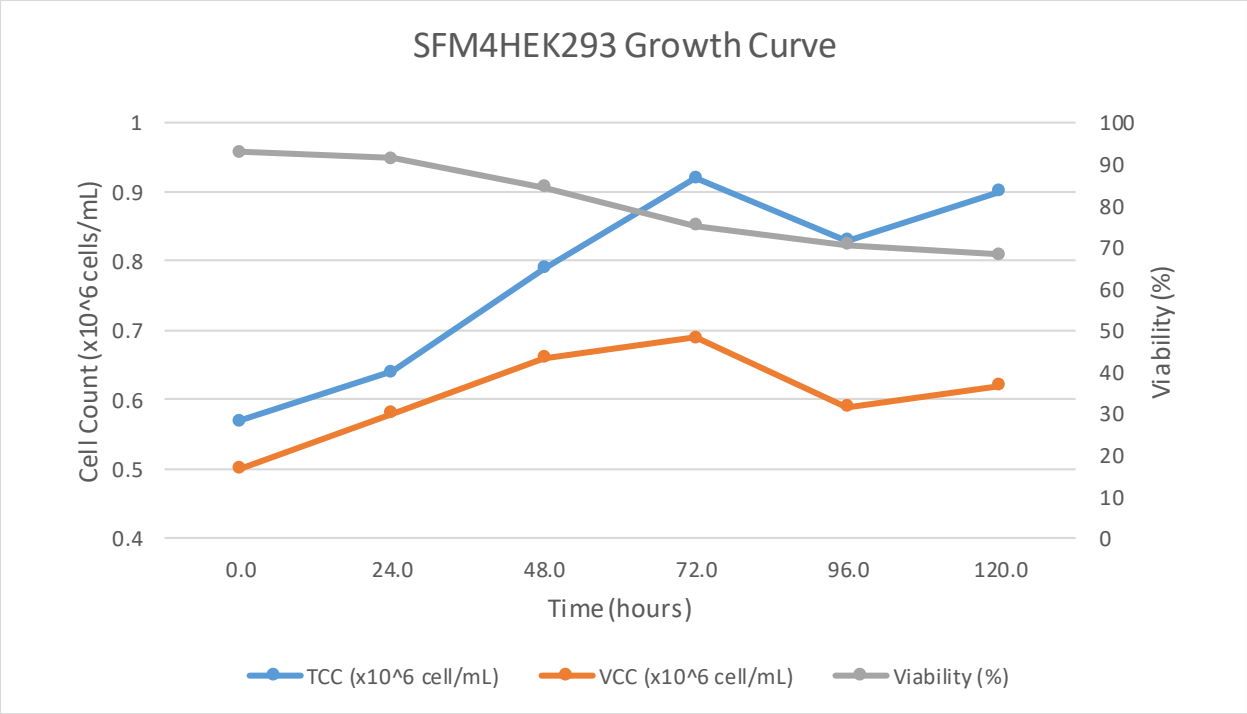


Figure 3.2: Adaptation schematic from adherent vial thaw to 5 L WAVE bag scale-up

Figure 3.2 shows the schematic for the suspension adaptation process. The adherent HEK293 cells were thawed and passaged in T-flasks while reducing the serum content as described in the materials and methods section. The adherent cells with 2.5% FBS were passaged

to a baffled shake flask with suspension medium without FBS. Once the cells were adapted to growth in a suspension culture, they were scaled up to a final production volume of five liters.

During adaptation to serum-free suspension medium, three media were tested: SFM4HEK293 (Thermo Fisher Scientific Part #: SH3052102), HyCell TransFx-H, and SFM4HEK293 TransFx (Thermo Fisher Scientific Part #: SH30860.02). The cells in the SFM4HEK293 TransFx media failed to stay in suspension; therefore, the medium was eliminated from subsequent testing. A growth curve for the HEK293 suspension cells in the HyCell TransFx-H and SFM4HEK293 medium was performed in 250 mL baffled shake flasks with 75 mL working volumes. The growth curve for each medium was performed in a single replicate and carried out for five days.



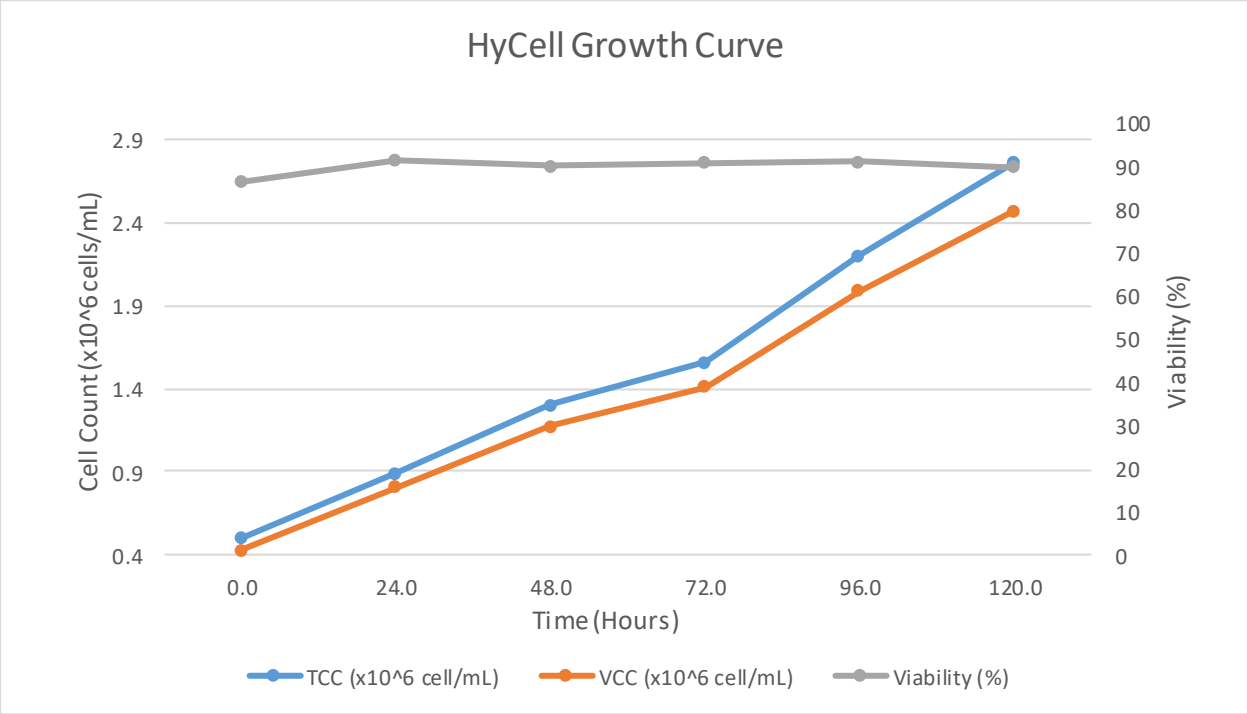
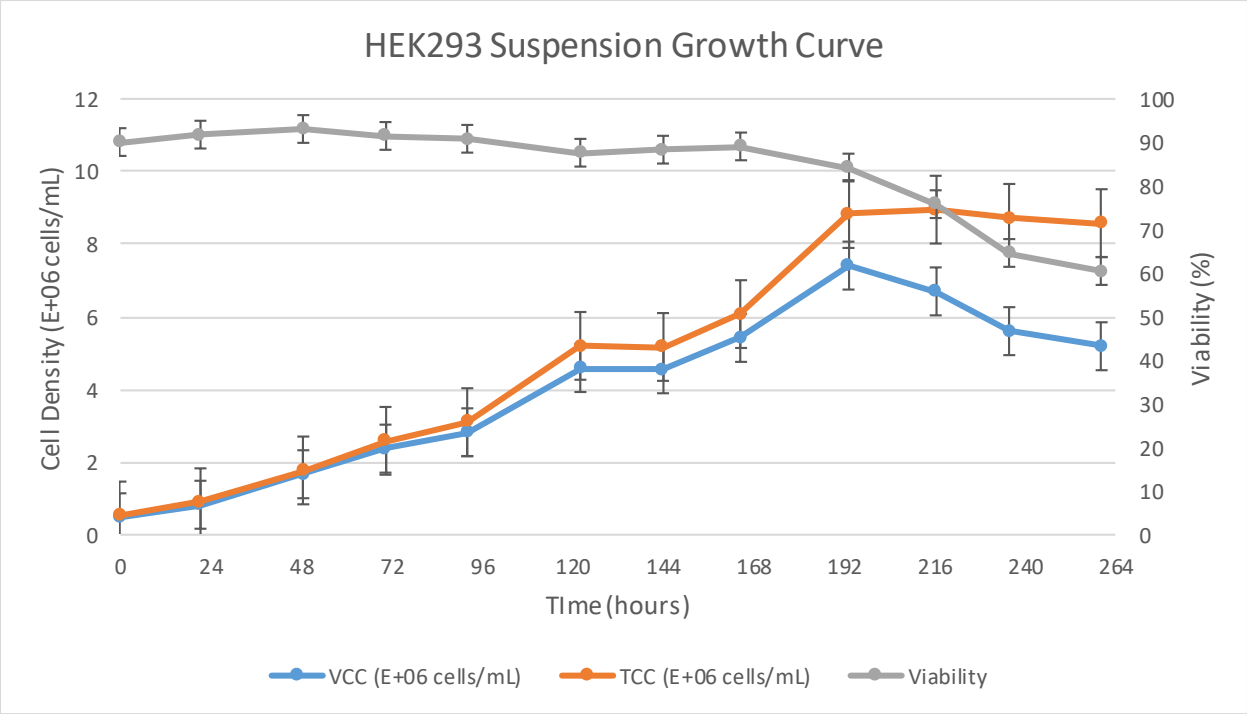


Figure 3.3: Growth curve with cell densities and viabilities of cells grown in SFM4HEK293 media and HyCellTransFx-H media

Based on the growth profile in Figure 3.3, cells in the Hycell TransFx-H medium demonstrated the best growth rate, cell densities, and viability. Cells in this medium also recovered from cryopreservation with minimal complications compared to the other two media. Further development work was carried out on the cells cultured in the HyCell TransFx-H media. After transitioning from 2.5% FBS containing media to the suspension media, there was an inherent clumping issue that caused the cells to fall out of suspension. To address this, Gibco Anti-Clumping Agent (Part #: 0010057AE) was added to the suspension media at a 1:500 parts concentration for the first couple of passages and the cells were strained with a sterile 70µm Falcon nylon mesh cell strainer to remove large clumps during passaging. This drastically reduced the clumping issue and allowed the cells to grow easily in the suspension culture. Once the cells in the HyCell medium demonstrated optimal growth characteristics, an 11-day growth curve shown in Figure 3.4 was carried out on the suspension cells in biological triplicates. Three 250 mL baffled flasks seeded at 0.5E+06 viable cells per mL with a working volume of 75 mL were used.



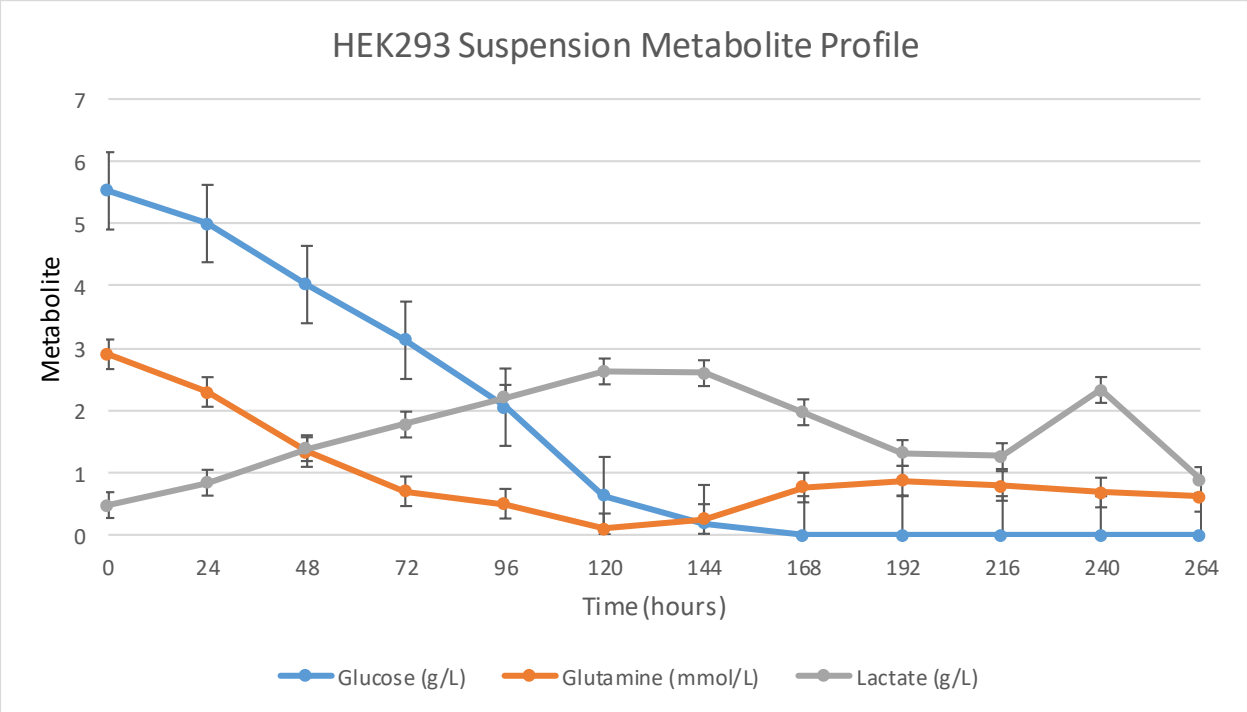


Figure 3.4: HycellTransFx-H 11-day growth curve and metabolite profile. Error bars represent the standard deviation of triplicate biological replicates.

The specific growth rate and doubling time of the optimized HyCell cell culture is 0.018 hr^{-1} and 35.6 hours, respectively. The cells were in the exponential growth phase from day 0-8, after which they entered stationary growth phase. The peak cell density was 7.41E+06 viable cells per mL and occurred on day 8. There appeared to be no clumping of cells during the first 5 days of the growth curve study, however the cells began to form macroscopic clumps in the later stage of the growth study. The media was supplemented with 4mm glutamine and the metabolite profile shows steady metabolism of glucose and glutamine over time. There was an increase in lactate as a by-product of cell culture. Compared to the adherent cells growth curve, the suspension cells appeared to grow slower with a doubling time of approximately four hours more. The adaptation and optimization process of the suspension cell line occurred over the course of 12 months.

Triple transfection optimization

For efficient introduction of DNA into a host cell, a stable cationic polymer such as polyethylenimine (PEI), liposomes, or electroporation must be utilized. In order for the DNA to bind to anionic cell surfaces, PEI works by condensing the DNA into positively charged particles. This allows for the PEI and DNA mixture to be taken up by the cell for the release of the DNA into the cytoplasm. PEI serves as an ideal transfection agent due to the fact that it is highly suitable for and tolerated by HEK293 cell lines, it is cost effective, and yields high levels of protein production in HEK293 cells (Longo et al.).

PEI Max is an almost fully hydrolyzed linear polyethylenimine with longer contiguous ethyleneimine segments compared to the popular linear polyethylenimine. Polysciences PEI Max (MW 40,000) is a high potency linear PEI built from the same MW 25,000 polymer backbone. It contains more than 11% additional protonatable nitrogens than the original PEI material with an

average of 581 repeating ethyleneimine segment units, which results in a dramatic increased length of free nitrogens (Longo et al.).

To produce AAV-2 in the suspension adapted HEK293 cells, several conditions had to be optimized to achieve high vector genome titers. Prior to evaluating the optimal transfection conditions, the method of lysing the cells described in detail in Chapter 6 was developed to ensure proper vector quantification. The conditions tested included determining the optimal molar ratios of the three plasmids for triple transfection, the ratio of transfection agent to total DNA present, the concentration of total DNA utilized during transfection, and the final transfection media. The vector genome per mL (vg/mL) from the cell lysates were quantified using qPCR as described in the materials and methods section

The order of addition of the components of the transfection cocktail was tested to determine if there was an impact on the vector yields. The orders of addition tested were: 1) plasmid DNA, PEI Max, and F17 Media, and 2) plasmid DNA, F17 Media, and PEI Max.

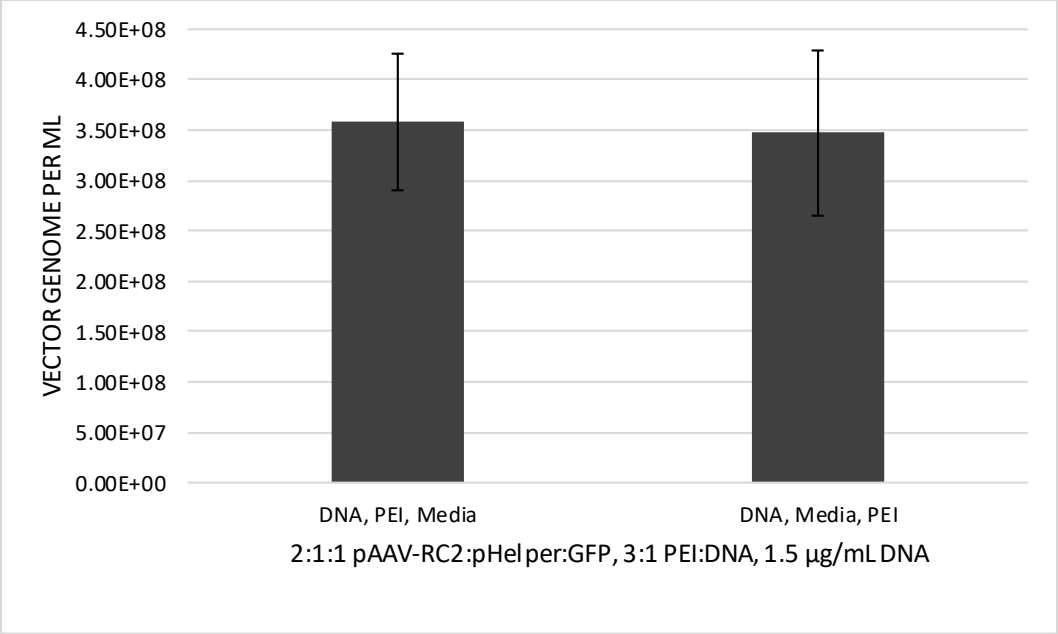


Figure 3.5: The effect of order of addition of the transfection cocktail components on vector genome per mL titer on suspension cells. 2:1:1 pAAV-RC2:pHelper:GFP plasmid molar ratio, 3:1 PEI:DNA ratio, and 1.5 µg/mL total DNA concentration were the transfection conditions used in 30 mL working volume. Error bars represent standard deviation of triplicate technical replicates. P-value for difference in vector genome production was 0.867, not statistically significant.

As seen in Figure 3.5, the first order of addition in which the PEI Max is added directly after the plasmids resulted in 3.58E+08 vg/mL while the second order of addition resulted in 3.47E+06 vg/mL. The results from the qPCR analysis concluded that order of addition did not significantly impact the final vector titers, however moving forward the second order of addition, plasmid DNA, F17 Media, and PEI Max, was used to ensure no PEI induced aggregation occurred when directly mixed with the plasmids. The growth media prior to transfection was also tested to determine its impact on vector genome yields. Prior to transfection the cells were grown in HyCellTransFx-H media and F17 media was used in preparation of the transfection cocktail. Another avenue of transitioning the cells to F17 media for growth prior to transfection and using the F17 media in the cocktail was explored.

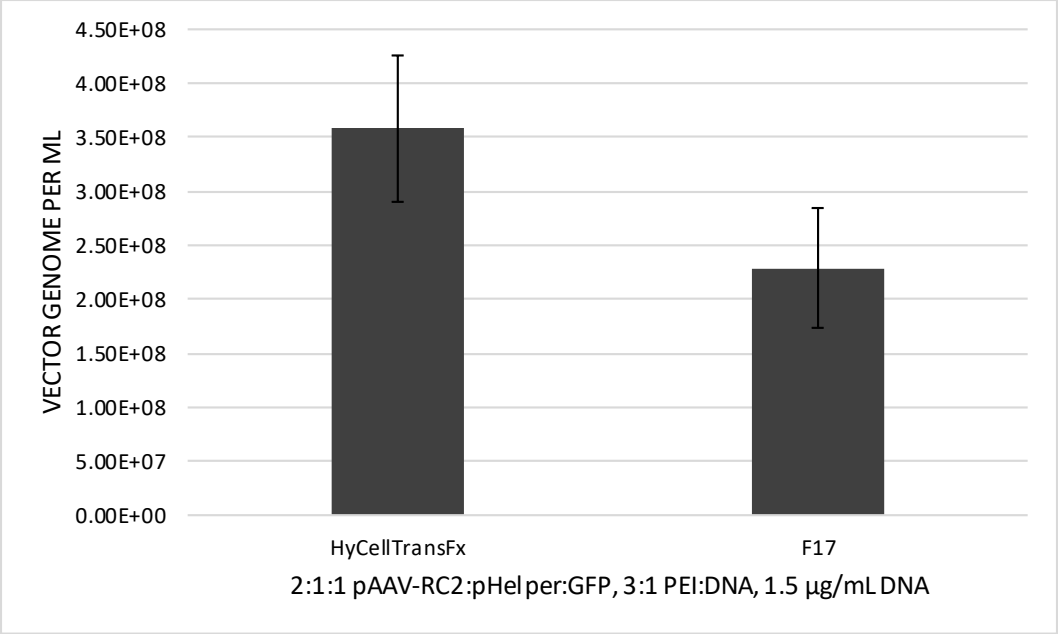


Figure 3.6: The effect of transfection media on vector genome per mL titer for suspension cells. Gibco HyCellTransFx-H and Thermo Fisher FreeStyle F17 Expression Medium were tested in 30 mL working volumes. 2:1:1 pAAV-RC2:pHelper:GFP plasmid molar ratio, 3:1 PEI:DNA ratio, and 1.5 µg/mL total DNA concentration were the transfection conditions used. Error bars represent standard deviation of triplicate technical replicates.

Although the cells experienced similar growth characteristics in the F17 medium as they did in the HyCellTransFx medium, their vector genome titers were different. Figure 3.6 suggests that the vector production for the cells grown and transfected in the HyCellTransFx-H media were higher than that of the cells grown and transfected in the F17 media. However, after statistical analysis the differences in the vector titers were deemed to be not quite statistically significant at the 0.05 alpha-level with a p-value of 0.063. Subsequently, cells were grown and transfected in the HyCellTransFx-H media, however the F17 media remained a component in the preparation of the transfection cocktail.

Optimization work was performed on both adherent and suspension cultures to ensure that the adapted suspension cells were producing as much, if not better AAV-2 titers. A partial factorial design of experiment (DOE) study was performed to determine how the interactions of the main variables affected the overall vector genome yields. The variables tested in the

suspension culture were plasmid molar ratios (1:1:1, 2:1:1, and 1:3:1 pAAV-RC2:pHelper:pAAV-GFP), PEI to DNA ratios (2:1, 3:1, and 4:1), DNA concentrations (1.0 μg/mL, 1.5 μg/mL, and 2.0 μg/mL DNA), and transfection temperature (34°C and 37°C). All variables except temperature were tested in the adherent culture. The vector genome per mL (vg/mL) from the cell lysates were quantified using qPCR as described in the materials and methods section. For a linear comparison between the suspension and adherent cultures, the vector genome per cell (vg/cell) production was used. The JMP Pro software was used to determine the combination of variables to test and an analysis of variance (ANOVA) analysis was performed to identify the optimal transfection conditions.

Table 3.1: Vector genome titer for design of experiment determined using qPCR in suspension cells.

Temperature (°C)	PEI:DNA	Plasmid DNA Ratio	DNA Concentration (μg/mL)	vg/mL	vg/cell
34	2:1	1:1:1	1.0	1.20E+08	1.24E+02
34	2:1	2:1:1	1.5	9.04E+07	8.78E+01
34	2:1	1:3:1	2.0	6.69E+07	6.57E+01
34	3:1	1:1:1	1.5	5.15E+07	4.38E+01
34	3:1	2:1:1	2.0	2.41E+07	2.65E+01
34	3:1	1:3:1	1.0	9.79E+07	8.10E+01
34	4:1	1:1:1	2.0	2.96E+07	3.63E+01
34	4:1	2:1:1	1.0	5.50E+07	5.04E+01
34	4:1	1:3:1	1.5	4.36E+07	4.16E+01
37	2:1	1:1:1	2.0	1.65E+08	1.48E+02
37	2:1	2:1:1	1.0	3.35E+08	3.04E+02
37	2:1	1:3:1	1.5	1.15E+08	1.12E+02
37	3:1	1:1:1	1.0	3.22E+08	2.36E+02
37	3:1	2:1:1	1.5	1.68E+08	1.52E+02
37	3:1	1:3:1	2.0	9.74E+07	1.03E+02
37	4:1	1:1:1	1.5	5.32E+07	4.58E+01
37	4:1	2:1:1	2.0	1.96E+07	2.41E+01
37	4:1	1:3:1	1.0	1.26E+08	9.10E+01

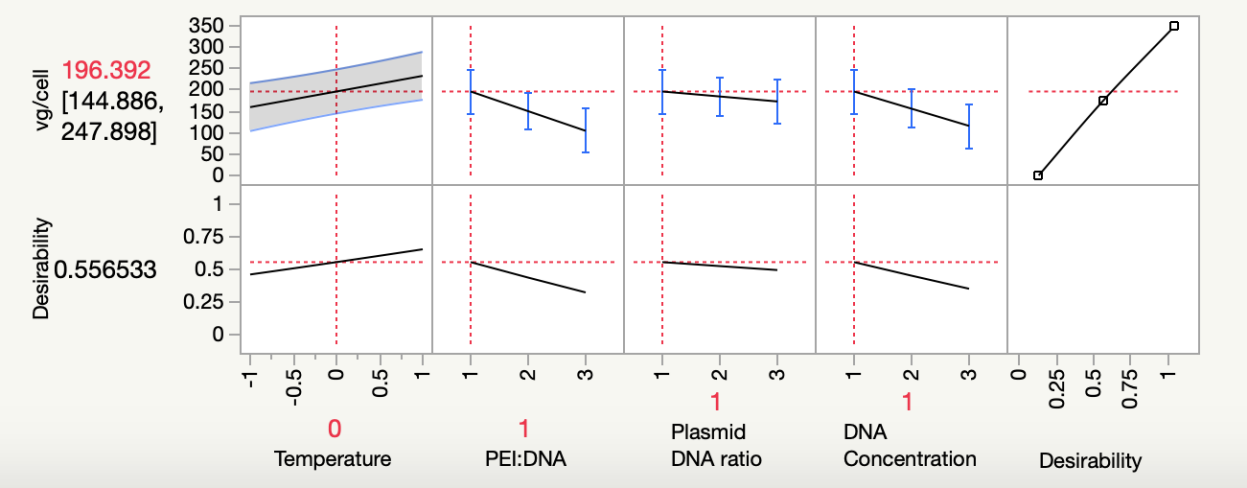


Figure 3.7: Prediction profile for design of experiment study on suspension cells evaluating the effects of temperature, PEI:DNA ratios, plasmid DNA ratios, and DNA concentrations on vector genome titer.

The vector genome data from the various transfection conditions analyzed in the DOE study shown in Table 3.1 was used to develop an ANOVA analysis in JMP. Figure 3.7 shows the prediction profile for the transfected suspension cells, with high desirability being ideal. According to the prediction profile, the ideal transfection conditions for the suspension culture was transfection at 37°C, 1:1:1 plasmid DNA ratio, 2:1 PEI:DNA ratio, and 1.0 μg/mL plasmid DNA concentration.

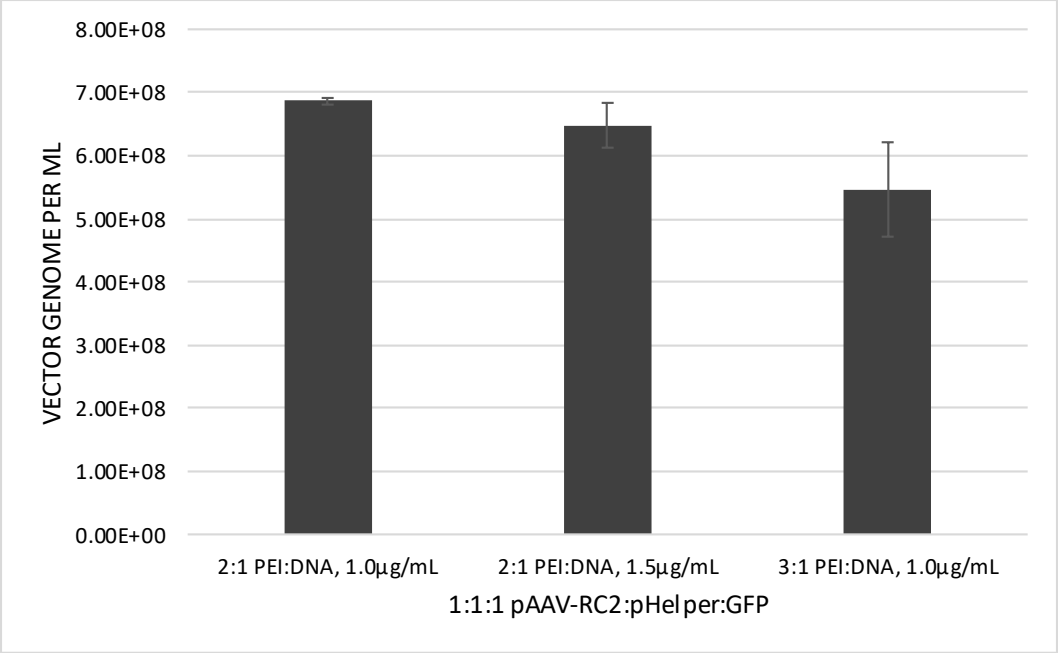


Figure 3.8: Evaluation of optimal condition identified by design of experiment prediction profile for suspension cells. As predicted, 1:1:1 pAAV-RC2:pHelper:GFP plasmid molar ratio, 2:1 PEI:DNA ratio, and 1.0 µg/mL total DNA concentration resulted in the highest vector genome titer. Error bars represent the standard deviation of triplicate biological replicates. P-value for the difference between first two conditions was 0.141. P-value for the difference between first and third condition is 0.006. Alpha-level = 0.05.

Subsequent transfections were performed in triplicates to confirm the prediction from the DOE analysis. Two different ratios of PEI Max to plasmid DNA and total DNA concentrations were tested to determine the impact on transfection efficiencies, while keeping the plasmid molar ratio at 1:1:1 pAAV-RC2:pHelper:pAAV-GFP. The ratios tested were 2:1, and 3:1 PEI:DNA and plasmid DNA concentrations of 1.0 µg/mL and 1.5 µg/mL. Figure 3.8 shows the effect of PEI and plasmid DNA concentrations on vector genome yields. As predicted, the PEI Max to DNA ratio of 2:1 produced the most vector genomes per mL when compared to the higher PEI ratio of 3:1. The lower plasmid DNA concentration of 1.0 µg/mL also resulted in a slightly higher vector genome titer than the 1.5 µg/mL plasmid DNA counterpart. However, a statistical test to indicate the statistical difference between the vector genome production between the 2:1 PEI to DNA ratio and 1.0 µg/mL DNA concentration and the 2:1 PEI DNA ratio and 1.5 µg/mL DNA

concentration resulted in a p-value of 0.141. At the 0.05 alpha-level, the difference in the two results was not considered to be statistically significant. The difference in the vector genome production for the 2:1 PEI to DNA ratio and the 3:1 PEI to DNA conditions resulted in a p-value of 0.006, so at the 0.05 alpha-level, the difference was statistically significant. The ideal condition of transfection at 37°C, 1:1:1 plasmid DNA ratio, 2:1 PEI to DNA ratio, 1.0 µg/mL total DNA concentration yielded 6.86E+08 vg/mL.

A DOE study was also performed on the adherent cells to identify the optimal transfection conditions. Twenty-four hours prior to transfection, 10 T-25 flasks were inoculated with 162,000 cells per cm^2 and placed in a Forma Series II Water Jacketed CO_2 Incubator at 5% CO_2, 37°C, and 80% humidity. It should be noted that the typical seeding density for adherent transfections used in this development was 126,000 cells per cm^2, and this DOE study was inadvertently seeded higher.

Table 3.2: Vector genome titer for design of experiment determined using qPCR in adherent cells.

PEI:DNA	Plasmid DNA Ratio	DNA Concentration (µg/1E+06 cells)	vg/mL	vg/cell
2:1	1:1:1	1.0	1.11E+09	8.09E+02
2:1	2:1:1	1.5	4.20E+09	3.53E+03
2:1	1:3:1	2.0	2.11E+09	1.90E+03
3:1	2:1:1	1.0	2.31E+08	2.05E+02
3:1	1:3:1	1.5	3.98E+08	3.51E+02
3:1	1:1:1	2.0	3.72E+08	3.00E+02
4:1	1:3:1	1.0	2.69E+08	2.10E+02
4:1	1:1:1	1.5	2.45E+08	2.19E+02
4:1	2:1:1	2.0	3.94E+08	2.85E+02

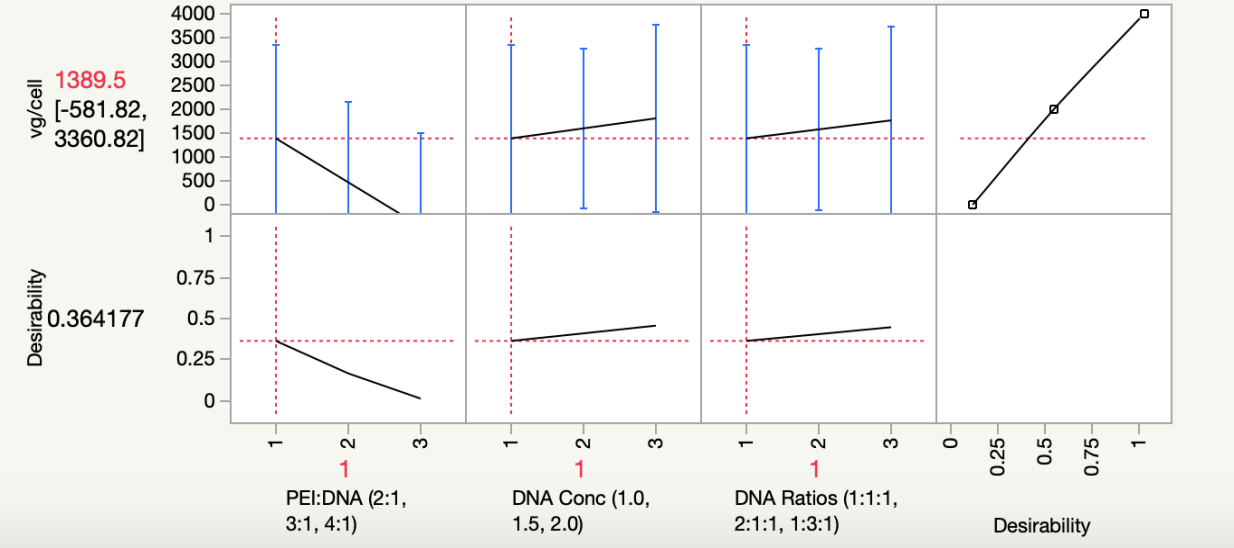


Figure 3.9: Prediction profile for design of experiment study on adherent cells evaluating the effects of PEI:DNA ratios, DNA concentrations, and plasmid DNA ratios on vector genome titer.

The ideal transfection conditions for the adherent cells in the prediction profile in Figure 3.9 were 2:1 PEI to DNA ratio, 2.0 µg/1E+06 cells DNA concentration, and 1:3:1 pAAV-RC2:pHelper:GFP plasmid molar ratio. To confirm the results, subsequent transfections were performed in biological triplicates.

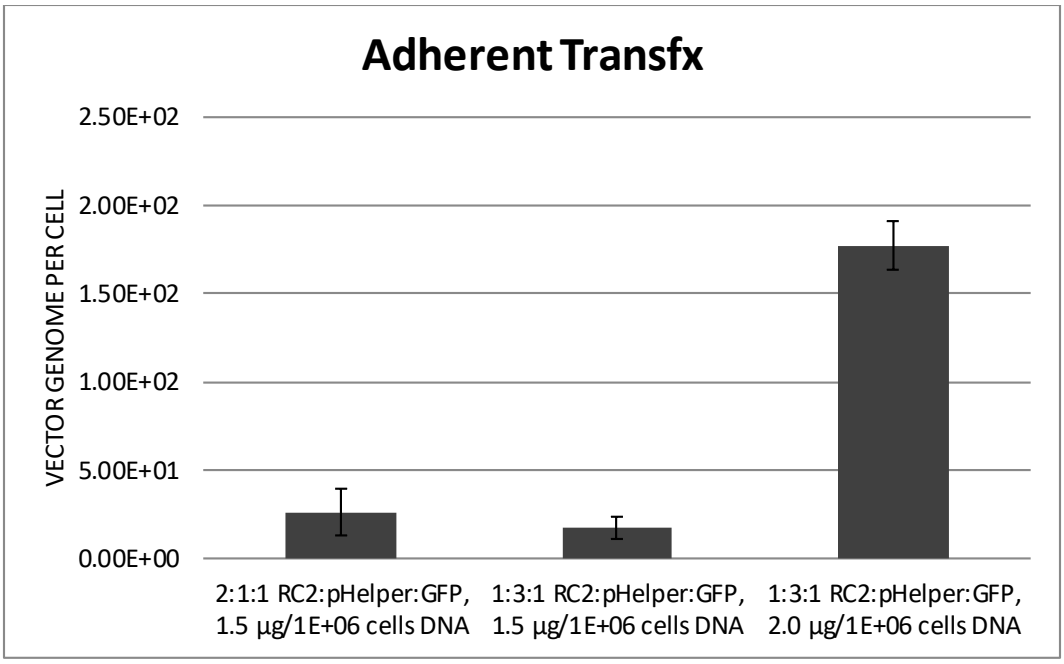


Figure 3.10: Evaluation of optimal condition identified by design of experiment prediction profile for the adherent cells. As predicted, 1:3:1 pAAV-RC2:pHelper:GFP plasmid molar ratio, 2:1 PEI:DNA ratio, and 2.0 µg/1E+06 total DNA concentration resulted in the highest vector genome titer. Error bars represent the standard deviation of triplicate biological replicates.

As seen in Figure 3.10, the predictions from the ANOVA analysis that the 1:3:1 pAAV-RC2:pHelper:GFP plasmid molar ratio, 2:1 PEI to DNA ratio, and 2.0 µg/1E+06 cells total plasmid DNA concentration appears to produce the highest vector yields in the adherent cells. A statistical test was performed to determine if the difference in vector genome production was statistically significant. The difference in the vector genome production between the 2:1:1 plasmid ratio and 1.5 µg/1E+06 cells condition and the 1:3:1 plasmid ratio and 2.0 µg/1E+06 cells resulted in a p-value of 0.001 which was determined to be statistically significant at the 0.05 alpha-level. The difference in vector genome production between the last two conditions in which the plasmid ratios were kept constant at 1:3:1 plasmid ratios and the DNA concentrations were 1.5 and 2.0 µg/1E+06 cells, a statistical test resulted in a p-value of 0.0003 so the difference was determined to be statistically significant. The vector genome per cell titers for the

identified optimal condition was 1.77E+02 vg/cell. These flasks were seeded at 126,000 cells per cm^2 24 hours prior to transfection.

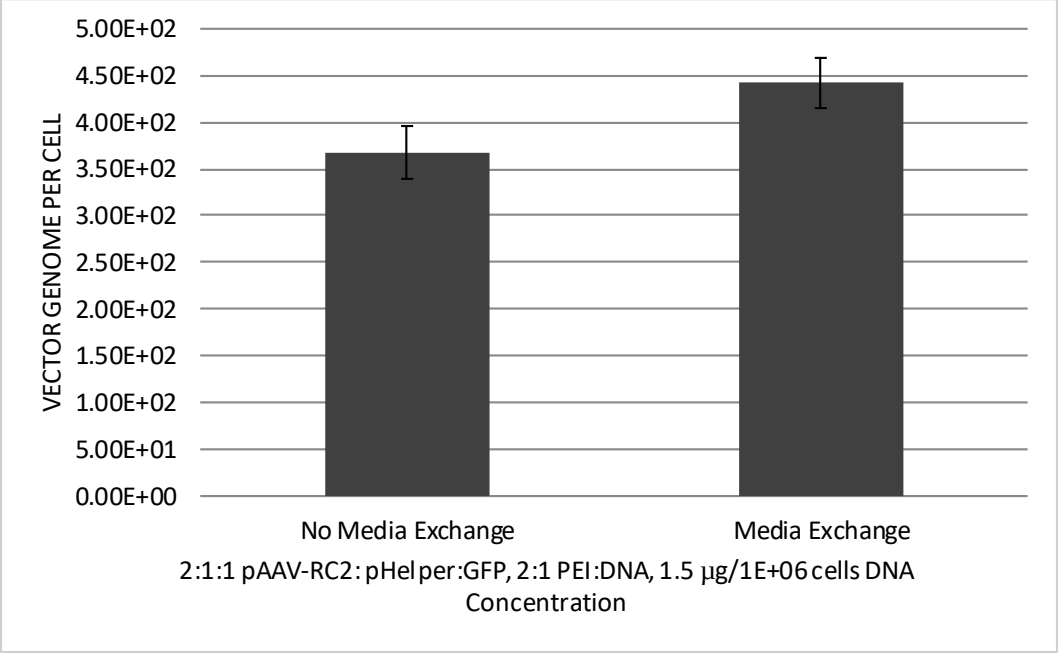


Figure 3.11: Impact of media exchange on vector production. T-25 flasks were seeded with 162,000 cells per cm^2 and transfected with 2:1:1 pAAV-RC2:pHelper:GFP, 2:1 PEI to DNA, and 1.5 µg/1E+06 cells DNA. Error bars represent the standard deviation of triplicate biological replicates.

To determine the impact of exchanging the media 24 hours post transfection on the resulting vector genome titer, a media exchange study was performed alongside the DOE study. Ideal transfection conditions had not been identified, so the six T-25 flasks were seeded at 162,000 cells per cm^2 and transfected with 2:1:1 pAAV-RC2:pHelper:GFP, 2:1 PEI to DNA ratio, and 1.5 µg/mL plasmid DNA concentration. Three of the six flasks were subjected to a media exchange while the other three flasks were left untouched. As shown in Figure 3.11, the average vector genome per cell titer from the flasks with media exchange was 4.42E+02 vg/cell and the average vector genome per cell titer from the flasks without media exchange was 3.68E+02 vg/cell. All subsequent adherent transfections were subjected to a media exchange 24 hours post transfection.

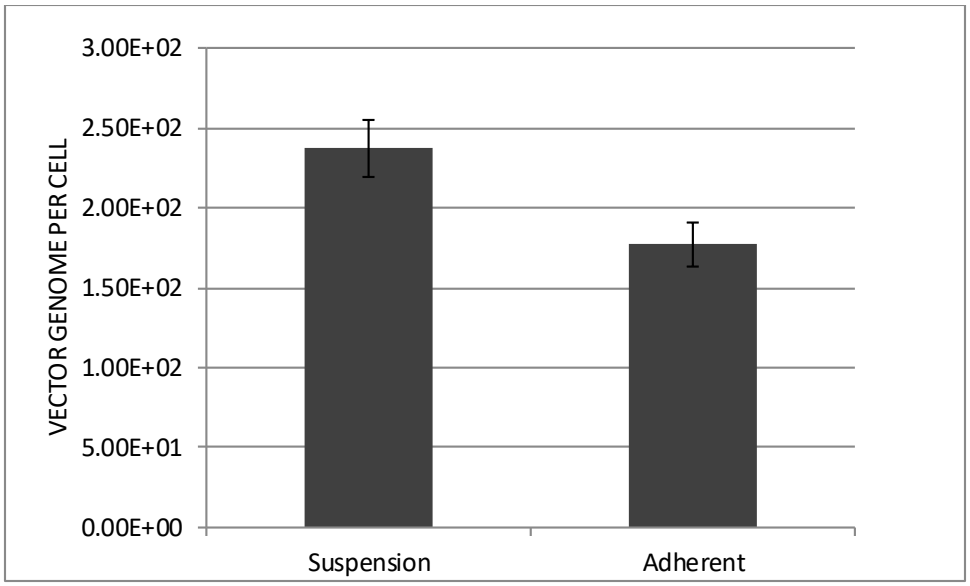


Figure 3.12: vector genome per cell titers for suspension and adherent cells transfected with identified optimal conditions. The suspension cells were transfected with 1:1:1 pAAV-RC2:pHelper:GFP plasmid molar ratio, 2:1 PEI:DNA ratio, and 1.0 μg/mL total DNA concentration and the adherent cells were transfected with 1:3:1 pAAV-RC2:pHelper:GFP plasmid molar ratio, 2:1 PEI:DNA ratio, and 2.0 μg/1E+06 cells total DNA concentration. Error bars represent the standard deviation of triplicate biological replicates. Statistical test for difference in means results in p-value = 0.0637.

A comparison of the suspension and adherent cells in Figure 3.12 shows the productivity on a per cell basis. The suspension cells yielded a vector genome per cell titer of 2.37E+02 vg/cell, while the adherent cells produced 1.77E+02 vg/cell. The suspension cells produced slightly more vector than the adherent cells, however a statistical test on the difference in means resulted in a p-value of 0.064 and at the 0.05 alpha-level, the difference was determined to not be statistically significant.

To determine the percent of total capsids produced by the suspension and adherent cells, an ELISA assay was performed.

Table 3.3: Comparison of capsid ratios and vector genome titers determined by ELISA and qPCR assays for suspension cells. The bolded data represents the optimal transfection condition identified.

Suspension Cells	vg/mL	Capsids/mL	Percent Full Capsids (%)
2:1 PEI:DNA, 1.0 μg/mL DNA, 1:1:1 RC2:pHelper:GFP	**6.80E+08**	**6.62E+10**	**1.03**
2:1 PEI:DNA, 1.0 μg/mL DNA, 1:1:1 RC2:pHelper:GFP	**6.88E+08**	**7.83E+10**	**0.88**
2:1 PEI:DNA, 1.0 μg/mL DNA, 1:1:1 RC2:pHelper:GFP	**6.90E+08**	**7.37E+10**	**0.94**
2:1 PEI:DNA, 1.5 μg/mL DNA, 1:1:1 RC2:pHelper:GFP	6.28E+08	7.70E+10	0.82
2:1 PEI:DNA, 1.5 μg/mL DNA, 1:1:1 RC2:pHelper:GFP	6.89E+08	9.06E+10	0.76
2:1 PEI:DNA, 1.5 μg/mL DNA, 1:1:1 RC2:pHelper:GFP	6.27E+08	1.01E+11	0.62
3:1 PEI:DNA, 1.0 μg/mL DNA, 1:1:1 RC2:pHelper:GFP	5.91E+08	7.95E+10	0.74
3:1 PEI:DNA, 1.0 μg/mL DNA, 1:1:1 RC2:pHelper:GFP	5.88E+08	6.39E+10	0.92
3:1 PEI:DNA, 1.0 μg/mL DNA, 1:1:1 RC2:pHelper:GFP	4.60E+08	7.77E+10	0.59

Table 3.4: Comparison of capsid ratios and vector genome titers determined by ELISA and qPCR assays for adherent cells. The bolded data represents the optimal transfection condition identified. The first listed optimal condition was an outlier, thus removed from average and standard deviation calculations, as well as statistical analysis.

Adherent Cells	vg/mL	Capsids/mL	Percent Full Capsids (%)
2:1 PEI:DNA, 1.5 μg/mL DNA, 2:1:1 RC2:pHelper:GFP	2.17E+07	7.36E+08	2.95
2:1 PEI:DNA, 1.5 μg/mL DNA, 2:1:1 RC2:pHelper:GFP	3.90E+07	1.37E+09	2.85
2:1 PEI:DNA, 1.5 μg/mL DNA, 2:1:1 RC2:pHelper:GFP	1.61E+07	8.76E+08	1.84
2:1 PEI:DNA, 1.5 μg/mL DNA, 1:3:1 RC2:pHelper:GFP	1.97E+07	1.46E+09	1.35
2:1 PEI:DNA, 1.5 μg/mL DNA, 1:3:1 RC2:pHelper:GFP	2.51E+07	1.32E+09	1.90
2:1 PEI:DNA, 1.5 μg/mL DNA, 1:3:1 RC2:pHelper:GFP	1.09E+07	1.14E+09	0.96
2:1 PEI:DNA, 2.0 μg/mL DNA, 1:3:1 RC2:pHelper:GFP	**1.61E+07**	**4.50E+09**	**0.36**
2:1 PEI:DNA, 2.0 μg/mL DNA, 1:3:1 RC2:pHelper:GFP	**1.59E+08**	**5.90E+09**	**2.69**
2:1 PEI:DNA, 2.0 μg/mL DNA, 1:3:1 RC2:pHelper:GFP	**1.48E+08**	**6.58E+09**	**2.25**

The ELISA assay measures the total capsid concentration, full and empty capsids, while the qPCR assay measures only the full capsids. The ELISA assay was performed on the samples analyzed in Figures 3.8 and 3.10. To determine the percent of full capsids produced by the cells, the vector genome per mL data from the qPCR assay was divided by the capsids per mL data from the ELISA assay. As shown in Table 3.3 and 3.4, the adherent cells transfected using the identified optimal conditions produced an average or 2.47% full capsids while the suspension cells transfected using the optimal conditions produced an average of 0.95% full capsids. However, the total vector genome titer for the suspension cells were slightly higher than the adherent cells but not quite significantly different. An ideal system should produce both high vector genomes and high percentage of full capsids. In the case of the suspension and adherent

cells being compared, the adherent cells would be the better prep since it has a higher percent of full capsid compared to the suspension prep, despite the lower vector production in the adherent cells. There is a huge concern about empty capsids increasing adaptive or innate immune response on clinical outcomes; however the definite effect is still unclear. Some recent studies suggest that empty capsids may enhance gene transfer by mitigating humoral immunity to AAV (Wright).

The transfection efficiencies for both the suspension and adherent cells are lower than expected with approximately 20% of the cells in suspension expressing GFP as seen in Figure A.1 in the Appendix and 15% of the adherent cells expressing GFP in Figure A.4. The low transfection efficiencies attribute to the low vector titers and improving the transfection efficiency and the uptake of AAV by the cells could improve the resulting vector titer.

CHAPTER 4

Process Scalability

Growth curve in WAVE bags

To demonstrate scalability, the suspension HEK293 cells were used to inoculate a 5L wave bag bioreactor and a growth curve was carried out. Several growth curve studies were performed to determine the ideal WAVE bag bioreactor settings to support sufficient cell growth. The cells were grown in a Sartorius Stedim Flexsafe RM 10L opt. SC (part #: DFO010L) WAVE bag bioreactor operated with a Sartorius Stedim BIOSTAT CultiBag RM controller. The temperature was set at 37°C, and the dissolved oxygen was controlled via cascade control to maintain the dissolved oxygen levels above 60%. There was no active pH control for the WAVE bag bioreactor, however the pH levels were measured both on-line and off-line. The CO_2 flow was set at 30 ccm to keep the CO_2 at 5.0%. The WAVE bag was rocked at 32 rocks/min throughout the duration of the growth curve on a 7.5° rocking angle. Several growth studies performed on the WAVE bag showed severe cell clumping and early cell apoptosis when rocked at a lower speed and smaller angle. Increasing the rocking speed showed signs of longer cell growth and decreased clumping. Off-line NOVA and BioHT analysis were performed to determine the metabolite data for the cell culture. A small-scale shake flask model was run in parallel to the 5L WAVE bag to determine how closely the cell growth data matched.

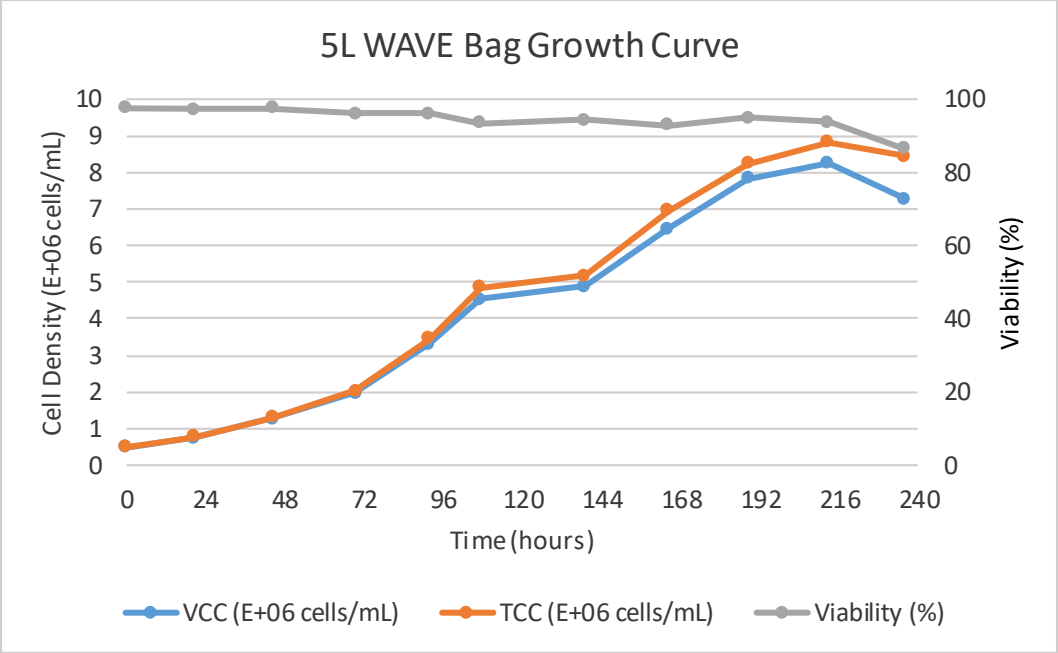


Figure 4.1: 5L WAVE bag bioreactor 10-day growth curve. The WAVE bag was seeded at 0.5E+06 cells per mL and grown in HyCellTransFx-H media supplemented with 4mM glutamine and 0.1% pluronic F68.

Prior to inoculating the WAVE bag, 4L of HyCellTransFx-H media supplemented with 4mM glutamine and 0.1% pluronic was batched into the WAVE bag 24 hours prior to allow the media equilibrate to the set conditions. The 5L WAVE bag was then seeded 24 hours after media to equilibration at a cell density of 0.5E+06 cells/mL, with a final working volume of 5L. The growth curve data of the WAVE bag in Figure 4.1 was collected over the span of 10 days. The cells grew increasingly from day 1-9. On day four, the cell culture was supplemented with an additional 3.5mM glutamine to provide nutrients to the cells. The cells maintained viabilities above 90% until day nine, after which they entered death phase. The specific growth rate and doubling time in the exponential growth phase is 0.021 hr^{-1} and 33.2 hours, respectively. The specific growth rate and doubling time are similar to that of the suspension growth curve reported in Chapter 3.

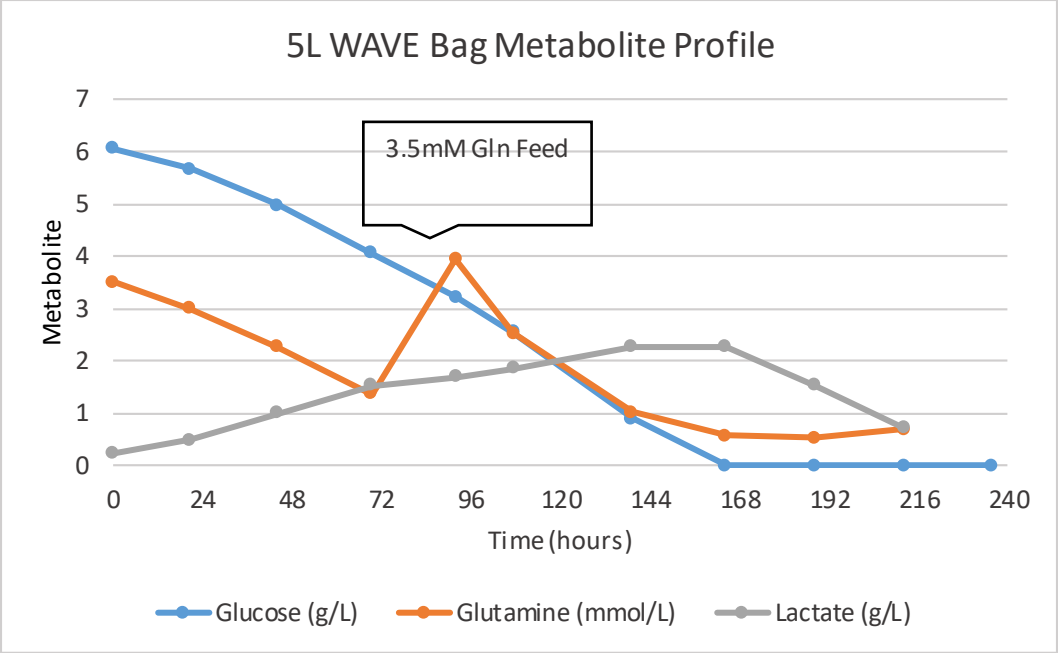


Figure 4.2: 5L WAVE bag bioreactor metabolite profile from 10-day growth curve. The cells were supplemented with an additional 3.5mM bolus glutamine feed on day 4.

The metabolite profile in Figure 4.2 shows a steady metabolism of glucose and production of lactate throughout the growth curve period. The spike in glutamine on day 4 is indicative of the 3.5mM bolus glutamine feed.

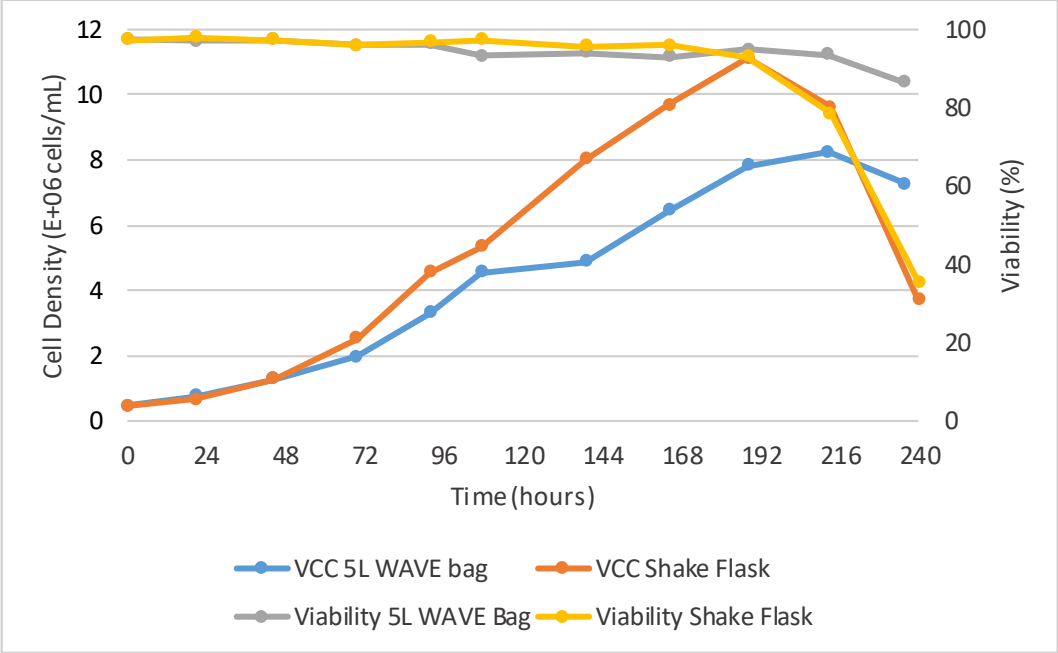


Figure 4.3: Comparison of 5L WAVE bag bioreactor growth profile to 250 mL small-scale shake flask model.

The 150mL small scale model compared to the 5L WAVE bag in Figure 4.3 shows that the cells grew faster at small scale than they did in the WAVE bag. The cells in the 250 mL shake flask reached a peak cell density of 11.13E+06 cells per mL on day eight, after which the cells entered death phase. The specific growth rate for the 250 mL shake flask was 0.024 hr^{-1} and the doubling time was approximately 29 hours. The cells in the 5L WAVE bag reached peak cell density of 8.25E+06 cells per mL on day nine before entering death phase. The cells in the 5L WAVE bag maintained high viability longer than the cells in the 250 mL shake flask.

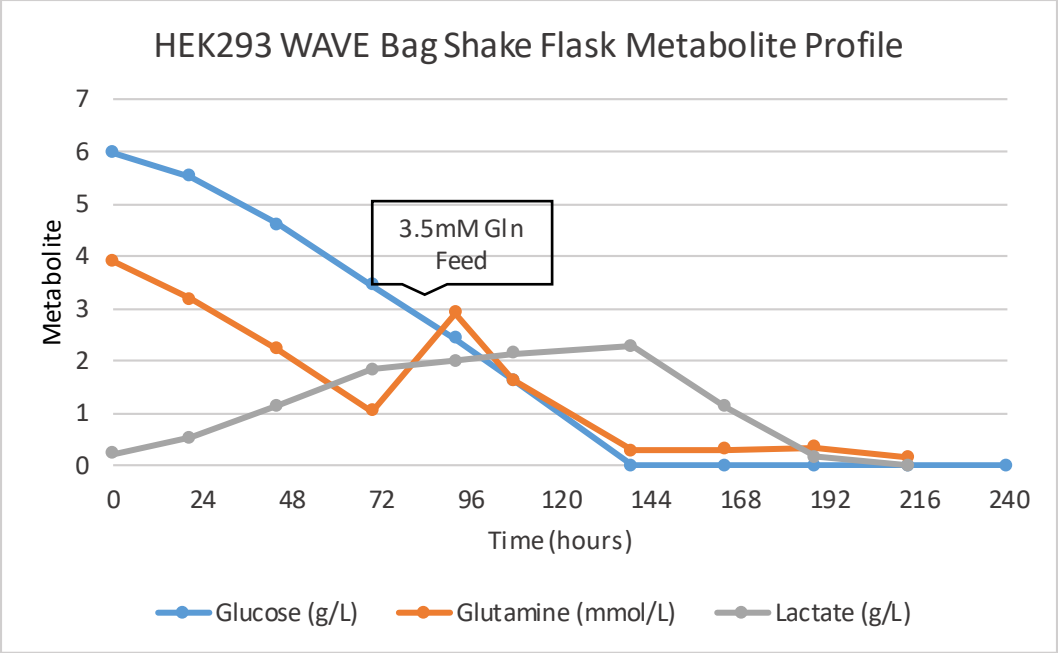


Figure 4.4: 150 mL shake flask metabolite profile from 10-day growth curve. The cells were supplemented with an additional 3.5mM bolus glutamine feed on day 4.

The metabolite profile for the 250 mL shake flask in Figure 4.4 was similar the metabolite profile of the 5L WAVE bag in Figure 4.3. The small-scale model was also given a 3.5mM bolus glutamine feed on day 4, thus attributing to the increased cell density and viability duration compared to the suspension growth curve of the cells in Chapter 3 Figure 3.4.

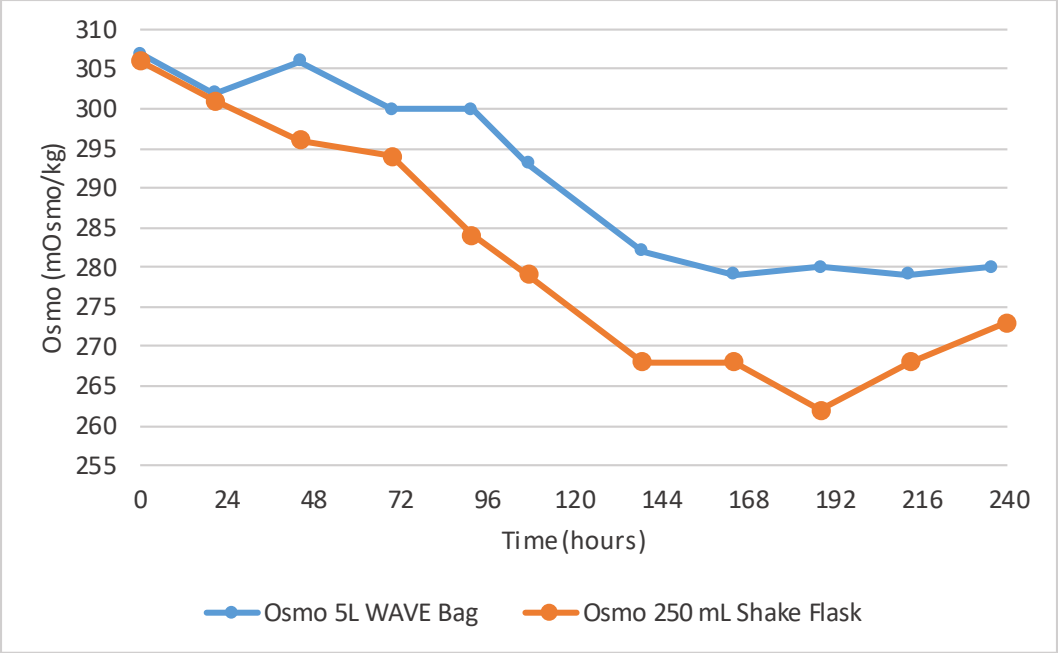


Figure 4.5: Osmolality measurements from 5L WAVE bag and 150 mL shake flask.

Osmolality measurement in Figure 4.5 were also taken throughout the run to determine the salt content of the media. During the growth phase the osmolality readings in the 5L WAVE bag ranged from 307 to 279 mOsmo/kg, while the osmolality measurements in the 150 mL shake flask ranged from 306 to 262 mOsmo/kg. Typically, osmolalities that exceed the range of 280 to 300 mOsmo/kg result in cell shrinkage, oxidative stress, cell cycle delay, and cell apoptosis (Christoph et al.). However, the low osmolality of the 150 mL shake flask cell culture demonstrates that there was no negative impact to cell growth in response to osmotic stress.

Production of AAV-2 in WAVE bag bioreactor

The ideal transfection conditions identified in the shake flask model were used to transfect the 5L WAVE bag for the production of AAV-2. The cells in the 5L WAVE bag were grown using the same settings in the growth curve experiment. The WAVE bag was inoculated at 0.5E+06 vc/mL and allowed to grow for approximately 29 hours until the cell count reached 1.0E+06 viable cells per mL before transfecting. The cells were transfected at 37°C, 1:1:1

plasmid DNA molar ratio, 2:1 PEI to DNA ratio, and 1.0 µg/mL plasmid DNA concentration and harvested 72 hours post transfection. The cells were lysed and quantified according to the lysis and qPCR methods in the materials and methods section. A small-scale shake flask model was transfected alongside the 5L WAVE bag to serve as a control.

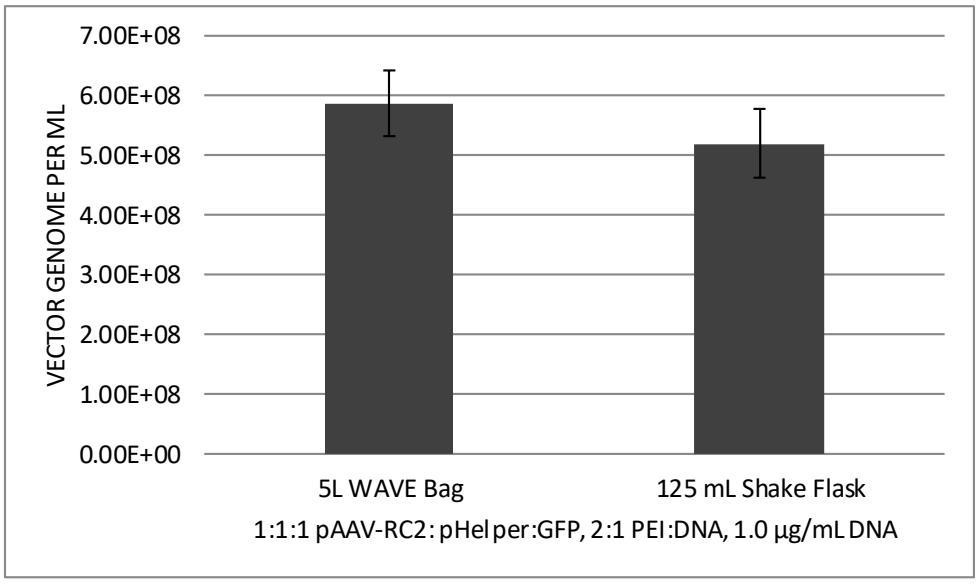


Figure 4.6: vector genome per mL titers for suspension cells in a 5L WAVE bag bioreactor and a 125 mL shake flask transfected with identified optimal conditions. The suspension cells were transfected with 1:1:1 pAAV-RC2:pHelper:GFP plasmid molar ratio, 2:1 PEI:DNA ratio, and 1.0 µg/mL total DNA. Error bars represent standard deviation of triplicate of technical replicates.

The vector genome per mL titer of the transfected cells in the 5L WAVE bag and the 125 mL shake flask shown in Figure 4.6 was 5.87E+08 vg/mL and 5.20E+08 vg/mL, respectively. The difference in vector production was deemed not statistically significant at the 0.05 alpha-level with a p-value of 0.219. This demonstrates that the suspension adapted HEK293 cell line is scalable in terms of growth and vector production.

CHAPTER 5

Cell Lysis

All cells possess a protein-lipid bilayer plasma membrane that acts as a barrier between the intracellular contents and the extracellular environment. The plasma membrane lipids are comprised of hydrophobic and hydrophilic moieties. These amphipathic lipids form a closed biomolecular sheet embedded with membrane proteins held by the hydrophobic core domains and peripheral proteins that bind the inner and outer bilayer surface through integral membrane protein interactions. The plasma membrane of animal cells is relatively easy to lyse due to the lack of an extracellular wall (Jahanshahi and Najafpour).

Cell lysis is the first step in protein extraction and purification, and several methods and techniques have been developed to ensure the best possible yields for extraction. The two methods of cell lysis can be categorized as mechanical or non-mechanical techniques as shown in Figure 5.1.

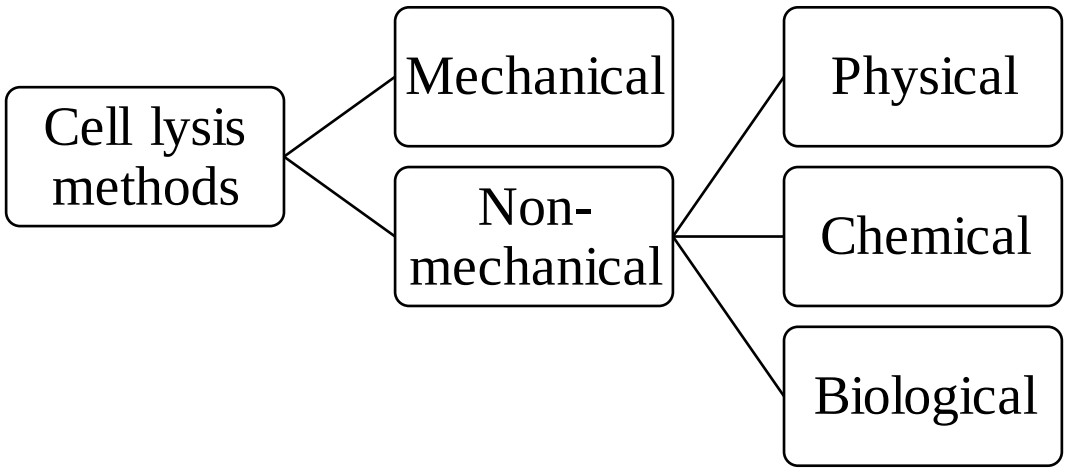


Figure 5.1: Classification of cell lysis methods

Mechanical Lysis

Mechanical lysis involves the use of shear force to physically break down the cell membrane. It utilizes a combination of high throughput and high-pressure techniques such as a homogenizer or a bead mill to achieve higher lysing efficiencies. Mechanical lysis is the most popular and commercial method of cell membrane lysis.

A homogenizer is a high-pressure system that is used for large scale microbial disruption in which cells in suspension are forced through a valve orifice at high pressure. Due to the high shear force at the orifice, cells are compressed at entry and expanded upon discharge, causing the disruption of the cell membrane. Cells are passed through the homogenizer at operating pressures as high as 1500 bar and complete cell disruption is achieved with multiple passes. This often results in the generation of heat which poses an issue in terms of enzyme degradation. This issue could be mitigated by the addition of cooling systems, however it has been reported that a combination of lysis methods such as a chemical pre-treatment prior to homogenization yields better results (Anton P. J. Middelberg).

A bead mill is another widely used laboratory scale cell lysis method. It involves the use of tiny glass, steel, or ceramic beads mixed with cell suspension and agitated at high speeds to disrupt the cell membrane. In this process, the shear force of the bead collisions breaks the cell membrane open and the intracellular components are released. This method can be used to efficiently lyse several cell types at high cell density, however separation and purification of the sample is increasingly difficult due to the production of small cell debris. In addition, the bead collisions also generate a significant amount of heat that may result in protein and RNA degradation (Taskova et al.).

Non-Mechanical Lysis

Unlike mechanical lysis, non-mechanical lysis uses methods other than shear force to disrupt the cell membrane. Non-mechanical lysis can be categorized as physical, chemical, or biological disruption techniques.

Physical Cell Disruption

Physical disruption does not involve contact; however, it utilizes external forces to break open the cell membrane. It uses forces such as heat and sound energy to achieve lysis. Thermal lysis is the process of repeated freeze/thaw cycles that causes ice crystals to form on the cell membrane, which helps break down the cell membrane as the temperature is increased. The high temperature denatures the membrane proteins and releases the intracellular organelles as a result. This process is time consuming since it requires the sample to be completely and uniformly frozen and thawed multiple times to achieve efficient lysis. And exposure to elevated temperatures for a prolonged period of time can result in DNA damage (Collis et al.)

At the micro-scale, thermal lysis is an attractive method for lysis because the high surface to volume ratio can lead to quick lysis since the heat dissipates quickly and the cell membranes rupture more effectively. However, at large scale this method becomes expensive and the higher temperatures poses greater issues in terms of damage to proteins and enzymes (Anton P.J. Middelberg).

Cavitation involves the formation and rupture of bubbles or cavities by reducing the pressure. A change in the local pressure causes the bubbles or cavities to collapse, thus releasing a great deal of mechanical energy through the media in the form of a shockwave. The high energy of the shockwave causes the cell membrane to disintegrate. A sonicator is a commonly used laboratory device for the generation of ultrasonic vibrations at 15-20kHz to generate a sonic

pressure wave. Ultrasonic cavitation can be used to lyse large biomass concentrations, however this technique produces small cell debris that require additional processing and it also generates large amounts of heat that have been reported to degrade enzymes (Mark et al.).

Osmotic shock is another type of physical cell disruption that involves the drastic change of the salt concentration surrounding the cell, which allows the cell membrane to become permeable to water. Lowering the salt concentration of the surrounding solution causes water to rush into the cell, causing the cells to swell and burst. The osmotic shock method is ideal for mammalian cells due to the fragile structure of their cell membrane (Y.-C. Chen et al.).

Chemical Cell Disruption

Chemical disruption methods utilize buffers to break the cell membrane through lysis buffers and detergents. Lysis buffers change the pH of the cell culture, which causes the disruption of the cell membrane, while detergents solubilize the cell membrane proteins and causes them the rupture, thus releasing the intracellular contents. Alkaline lysis buffers consist mainly of OH^- ions, which react with the fatty acid-glycerol ester bonds on the cell membranes subsequently making it permeable. The lysis buffers can also consist of sodium dodecyl sulphate, which solubilizes the proteins on the cell membrane. Alkaline lysis is a slow process that requires incubation, however it is ideal for isolating plasmid DNA from bacteria (Feliciello and Chinali). Detergent lysis use surfactants that disrupt the hydrophobic-hydrophilic interactions on the cell membrane lipid bilayer. Non-ionic detergents such as the Triton-X and Tween series are most commonly used for lysing mammalian cells because they are less damaging to proteins and enzymes (Sharma et al.).

Biological Cell Disruption

Biological cell disruption uses enzymes for large-scale lysis with high degree of specificity. Lysozymes can be used for bacterial cell lysis, chitinase for yeast cell lysis, and proteinase K for isolating genomic DNA. These enzymes can be used in combination with detergents to disrupt the cell wall and cell membrane.

HEK293 Suspension Cell Lysis

Prior to initiating a lysis study, it was determined that incomplete lysis was a contributing factor in the low vector genome titers yields and the method of cell lysis used had a great impact on the resulting qPCR results for rAAV quantitation. It was empirical to develop a standard lysis protocol that was scalable and would result in complete lysis of the transfected cells. Due to limited resources, only non-mechanical lysis techniques were evaluated. Thermal, chemical, and biological lysis methods were evaluated.

The freeze thaw thermal lysis method consisted of four cycles of freezing in an ethanol ice bath and complete thaw at 37°C in a heated bead bath. The chemical lysis methods tested consisted of 0.5% and 1.0% Triton X-100 (Sigma-Aldrich Part #: 9036-19-5) concentrations incubated for two hours, and 0.5% and 1.0% Sodium Deoxycholate concentrations incubated for two hours. The chemical lysis methods were also tested in the presence of benzonase, a biochemical endonuclease that cleaves DNA and RNA to reduce the nucleic acid load for downstream processing and for removal of residual plasmids that can interfere with qPCR results.

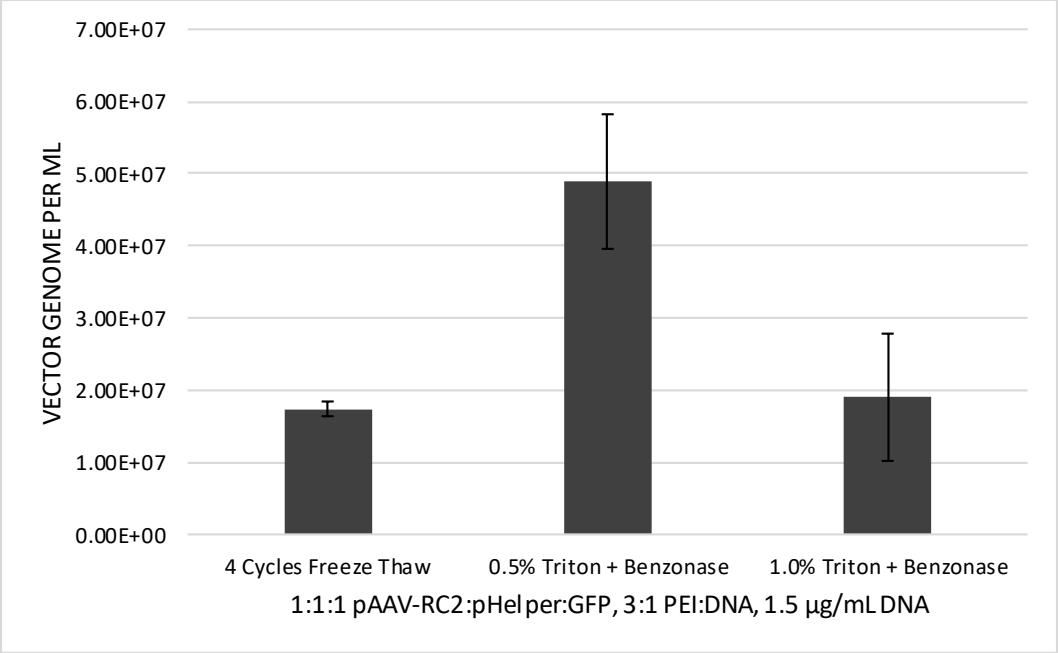


Figure 5.2: Impact of lysis conditions on vector yields on suspension adapted HEK293 cells. One mL aliquots were used from a 30 mL shake flask were lysed using different methods. Chemical lysis methods were incubated for two hours at 37°C. Four freeze thaw cycles in an ethanol and dry ice bath were performed for thermal lysis. Error bars represent standard deviation from replicates of technical triplicates.

The lysis of the suspension adapted HEK293 cells in Figure 5.2 shows that 0.5% Triton X-100 + 25 units per mL benzonase outperforms 1.0% Triton + benzonase. The difference between the vector titer from the 0.5% Triton X-100 and 1.0% Triton X-100 was determined to be statistically significant at the 0.05 alpha-level with a p-value 0.016. The thermal lysis method did not yield in high vector titers compared to the 0.5% Triton + bensonase lysis, and it was deemed to be the least scalable method of lysis. The difference between the vector titers were determined to be statistically significant at the 0.05 alpha-level with a p-value of 0.004. The lysis was concluded to be inefficient due to the low vector titer reported, so other methods were evaluated.

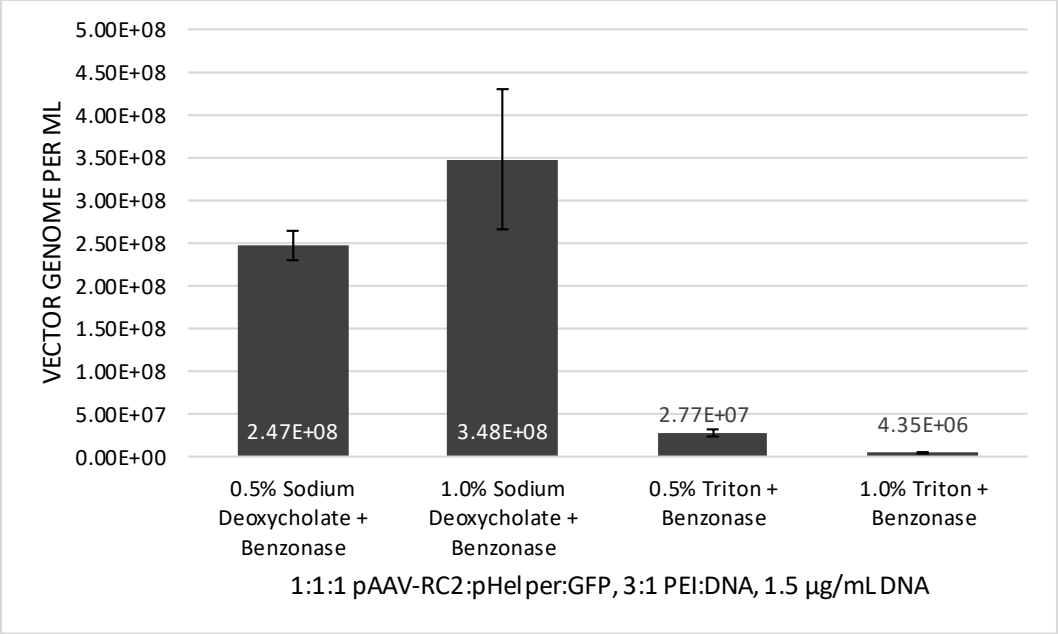


Figure 5.3: Effect of Sodium Deoxycholate and Triton X-100 at 0.5% and 1.0% final concentration on vector yields. One mL aliquots were used from a 30 mL shake flask were lysed using different methods. Chemical lysis methods were incubated for two hours at 37°C. Error bars represent standard deviation of replicates of technical triplicates.

In addition to triton, sodium deoxycholate was analyzed to determine its lysis efficiency. 0.5% and 1.0% sodium deoxycholate and 0.5% and 1.0% triton X-100, all incubated for two hours at 37°C followed by a benzonase treatment were evaluated. Figure 5.3 shows that 1.0% sodium deoxycholate + benzonase resulted in the best lysis with the highest recovery of vector genomes. However, there was no statistical difference between the 0.5% sodium deoxycholate and 1.0% sodium deoxycholate vector production results with a p-value of 0.105 at a 0.05 alpha-level. There was a statistical difference between the sodium deoxycholate and triton x-100 vector titers. Moving forward, cells were lysed using 1.0% sodium deoxycholate lysis described in the materials and methods section.

CHAPTER 6

Conclusion and Future Work

This report describes the scalable process of production of AAV-2 in serum-free suspension cultures suitable to meet the increasing demand for pre-clinical and manufacturing demands for AAV-2 material. The process of production from vial thaw to harvest can be completed in 2-4 weeks depending on the scale of the final growth vessel. All development work performed on the suspension-adapted cells have resulted in improves growth and vector production to make them comparable to the adherent cells with added ease of scalability. Suspension cells seeded and transfected at cell densities of 1E+06 cells/mL yielded vector genome titers of 6.86E+08 vg/mL and 2.37E+02 vg/cell, while the original adherent cells yielded titers of 1.77E+02 vg/cell when seeded with 126,000 cells/cm^2. Compared to the vector titers reported in literature of titers higher than 1E+11 vg/mL, quite a bit of developmental work still needs to be done on optimizing the transfection of the suspension-adapted cells. Other avenues of approach such as increasing the seeding density should be explored. An accidental 0.3 fold increase in the seeding density of the adherent cells resulted in a 29.7 fold increase in the resulting vector genome per cell titer. In addition, the lysis method utilized in the development of this paper demonstrated less than ideal lysis efficiency which resulted in incomplete lysis and lower than expected vector titer measurements. An ideal method of lysing the HEK293 cells is currently being developed by the BTEC downstream processing team on transfected material provided from this development. Further development work such as isolating a monoclonal cell line from the polyclonal HEK293 suspension pool and selecting for ideal growth with approximately 24 hours doubling time and high AAV-2 productivity could be done to improve the quality of the cell line. To increase the percent of full to empty capsids, a serial transfection

approach could be evaluated, where the transfer plasmid is added 24 hours after the addition on the helper and *rep/cap* plasmids. Moreover, a metabolomics study would be beneficial to determine the key cellular and media nutrient required to improve transfection efficiencies in order to increase the percentage of cells expressing GFP.

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APPENDIX

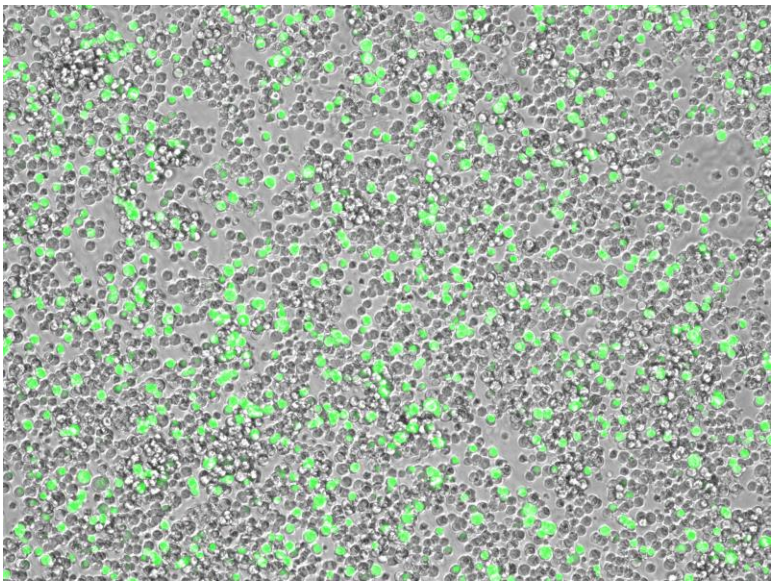

Figure A1: Transfection efficiency of suspension cells expressing GFP. Cells transfected using optimal conditions 1:1:1 pAAV-RC2:pHelper:GFP plasmid molar ratio, 2:1 PEI:DNA ratio, and 1.0 µg/mL total DNA concentration. 20 % of cells transfected.

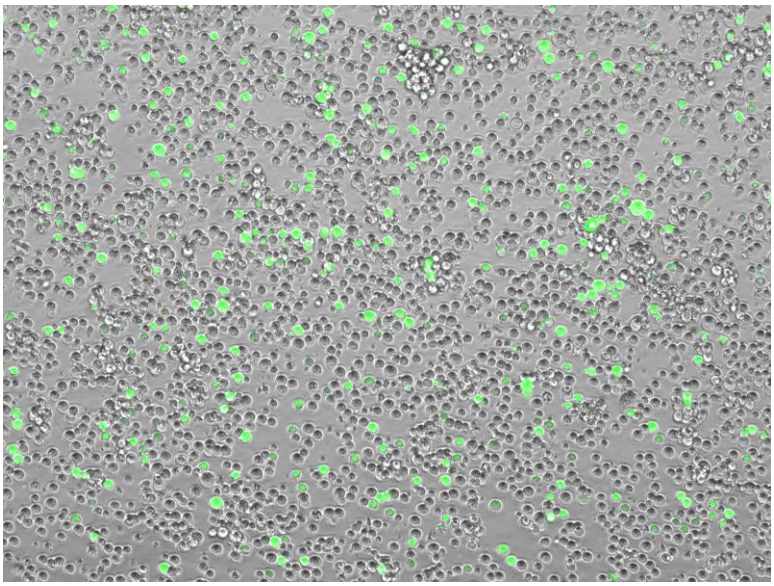

Figure A2: Transfection efficiency of suspension cells expressing GFP. Cells transfected using 1:1:1 pAAV-RC2:pHelper:GFP plasmid molar ratio, 2:1 PEI:DNA ratio, and 1.5 µg/mL total DNA concentration. 10 % of cells transfected.

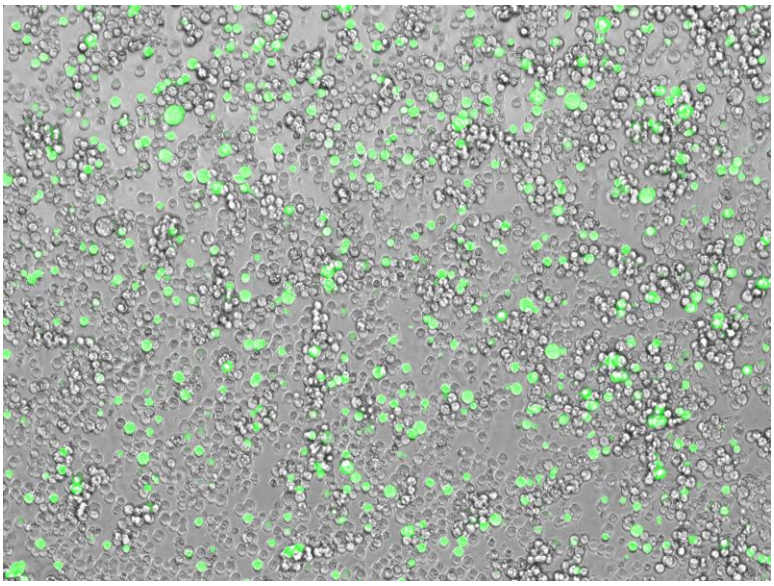

Figure A3: Transfection efficiency of suspension cells expressing GFP. Cells transfected using 1:1:1 pAAV-RC2:pHelper:GFP plasmid molar ratio, 3:1 PEI:DNA ratio, and 1.0 µg/mL total DNA concentration. 15 % of cells transfected.

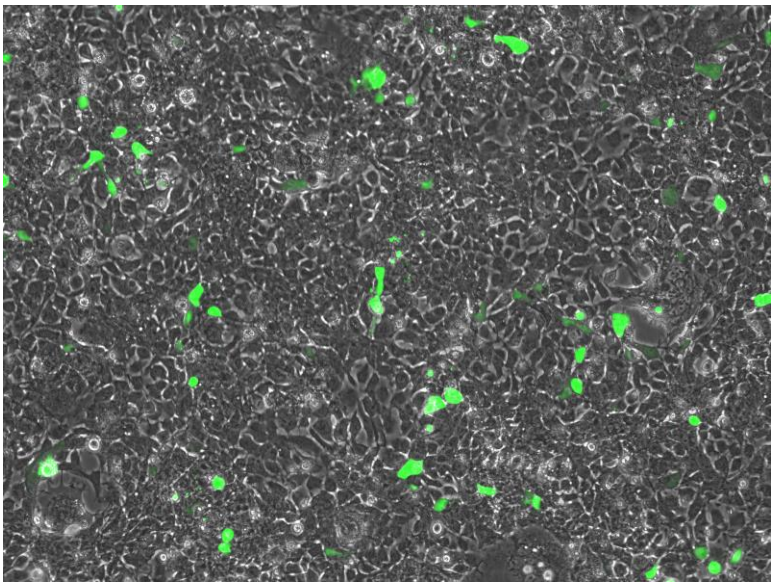

Figure A4: Transfection efficiency of adherent cells expressing GFP. Cells transfected using 2:1:1 pAAV-RC2:pHelper:GFP plasmid molar ratio, 2:1 PEI:DNA ratio, and 1.5 µg/mL total DNA concentration. <5 % of cells transfected.

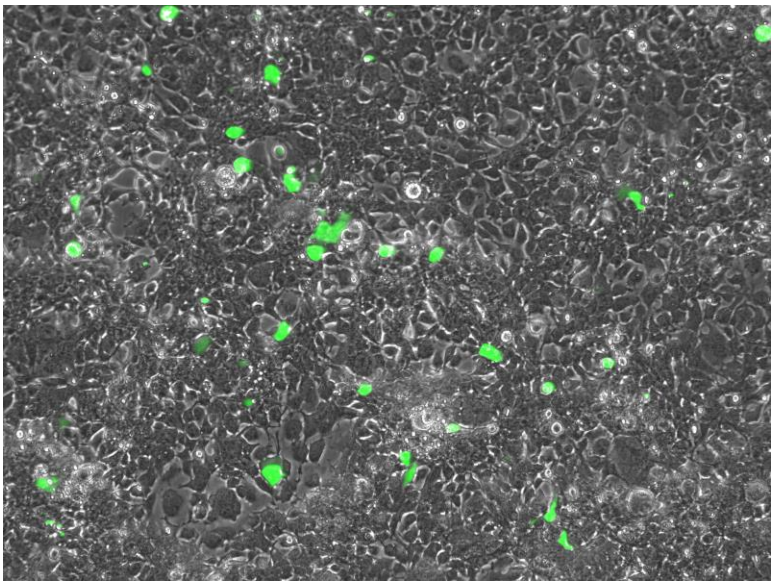

Figure A5: Transfection efficiency of adherent cells expressing GFP. Cells transfected using 1:3:1 pAAV-RC2:pHelper:GFP plasmid molar ratio, 2:1 PEI:DNA ratio, and 1.5 µg/mL total DNA concentration. <5 % of cells transfected.

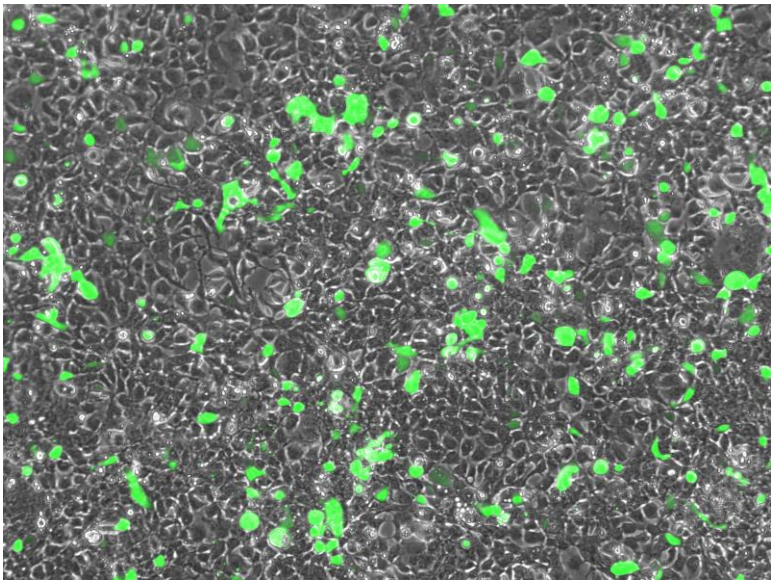

Figure A6: Transfection efficiency of adherent cells expressing GFP. Cells transfected using optimal conditions 1:3:1 pAAV-RC2:pHelper:GFP plasmid molar ratio, 2:1 PEI:DNA ratio, and 2.0 µg/mL total DNA concentration. 15 % of cells transfected.