Development of a High-Throughput Screening Approach to Identify Production Enhancers of Adeno-Associated Virus

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Abstract

Gene therapy has emerged as a revolutionary approach for treating genetic disorders, holding great promise for improving patient outcomes. Among the various viral vectors used for delivery of therapeutic transgenes, Adeno-Associated Viruses (AAVs) have gained prominence due to their favorable characteristics including low immunogenicity, long-term gene expression, and the ability to target both dividing and non-dividing cells. However, AAV's are associated with the high costs of production and challenges with production of a high-quality virus, limiting AAV's utilization and widespread use. In this study, we aimed to develop a high-throughput screening assay targeting AAV production enhancers, thus addressing the manufacturing obstacles and advancing the affordability and accessibility of gene therapies.

To help overcome the limitations and expenses associated with AAV manufacturing, an innovative high-throughput screening assay was developed with the intent to identify cell culture additives/conditions which maximize AAV production. We optimized various parameters, including the transgene, producer and reporter cell lines, harvest timings and methods, and transduction techniques. The optimized screening assay was employed to evaluate novel compounds across several timings of addition, for their ability to enhance AAV production. Notably, several compounds indicated transfection enhancing capabilities up to 3.4-fold and the developed assays final variability was below 14%. Additionally, compound combinations were assessed to uncover potential additive and synergistic effects that could further enhance AAV productivity.

In conclusion, our study presents a significant advancement in targeting the manufacturing challenges associated with AAV. By utilizing an optimized high-throughput screening assay,

researchers and manufacturers can identify compounds that enhance AAV production, paving the way for cost-effective and scalable manufacturing processes. Ultimately, this progress holds the potential to improve the affordability, accessibility, and impact of gene therapies for patients worldwide.

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%CV – Coefficient of variation percent

DI water – deionized water

DMEM - Dulbecco's modified Eagle medium

DMSO – Dimethyl Sulfoxide

DoE – Design of Experiment

F/T – freeze-thaw cycle

FBS – Fetal bovine serum

GFP – green fluorescent protein

GOI – gene of interest

ITR – inverted terminal repeat

LN2 – liquid nitrogen

MOI - multiplicity of infection

ORF – open reading frame

PBS – Phosphate buffered saline

PI - proteosome inhibitor

RLS - RIG-I-like receptor

RLU – relative light unit

scAAV - self-complimentary AAV

SCID - severe combined immunodeficiency

SF – Serum-free

SMA – spinal muscular atrophy

TC – Tissue culture

TLR – Toll-like receptor

VG – vector genome

VG – Vector genomes

VSe – viral sensitizer

VP – viral protein

WFI – water for injection

Chapter 1: Introduction

1.1 History of gene therapy

There are several definitions of gene therapy, but the U.S. Food and Drug Administration (FDA) defines gene therapy as a technique that modifies genes to treat or cure a disease [1]. The concept of gene therapy arose during the 1960-1970s. In 1972, Theodore Friedmann and Richard Roblin proposed that "good" DNA could be used to replace the defective DNA in organisms that suffer from genetic conditions [2]. Prior to which, protein and enzyme therapy were the standard of care for genetic conditions. Unfortunately, these approaches are associated with short half-life in body, immunogenicity and allergic reactions, high cost, delivery challenges and limited target specificity [3].

In 1989, a clinical protocol was approved for the insertion of a foreign gene into immune cells of an individual suffering from cancer, as a form of treatment [4]. Shortly after, gene therapy was performed for the first time in 1990 on a four-year-old patient that was born with severe combined immunodeficiency (SCID). This novel procedure proved to be a great success, allowing the individual to lead a normal life, despite being born with SCID. Before the gene therapy approach, bone marrow transplantation was used. Since this ground-breaking success in 1990, hundreds of human gene therapy clinical trials have been conducted, costing billions of dollars [4]. So far, cancer has been the main target for gene therapy, comprising of over 60% of all ongoing clinical trials for gene therapies worldwide. Due to high manufacturing costs and poor *in vivo* clearance associated with enzyme and protein replacement therapy, gene therapy presents as a potent alternative due to its higher efficacy and substantially reduced therapeutic doses [5]. Thus, making gene therapy potentially the new standard of care for the treatment of genetic disorders.

Generally, gene therapy can be divided into two main categories – germline and somatic gene therapy. Somatic gene therapy involves genetic material inserted in target cells and the change is not passed on to the offspring, whereas germline gene therapy is either used to modify genomes of germ cells - oocytes and spermatozoa or upon fertilization and fusion of gametes. Upon fertilization, as the zygote replicates, the modified gene is passed onto all resulting cell populations during embryonic development of the offspring. This could theoretically counteract hereditary diseases, making such germline gene therapy highly potent treatment for genetic disorders. While there are benefits and setbacks associated with both approaches, altering genomes of germ cells raises ethical concerns, and is forbidden in several countries due to the unknown risks associated with this novel approach [6].

Gene therapies may be delivered using diverse tools, such as, CRISPR/Cas9, virus and non-virus mechanisms. Though the specifics vary depending on the method, the first step in the administration of gene therapy is determination of the disease-causing mutant gene. Next, the corresponding healthy gene, which is called therapeutic gene or transgene is cloned into a vector. A vector is a gene delivery vehicle that delivers therapeutics to target cells. Once the vector reaches the nucleus, the genetic material gets integrated into DNA, or exists extrachromosomally (for example as episomes) and corrects the defective gene. Gene therapy focuses on correcting a disease-causing gene, which may include [7]:

- Inactivation (silencing) of a disease-causing gene.
- Replacing a disease-causing gene with a healthy counterpart.
- Introducing a new or modified gene in the DNA to help treat a disease.
- Gene editing for permanent change.

The most crucial step in gene therapy is choosing the correct type of vector. There are numerous requirements for the ideal vector and determining the best one heavily relies on multiple parameters such as the target cell type and its characteristics, required duration of expression and the size of the genetic material to be incorporated in the vector [5].

There are two main types of vectors: viral and non-viral vectors, each bearing its own sets of advantages and disadvantages:

- Non-viral vectors are naked DNA, chemical and particle based, and they are administered by direct administration (plasmid DNA, chemical, physical, or naked DNA). Most cardiovascular clinical trials rely on non-viral vectors as their method of gene transfer [5]. There are several advantages associated with using non-viral vectors such as: low pathogenicity, low cost and ease of production as well as biosafety. Unfortunately, non-viral vectors are associated with poor delivery efficiency which translates to low transient expression of their transgenes [8].
- Viral vectors rely on viruses for delivery of genetic material. Viral therapy is associated with high transduction efficiency and transgene expression can be regulated by the virus. The use of viral vectors for gene therapy can be targeted against specific cell types, such as mitotic and non-mitotic cells. The natural ability of viruses to enter cells and deliver the genetic material, makes them ideal vectors for gene therapy. However, viral vectors are associated with difficulties with production of high titers, high cost, limitations in packaging capacity and safety concerns [9]. One of main drawbacks of using viral vectors is in their immunogenicity and cytotoxicity. The first related fatality of gene therapy clinical trial was related to the inflammatory reaction towards Adenovirus that was used as a viral vector [5].

1.2 Viral vectors

There are three main components to viral vectors. First one is protein capsid and/or envelope that encapsidates the genetic material, defines vector tropism and antigen recognition. Second is a transgene of interest which serves a desired effect once expressed in target tissue. Last component is an "expression cassette", the combined enhancer/promoter/auxiliary elements that control expression of the desired transgene [10].

The design aspects for these three components are different for each viral vector platform, and each has unique considerations, strengths and weaknesses [11]. Three main viral vector platforms that have been widely used for efficacious gene therapy and regulatory approval are based on Adenovirus (Ad), lentivirus (LV,) and Adeno-associated virus (AAV).

LVs are a subclass of retroviral vectors with single-stranded diploid RNA genomes which is converted into dsDNA post entry. Unlike Adenoviruses and AAVs, retroviral dsDNA genome copies into host chromosomes to produce sustained gene expression. After cell division, the genetic material carried introduced into the genome by retroviruses can be transmitted to the daughter cells [12]. Unfortunately, LVs can insert their cargo randomly into the host genome, increasing the risk of insertional mutagenesis. The LV's ability to hold larger genomic payloads (up to 9 Kb), as well as being mildly immunogenic, high transduction efficiency, *in vivo* and *in vitro*, along with the ability to sustain long term expression of the delivered gene due to genomic incorporation make LVs a desirable vector for gene therapy [13].

Adenoviruses (Ads) are non-enveloped viruses known to cause infections in the upper respiratory tract. They consist of icosahedral protein accommodating 37 kb of linear, double-stranded DNA (dsDNA) genome, falling into class I of Baltimore classification [14]. There are

several advantages of using Ad such as: high transduction efficiency in both mitotic and quiescent cells, epichromosomal persistence in the host cell, availability of scalable production systems, and broad tropism. Ads are strongly immunogenic which decreases the efficacy of gene therapy. There is a strong existing immunity to Ads in the general population of all ages. This translates to one of the main challenges in Ad vector development – to overcome existing viral immunity, since the immune response to capsid proteins is life-threatening [11].

Adeno-Associated Viruses (AAVs) are similar to Adenoviruses but are less efficient at replicating [12]. AAV belongs to the Dependovirus sub-family, which is a group of viruses that can't replicate productively in the host cell without co-infection with a helper virus. Known helper viruses for AAVs include Ads and Herpes Simplex Virus (HSV). Historically, Dependoviruses were found to be associated with adenoviruses hence the reason why they are called Adeno-Associated Viruses. AAVs are widely distributed and common, with more than 90% of adults having antibodies to AAVs. Without helper virus, AAV establishes a latent infection, when the helper virus is present, AAV replicates at a much higher rate. Native AAVs can integrate its genetic payload into the host genome, but most AAVs, including recombinant AAVs used for gene therapy, remain in episomal form [15]. Due to AAV's low immunogenicity as well as ability to either integrate to host genome or stay in episomal form, AAV presents as a potent and more attractive means for gene therapy, as compared to Ad or LV.

1.3 AAV as a Viral Vector

Wild-type AAV is a small, non-pathogenic single-stranded DNA virus. AAV has a 4.7 kb genome which consists of two large open reading frames (ORFs) encoding two proteins, namely the nonstructural *rep* and the structural *cap.* ORFs are flanked by inverted terminal repeats (ITRs) that play a crucial role in AAV replication. It is important to understand AAV capsid assembly

because production of this virus is expensive, and administration of high doses can cause an adverse immune response [16]. AAV's capsid is comprised of 3 viral structural proteins (VP), VP1, VP2 and VP3, all of which are encoded by the *cap* ORF. These proteins are crucially organized in a ratio of 1:1:10 VP1 to VP2 and to VP3 respectively, making a total of 60 molecules for the capsid. It was found that VP1 and VP2 play crucial roles in endosomal trafficking and escape, nuclear localization, and genome release [11]. The *rep* region encodes four proteins designated as Rep 78, Rep 68, Rep 52, and Rep 40 based on the apparent molecular mass of the protein. The Rep proteins play an important role in viral genome replication and are crucial to AAV packaging. Depending on Rep proteins, AAV can either integrate its genetic payload into the host genome, specifically, within a defined region of chromosome 19, for humans or be in episomal form [15].

The use of AAV as a gene therapy vector confers a set of advantages, which include broad tropism of serotypes that can target most tissues and cell types for gene delivery. While there are hundreds of naturally occurring variants of AAV, there are 7 commonly used serotypes. These are AAV1, 2, 5, 6, 8, 9, and DJ. [17]. Variable VP regions on the capsid surface dictate the primary tropism of each serotype. It is important to note that by definition a new serotype is a newly isolated virus that does not react with neutralizing antibodies specific to other existing and characterized serotypes. Based on that definition, only AAV1-5 and AAV7-9 can be defined as true serotypes. AAV6 does not fit in this definition because the serological profile of AAV6 is nearly identical to AAV1 [18]. Most serotypes have specific tissue they are best at transducing [17]:

Tissue	AAV serotype
Liver	AAV8, AAV9
Skeletal muscle	AAV1, AAV6, AAV8, AAV9
Eye Retinal Pigment Epithelium (RPE)	AAV4, AAV5
Photoreceptor cells	AAV5
Kidney	AAV2
Pancreas	AAV8
Lungs	AAV9
Heart	AAV8
CNS	AAV1, AAV4, AAV5

Table 1. AAV serotypes and their tropism

Besides the natural serotypes of AAV, there have been successful attempts at creating synthetic serotypes. The field of AAV vectorology has advanced to the stage where the capsid can be tailored to transduce specific cell types. A great example would be AAV-DJ – a chimera of AAV2, AAV8 and AAV9. To synthesize AAV-DJ, DNA shifting was performed on eight different AAV serotypes - AAV2, 4, 5, 8, 9, bird AAV, bovine AAV and goat AAV [19]. As a result, rAAV-DJ vectors mediate superior *in vitro* transduction efficacies compared to other wild type AAV serotypes. AAV2 is the most studied and most commonly used serotype for *in vitro* applications. When it comes to *in vivo,* AAV8 performs the best [16]. One of interesting features of AAV8 is the distribution of the virus in cell culture media and lysates. Unlike AAV2 that stays within the cell throughout the whole cycle of infection and virus maturation, AAV8 is commonly found in the culture media [20]

Recombinant AAVs (rAAVs) have been successfully used in clinical trials for the treatment of a broad range of rare genetic disorders. Currently, there are three AAV-based biologics that have been approved: Luxturna by both FDA and EMA, Zolgensma by the FDA and Glybera by the EMA [21]. With its controversial $2.1 million price tag, Zolgensma is the most expensive drug on the market [22]. Zolgensma is a one-time gene therapy treatment for patients with spinal muscular atrophy (SMA). Spinraza, a non-AAV alternative to Zolgensma, costs USD $750k for the first year

and USD $350k for every following year [23]. The extravagant price of gene therapy can be attributed to several factors. Biopharmaceutical companies spend many years on research and development of drugs and go through clinical trials. It takes around 12 years for FDA to give marketing approval for therapeutic agents, and statistically, only 9.6% of drugs entering phase I of clinical trials will reach the market [24]. Additionally, gene therapies are targeted, meaning that the market for them is very small. Approximately one in 25,000 children and adults in the USA are diagnosed with SMA while other conditions such as asthma affect an estimated 262 million people worldwide with 461k lethal cases worldwide [25]. Additionally, production of rAAVs is cell culture based, making large scale production of AAV very expensive. This is so because only 5-30% of viral particles harvested from cells have the vector genome of interest and even smaller percentage can effectively transduce cells [16]. A large percentage of harvested viral particles are either empty, partially empty or contain non-transgene DNA are generally considered nonfunctional as they lead to an increased likelihood of post administration immunogenicity. Removing defective rAAVs particles in the downstream process reduces vector genome titer, posing another barrier in cost effective production [16]. There is a huge market for rAAV mediated gene therapy. As such, overcoming barriers such as quick yet accurate detection of competent virus particles to determine accurate viral titers, as well as more efficient production will turn a stone in making AAV gene therapy more commonly available and affordable.

1.4 Production of AAV in cell culture

There are two main methods of AAV production in mammalian cell culture: using co-infection with a helper virus or performing triple transfection with all the required plasmids to make AAV. There are three genetic structures needed for rAAV production. First, is the genomic construct which is limited to approximately 4.7 kb and must encode the AAV ITR flanking an

expression cassette for the transgene of interest. Secondly is a Rep/Cap construct which encoded the AAV *rep* and *cap* genes for genome replication and virion assembly. The common reverse genetics systems rely on the AAV2 *rep* gene while the *cap* gene is serotype specific. The third requirement is the helper virus genes that are crucial for rAAV production. This can be introduced either through infection with a wild-type helper virus or through transfection with a plasmid containing Ad genes: *E1A, E1B, VA, E4,* and *E2A.* The latter method is generally preferred because it allows production of rAAV without any virus contaminations from the helper virus, which is important for the safe use as a gene therapy product [16].

The most used method of rAAV production is via triple plasmid transfection of HEK293 cells. The delivery of plasmids is assisted by transfection reagent such as polyethyleneimine (PEI). PEI binds to the DNA via ionic interactions enabling the cellular intake. PEI is bound on cell membrane and then internalized via endosomes. PEI then "releases" plasmids into the cell. Where they are subsequently transported to the nuclei. It is important to keep in mind however that PEI is toxic to the producing cells and its performance is sensitive to changes in pH. Alternatively, rAAV can be produced in insect cells Sf9 which can be grown in serum-free medium [16]. Cells are infected with recombinant baculovirus-based (rBv) vectors that carry the transgene of interest and *rep/cap* genes. The use of baculovirus for rAAV production requires a relatively small multiplicity of infection (MOI) of 2-5. During the infection, rBv lyses cells and continues to infect uninfected cells until all the cells are infected. This poses as an advantage for the use of baculovirus in the production of AAV with higher yields. On the other hand, these cells are of non-mammalian origin which is associated with unwanted biohazard risks and AAV virion produced in Sf9 cells tend to be less potent than their mammalian counterparts. The third method of rAAV production is using replication deficient recombinant HSV (rHSV) to infect either HEK293 or baby hamster

kidney cells (BHK). This method is similar to the baculovirus approach, the main difference is the type of helper virus used and cell lines used for production [16].

1.5 AAV and cellular pathways

To understand the production of AAV in cell culture it is important to understand the pathways underlying the infection of cells by AAV. AAV replication is initiated by host cell co-infection with AAV and a helper virus (either Ad or HSV). Upon translocation into the nucleus, the viral genome is released from the capsid and must undergo second-strand synthesis prior to initiating the expression of AAV proteins involved in assembly and replication.

The cellular entry of nonenveloped viruses, such as AAV, is initiated by interaction between capsid and cell surface glycan receptors [17]. Different rAAV serotypes use different primary glycan receptors and co-receptors, however the most common ones are heparin sulphate and sialic acid [26]. In 2016, a type I transmembrane protein KIAA0319L was identified as an essential host factor required for rAAV transduction. This host factor was named the universal AAV receptor (AAVR) due to its requirement for the transduction of majority of natural as well as engineered rAAV capsids. KIAA0319L is involved in rAAV post-attachment and in Golgi trafficking.

Binding of rAAV to the cell surface receptors causes it to be internalized via receptor-mediated endocytosis. There are number of host factors that limit replication efficiency, one study estimated that only about 30% of AAV particles will reach the nucleus following the cellular uptake [27]. rAAV vector is internalized, trafficked through the endosomal pathways to the microtubule organizing center. rAAV then escapes from the endosome and is trafficked to the nucleus, where it uncoats, exposing the genome for second-strand synthesis and transcription. Real-time single molecule imaging has shown that 13% of rAAV2 are rapidly internalized by Hela cells within 1.2

sec after contacting the cell surface. Within 15 min at least one rAAV particle was detected in the nucleus of half of the cells [28]. There are several mechanisms of rAAV endocytosis that include clathrin and caveolin-dependent as well as -independent mechanisms. Use of a particular route depends on the host cell type, capsid protein and the availability of receptor, which makes multiple routes of rAAV internalization. Degradation of rAAV particles is caused by ubiquitin-protease system and it can cause unproductive transduction.

After escaping endosomal compartments and Golgi, into the cytoplasm, rAAV particles accumulate around perinuclear space and can be transported into the nucleus via nuclear pore complexes (NPCs) which is a major rate-limiting step [29]. Once inside the nucleus, the rAAV genome is released from the capsid. Unlike wildtype AAV, rAAV can't produce transducing particles due to lack of *rep* and *cap* gene expression. This is caused by lack of complimentary strand synthesis – single stranded AAV genome needs to be converted into dsDNA. Second-strand synthesis is one of the rate-limiting steps during rAAV transduction that can be overcome by co-infection with a helper virus or transduction with a helper plasmid. Additionally, dsDNA is highly unstable which is another limiting factor [26]. Another method to overcome second-strand synthesis limitation is by using self-complimentary AAV (scAAV). Mutated ITRs in scAAV enable molecular recombination leading to circularization of a vector genome. Unfortunately, using scAAV limits therapeutic transgene to approximately 1.5 kb which is not enough for most gene therapies [17].

It is also important to note the role of rAAV capsid in the transgene expression. A point mutation at a conserved site in the AAV8 capsid caused the mutant vector defective for mRNA synthesis. Comparison between different serotypes showed that the difference in genome release efficiency causes various transduction efficiency of different serotypes in various cell types [17].

There are several ways to enhance rAAV transduction, however, some transduction enhancers are serotype specific. It is known that proteasome causes degradation of AAV plasmid during cytoplasmic trafficking which makes inhibitors of the degradation, transduction enhancers of AAV [30]. Interestingly, proteasome inhibitors such as MG132 are strong activators of misfolded protein/ER stress pathways, and it was shown that ER stress induction potentiates AAV2 transduction [30]. Heat shock is another known ER stress inductor making it a transduction enhancer. It was identified that DNA synthesis inhibitors such as hydroxyurea enhance AAV transduction [31]. It is important to point out the abundance of various cellular antiviral defense mechanisms that decrease AAV transduction, such as APOBEC3 proteins which work via damaging the viral DNA. Additionally, immune clearance and innate immune response are activated by AAV transduction resulting in activation and recruitment of various immune cells such as inflammatory cytokines, chemokines and cytotoxic T-lymphocytes. Activation of the abovementioned systems results in recognition and elimination of AAV-transduced cells effectively limiting the extent and duration of the transgene expression.

1.6 AAV production enhancers

Vectors based on AAV are the subject of increasing interest as research tools and agents for *in vivo* gene therapy. With the increase of numbers of rAAV clinical trials, it is important to address production efficiency of rAAV. One such strategy is to use high-throughput screens to identify small molecules enhancers of virus production which could be employed in cell-based production platforms. A prime example of such an approach is the discovery and development of a class of production enhancers termed viral sensitizers (VSes). These are molecules that target various systems and mechanisms in infected cells resulting in virus replication and spread enhancement as much as 1000-fold with Vesicular Stomatitis Virus (VSV) [32]. Performing high-throughput

screening of libraries targeting rAAV production enhancers would require a robust, fast, and reliable detection method.

During AAV vector generation in cell culture, empty or partially empty capsids are being formed. Viral particles that are either empty or contain a truncated transgene are generally considered nonfunctional and lead to an increased likelihood of post administration immunogenicity. qPCR is a commonly used method for rAAV detection and quantification; however, it is limited due to ITR's high GC content and qPCR primer inefficiency. ELISA can be used to confirm qPCR results. Unfortunately, ELISA is expensive and involves a lot of labor. Main disadvantage of using qPCR as a method of AAV detection is the inability to quantify the infectious AAV particles. The ideal method for rAAV detection and quantification using the high-throughput format will be able to quantify crude unpurified samples quickly and reliably. Transduction is a great candidate for the detection and quantification method since it gives a valuable insight on the number of infectious particles of AAV.

There are a countless number of intracellular mechanisms that might affect the production of AAV, and the compounds discovered after performing the high-throughput screen targeting AAV production enhancers will be targeting one of these intracellular mechanisms. There are several categories of cellular mechanisms which defend against viral infections consequently may play a role downregulating the production of AAV in manufacturing platforms:

- Innate immune response: the AAV infection and plasmid transfection can trigger the immune response in the form of release of inflammatory cytokines directly affecting production and transduction efficiencies.
- Type I interferon response plays a crucial role in the hosts immune response to the viral infection. The cells infected by AAV can trigger production of type I interferon

inducing the antiviral state in the neighboring cells and inhibiting the protein synthesis and viral replication.

- Additionally, AAV infection can induce apoptosis in infected cells causing cell death consequently reducing the number of producer cells [33].
- In addition to apoptosis, activation of autophagy pathways can target and degrade AAV particles.
- Restriction factors play an important role in inhibition of viral replication. There are several restriction factors aimed to restrict AAV replication, such as SAMHD1 and APOBEC3 proteins. These factors can interfere with various stages of AAV production cycle, including viral entry, transcription, and genome integration [34].
- RNA interference (RNAi) often gets activated by viral infection. RNAi is a cellular defense mechanism that degrades viral RNA effectively preventing AAV production [16], [18].

1.7 High-throughput screening

When setting up a high-throughput screen, it is important to keep in mind the purpose of the screen and aim to optimize all the essential steps. An ideal high-throughput screen is fast, robust and reliable while also being cost-efficient. Additionally, it is important to set a threshold of an acceptable %CV to aim for.

After performing the screen and identifying potential candidates, it is crucial to vigorously assess the selected compounds and ensure they indeed have the production enhancing effect while being not toxic or mutagenic. Additionally, it might be beneficial to assess things like drug-drug combinations since some compounds when combined may have synergistic effects on virus production. A great high-throughput assay would allow for testing for all these parameters.

1.8 Hypothesis and Amis:

1.8.1 Hypothesis:

We hypothesize that optimized parameters of AAV production will allow a high-throughput drug screen targeting production enhancers.

1.8.2 Aims:

1. Develop a high throughout method of detection and quantification of AAV.
2. Develop HTS assay for production of AAV and detection of production enhancers.
3. Maintain a %CV of 20% or lower for baseline production and quantification.

Chapter 2: Materials and Methods

2.1 Determination of the optimal transgene

HEK293 cells were seeded in complete media in 96-well plates to be 70% confluent by the time transfection. Cells were transfected with AAV2-scNluc, AAV2-GFP, AAV2-Fluc, AAV8-scNluc, AAV8-Nluc, AAV8-GFP, AAV8-Fluc according to AAV transfection protocol. AAV2 and AAV8 samples were harvested 72 h and 96 h post transfection via 3 cycles of freeze-thaws at -80°C. HEK293T were seeded in 96-well to be 70% confluent by the time of transduction. HEK293T were transduced with appropriately diluted samples:

- AAV2-scNluc samples and background were dilute 1:50.
- AAV2-GFP samples and background were dilute 1:2.
- AAV2-Fluc samples and background were dilute 1:10.
- AAV8-scNluc samples and background were dilute 1:2000.
- AAV8-Nluc samples and background were dilute 1:2000.
- AAV8-GFP undilute samples and background were used.
- AAV8-Fluc undilute samples and background were used.

AAV2 and AAV8 transduction was quantified 72 h and 96 h post transduction respectively according to the abovementioned quantification protocols.

2.2 Determination of the optimal producer cell line

HEK293, HEK293T, HT1080 and Hela were seeded in complete media in 96-well plates to be 70% confluent by the time of transfection. Cells were transfected with AAV2-Fluc and AAV8-GFP according to the AAV transfection protocol. The virus samples were harvested in 72 h post

transfection for AAV2 and 96 h post transfection for AAV8 via 3 cycles of freeze-thaws at -80°C. HEK293T seeded in 96-well plates in complete media to be 70% confluent by the time of transduction were transduced with 1:10 diluted samples of AAV2-Fluc and undiluted samples of AAV8-GFP. AAV2 and AAV8 were quantified 72 h and 96 h post transduction respectively according to abovementioned quantification protocols.

2.3 Determination of the optimal time of harvest

HEK293 cells were seeded in complete media in 96-well plates to be 70% confluent by the time transfection. Cells were transfected with AAV2-GFP, AAV2-Fluc and AAV8-GFP according to AAV transfection protocol. AAV2 samples were harvested 24 h, 48 h and 72 h post transfection via 3 cycles of freeze-thaws at -80°C. AAV8 samples were harvested 48 h, 72 h, 96 h and 120 h post transfection via 3 cycles of freeze-thaws at -80°C. HEK293T were seeded in 96-well to be 70% confluent by the time of transduction. HEK293T were transduced with 1:10 diluted samples of AAV2-Fluc, 1:2 sample dilution for AAV2-GFP and undiluted samples of AAV8-GFP. AAV2 and AAV8 were quantified 72 h and 96 h post transduction respectively according to the abovementioned quantification protocols.

2.4 AAV harvest optimization

HEK293 cells were seeded in complete media in 96-well plates to be 70% confluent by the time transfection. Cells were transfected with AAV2-Fluc according to AAV transfection protocol. Three methods of cell lysis were assessed:

- Using -80C° freezer. For the freezing cycle, plates were placed in -80C° freezer for an hour. For the thawing cycles, the plates were placed in +30C° water bath. The freeze-thawing cycle was performed three times.

- Using Liquid nitrogen. For the freezing cycle, plates were placed in vapour phase of the liquid nitrogen for an hour. For the thawing cycles, the plates were placed in +30C° water bath. The freeze-thawing cycle was performed three times.
- Using chemical lysis buffer. Various dilutions of the lysis buffer were added to the corresponding wells of the microplate and the samples were incubated for 7 h in 37°C 5% CO2 incubator.

The samples were spun down for 5 min 1500 rpm at 4°C. HEK293t were seeded to be 70% confluent by the time of transduction. AAV2 samples were dilute 1:10 for the transduction and the transgene signal was quantified 72h post transduction according to the abovementioned firefly luciferase quantification protocol.

2.5 Effect of freeze-thawing on AAV potency

AAV2-Fluc virus stock was produced, purified and concentrated according to large scale AAV production and harvesting protocol. Aliquoted AAV stocks were freeze-thawed 1-4 times in -80°C – room temperature conditions. HEK293T were seeded in 96-well to be 70% confluent by the time of transduction. Freeze-thawed concentrated purified AAV2-Fluc stocks were dilute in 10-fold steps and HEK293T cells were transduced. The transgene signal was quantified 72 h post transduction according to the abovementioned firefly luciferase quantification protocol.

2.6 Optimal AAV reporter cell line

AAV2-Fluc and AAV8-GFP virus stocks were produced, purified and concentrated according to large scale AAV production and harvesting protocol. HEK293, HEK293T, HT1080 and Hela were seeded in complete media in 96-well plates to be 70% confluent by the time of transduction. The cells were transduced with AAV2-Fluc and AAV8-GFP concentrated stocks at

various dilutions. AAV2 and AAV8 were quantified 72 h and 96 h post transduction respectively according to abovementioned quantification protocols.

2.7 Optimal AAV2 transduction dilution and read time

HEK293 cells were seeded in complete media in 96-well plates to be 70% confluent by the time transfection. Cells were transfected with AAV2-Fluc according to AAV transfection protocol and the virus was harvested 72 h post transfection via 3 cycles of freeze-thaws at -80°C. HEK293T were seeded in 96-well to be 70% confluent by the time of transduction and the cells were transduced with various dilutions of crude AAV2-Fluc: undilute, 1 in 2, 1 in 5, 1 in 10 transduction dilution. Additionally, HEK293T were transduced with purified concentrated AAV2-Fluc stocks with 10-folid dilution steps. The transgene signal was quantified 24 h, 48 h, and 72 h post transduction according to the abovementioned firefly luciferase quantification protocol.

2.8 Optimal AAV8 transduction dilution and read time

HEK293 cells were seeded in complete media in 96-well plates to be 70% confluent by the time transfection. Cells were transfected with AAV8-GFP according to AAV transfection protocol and the virus was harvested 96 h post transfection via 3 cycles of freeze-thaws at -80°C. HEK293T were seeded in 96-well to be 70% confluent by the time of transduction and the cells were transduced with various dilutions of crude AAV8-GFP: undilute, 1 in 2, 1 in 5 transduction dilution and the undiluted background was assessed. The transgene signal was quantified 48 h, 72 h, 96 h, 120 h, 160 h post transduction according to the abovementioned GFP quantification protocol.

2.9 AAV production and transduction robustness experiment

HEK293 cells were seeded in 4x96w to be 70% by the time of transfection. The plates were transfected with AAV2-Fluc according to the small scale AAV production protocol. In 72 h post

transfection, the cells were freeze-thawed three times. After finishing a third round of freeze-thawing, the plates were spun down 1500 rpm 5 min and 20 uL of supernatant from each production plate (A, B, C, D) were transferred into four new daughter plates (A to A1, A2, A3 and A4, etc.) to make a total of 16 daughter plates. See Figure 1 for a schematic description of the experiment. The plates were organized in four batches: Batch 1 consisted of plates A1, B1, C1, D2 and so on. Every consecutive week a bath was thawed and HEK293T cells were transduced according to transduction protocol. 72 h post transduction the -Fluc transgene was quantified according to Firefly luciferase quantification protocol.

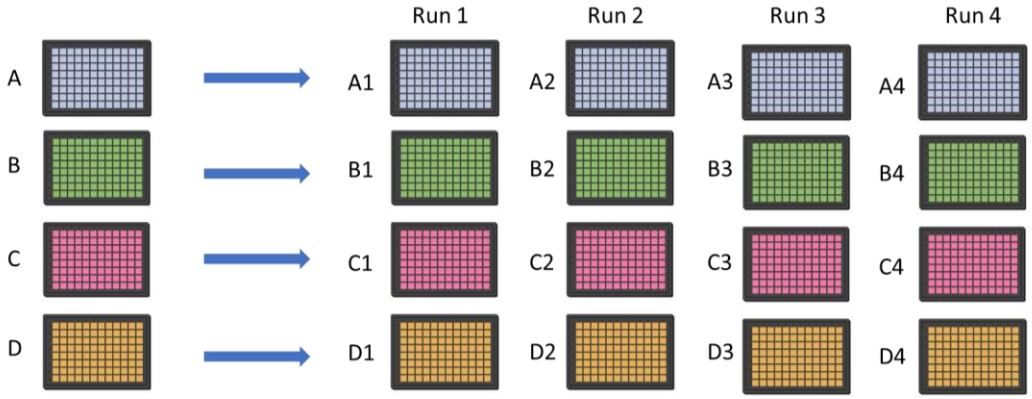


Figure 1. Schematic description of the robustness test experiment of AAV production and transduction.

2.10 Determination of the optimal time for transfection enhancing drug treatment

HEK293 were seeded in 96-well to be 70% confluent by the time of transfection. Impact of VS003 on AAV2-Fluc production was assessed. For this approach a different transfection protocol was used that is not small scale AAV production protocol. The small scale AAV production protocol entails a complete media swap at the time of the transfection to the media containing the plasmids with the transfection reagent. Due to 4 h pre-transfection drug treatment condition, that was not the option. Instead, the plasmid mix was combined to be at 10x concentration and 10 uL

of the plasmid mix was added to 100 uL of cell media making the plasmid concentration 1x. The drug treatments calculations were adjusted to the appropriate volumes. The untreated transfected baseline production control was assessed as well as three times of drug treatment:

- 4 h pre-transfection drug treatment. HEK293 were 4 h pre-transfection treated with VS003 at various concentrations. At the time of transfection, the cells were triple transfected with AAV2-Fluc. 72 h post transfection the samples were harvested via 3 cycles of freeze-thaws.
- Co-transfection drug treatment. HEK293 were co-transfection treated with VS003 at various concentrations. At the time of transfection, the cells were triple transfected with AAV2-Fluc. 72 h post transfection the samples were harvested via 3 cycles of freeze-thaws.
- 18 h post-transfection drug treatment. HEK293 were triple transfected with AAV2. 18 h post transfection, the cells were treated with VS003 at various concentrations. 72 h post transfection the samples were harvested via 3 cycles of freeze-thaws.

HEK293T were seeded in 96-well to be 70% confluent by the time of transduction. The cells were transduced with 1:10 diluted crude samples. The transgene signal was quantified 72 h post transduction according to Firefly luciferase quantification protocol.

2.11 Validation of the high-throughput screen

HEK293 were seeded in 96-well to be 70% confluent by the time of transfection. The cells were transfected with AAV2-Fluc according to the small scale AAV production protocol. Additionally, the transfected cells were co-transfection treated with VS001, VS002, VS003, VS004, VS005, VS006, VS0011, VS0041 and Pevonedistat. 72 h post transfection the samples were freeze-thawed three times. HEK293T were seeded in 96-well to be 70% confluent by the

time of transduction. HEK293T were transduced with 1:10 diluted samples and the transgene signal was quantified 72 h post transduction according to Firefly luciferase quantification protocol.

2.12 Using drug-drug combo for AAV production enhancement

HEK293 were seeded in 96-well to be 70% confluent by the time of transfection. The cells were transfected with AAV2-Fluc and AAV8-GFP according to the small scale AAV production protocol. Additionally, the cells were co-transfection treated with various concentrations and combinations of VS003 and Pevonedistat. AAV2 and AAV8 samples 72 h and 96 h via three freeze-thaws. HEK293T were seeded in 96-well to be 70% confluent by the time of transduction. AAV2-Fluc samples were dilute 1:10 for the transduction and AAV8 samples were used undilute. The AAV2-Fluc transgene signal was quantified 72 h post transduction and AAV8-GFP was quantified 96 h post transduction according to the abovementioned detection protocols.

2.13 Use of transduction enhancers

Several drugs were used as transduction enhancers: Hydroxyurea (Thermo Scientific; A10831.06), MG132 (Thermo Scientific; j63250.#0), VS001, VS003, and Pevonedistat (Adooq Bioscience; A11260). HEK293T were seeded in 96-well to be 70% confluent by the time of transduction. The cells were 4 h pre-transduction treated with the abovementioned compounds at various concentrations. In 4 h, the media was gently aspirated, and the fresh complete media was added. Followed by the media addition, the cells were transduced with the AAV2-Fluc and AAV8-GFP. The AAV2-Fluc transgene signal was quantified 72 h post transduction and AAV8-GFP was quantified 96 h post transduction according to the abovementioned detection protocols.

2.14 Drug carryover and impact on transduction

HEK293 were seeded in 96-well to be 70% confluent by the time of drug treatment. The untransfected cells were treated with VS002, VS003, VS006 and Pevonedistat. 72 h post transfection, the cells were freeze-thawed three times. HEK293T were seeded in 96-well to be 70% confluent by the time of transduction. HEK293T were 4 h pre-transduction treated with two types of samples: 72 h samples and freshly diluted chemical compounds. 4 h post drug treatment, the media was changed, and the cells were transduced with diluted concentrated purified stocks of AAV2-Fluc. The transgene signal was quantified 72 h post transduction according to Firefly luciferase quantification protocol.

2.15 Effect of AAV2 storage at 4C° for extended period

AAV2-Fluc virus stock was produced, purified and concentrated according to large scale AAV production and harvesting protocol. Aliquoted AAV stocks were incubated in 4°C fridge for various periods of time. HEK293T were seeded in 96-well to be 70% confluent by the time of transduction. The cells were transduced with the abovementioned AAV2-Fluc stocks stored in 4°C in 10-fold dilution steps. The transgene signal was quantified 72 h post transduction according to Firefly luciferase quantification protocol.

2.16 Cell lines used

- HEK293 - Embryonic Kidney; Human (Homo sapiens). Acquired from ATCC CRL-1573.
- HEK293T - Embryonic Kidney Cells; Human (Homo sapiens). Acquired from ATCC - CRL - 3216.
- Hela - Cervical Adenocarcinoma; Human. Acquired from ATCC CCL-2.
- HT1080 - Fibrosarcoma; Human (Homo sapiens). Acquired from ATCC CCL-121.

2.17 Tissue culturing protocol

All the cell lines described in this study were passaged twice per week for the routine cell passaging and for seeding in the microplates for the experiments. The routine cell passaging was performed in T175 flasks (Thermo Fisher; 159910) and seeded in various microplates depending on the nature of the experiment. For all experiments involving cell cultures in this study the complete media was used – DMEM (Gibco; 11995-065), 5% FBS (Avantor; 97068-085), 1% penicillin-streptomycin (Gibco; 15140-122). To passage the cells, there were several key components: PBS (Gibco; 20012-027), Trypsin (Gibco; 25300-054), and complete media.

At the day of cell passaging, the cells confluency was assessed using a microscope. Media overlaying the cells was aspirated with a Pasteur pipette and 5 mL of PBS was added. The flask was gently rotated ensuring the PBS washes the whole surface of the flask evenly. After that, the PBS was gently aspirated, and 4 mL of room temperature trypsin was added to the cells and the flasks were incubated at 37°C 5% CO2 for 5-10 minutes. After visually confirming that the cells lifted, 10 mL of room temperature complete media was added to the flask. The cells were gently pipetted up and down to break clumping and the mixture was transferred into a separate tube. The viability and cell count were assessed using Invitrogen Countess 3 cell counter. Various volumes of cell mixtures were added to the flasks or microplates to achieve desired confluency.

2.18 List of used plasmids:

Plasmid name	Source
pAAV-RC2	Cell Biolabs VPK-402 - VPK-422
pAAV-GFP	Cell Biolabs VPK-402 - AAV-400
pHelper	Cell Biolabs VPK-402 - 340202
pAAV-RC8	In-house plasmid stock
pAAV-Fluc	In-house plasmid stock
pAAV-scNluc	In-house plasmid stock
pAAV-nLuc	In-house plasmid stock

Table 2. List of plasmids used in the study.

2.19 Diagnostic digest protocol

The diagnostic digest protocol requires enzymes that are plasmid-specific for the plasmid linearization. In thus study, all plasmids were designed to have BamHI and EcoRI cut sites. The table below shows a master mix recipe for the diagnostic digest preparation as well as the materials needed:

Reagent	Source	Required volume
BamHI enzyme	NEB; R3136S	1 uL
EcoRI enzyme	NEB; R3101S	1 uL
CutSmart buffer	NEB; B6004	2 uL
MilliQ water	Invitrogen; 10977015	Top up to 20 uL total
DNA sample	---	1 ng for purified or 5 ng for gel extracted DNA

Table 3. Master mix protocol for the plasmid diagnostic digest

Once prepared, the samples were placed in the thermocycler (Biorad; T100) to be incubated for 30-40 min at 37°C. After the incubation, the agarose gel electrophoresis was performed on the samples to separate the bands. The results of the gel were compared to the virtual digest to confirm the presence and the correct size of the insert of interest.

2.20 Bacterial transformation

Stbl3 *E.coli* (Invitrogen; C737303) was used for the bacterial transformation. To 25 uL of bacteria, 2 uL of 25 ng/uL plasmid was added. The tube was flicked 5 times and incubated on ice

for 30 min. Following that, the samples were 42°C heat shocked for 30 seconds and placed on ice for 5 min. 950 uL of LB medium was added and the tube was shaken 250 rpm for 1 hour at 37°C. 100 uL of the sample was spread on pre-warmed up selection plate which was placed in the incubator. The incubator temperature was variable: plasmids containing ITR fragments (pAAV-RC2 and RC8) were incubated at 30°C, all other plasmids were incubated at 37°C for 24 h.

2.21 Bacterial isolation

After incubating the selection plates in the incubator for 24 h, the bacteria were isolated via colony picking. It's a process of selecting a single colony of pure single-strain bacteria for further duplication. A suitable colony was isolated via scraping by the sterile pipette tip which was ejected into culture test tubes (Fisherbrand; 14-956-1J) containing 10 mL of LB broth. The tubes were incubated overnight at 30°C.

2.22 Bacterial glycerol stocks long-term storage

The transformed bacterial culture was converted to glycerol stocks. This was done to avoid unnecessary bacterial transformation for every plasmid preparation which is associated with potential impurities and genome mutation. To make glycerol bacterial stocks, glycerol (Thermo Scienficic; #17904) was dilute to 50% concentration in sterile DI water. The overnight liquid culture of transformed bacteria was combined in 1:1 ratio with 50% glycerol, mixed well, and aliquoted in cryovials to be stored in -80°C freezer. The glycerol storage in -80°C is essential for plasmid long-term storage and avoids unnecessary bacterial transformation while keeping the bacterial cultures ready to produce desired plasmids overnight.

2.23 Plasmid production and purification

To produce plasmids in large quantities, 500 mL of LB media (see solution preparation section for more information) was added per 1 L baffled flasks. Using a sterile pipette tip, the bacterial glycerol stock of choice was scraped, and the pipette tip was dropped into the flask with media. The antibiotic was added to the flask to be at the final concentration of 1X. The inoculated culture was incubated at 30°C for 18-24 h in a shaking incubator (New Brunswick Scientific; G-25).

The plasmids were purified using commercial bacterial purification kit (QIAGEN Plasmid Mini Kit #12123) as per manufacturers protocol. Once eluted, plasmid concentrations were quantified (Tecan; Spark Cyto) and the restriction digest was performed to confirm plasmid's structure according to the restriction digest protocol. After the restriction digest, multiple batches of the same plasmid were combined to make a stock of 5 mL of concentrated plasmids, it was aliquoted in properly labeled microcentrifuge tubes and stored in -20°C to be thawed no more than three times. Even when the plasmids are prepared the same way, there is a batch-associated variability, so it is generally recommended to produce a large volume of plasmids in situation where low %CV is important between experiments.

2.24 AAV transfection protocol

A three-plasmid transfection system was used for production of both AAV2 and AAV8. The transfection reagent, PEI pro (Polyplus; 101000033) was used in 1:1 ratio with total amount of DNA. For the triple transfection, 1:1:1 molar ratio of plasmids (pAAV-transgene, pAAV-RC2 or -RC8, and pHelper) was used. To ensure effective transfection, we utilized 1.5 ug of DNA per 1e6 cells.

To begin the transfection process, we used 70% confluent cells and performed a full media swap. The transfection media, containing the required plasmids, were prepared in several steps. Initially, an appropriate volume of plasmids were added to a tube with serum-free DMEM and the tube was vortexed. Simultaneously, we added the suitable volume of PEI pro to a separate tube that was vortexed. Two solutions were combined, vortexed briefly and incubated at room temperature for 15 min. After the incubation period, the plasmid mix was gently added to a flask containing complete media, the mixture was mixed and incubated for an additional 15 min at room temperature. Following this incubation step, the media on the cells was aspirated and replaced with the media containing the plasmids.

2.25 Large scale AAV production and harvesting

To produce AAV in large scale, HEK293 cells were used as producer cell line. The cells were seeded in T175 flasks to be 70% confluent by the time of transfection. Cells were grown in complete media. Cells were transfected according to AAV transfection protocol.

The harvest time was variable for different AAV serotypes. AAV2 was harvested 72 h post transfection and AAV8 was harvested in 96 h. At the appropriate time of harvest, the cells were dethatched by adding $\frac{1}{80}$ volume of 0.5M EDTA (Invitrogen; 15575-038) and incubated at room temperature for 15 min. After the cells were lifted, the virus was purified using commercial purification kit (Takara AAVpro® Purification Kit Maxi; #6666) as per manufacturers protocol. Once the virus was purified, it was aliquoted in qPCR flat cap tubes 15 uL each and stored in -80°C. Aliquoting in small tubes was done to avoid negative impact of freeze-thawing concentrated virus stocks several times. Having individual tubes with 15 uL in each allowed to thaw the separate virus stocks for every experiment which positively affected the overall variability of this study.

2.26 Small scale AAV production and harvesting

To produce AAV in small scale, HEK293 cells were used as producer cell line. The cells were seeded in TC-treated 96-well microplates (Avantor; 10062-800) to be 70% confluent by the time of transfection. Cells were grown in complete media. Cells were transfected according to AAV transfection protocol.

Additionally, HEK293T, Hela, and HT1080 were tested for their AAV production capabilities. The seeding parameters, media used, and transfection parameters were the same as it was previously described in HEK293 context.

The harvest time was variable for different AAV serotypes. AAV2 was harvested 72 h post transfection and AAV8 was harvested in 96 h. The main method of harvesting was via freeze-thawing plates in -80°C and 37°C. At the time of harvest, microplates were sealed with plate sealers (Thermo Scientific; 5701) and placed in the -80°C freezer. For optimal freezing time it was ensured not to stack the plates. After 1h in the freezer, the plates were placed in hermetic bags in 30°C water bath for 10 min. The freeze-thawing cycle was repeated twice for the total number of three freeze-thaw cycles.

Additionally, two methods of cell lysis were tested: using LN2 and using lysis buffer. LN2 method is similar to freezing in -80°C freezer with one difference of using vapour phase of liquid nitrogen as a freezing medium. Plates were sealed and frozen in a vapour of liquid nitrogen for 1h followed by thawing in hermetic bags in 30°C water bath for 10 min. The freeze-thawing cycle was repeated twice for the total number of three freeze-thaw cycles.

Lastly, a method of chemical lysing was tested. For this approach, commercial AAV lysis buffer (Gibco; 100097742) was used. At the time of harvest, various dilutions of the lysis buffer

were added to the wells of the microplate starting from 1:10 to 1:2000. The plates were incubated for 7 h in the incubator and the cell lysates were harvested for the further transduction quantification.

2.27 Transduction assay

HEK293T cells were used as reporter cell line in the transduction assay. The cells were seeded in TC-treated 96-well microplates to be 70% confluent by the time of transduction. Cells were grown in complete media as per tissue culture protocol. At the time of transduction, the samples were diluted in SF-DMEM, and the reporter cells were transduced. The quantification time for the transgene signal was serotype specific. AAV2 was quantified 72 h post transduction and AAV8 was quantified in 96 h. The transduction read time was optimized via reading transgene reporter signal throughout a time course experiment.

Additionally, HEK293, Hela and HT1080 were tested as reporter cell lines for transduction assay. The seeding parameters, media used, and transduction parameters were the same as it was previously described in HEK293 context.

2.28 Firefly luciferase protocol

For all luminescence assays described in this study, white bottom 96-well microplates were used (ThermoScientific; 136101). To quantify AAVs containing -Fluc transgene, the firefly luciferase assay was used. At the appropriate transduction read time, a 2 mg/mL solution of D-Luciferin (Thermo Scientific; 88294) was made in PBS. It is essential to handle luciferin in absence of light due to its light sensitivity. 20 uL of 2 mg/mL solution was automatically dispensed by the plate reader (Tecan; Spark Cyto) to 100 uL in every well making it 0.33 mg/mL working

concentration. The plates were shaken by the plate reader for 10 seconds and relative light unit (RLU) readings were obtained after 90 second delay.

2.29 GFP event counting protocol

For the GFP event counting, the Tecan Spark Cyto plate reader was used in the fluorescence imaging mode. 4x objective was used for acquisition of 4 fields per well of a 96-well plate. For the GFP event counting analysis, Tecan SparkCyto ImageAnalyzer was used. The sensitivity % threshold was set to 90 and object length was set to 10 um for minimum and 60 um for maximum.

2.30 Nanoluc protocol

To quantify AAVs containing -nluc or -scnLuc transgenes, the Nano-Glo® Luciferase assay used. The commercial kit was used (Promega Nano-Glo® Luciferase Assay System; #N1120). At the appropriate read time, the substrate was prepared according to manufacturer's protocol and 1:1 mixed with the samples. The plates were shaken by the plate reader and the relative light unit (RLU) readings were obtained after 3 min delay.

2.31 Drug reconstitution

Most chemical compounds used in this study were reconstituted in DMSO (Fisher Bioreagents; BP-231-100) with the exception of VS005 which was reconstituted in WFI (Gibco; A1287301). Compounds molecular weight was used to prepare concentrated stocks (10-200 mM) and the samples were aliquoted in appropriately labeled microcentrifuge tubes which were stored in the -80°C freezer. At the time of drug treatment, the compounds were diluted to the required concentration ensuring the final concentration of DMSO is less or equal to 1%.

2.32 Solution preparation

LB media – Nutrient-rich broth used for liquid bacteria culturing. For 1 L liquid LB media combined: NaCl 10 g (Thermo Fisher Scientific #S27110), 10 g Tryptone (AG Scientific; #T-2773), 5 g Yeast extract (RPI Research Products International; #Y20020), 1 L DI H20 and the mixture was autoclaved.

2.33 Statistical methods used

To determine the %CV of the population, the average of the samples was divided by the standard deviation of the samples and the outcome of that was multiplied by 100% to get a final %CV value.

For the graphs with p-values the two-tailed homeostatic t-test was performed.

Chapter 3: Results

Unless otherwise noted, the production and detection of AAV was performed similarly throughout the course of the project.

AAV2 was produced via triple-transfection of HEK293. 72 h post transfection, the samples were freeze-thawed three times in -80C° freezer and +30C° water bath. HEK293T were transduced with samples dilute 1:10 and the transgene was quantified 72 h post transduction.

AAV8 was produced via triple-transfection of HEK293. 96 h post transfection, the samples were freeze-thawed three times in -80C° freezer and +30C° water bath. HEK293T were transduced with undilute samples, and the transgene was quantified 96 h post transduction.

3.1 Determination of the optimal transgene

Several transgenes including secreted Nanoluciferase (-scNluc), -Nanoluciferase (nLuc), Green Fluorescent Protein (-GFP), and Firefly luciferase (-Fluc) were compared for optimal detection of AAV2 and AAV8 serotypes and assessed for sensitivity across dilutions. The ideal transgene would have a low background signal and high sensitivity of detection across several orders of magnitude. HEK293 cells seeded in 96-well microplates were transfected with either AAV2 or AAV8 Rep/Cap contructs, pHelper and a genomic construct containing one of the abovementioned transgenes. AAV2 and AAV8 were harvested 72 h and 96 h post transfection respectively, HEK293T were transduced at various dilutions and the transgene and background signals were quantified.

For AAV2 (Figure 3.1 A-C), it was found that the most sensitive method of detection involved -scNluc transgene where a 1:50 dilution of the sample produced a signal several orders of magnitude above the background (Figure 3.1 A). The -GPF transgene allowed for detection and

quantification of AAV2, however the background signal was only ten-fold lower from the samples signal and required use of a relatively concentrated sample (1:2 dilution) (Figure 3.1 B). Using -Fluc transgene allowed for accurate quantification of AAV2 at 1:10 dilution, with a moderate signal to noise ratio (Figure 3.1 C).

When it comes to AAV8 (Figure 3.1 D-G), both -scNluc and -Nluc samples were dilute 1:2000 for detection, however the background signal values were approaching abovementioned samples (Figure 3.1 D and E). Similarly to -scNluc and -nLuc, using -Fluc transgene was associated with a high background signal making it hard to distinguish between the background noise and the transgene signal (Figure 3.1 F). It was found that -GFP was the most optimal transgene for AAV8 detection due to distinct half-log difference between sample signal and the background (Figure 3.1 G).

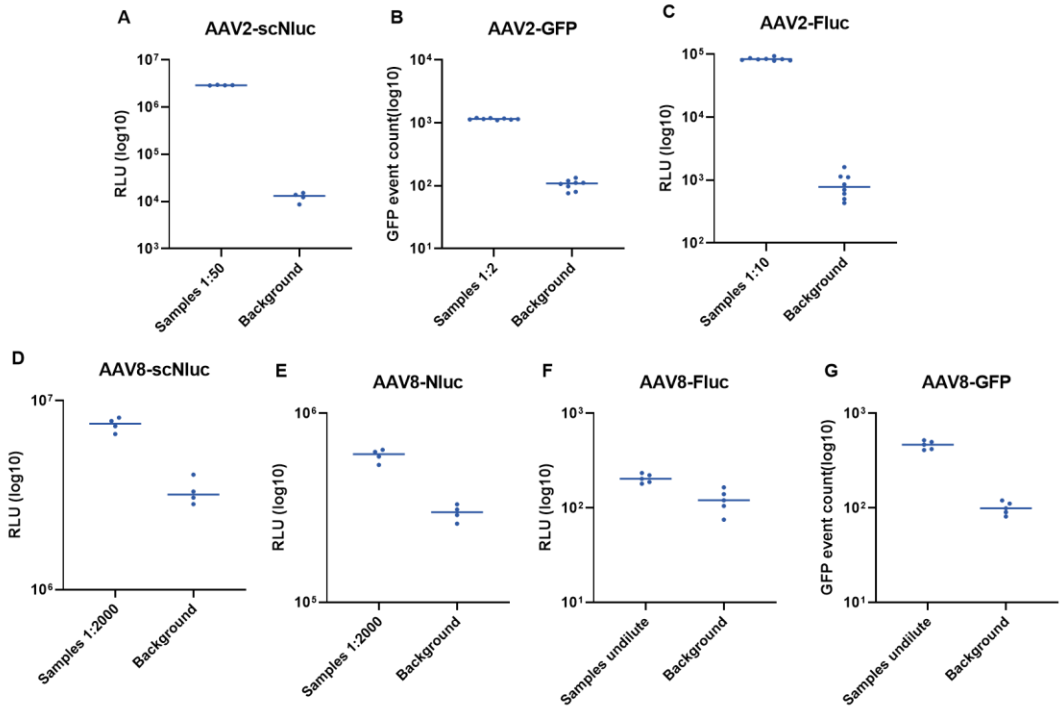


Figure 3.1 Fluc transgene was found to be the optimal transgene for AAV2 detection.

Quantification of AAV2 and AAV8 containing various transgenes via transduction method. HEK293 were triple transfected with AAV2 and AAV8 and harvested at 72 h and 96 h post transfection respectively via 3 cycles of freeze-thawing. HEK293T were transduced at various dilutions. AAV2 and AAV8 were quantified 72 h and 96 h post transduction respectively. (A) Quantification of AAV2-scNluc via transduction of HEK293T with 1:50 diluted samples as well as the background control. Dots represent technical replicates, n=4. (B) Quantification of AAV2-GFP via transduction of HEK293T with 1:2 diluted samples as well as the background control. Dots represent technical replicates, n=8. (C) Quantification of AAV2-Fluc via transduction of HEK293T with 1:10 diluted samples as well as the background control. Dots represent technical replicates, n=8. (D) Quantification of AAV8-scNluc via transduction of HEK293T with 1:2000 diluted samples as well as the background control. Dots represent technical replicates, n=4. (E) Quantification of AAV8-Nluc via transduction of HEK293T with 1:2000 diluted samples as well as the background control. Dots represent technical replicates, n=4.

3.2 Determination of the optimal producer cell line

Following our observations of various transgenes for detection of AAV, various producer lines were investigated for their ability to generate rAAV particles. Based on the results from Figure 3.1, production of AAV2-Fluc and AAV8-GFP was assessed. The ideal producer cell line would have a high yield of AAV production. Four cell lines were assessed: HEK293, HEK293T, Hela, and HT1080. Both AAV serotypes were produced in all four cell lines, the virus was harvested and HEK293T were transduced with a 1:10 sample dilution for AAV2 and undiluted sample for AAV8.

For AAV2, both HEK293 and HEK293T showed a substantial amount of virus production with HEK293 producing more virus compared to HEK293T. Both Hela and HT1080 showed insignificant number of produced AAV2-Fluc (Figure 3.2 A). HEK293 was found to be the optimal producer cell line for AAV2-Fluc.

Similarly, both HEK293 and HEK293T produced more AAV8-GFP compared to Hela and HT1080 (Figure 3.2 B). HEK293 was found to be the optimal producer cell line for AAV8-GFP.

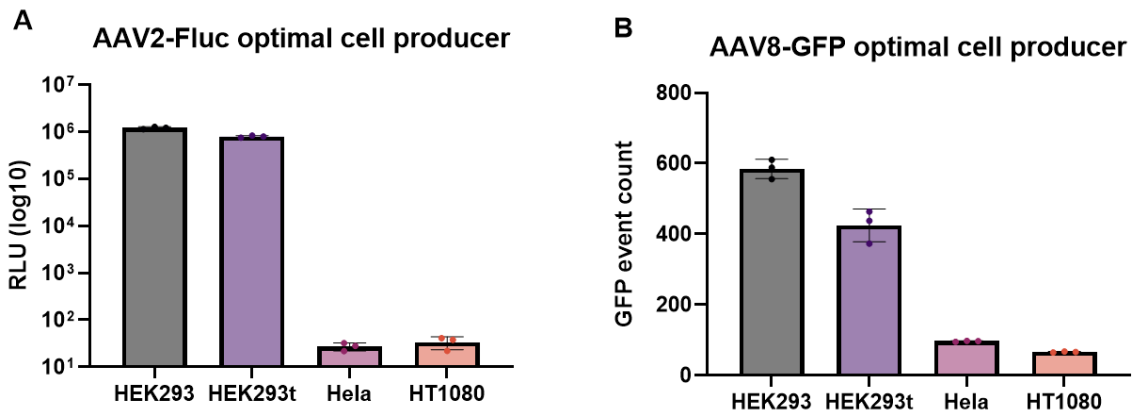


Figure 3.2 HEK293 were found to be most optimal producer cell lines for both AAV2 and AAV8.

Transduction quantification of AAV2-Fluc and AAV8-GFP produced in various cell lines via triple transfection method. The virus samples were harvested in 72 h post transfection for AAV2 and 96h post transfection for AAV8 via 3 cycles of freeze-thawing. HEK293T were transduced with 1:10 diluted samples of AAV2-Fluc and undiluted samples of AAV8-GFP. AAV2 and AAV8 were quantified 72 h and 96 h post transduction respectively. (A) Transduction quantification of AAV2-Fluc produced in HEK293, HEK293T, Hela, HT1080. HEK293T were transduced with 1:10 diluted samples harvested from four cell lines. Dots represent 3 biological replicates (mean of n= 12 technical replicates). (B) Transduction quantification of AAV8-GFP produced in HEK293, HEK293T, Hela, HT1080. HEK293T were transduced with undiluted samples harvested from four cell lines. Dots represent 3 biological replicates (mean of n= 12 technical replicates).

3.3 Determination of the optimal time of harvest

To further optimize the method of high-throughput screening for AAV production enhancers, we set out to optimize the time of virus harvest to maximize the viral production and decrease unwanted variability. The optimal harvest time would have a high yield of virus production with low variability. AAV2-Fluc and AAV8-GFP were produced in HEK293 cells and the virus was harvested at various timepoints allowing to assess the virus production over time. HEK293T were transduced at various dilutions: 1:10 sample dilution for AAV2-Fluc, 1:2 sample dilution for AAV2-GFP and undiluted transduction for AAV8-GFP.

For both AAV2-Fluc and AAV2-GFP the virus production was increasing with time and the 72 h samples had the most virus detected compared to 24 h and 48 h. For both AAV2-Fluc and AAV2-GFP the variability was found to be the lowest at the latest time point: 14.5% for the -Fluc transgene and 11.8% for the -GFP transgene (Figure 3.3 A and B). Overall, it was found that the optimal harvest time for AAV2 is 72 h.

Similarly, AAV8-GFP production was also increasing with time (Figure 3.3, C). It was found that the most virus was produced in 120h post transfection which was also the time point with the lowest %CV of 5.6%. 96 h was found to be the optimal time of harvest of AAV8-GFP.

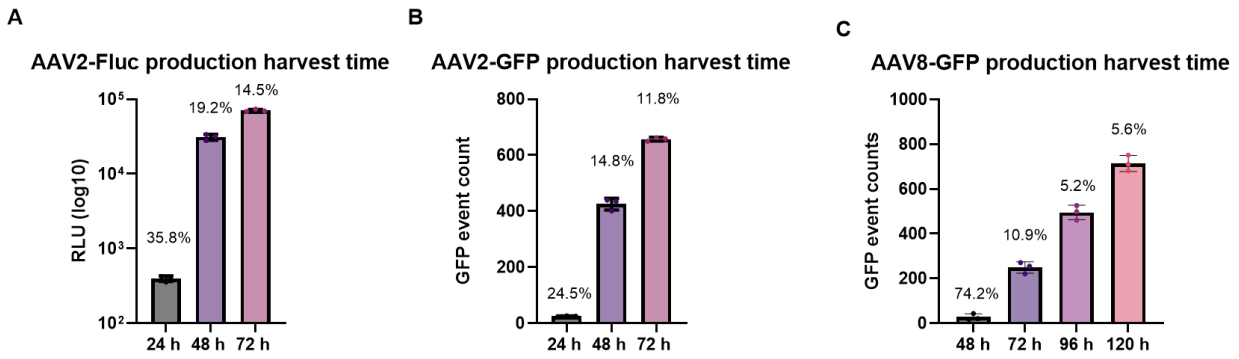


Figure 3.3 Optimal production harvest time of AAV2 and AAV8

Transduction quantification of AAV2 and AAV8 harvested at various time points post transfection. HEK293 were triple transfected with AAV2 and AAV8 and harvested at various time points via 3 cycles of freeze-thawing. HEK293T were transduced at various dilutions. HEK293T were transduced with 1:10 diluted samples of AAV2-Fluc, 1:2 sample dilution for AAV2-GFP and undiluted samples of AAV8-GFP. AAV2 and AAV8 were quantified 72 h and 96 h post transduction respectively. (A) Transduction quantification of AAV2-Fluc produced in HEK293 and harvested in 24, 48, 72 h post transfection. HEK293T were transduced with 1:10 diluted samples. Dots represent 3 biological replicates (mean of n= 12 technical replicates). The calculated %CV are 35.8% for 24 h samples, 19.2% for 48 h samples, and 14.5% for 72 h samples. (B) Transduction quantification of AAV2-GFP produced in HEK293 and harvested in 24, 48, 72 h post transfection. HEK293T were transduced with 1:2 diluted samples. Dots represent 3 biological replicates (mean of n= 12 technical replicates). The calculated %CV are 24.5% for 24h samples, 14.8% for 48 h samples, and 11.8% for 72 h samples. (C) Transduction quantification of AAV8-GFP produced in HEK293 and harvested in 48, 72, 96, and 120 h post transfection. HEK293T were transduced undiluted samples. Dots represent 3 biological replicates (mean of n= 6 technical replicates). The calculated %CV are 74.2% for 48 h samples, 10.9% for 72 h samples, 5.2% for 96 h samples, and 5.6% for 120 h samples.

3.4 AAV harvest optimization

After addressing the optimal transgene for AAV serotypes, optimal producer cell line and time of harvest, the optimal harvest method was assessed. Unlike AAV2, AAV8 is secreted by cells, however it is a common practice to lyse cells in order to maximize the virus harvesting. With that in mind, we decided to compare methods of freeze-thawing in -80C° and Liquid Nitrogen as well as chemical methods of cell lysis via using lysis buffer. The optimal harvesting method will allow for a fast release of the virus particles from the cells while also having a low coefficient of variation. HEK293 were transfected with AAV2-Fluc and the virus was harvested at 72 h via three-time freeze-thaws using -80C°, Liquid Nitrogen in conjunction with a 30C° water bath, or by addition of lysis buffer (chemical lysis). The lysis buffer was added for 7 h followed by transduction of the reporter cells. HEK293T were transduced with the harvested samples at 1:10 dilution and the reporter gene was quantified 72 h post transduction. Additionally, the impact of the number of freeze-thaws on the harvesting efficiency was examined. For this experiment HEK293 were transfected with AAV2-Fluc and harvested in 72 h via freeze-thawing once, twice, and three times in -80C°.

The results indicate that both -80C° and Liquid Nitrogen freezing methods can be used for cell lysis in order to release AAV particles with -80C° method yielding higher results but slightly higher variability (Figure 3.4 A). The -80C° harvesting method is a preferable method of harvesting AAV.

The transduction results of the samples harvested using the chemical lysis method were significantly lower compared to the control – samples harvested using the -80C° freeze-thaw method. On top of the decrease in the transduction results, the samples harvested with a lysis buffer demonstrated a greater degree of variability. After visually looking at the reporter cells transduced

with the samples harvested with the lysis buffer, it was noted that the reporter cells were lysed. To determine if the lysis buffer could be sufficiently diluted to allow for subsequent transduction of monolayers, samples were assessed across a wide range of dilutions up to 1:2000. All dilutions produced similar results.

While the overall -Fluc signal was similar between 1X, 2X, and 3X freeze/thaw cycles, it was noted that the variability decreased with increasing freeze thaws (Figure 3.4 B). Given the overall observations, it was determined that the 3X freeze/thaw using -80C° was the optimal harvest method for high-throughput screening.

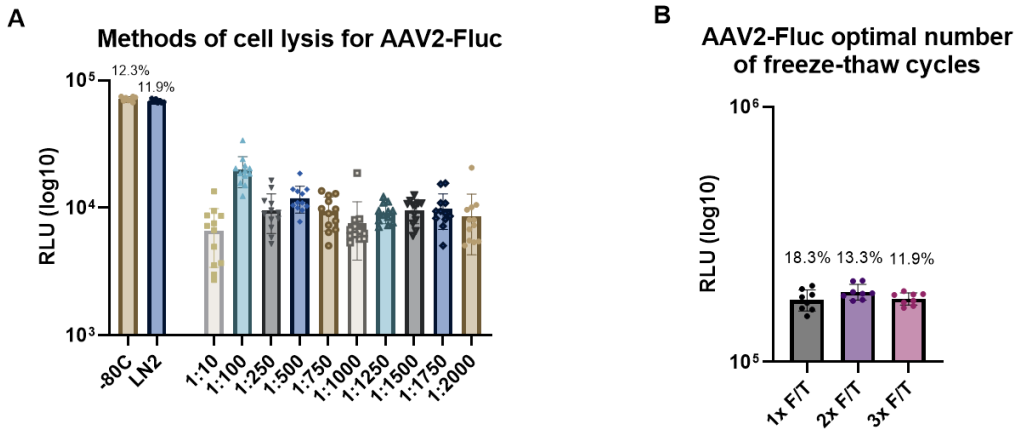


Figure 3.4 Cell lysis optimization.

Transduction quantification of samples harvested using various methods of cell lysis. HEK293 were triple transfected with AAV2-Fluc and harvested via 3 cycles of freeze-thawing in -80C°, Liquid Nitrogen as well as by addition of the lysis buffer (Figure A). HEK293 were triple transfected with AAV2-Fluc and harvested via various number of freeze-thaws in -80C° (Figure B). HEK293T were transduced with 1:10 diluted samples of AAV2-Fluc and the transgene was quantified 72 h post transduction. (A) Transduction quantification of AAV2-Fluc produced in HEK293 and harvested 72 h post transfection. Various methods of virus harvesting were compared: three cycles of freeze-thawing in -80C° and Liquid Nitrogen as well as by addition of lysis buffer at various dilutions. HEK293T were transduced with 1:10 diluted samples: -80C° n= 8 (technical replicates); Liquid Nitrogen n= 8 (technical replicates); lysis buffer n= 12 (technical replicates). The calculated %CV are 12.3% for -80C° method of cell lysis, and 11.9% for Liquid Nitrogen method of freeze-thawing. (B) Transduction quantification of AAV2-Fluc produced in HEK293 and harvested 72 h post transfection. Samples were harvested via freeze-thawing once, twice and

three times in -80C° and HEK293T were transduced with 1:10 diluted samples, n = 8 (technical replicates). The calculated %CV are 18.3% for one time freeze-thaw, 13.3% two-time freeze-thaw, and 11.9% for three-time freeze-thaw.

3.5 Effect of freeze-thawing on AAV stability

To address the question of freeze-thaw associated stability decrease of AAV, we compared the impact of several cycles of freeze-thawing on purified concentrated stocks of AAV2-Fluc and transduced HEK293T with 10-fold diluted virus samples.

As shown in Figure 3.5 there is a small yet notable decrease in stability of AAV2-Fluc associated with several cycles of freeze-thawing.

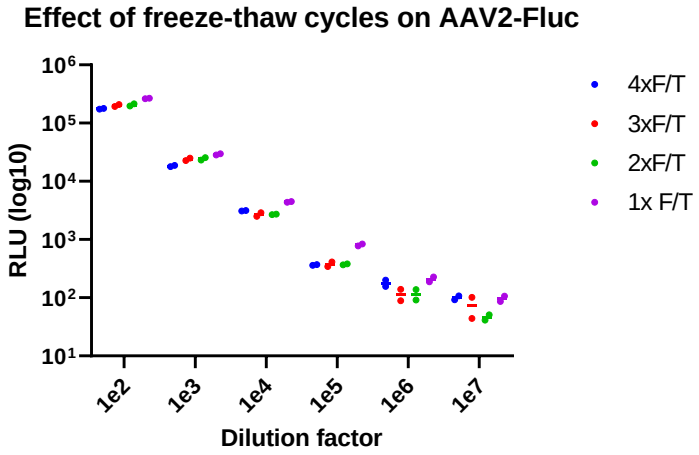


Figure 3.5 Freeze-thawing does not have a drastic impact on AAV2-Fluc stability.

Transduction quantification of HEK293T transduced with concentrated AAV2-Fluc freeze-thawed 1-4 times. AAV2-Fluc was produced and purified using commercial purification kit. Concentrated virus was freeze-thawed 1-4 times and HEK293T were transduced with various dilutions of the freeze-thawed samples. The transgene was quantified 72 h post transduction. Dots represent n = 2 technical replicates.

3.6 Optimal AAV reporter cell line

Pursuing the low variability and high sensitivity of detection, several cell lines were evaluated as reporter for AAV2-Fluc and AAV8-GFP quantification. An optimal reporter cell line

would not only allow for a high sensitivity of detection, but also would allow for an accurate detection over a range of sample dilutions. Four cell lines were assessed: HEK293, HEK293T, Hela, and HT1080. Both AAV serotypes were produced and purified using commercial purification kit. All four abovementioned cell lines were transduced at various dilutions and the transgene signal was quantified 72 h post transduction for AAV2 and 96 h post transduction for AAV8.

While all four cell lines showed response to AAV2 transduction, use of HEK293T allowed for the highest sensitivity of detection of -Fluc transgene. Similarly to AAV2, AAV8-GFP was quantified with the highest sensitivity in HEK293T cell line (Figure 3.6 A and B). Overall, HEK293T was found to be the optimal cell line for detection of both AAV2 and AAV8.

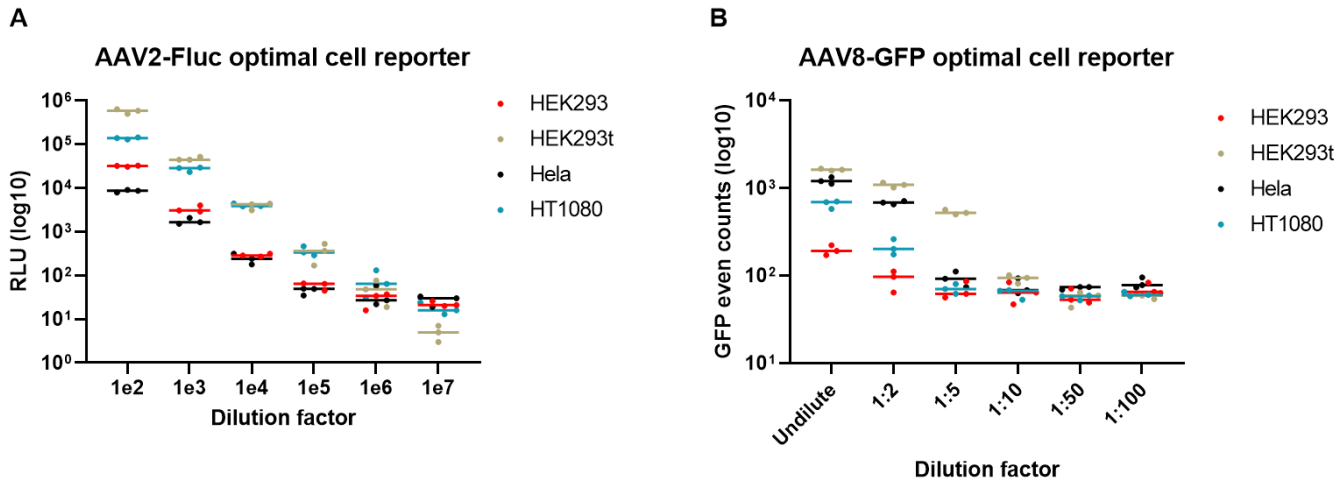


Figure 3.6 HEK293T is the optimal reporter cell line for both AAV2 and AAV8.

Transduction quantification of AAV2-Fluc and AAV8-GFP in four cell lines. Both AAV2-Fluc and AAV8-GFP were produced and purified using commercial purification kit. HEK293, HEK293T, Hela, and HT1080 were transduced with the purified samples of AAV2 and AAV8 at various dilutions. The transgene signals were quantified 72 h post transduction for AAV2 and 96 h post transduction for AAV8. (A) Transduction quantification of concentrated AAV2-Fluc. HEK293, HEK293T, Hela and HT1080 were transduced with the 10-fold diluted samples of AAV2-Fluc. Dots represent n = 3 technical replicates. (B) Transduction quantification of concentrated AAV8-GFP. HEK293, HEK293T, Hela and HT1080 were transduced with the 10-fold diluted samples of AAV2-Fluc. Dots represent n = 3 technical replicates.

3.7 Optimal AAV2 transduction dilution and read time

After optimizing the reporter cell line described in Figure 3.6, transduction parameters, such as dilution factor and transduction read time were evaluated. Optimal transduction read time would allow for an accurate detection of AAV2 and the optimal transduction dilution would allow for optimal detection of crude AAV2 samples within the linear range of the standard curve. HEK293 were triple transfected with AAV2-Fluc, and the virus was harvested 72 h post transfection. HEK293T were transduced at several dilutions and the transgene signal was read at various timepoints.

For AAV2-Fluc, 48 h post transduction read time resulted in higher RLU, but 72 h read time allowed for a lower variability of 12.7% compared to 14% of 48 h, making 72 h transduction read time optimal time of AAV2-Fluc transduction quantification (Figure 3.7 A). For optimal transduction dilution of AAV2-Fluc crude samples, 1:10 dilution was found to be optimal allowing for low variability of detection of 11%. Additionally, 1:10 transduction sample dilution allowed for accurate quantification of samples throughput various orders of magnitude of the standard curve while staying within the linear range (Figure 3.7 B and C).

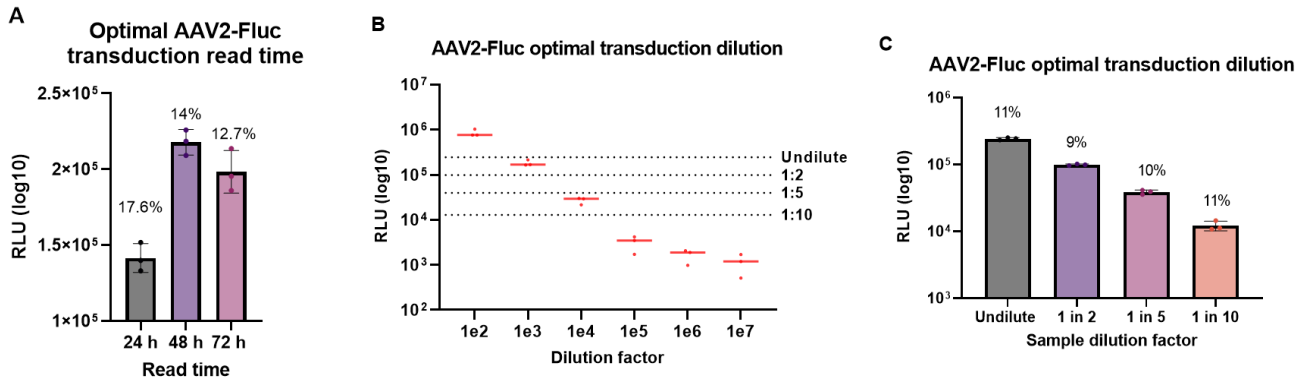


Figure 3.7 Optimization of transduction parameters of AAV2-Fluc.

Transduction quantification of various dilutions of AAV2-Fluc at different transduction read times. HEK293 were triple transfected with AAV2-Fluc and the virus was harvested 72 h post transfection. HEK293T were transduced with 1:10 diluted crude samples of AAV2-Fluc and the transgene signal was quantified 24 h, 48 h, and 72 h post transduction (Figure A). HEK293T were transduced with undilute, 1:2, 1:5, and 1:10 diluted crude samples of AAV2-Fluc represented by dotted lines (Figure B, C). Additionally, HEK293T were transduced with standard curve - purified AAV2-Fluc at various dilutions represented in red (Figure B). (A) Transduction quantification of AAV2-Fluc read at different time points. HEK293 were used to produce the virus and HEK293T were transduced at 1:10 sample dilution. The transgene signal was quantified 24, 48, and 72 h post transduction. The calculated %CV are 17.6% for 24 h samples, 14% for 48 h samples, and 12.7% for 72 h samples. Dots represent 3 biological replicates (n= 16 technical replicates). (B) Transduction quantification of various dilutions of AAV2-Fluc. The samples were produced in HEK293. For the transduction, crude AAV2-Fluc samples were dilute 1:2, 1:5, 1:10 as well as undilute represented by the horizontal dotted lines. Additionally, HEK293T were transduced with purified concentrated AAV2-Fluc at 10-fold dilution steps represented in red marks. Dots represent 3 biological replicates (n= 16 technical replicates). (C) In-depth look at the transduction quantification of crude AAV2 samples from Figure 3.7 B. The graph shows the variability of each dilution. Dots represent 3 biological replicates (n= 16 technical replicates).

3.8 Optimal AAV8 transduction dilution and read time

Similarly to the Figure 3.6, transduction parameters for AAV8 were assessed. The samples were produced in HEK293, harvested, and HEK293T were transduced at several dilutions and the transgene signal was quantified at various timepoints.

While reading AAV8-GFP, 160 h post transduction read time resulted in the highest yield, however it was decided that the 96 h transduction read time was optimal, as this timepoint provided sufficient signal and low variability making it more amenable timepoint for HTS (Figure 3.8 A). When it comes to the optimal sample dilution for AAV8-GFP transduction, the undilute transduction resulted in lowest variability of 10.3% (Figure 3.8 B). Additionally, use of undiluted samples for AAV8-GFP transduction allowed for a distinct differentiation between the sample signal and the background noise.

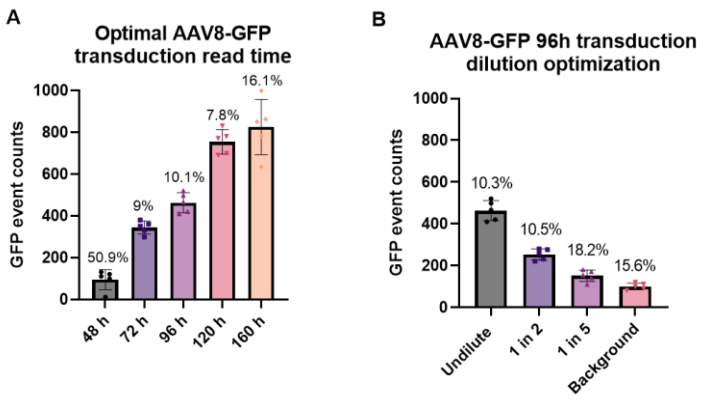


Figure 3.8 Optimization of transduction parameters for AAV8-GFP

HEK293 were triple transfected with AAV8-GFP, and the virus was harvested 96 h post transfection. HEK293T were transduced with undilute crude samples, and the transgene signal was quantified at various times (Figure A). HEK293T were transduced with various dilutions of the crude samples, and the transgene signal was quantified 96 h post transduction (Figure B). (A) Transduction quantification of AAV8-GFP read at different time points. HEK293 were used to produce the virus and HEK293T were transduced with undiluted samples. The transgene signal was quantified in 48, 72, 96, 120 and 160 h post transduction. The calculated %CV are 50.6% for 48 h samples, 9% for 72 h samples, 10.1% for 96 h samples, 7.8% for 120 h samples, and 16.1% for samples read 160 h post transduction. Dots represent n = 5 technical replicates. (B) Transduction quantification of various dilutions of AAV8-GFP. The samples were produced in HEK293. For the transduction, HEK293T were transduced with undiluted crude samples, 1:2, 1:5 diluted, and the undilute background were assessed. Dots represent n = 5 technical replicates.

3.9 AAV production and transduction robustness test

In this experiment the variability of the proposed assay using the optimized parameters determined above was assessed. See Figure 1 for a schematic description of the robustness test experiment. Briefly, four microplates (A-D) with HEK293 were transfected with AAV2-Fluc and harvested 72 h post transfection. The samples from each microplate were transferred into 4 separate microplates (A1, A2, A3, A4) to be sealed and frozen creating 16 daughter plates. Every week a set of microplates was thawed (A1, B1, C1, D1) and HEK293T were transduced at 1:10 sample dilution to assess production and transduction variability.

The experimental setup allowed to assess not only the assays transfection variability, but also the variability associated with the transduction. The overall %CV was in the range of 14-16% which is below the set threshold %CV of 20%. When looking at the results shown on the Figure 3.9 A-D, a similar trend can be observed with the plate D1-4 being lower throughout all four validation runs compared to the rest of the samples. Additionally, we observed that the daughter plates from the same transfection plates have a similar variability throughout the course of transduction validation signifying the transduction robustness. These results confirm the robustness of the developed assay.

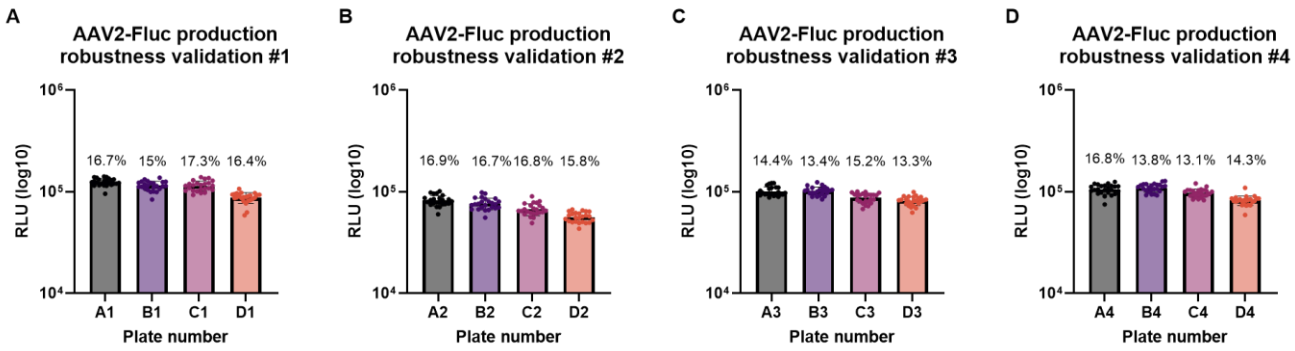


Figure 3.9 The developed method AAV production and transduction was found to be reliable and with low degree of variability.

Transduction quantification of AAV2-Fluc. Four 96-well microplates with HEK293 were triple transfected with AAV2-Fluc and in 72 h the samples were freeze-thawed three times. Samples from every production plate were aliquoted into four separate plates to be frozen. Every week for four weeks, four plates were thawed and HEK293T were transduced with samples dilute 1:10. The transgene signal was quantified 72 h post transduction. (A-D) Transduction quantification of AAV2-Fluc samples generated in the scope of AAV production and transduction robustness experiment. The numbers on bars represent the %CV. Dots represent n = 24 technical replicates.

3.10 Use of Assay for screening of small molecules

To test the assay for discovery of small molecule enhancers of AAV production, the developed assay was used to evaluate a molecule which had been previously identified to enhance AAV production in several HEK platforms (VS-003, Virica Biotech). Three timings of compound

addition were evaluated: 4 h pre-transfection, co-transfection and 18 h post transfection drug treatment. HEK293 were transfected with AAV2-Fluc and treated with VS003 at various concentrations and timepoints. Additionally, for every tested treatment time the baseline virus production of untreated transfected HEK293 was assessed. The virus was harvested and HEK293T were transduced with 1:10 diluted samples.

When looking at the 4 h pre-transfection treatment results, it can be seen that VS003 has a bell-curve shaped impact on AAV2-Fluc transfection from 5-35 uM, with 50 uM demonstrating no increase on AAV2-Fluc production (Figure 3.10 A). Interestingly, co-transfection treatment results showed a similar trend, but unlike in the 4 h pre-treatment experiment, the highest treated concentration positively impacted the virus production (Figure 3.10 B). Additionally, there is a visible dose-dependent impact of VS003 on AAV2-Fluc transfection enhancement with cells treated 18 h post transfection: with the increase of the drug concentration the viral output increases as well (Figure 3.10 C).

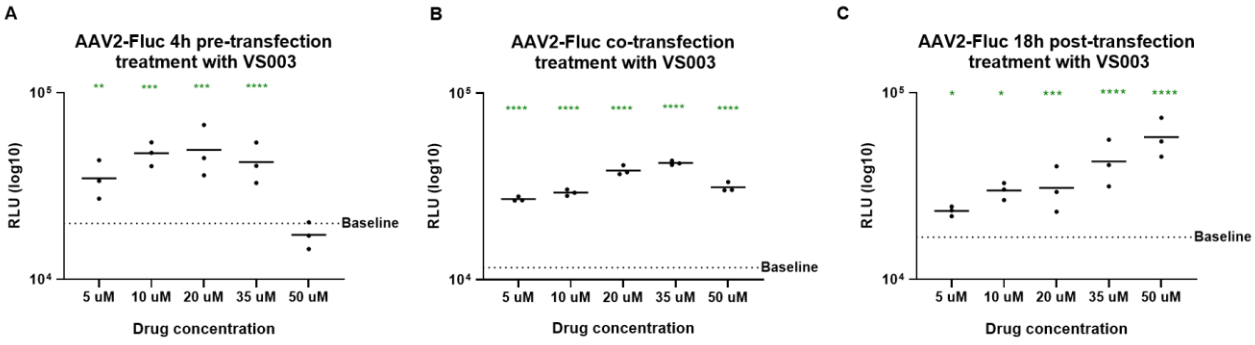


Figure 3.10 Co-transfection drug treatment is the optimal treatment time to screen for AAV2-Fluc production enhancers.

HEK293 were treated with VS003 4 h pre-transfection, co-transfection, and 18 h post transfection treated, and the treated cells were triple transfected with AAV2-Fluc. The virus was harvested 72 h post transfection and freeze-thawed three times. HEK293T were transduced with 1:10 diluted samples and the transgene signal was quantified 72 h post transduction. (A) Transduction quantification of HEK293 4 h pre-transfection treated with VS003 and transfected with AAV2-

Fluc. HEK293T cells were transduced at 1:10 dilution and the transgene signal was quantified 72 h post transduction; dots represent 3 biological replicates (mean of n = 8 technical replicates). (B) Transduction quantification of HEK293 co-transfection treated with VS003 and transfected with AAV2-Fluc. HEK293T cells were transduced at 1:10 dilution and the transgene signal was quantified 72 h post transduction; dots represent 3 biological replicates (mean of n = 8 technical replicates). (C) Transduction quantification of HEK293 transfected with AAV2-Fluc and 18 h post-transfection treated with VS003. HEK293T cells were transduced at 1:10 dilution and the transgene signal was quantified 72 h post transduction; dots represent 3 biological replicates (mean of n = 8 technical replicates).

3.11 Validation of the high-throughput screen

To validate the proposed high-throughput screening assay targeting AAV production enhancers, 9 small molecules were evaluated for their ability to boost production of AAV2. HEK293 were transfected with AAV2-Fluc, and co-transfection treated with various concentrations of 8 of Virica Biotech's VSE molecules, which are known to suppress innate anti-viral pathways, (VS001, VS002, VS003, VS004, VS005, VS006, VS0011, VS0041) and Pevonedistat which inhibits the neddylation pathway. The virus was harvested 72 h post transfection and HEK293T were transduced with 1:10 diluted crude samples.

As can be seen on Figure 3.11, not all compounds showed AAV production boosting capabilities. VS001 had no significant impact on AAV2 at 20 uM but all higher concentrations significantly decreased amount of produced virus when compared to the baseline (Figure 3.11 A). When it comes to VS002, it showed a boost of AAV production over a broad concentration range (Figure 3.11 B). VS003 had a positive impact on AAV2 production enhancing the viral output up to 2-fold when treated at 2 uM (Figure 3.11 C). Similarly to VS001, VS005 showed non-significant impact on AAV2 at low concentrations but all higher concentrations significantly decreased amount of produced virus when compared to the baseline (Figure 3.11 E). Treatment of HEK293 with other compounds such as VS004, VS006, VS0011 and VS0041 did not have negative impact

on the amount of produced virus when compared to the baseline production (Figure 3.11 D, F, G, H). Interestingly, co-transfection treatment with Pevonedistat showed a great enhancement of virus output up to 5-fold at a broad range of concentrations (Figure 3.11 I).

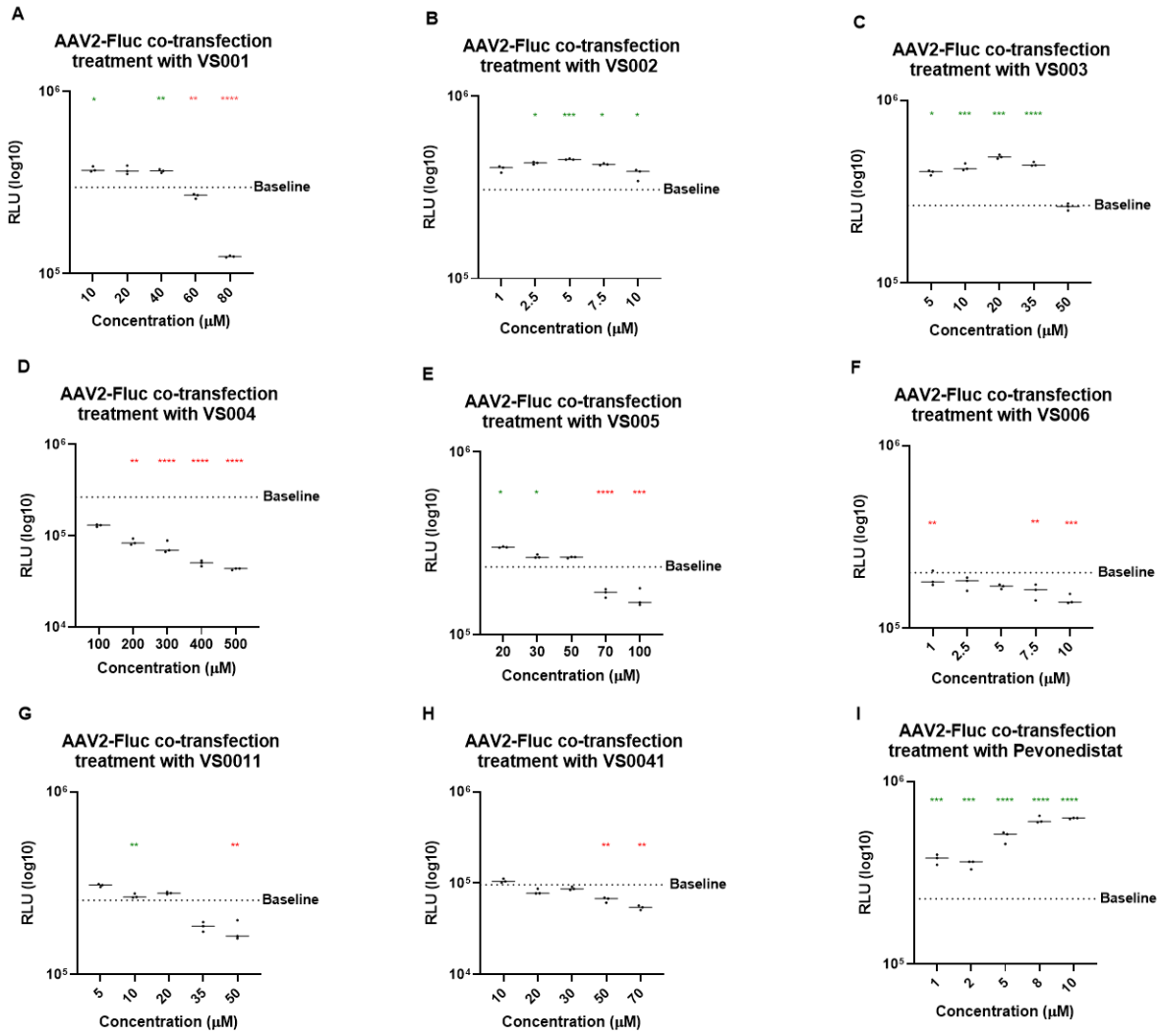


Figure 3.11 Co-transfection drug treatment identifies potential transfection enhancers for AAV2-Fluc.

HEK293 were triple transfected with AAV2-Fluc and co-transfection treated with various compounds. 72h post transfection the samples were harvested via three freeze-thaws. HEK293T were transduced with 1:10 diluted samples and the transgene signal was quantified 72 h post transduction. (A-I) Transduction quantification of HEK293 transfected with AAV2-Fluc and co-transfection treated with chemicals looking for their ability to enhance AAV production. Cells were transfected, treated with VS001, VS002, VS003, VS004, VS005, VS006, VS0011, VS0041 and

Pevonedistat and the virus was harvested 72 h post transfection. HEK293T were transduced with 1:10 dilute crude samples. The baseline represents the transduction of HEK293T with samples harvested from transfected untreated HEK293. Dots represent 3 biological replicates (mean of n = 8 technical replicates).

3.12 Using drug-drug combination for AAV production enhancement

Next the optimized assay was used to assess drug-drug combination on AAV production driven by a possibility of either additive or synergistic enhancement effects. HEK293 cells were transfected with AAV2-Fluc and AAV8-GFP and co-transfection treated with various concentrations and combinations of VS003 and Pevonedistat. The virus was harvested and HEK293T were transduced with 1:10 sample dilution for AAV2 and transduced undiluted for AAV8.

When looking at the impact of VS003 and Pevonedistat on AAV2 transfection, it can be seen that both drugs separately enhanced virus production to the same degree when compared to the baseline (Figure 3.12 A). When combined, the drug combo showed the same enhancement as the samples treated with either Pevonedistat or VS003. Interestingly, the samples treated with a combination of Pevonedistat 5 uM and VS003 20 uM did not show transfection enhancement staying on the baseline level.

When it comes to AAV8, Pevonedistat alone showed a significant production enhancement compared to the baseline. On the other hand, VS003 did not show any enhancement of AAV production, in fact the transduction results of the AAV8-GFP harvested from the cells treated with VS003 alone were lower than the baseline (Figure 3.12 B). The drug combination showed the same enhancement as the samples treated with the Pevonedistat only.

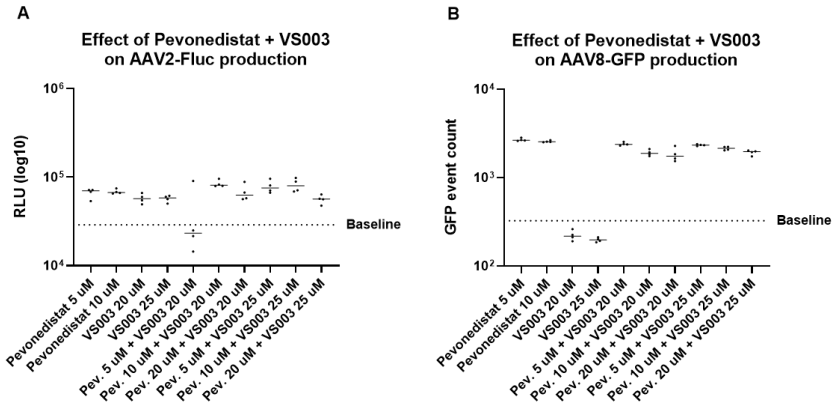


Figure 3.12 Transduction quantification of the impact of VS003 and Pevonedistat drug combination on AAV2-Fluc production.

HEK293 were transfected with AAV2-Fluc and AAV8-GFP and co-transfection treated with various concentrations and combinations of VS003 and Pevonedistat. AAV2 and AAV8 samples were harvested 72 h and 96 h post transfection respectively via three times freeze-thaw. HEK293T were transduced with undiluted AAV8 samples and 1:10 diluted AAV2 samples and the transgene signals were quantified in 96 h and 72 h post transduction respectively. (A) Transduction quantification of HEK293 transfected with AAV2-Fluc and co-transfection treated with various concentrations and combinations of VS003 and Pevonedistat. The virus was harvested 72 h post transfection. HEK293T were transduced with 1:10 dilute crude samples n = 4 (technical replicates). The baseline represents the transduction of HEK293T with samples harvested from transfected untreated HEK293. (B) Transduction quantification of HEK293 transfected with AAV8-GFP and co-transfection treated with various concentrations and combinations of VS003 and Pevonedistat. The virus was harvested 96 h post transfection. HEK293T were transduced undiluted crude samples n = 4 (technical replicates). The baseline represents the transduction of HEK293T with samples harvested from transfected untreated HEK293.

3.13 Use of transduction enhancers boosts AAV transduction

Capitalizing on the generation of both production and transduction assays, the transduction component of the HTS assay was assessed for its ability to identify AAV transduction enhancers. To address this question, HEK293T cell were treated with various compounds for 4 h pre-transduction: VS003, VS005, Pevonedistat, and Hydroxyurea. At the time of transduction, the media swap was performed, and the cells were transduced with diluted sample of purified and concentrated AAV2-Fluc and AAV8-GFP. There were several parameters tested: impact of various

concentrations of the compounds on AAV transduction, untreated transduction baseline control as well as the background control for the AAV8-GFP.

As it can be seen on the Figure 3.13 A-D there is a distinct impact of chemicals on AAV2-Fluc transduction efficiency. VS003 showed a dose-dependent increase in the transduction when compared to the baseline (Figure 3.13 A). VS005 however did not show transduction enhancement capabilities in AAV2-Fluc context with treated concentrations showing the same level of transduction as the baseline control (Figure 3.13 B). On the other hand, both Pevonedistat and Hydroxyurea (a known transduction enhancer) showed a boost of transduction throughout the whole panel of treated concentrations (Figure 3.13 C and D). Notably, Pevonedistat had a stronger impact on AAV2-Fluc transduction compared to both VS003 and Hydroxyurea surpassing the mark of 1e6 RLU in samples treated above 40 uM.

AAV8-GFP was also affected by the chemicals. VS003 had minor transduction enhancement effect on AAV8-Fluc when compared to the baseline control (Figure 3.13 E). VS005 however did not show significant transduction enhancements capabilities when used in the context of AAV8 (Figure 3.13 F). Interestingly, both Pevonedistat and Hydroxyurea had a strong transduction enhancing impact on AAV8 (Figure 3.13 G and H). Notably, the background control for all conditions was significantly lower compared to the treated samples as well as the baseline control.

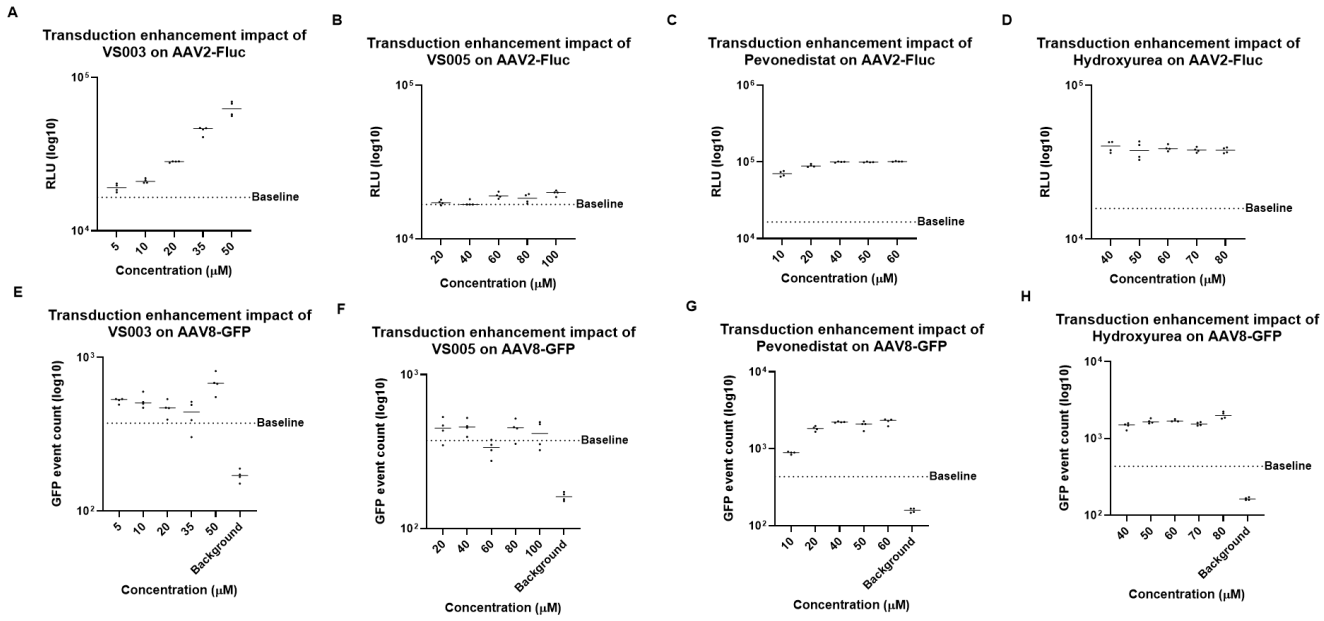


Figure 3.13 Determination of the impact of transduction enhancers on transduction of AAV2 and AAV8.

HEK293T were 4 h pre-transduction treated with various compounds. The culture media was replaced 4 h after the treatment and the cells were transduced with AAV2-Fluc and AAV8-GFP. The transgene signal was quantified 72 h and 96 h post transduction for AAV2 and AAV8 respectively. (A-D) Transduction quantification of HEK293T treated with chemical compounds at various concentrations and transduced with AAV2-Fluc. HEK293T were 4 h pre-transduction treated with VS003, VS005, Pevonedistat, and Hydroxyurea (A-D respectively). At the time of transduction, the media was changed, and the cells were transduced with dilute sample of purified AAV2-Fluc, n = 4 (technical replicates). The baseline represents the transduction of untreated HEK293T. (E-H) Transduction quantification of HEK293T treated with chemical compounds at various concentrations and transduced with AAV8-GFP. HEK293T were 4 h pre-transduction treated with VS003, VS005, Pevonedistat, and Hydroxyurea (E-H respectively). At the time of transduction, the media was changed, and the cells were transduced with dilute sample of purified AAV8-GFP, n = 4 (technical replicates). The baseline represents the transduction of untreated HEK293T. The background represents the background noise quantification acquired by transducing wells of a microplate without cells.

3.14 Drug carryover and impact on transduction

Given the transduction enhancing capabilities of compounds described in Figure 3.13, the potential of drug carryover from the production drug treatment into the transduction assay was assessed. The experiment was performed on untransfected HEK293 treated with VS002, VS003, VS006 and Pevonedistat. See materials and methods for in-depth protocol. Briefly, HEK293 were treated with the chemical compounds, at 72 h the cells were freeze-thawed, and samples were collected – these samples were labeled 72 h samples. HEK293T were treated for 4 h pre-transduction with two types of samples: 72 h samples and freshly diluted chemical compounds. 4 h post drug treatment, the media was changed, and the cells were transduced with diluted concentrated purified stocks of AAV2-Fluc.

This experiment suggests that some chemical compounds might be carried over and have a transduction enhancing effect. Figures 3.14 A-C suggest that while VS002, VS003 and VS006 can be used as transduction enhancer, they do not get carried over to the transduction when used as per the HTS assay (incubation on cells for 72 hours with 3X freeze-thaw). Interestingly, Pevonedistat 72 h samples showed transduction enhancing capabilities throughout the whole panel of concentrations. The freeze-thawed samples resulted in decreased transduction enhancing capabilities when compared to treatment with the freshly diluted chemical compound. Out of all four tested compounds, only Pevonedistat was carried over to the transduction.

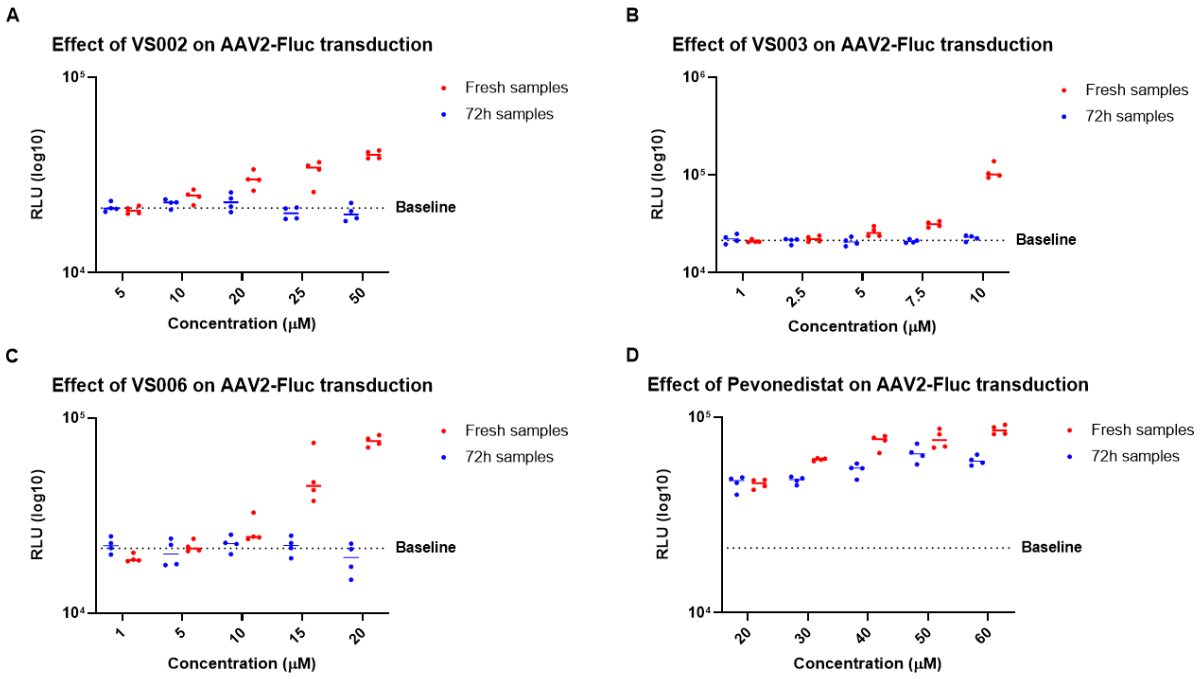


Figure 3.14 Determination of the transduction enhancing impact of the drug carryover.

Untransfected HEK293 were treated with various chemical compounds and in 72 h the cells were freeze-thawed three times. HEK293T were treated with harvested samples and freshly prepared chemical compounds. In four hours, the media was changed, and the cells were transduced with dilute stocks of purified AAV2-Fluc. The transgene signal was quantified 72 h post transduction. (A-D) Transduction quantification of HEK293T 4 h pre-transduction treated with VS002, VS003, VS006 and Pevonedistat. 72 h samples represent supernatants collected from untransfected HEK293 treated with chemical compounds and harvested 72 h post treatment with 3 cycles of freeze-thawing. Fresh samples represent samples prepared at the time of transduction drug treatment. At the time of transduction, the media was changed, and the cells were transduced with diluted concentrated purified stocks of AAV2-Fluc n = 4 (technical replicates).

3.15 Effect of AAV2 storage at 4C° for extended period

Considering the need for continual use of a purified standard curve stock for HTS screening, the stability of purified AAV was assessed when stored at 4C° for an extended period of time. To address this question, HEK293T cells were transduced with purified concentrated AAV2-Fluc stored at 4C° for various periods of time.

The comparison between concentrated purified stocks of AAV2-Fluc stored for 0-16 weeks at 4C° can be seen on Figure 3.16 A. There is a notable decrease in potency associated with 4C°

storage. The potency shift caused by short to long term 4°C storage is seen in Figure 3.16 B There is a notable difference between the freshest and oldest virus stock. After calculating, it was determined that AAV2-Fluc on average loses 1e5 RLU/week while being stored at 4C°.

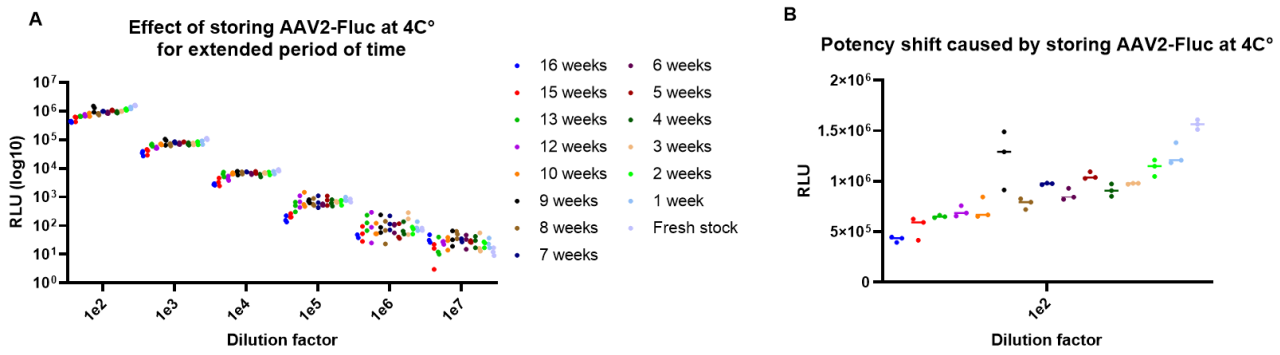


Figure 3.15 Determination of the transduction enhancing impact of the drug carryover.

Transduction quantification of AAV2-Fluc samples sored in 4C° for extended period of time. Purified concentrated AAV2-Fluc stocks were stored in 4C° for various periods of time and HEK293T were transduced with the samples at various dilutions. The transgene signal was quantified 72 h post transduction. (A) Transduction quantification of HEK293T transduced with purified concentrated AAV2-Fluc samples stored at 4C° for various periods of time, n = 3 (technical replicates). (B) In-depth look at the effect of storing AAV-Fluc at 4C° from Figure 3.11 A. The graph shows the variability of the samples focusing only on the highest transduction dilution, n = 3 (technical replicates).

Chapter 4: Discussion

Throughout the course of this project, the goal was to develop an AAV production and quantification assay that would achieve a final %CV of below 20%, to enable high-throughput screening and/or DoE efforts. Aimed by that, we set to optimize the production and quantification parameters of AAV. The goal was to establish an assay that would allow for robust and effective method of screening for AAV production enhancers.

4.1 Transgene optimization

Pursuing low variability and high sensitivity of detection, several reporter transgenes were assessed for both AAV2 and AAV8. An ideal transgene would have a distinct difference between the sample and the background signals over several orders of magnitude. AAV2-scNluc was found to be extremely sensitive allowing for significant sample dilution and good signal-to-noise characteristics (Figure 3.1 A). However, the technical complications as well as the cost of the nLuc substrate were not ideal for high-throughput screening, which requires easy-to-use and low-cost reagents. The GFP transgene performed well, however the detection time was found to be rather slow taking 9 minutes per 96-well plate and coupled with a less-than-ideal dynamic range. The AAV2-Fluc was determined to be the optimal transgene: it takes 4 minutes per microplate, good signa-to-noise ratio, and overall -Fluc signal is very sensitive making it necessary to dilute the samples 1:10 for the transduction.

To optimize AAV8 detection, four transgenes were compared: -scnLuc, -nluc, -Fluc, and -GFP. Due to AAV8's secreted nature and abundance in the medium, and the high stability and sensitivity of nLuc, there were significant issues with background carry-over of both scnLuc- and nLuc in non-purified AAV samples (data not shown) [35]. Only -GFP transgene allowed for good

separation between the sample signal and the background since the GFP assay relied on event count rather than acquiring a total fluorescence (which was indistinguishable from background).

Split reporter assays would be a potential solution to the sample carryover issue faced with the -nLuc and -scnLuc constructs. In such assays, the reporter protein is split into complimentary fragments that are designed to have minimal to no activity when they are separated. The reporter cell line is stably expressing one of the fragments that cannot generate a detectible signal on its own. The desired AAV transgene is designed to encode for the complimentary fragment that is synthesized as a product of transduction. Both complimentary fragments can interact in a close proximity restoring the functional reporter protein [36]. Depending on the reporter protein used, the detection signal can be in various forms, such as fluorescence or luminescence. This approach would be very effective for the detection of secreted AAV serotypes such as AAV8.

4.2 AAV production optimization

The next step step of optimization was focused on the optimal producer cell line. Commonly used AAV producer cell lines in the industry are immortalized Human Embryonic Kidney 293 cells (HEK293) and HEK293T. Immortalized cervical cancer cell line Hela is also used in the industry for AAV production, however for this cell line the AAV-HSV coinfection approach is used [16]. Additionally, HT1080 was assessed for AAV production. HT1080 is fibrosarcoma cell line that is commonly used for AAV transduction [37]. When choosing the best suited cell line for production of AAV in the high-throughput screening format, it is important to keep in mind the overall goal of the project. After establishing and eventually performing a screen targeting AAV production enhancers, the goal is to use the successful candidates in industrial settings. AAV is commonly produced in bioreactors with all parameters optimized to yield the highest possible amount of virus produced [16]. In those settings, it is important to use compounds

that will help with virus production even in the most saturated systems. Guided by this idea, we wanted to identify the cell line that would yield the highest quantity of AAV making the compounds screened in such platform relevant to the optimized industrial manufacturing processes.

AAV vectors require co-infection with a helper virus such as HSV or expression of helper proteins provided either by the plasmid during the transfection or by the cell lines. It was expected for both HEK293 and HEK293T to be the optimal producers of AAV not only because these cell lines have high transfection efficiency, but also because both HEK293 and HEK293T stably express Adenovirus 5 DNA. For proper rAAV production it is required to have all the helper genes including E1A, E1B, VA, E2A or E4. HEK293 and their derivatives such as HEK293T cells, stably express E1A and E1B proteins which are essential for AAV production [16]. It was expected that both HEK293 and HEK293T would be good producers of AAV since the helper plasmid only encodes for E2A, E4 and VA genes. After comparing all four cell lines, it was apparent that HEK293 yielded the highest amount of AAV2 and AAV8 produced making it the optimal produced cell line in the 96-well microplate format (Figure 2).

When AAV is produced in bioreactors, the average yield of AAV vector genomes (vg) is in a range of 1e4-1e5 vg/cell from crude harvest [16], [38]. We performed qPCR titration of AAV harvested from HEK293 and remarkably the small-scale production of AAV in HEK293 on average yielded 4e4 vg/cell in line with industrial processes.

4.3 Harvest optimization

4.3.1 Harvest time optimization

After determining the optimal cell producer for AAV2-Fluc and AAV8-GFP, optimal time of harvest was established. Both AAV2-GFP and AAV2-Fluc performed very similarly peaking at

72 h post transfection. On the other hand, AAV8-GFP did not reach the plateau even 120 h post transfection. This is expected since the cells are increasing the amount of AAV produced with time. It is important to keep in mind that the overall quality of the produced virus is expected to decrease with time since the producer cells have more time to activate the antiviral mechanisms.

These findings correlate to the literature as it is common practice to harvest AAV after 48-72 h post transfection [38]. In large scale, it is important to harvest rAAV within that window. While prolonged production comes with the benefit of higher virus titer, it comes with disadvantage of unwanted truncated and/or empty AAV capsids.

4.3.2 Optimal method of harvest

When it comes to the optimal method of harvesting, several methods were evaluated including physical (freeze/thaw) and chemical (lysis buffer) methods. In the industry, a common method of harvesting AAV is via implementing AAV lysis buffer. While exact ingredients are not disclosed, most of these formulations are detergent based. In the industrial settings, a commonly used method of cell lysis in large flasks and bioreactors is via addition of the detergents such as Tween-20 or Triton X-100 which lyses the cells releasing AAV in the media [38]. The next step is column separation where AAV binds to the affinity column allowing it to be thoroughly washed ensuring all the lysis buffer is removed. In the case of a high-throughput screen, it is impossible to wash every well of the microplate to remove the buffer. After treating the cells with the various concentrations of the lysis buffer, HEK293T cells were transduced with the samples. Interestingly, all samples containing lysis buffer had lower signal compared to the samples freeze-thawed in -80C° suggesting lysis buffer carryover to the transduction and affecting the reporter cells (Figure 4A). Given the AAV2 transgene optimization data (Figure 3.1 C), the AAV2-Fluc background was approximately 1e4 RLU which was found to be similar when lysis buffer was used to release the

virus particles across all dilutions tested (Figure 3.4 A). Additionally, after visually examining the transduced HEK293T cells with samples containing lysis buffer, it was found that the reporter cells were lysed. Given the similar transduction results compared to AAV2-Fluc background data and the visual observation, it is safe to say that the quantified signal is nothing else but -Fluc background carryover.

For physical freeze/thaw, both -80C° and Liquid Nitrogen methods of freeze-thawing were assessed with the thought that the latter would speed up the process allowing for a faster rate of plate processing. However, freezing the microplates in the -80C° freezer is not only faster compared to Liquid Nitrogen, but also easier since the Liquid Nitrogen requires quite a sophisticated setup and constant refinement. It is noteworthy that AAV8 is predominantly found in media with only fraction of the produced virus staying in the cells [35]. In our hands, we found that while majority of AAV8 is indeed in media, there was a substantial amount of virus left in the cells. Aiming to maximize the quantity of produced AAV8, we were performing cycles of freeze-thawing to harvest as much virus as possible.

Previous research done by Vandenberghe et al. focused on evaluating the impact of serum in media on distribution of various serotypes in media or cell lysates [39]. While the majority of tested serotypes had higher yields when produced in serum containing media, AAV5 yielded much higher results when produced in serum starved cells. Similarly to our observations, AAV2 was predominantly lysate associated and the majority of AAV8 was found in medium [39].

When we investigated the required number of freeze-thaw cycles, it was found that 3 cycles were the optimal condition due to the lowest variability of 11.9% when compared to one and two-time freeze-thaws. Interestingly, 2x F/T condition showed higher transduction results compared to one and three-time freeze-thaw cycles (Figure 3.4 B). This is caused by not having all the virus

released by one freeze-thaw cycle and by the harmful nature of three cycles of freeze-thawing on the AAV capsids resulting in decrease in titer. However, it was determined that the lower variability seen with the 3X freeze thaw was worth the slight loss in signal associated with the extra round of physical disruption.

To address the question of the harm of freeze-thawing associated stress on AAV, purified AAV2-Fluc was subjected to multiple cycles of freeze-thawing and quantified by transduction assay. This experiment was performed in the light of data discovered and shown in Figure 3.4 B. Of concern was the harmful nature of the freeze-thawing on the AAV capsid. As shown in Figure 3.5, there is moderate, yet notable impact of freeze-thawing on the transduction results of the AAV. Historically, both enveloped and non-enveloped DNA viruses tend to be more resilient to freeze-thawing compared to RNA viruses. RNA viruses tend to undergo more severe structural changes and degradation caused by freeze-thawing stress [40].

4.4 AAV detection optimization

When looking for the optimal reporter cell line, four cell lines were compared: HEK293, HEK293T, Hela and HT1080. HEK293T are a derivative from HEK293 cells that have been modified to express the SV40 T antigen allowing them not only to produce AAV more efficiently, but also making them a good option for AAV transduction [16]. Interestingly, Hela was found to be the second-best cell line for AAV8 detection while it was found to be the least favorable cell line for AAV2 detection (Figure 3.6). Hela expresses heparin sulphate proteoglycans receptors and both AAV2 and AAV8 are known to use the abovementioned receptors for cellular entry [16]. Additionally, fibrosarcoma cell line HT1080 is commonly used for AAV transduction [37].

After determining HEK293T as an optimal reporter cell line for both AAV2 and AAV8, optimal transduction parameters such as dilution and transduction read time were assessed. Even though AAV2-Fluc transduction peaked at 48 h, the lowest variability was observed at 72 h post transduction granting this time point as optimal read time for AAV2-Fluc (Figure 3.7). When it comes to the optimal sample dilution, even though all tested dilutions could be accurately detected within the standard curve, the optimal sample dilution for the transduction was 1:10. In hopes of potential compounds enhancing AAV transfection up to 10-fold or even more, the assay needed to be able to detect and quantify those hits. Using undiluted transduction dilution would leave only a narrow window of concentrations that would be quantifiable within the linear range of the standard curve. The lowest dilution on the other hand allowed for the detection of a potential 20-fold production enhancement (Figure 3.7). Additionally, 1:10 transduction sample dilution variability of 11% was passing the set goal of assay variability below 20%.

When it comes to optimal time and dilution for AAV8 transduction, the data indicated that with increasing transduction time the AAV8-GFP fluorescence signal kept increasing. For the highest transduction results 160 h post transduction read time could be used (Figure 3.8). However, core principles of high-throughput screen are being able to screen chemicals in a timely fashion which impacted the decision of detection and quantification of AAV8 at 96 h post transduction. After comparing various transduction dilutions, it was found that undiluted transduction was the optimal method of AAV8 transduction.

4.4.1 Alternative approach to achieve transduction enhancement

Chen et al. discovered an innovative method of boosting transduction efficiency of AAV [41]. Their method revolves around freezing the reporter cells in a freezing medium containing AAV vectors. They stored the cells at −80°C for 6h and froze in liquid nitrogen for 16 h. After

thawing the cells, they were placed in microplates in the incubator at 37°C for further culture [41]. Later, the cells were transduced with the same virus and the same MOI but excluding freeze-thaw step. This method allows for an increase of AAVs transduction efficiency in human aortic endothelial cells up to 23-fold and in human renal proximal tubular epithelial cells up to 128-fold [41]. This approach might help with the problem of poor AAV8 transduction efficiency. HEK293T can be produced in T175 flasks, transduced with AAV8 and frozen in cryogenic medium in multiple vials according to the abovementioned paper. A week before the screen, the cells can be thawed, passaged, and seeded in the microplates. After transfecting, treating, and harvesting the virus, the freeze-thawed cells can be transduced further boosting the transduction. Before it is implemented, this method will need to be firmly tested to determine the variability associated with the use of transduced freeze-thawed cells.

4.5 Robustness validation of the developed assay

After optimizing the production and detection of AAV, the overall variability of the developed assay was evaluated and benchmarked against the stated goal of below 20% CV. The production and transduction robustness experiment as described in Figure 3.9 was used to assess the variability of every step of this assay. Looking at Figure 3.9, it can be seen that the trends between all four validation runs remain the same: transduction results of plates D1-D4 showed consistently lowest results. Additionally, the overall variability between the transduction and production parameters on average was 15.3% which is much lower than our set threshold of 20%. While the average transduction was variable between the validation runs, the overall variability was consistent. In cell-based high-throughput screens it is a common practice to freeze large quantities of the reporter cells to be thawed before each run. In those instances, the cells are thawed to be passaged twice before being seeded in the microplates. This approach allows us to control

the passage number of the reporter cells used in each screen. In the experiments described here, this practice was not observed, as the same cell population was used at multiple passages throughout the course of the robustness test.

4.6 Cellular mechanisms of antiviral defence throughout the course of AAV production

4.6.1 Pre-transfection drug treatment

One last piece that required the optimization was the optimal time of drug treatment. There is no such thing as the ideal treatment time since the optimal drug addition time depends on the mechanism of action of the chosen compound. The cellular antiviral mechanisms are instantly activated in response to transfection and peak approximately 6 h post transfection. This takes place in the form of increase of INFB1, CCL5, IL8, cAMP and many others [42]. Both cAMP and INFB1 expressions are upregulated throughout the course of the transfection. It is expected that the compounds that affect the abovementioned cellular antiviral responses would have an impact on the viral yield. These pathways can be downregulated with the chemical treatment either during the time of transfection or before the transfection which is why we tested 4 h pre-transfection drug treatment. VS003 showed a statistically significant dose-dependent increase in production of AAV2-Fluc.

It was discovered that EGR1 expression is significantly upregulated within the first 12 h of the transfection period [42]. EGR1 is known for its role in cellular stress response, transcriptional regulation and immune response by activation of various immune cells [42]. Interestingly, EGR1 is not expressed by the producer cell lines 24 h post transfection. This signifies

the importance of the drug treatment time: compounds affecting EGR1 might have little impact on the viral yield when treated after the 24 h mark.

4.6.2 Co-transfection drug treatment

Using co-transfection treatment not only resulted in the most significant outcome, but also was the least technologically challenging approach. Co-transfection drug treatment resulted in the most statistically significant outcome when compared to 4 h pre- and 18 h post-transfection drug treatments. While performing a high-throughput screen, it is crucial to minimize the time needed. Since the cells get transfected and treated simultaneously, this approach not only reduces the time spent per plate, but it is also relevant biologically. As it was previously mentioned, the antiviral response mechanisms peak approximately 6 h post transfection and the compounds that downregulate the immune response are expected to result in boost of AAV production. In this experiment, VS003, a known suppressor of the innate antiviral response, was used.

It is known that AAV can trigger the activation of pattern recognition receptors in host cells escalating the activation of the cellular innate immune responses. There are several different pattern recognition receptors associated with viral infections including TLR – Toll-like receptors; RLSs - RIG-I-like receptors [43]. Additionally, AAV can interact with cytosolic DNA sensors, such as cGAS-STING pathways after cell entry. The interaction between AAV and the abovementioned pattern recognition receptors triggers cytokine and chemokine release, type I interferon response, activation of adaptive immune response and activation of various transcription factors such as NF-kB. As was previously discussed, NF-kB network is involved in the regulation of gene expression. After activation of NF-kB it can promote transcription of IFNFB1 leading to increased IFNB1 production. By downregulating NF-kB we are consequently downregulating its impact on production of IFNB1 which is a known inhibitor of viral replication and enhancer of the immune

response [42]. Knowing the impact of NF-kB activation on AAV, it is safe to hypothesize that other compounds downregulating this pathway would positively impact the overall yield of produced AAV.

Not all the compounds are expected to be present throughout the course of the transfection, it would be safe to assume that some compounds would be either metabolized or degraded. It might be beneficial to assess the impact of the abovementioned compound on cells when treated at lower doses throughout the whole production course.

4.6.3 Post-transfection drug treatment

AAV packaging starts about 12h post transfection when the cellular response to transfection is significantly decreased [16]. Depending on the mechanism of action of the compounds, 18h post transfection treatment is expected to have a significant boost in virus yield. A great example would be sodium butyrate that showed a significant increase in rAAV capsid production when cells are treated 18 h post transfection [44]. This was the rationale for doing 18 h post transfection drug treatment. There are a number of different pathways and cellular antiviral mechanisms that are activated after transfection. IL34 would be a good example, it was discovered that IL34 inflammatory response is upregulated 24 h post transfection [42]. IL34 is responsible for immune regulation and tissue homeostasis, and it was shown to regulate the differentiation and activation of monocytes and macrophages which play a key role in antiviral defense. It would be safe to hypothesize that compounds that downregulate IL34 expression might have a positive impact on the AAV production yields. In this case, VS003 showed a statistically significant dose-dependent increase of the AAV2-Fluc production.

4.7 Impact of VSes on AAV production at various times of drug treatment

Having validated the assay with VS-003, a variety of innate antiviral suppressors (supplied by Virica Biotech) with different mechanisms of action, were assessed at various concentrations and at different times of addition Figure 3.11 represents the co-transfection treatment of HEK293 with nine compounds. Additionally, Supplementary Figure 1 represents 4 h pre-transfection treatment and Supplementary Figure 2 represents 18 h post-transfection treatment.

Of the additional compounds tested, Pevonedistat demonstrated the most robust and consistent results. Pevonedistat, also known as MLN4924, is a small molecule inhibitor of the NEDD8-activating enzyme (NAE). It alters the ubiquitination and proteasomal degradation of cellular proteins [45]. Pevonedistat did not enhance AAV2 production when used prior to transfection – Supplementary Figure 1. However, there was a statistically significant production boost when the cells were treated at time of, or 18 hours-post transfection. When used at the time of transfection, Pevonedistat showed a statistically significant increase in viral output.

In addition to Pevonedistat, VS002 and VS003 showed a statistically significant increase in AAV2-Fluc production. These results were expected since both VS002 and VS003 are impacting a similar pathway (Virica Biotech). It is worth mentioning that both VS002 and VS003 showed statistically significant production enhancing results when the cells were treated 18 h post transfection. When the cells were 4 h pre-transfection treated however, the impact of VS002 and VS003 was statistically significant but the viral output was not enhanced by VS002 and enhanced slightly by VS003. This gives clues about the mechanisms of action within the cell. Both VS002 and VS003 downregulate a key signaling pathway which plays a crucial role in cellular antiviral response. One of the areas where this pathway is involved in the regulation of gene expression. Upon activation of, it can promote transcription of IFNFB1 leading to increased IFNB1

production. By downregulating this pathway, the compounds are consequently downregulating the production of IFNB1 which is a known inhibitor of viral replication and enhancer of the immune response [42].

4.8 Various transfection approaches and their impact on viral yield and the plate variability

It is worth mentioning the impact of the transfection approach on the viral titer. When looking at the baseline production using small scale AAV production protocol, the baseline RLU is approximately 5e5 RLU – Figure 3.11. In this case, the plasmids are combined with the transfection reagent in media and after incubation the media covering the cells is completely replaced by the media containing plasmids. This approach on average shows approximately 12-14%CV and the baseline virus production is approximately 5e5 RLU. Interestingly, when the plates were transfected to initially assess the impact of VS003 on AAV2-Fluc production when treated 4 h pre, co- and 18 h post transfection, the assay needed to be adjusted and used a different method of transfection. In this case, the 4-hour pre-treatment negated the ability to perform a full media swap at time of transfection. Instead, all the necessary plasmids to produce AAV2-Fluc were combined with the transfection reagent to be at 10x concentration, and that concentrated plasmid mix was added to the cells to be at 1x final plasmid concentration – Figure 3.10. While such an approach permitted omitting the media swap, the data indicated that approach to be much more variable compared to a full media transfection swap. In such an approach, the baseline %CV was found to be 30-40% and the baseline virus production was approximately 3e4 RLU compared to 5e5 RLU results of a complete media swap approach. These observations signify the importance of optimizing every step of the protocol for the high-throughput screen.

4.9 Impact of drug combinations on AAV production yield

Intrigued by the production boosting capabilities of Pevonedistat and VS003, it was hypothesized that a combination of these compounds might lead to either additive or synergistic production enhancement. Additionally, VS003 and Pevonedistat impact different cellular mechanisms. Driven by that hypothesis, HEK293 cell were transfected with both AAV2-Fluc and AAV8-GFP producing plasmids and co-transfection treated with various concentrations and combinations of VS003 and Pevonedistat. Unfortunately, the impact on AAV2-Fluc of VS003 and Pevonedistat alone was the same as the combinations of those two. Interestingly, VS003 did not show production enhancing capabilities in AAV8-GFP context signifying the potential serotype-specific nature of the potential screening hits.

There is a vast number of various cellular pathways and different mediators of antiviral response which when downregulated are expected to enhance the viral production yields. For the drug combinations, it is important for the compounds involved impact different cellular mechanisms ensuring the desired impact on the virus yield. For example, we can hypothesize that combination of compounds targeting OASL and OFIT2 proteins would enhance the viral yields. OASL protein inhibits viral protein synthesis and OFIT2 protein is responsible for inhibition of viral replication and the viral production yields might be boosted when these two proteins are impacted by the chemical compounds [42].

4.10 AAV and transduction enhancers

Since AAV8 is a poor transducer *in vitro*, every additional boost was deemed necessary. Initially, we hypothesized that using a class of chemicals called transduction enhancers will benefit AAV8 transduction. We tested the impact of VS003, VS005, Pevonedistat, Hydroxyurea (Figure 3.13) on AAV2-Fluc and AAV8-GFP transduction. Hydroxyurea has a known impact on AAV

transduction, it significantly boosts the transduction efficiency of both dividing and non-dividing cells [46]. Hydroxyurea treatment not only selectively inhibits DNA replication consequently inducing cell cycle arrest, but it also leads to DNA damage which triggers the activation of DNA repair pathways [47]. The relationship between DNA damage and AAV transduction enhancement is an area of ongoing research. However, it is hypothesized that since hydroxyurea damages DNA, it triggers the activation of cellular DNA repair pathways, specifically homologous recombination and non-homologous end joining. Both mechanisms involve the recruitment of several proteins and repair factors which may facilitate the processing or integration of AAV genomes consequently increasing the transduction efficiency [47].

During AAV-mediated transduction, the cellular ubiquitin-proteosome system recognizes and target for degradation transgenes that are foreign proteins to the host cell. Ubiquitination signals for the proteosome to degrade the transgene product which regulates the rates of transduction. The ubiquitinated AAV capsid proteins accumulate after the proteosome inhibitor (PI) treatment which suggests that PI prevents the degradation of ubiquitinated AAV capsids which leads to the increase in transgene expression levels [48]. According to previous studies, bortezomib, a proteasome inhibitor, is one of the leading candidates for enhancing transduction of AAV. Bortezomib has demonstrated the increase expression of a clinically relevant transgene 3-6-fold in an animal model [29]. MG132 belongs to the class of proteosome inhibitors, and we wanted to investigate the impact of MG132 on AAV8-GFP and AAV8-scNluc transduction (Supplementary Figure 3). Interestingly, the cells treated with 20-100 mM of MG132 significantly increased the transduction of both AAV8-GFP and AAV8-scNluc. A similar trend was observed when the cells were treated with Pevonedistat which is indirect proteasome inhibitor and

transduced with AAV8-GFP (Figure 3.13 G) and AAV2-Fluc (Figure 3.13 C). These observations emphasize the important role of proteasome in cellular antiviral response.

4.11 Drug carryover experiment

When the compound libraries are screened, the common drug concentration used is 10 uM [49]. Since AAV2-Fluc samples are diluted 1:10 for the transduction, there is a risk of treating the reporter cells with 1uM of the screen compounds. It is important to keep in mind that not all compounds can be fully metabolized by the cells making them a potential cause of false positives of the screen. These outliers can be easily detected in the compound validation stage. Seeing the transduction enhancing capabilities of VS003 and Pevonedistat, the potential issue of the drug carryover was addressed. Instead of transfecting the HEK293 and co-transfection treating with the compounds, a mock transfection was performed, followed by the compound treatment. The harvesting method mimicked the usual method of cell lysis since compounds may be chemically unstable and might not be able to withstand the freeze-thawing cycles. Treating the reporter cells with freshly prepared compounds and with the supernatants harvested from the mock transfection treatment allowed drug carryover question to be addressed. VS002, VS003 and VS006 showed transduction enhancing capabilities when the cells were treated at high concentrations of freshly prepared compounds (Figure 3.14 A-C). Intriguingly, all the three abovementioned compounds are either metabolized by HEK293 or can't withstand the stress of going through three cycles of freeze-thawing since the cells treated with the equivalent mock transfection samples did not show transduction enhancement of AAV2-Fluc. Pevonedistat, however, was not affected by freeze-thaw cycles since it showed similar levels of transduction enhancement compared to the freshly diluted compound samples. This highlights the importance of assessing any screen hits for transduction enhancements during compound validation.

Use of transduction enhancers can be seen as a potential solution to poor transduction of AAV8. However, it is important to keep in mind potential drug-drug interactions and the unwanted bias that might come from that experimental setup. In the light of the drug carryover results, the drug-drug interaction is a concern that must be taken into account.

4.11 Stability of AAV

Lastly, we investigated the impact of storing concentrated purified AAV2 at 4C°. This is important because we were concerned about the impact of freeze-thawing on the virus described in Figure 3.5. Expectedly, we saw a distinct decrease in AAV2-Fluc transduction efficiency associated with time stored at 4C° (Figure 3.15). After calculating, it was found that on average AAV2-Fluc loses 1e5 RLU/week while being stored at 4C°. These data persuaded us to store our virus stocks in -80C° since the impact of one freeze-thaw is less drastic compared to prolonged storage at 4C°. There are multiple factors that cause the decrease in AAV titers when stored at 4C° for prolonged time. Oxidation is one of the causes of chemical modifications that impact the capsid proteins decreasing the virus potency [50]. To minimize the impact of freeze-thawing, various buffers can be incorporated. Previous studies by Yuechuan Xu et al. showed that the addition of 10% sucrose and 0.1% poloxamer 188 decreased the leakage of ssDNA in AAV samples after freeze-thaw cycles indicating the success of preventing titer loss [51]. Additionally, pH plays an important role in AAV's viability. According to previous studies, various AAV serotypes are most stable at different pH. As such, AAV1, AAV2 and AAV8 are most stable at pH 5.5 while AAV5 is most stable at pH 7.5 [52].

4.12 Serotype-specific differences in AAV production and screening

There is little evidence of any substantial mechanistic differences between triple transfection method of production of various AAV serotypes. When the producer cell line is

transfected with plasmids assembling AAV2-Fluc or plasmids encoding for AAV8-GFP, the serotype-specificity of the *pAAV-RepCap* transfection plasmid lies solely in the -*cap* region. Therefore, the cellular processes undergone by the plasmids in the producer cell line are the same for both AAV2-Fluc and AAV8-GFP transfections. The serotype-specific differences appear during the genome packaging. It is important to consider the secreted nature of AAV8 compared to AAV2 which is accumulated within the nucleus of the cell. With that in mind, when it comes to performing a high-throughput screen targeting AAV production enhancers, there is no substantial difference in which AAV serotype is used. Without doubt, there will be compounds that will show serotype-specific production enhancement, but those compounds will be targeting specific cellular pathways involved in specific serotypes of AAV. On example of the secreted nature of AAV8, it would be safe to assume that some compounds would show production enhancement specifically for AAV8 enhancing the cellular release. The ideal compound would be targeting cellular pathways fundamentally involved in AAV replication and assembly and acting in a serotype agnostic manner.

Since there is no substantial cellular mechanistic difference between various AAV serotypes and given the ease and low variability associated with AAV2, it would be wise to perform a screen using this AAV serotype. After a high-throughput drug screen comes a stage of validations where the chemical compounds are assessed in depth to determine their working range, cytotoxicity, potential impact on the reporter system, mutagenicity and a number of other parameters. During the validation stage various other AAV serotypes can be tested to universal impact on AAV production.

4.13 System limitations

Since the beginning of gene therapies, the methods for producing and harvesting gene therapy vectors have undergone continuous refinement and enhancement. With the current

approach of bioreactor-based viral vector production, we have nearly exhausted opportunities for further process improvements. Therefore, the primary objective of this project was to identify the essential components of a high-throughput screening method capable of detecting AAV production enhancers, thereby increasing production yields.

Given that every parameter of the current bioreactor-based production process has been optimized to its maximum potential, a valid concern arises regarding system saturation, potentially limiting further improvements. It is reasonable to assume that any compounds introduced may only augment the final virus output by a modest factor.

Although our developed assay demonstrates the ability to detect orders of magnitude of AAV production enhancement in the microplate format, it would be overly optimistic to expect the same level of improvement in the bioreactor format. Rigorous testing and validation of these parameters will be imperative. However, even if it was possible to achieve a 3-5 times enhancement in AAV production, it would represent a significant breakthrough in addressing the high production costs associated with AAV, ultimately leading to more affordable treatments for a larger number of patients.

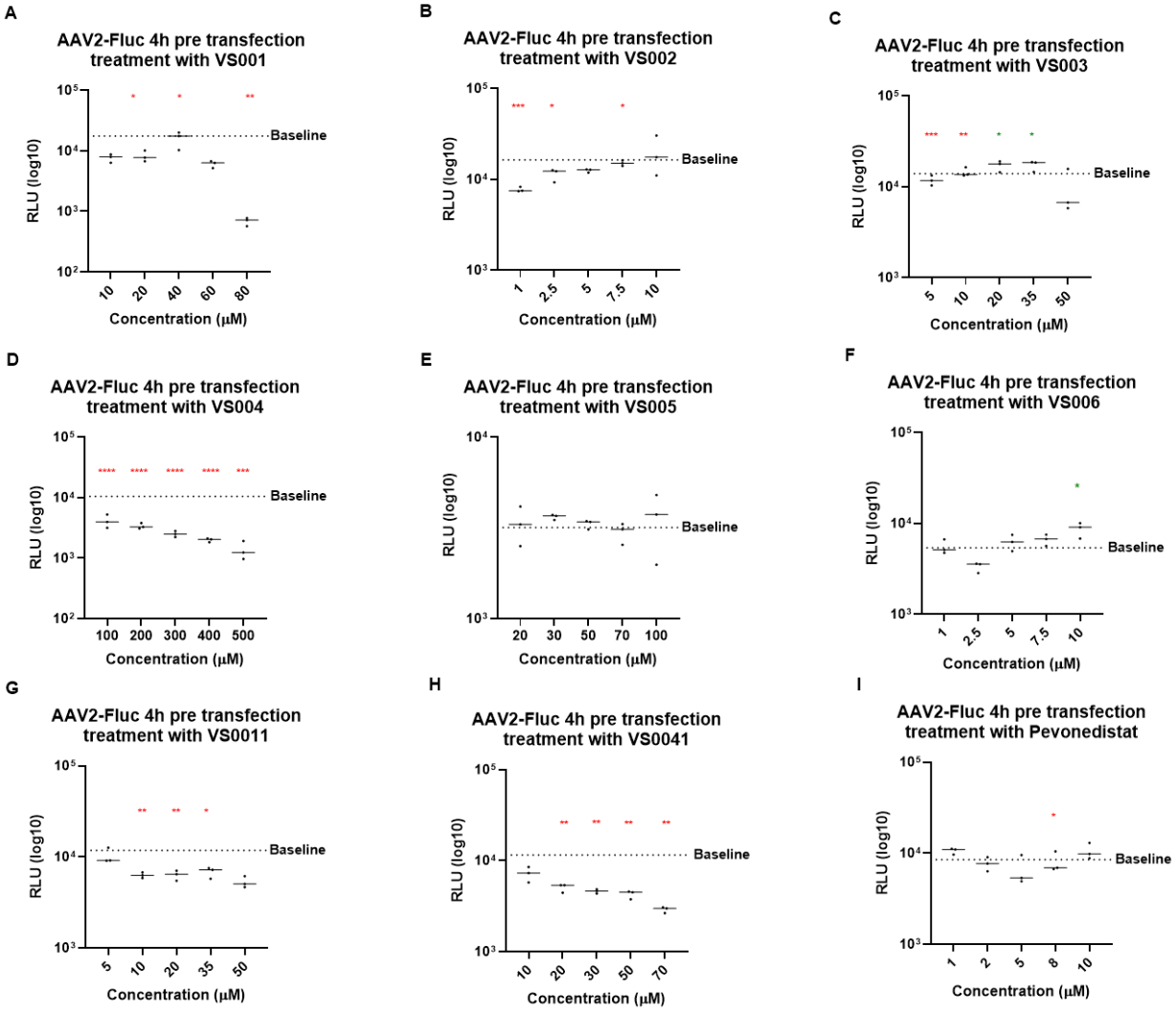
Chapter 5: Conclusion

We hypothesized that by optimizing all the parameters of the screen, it would allow for the development of a robust and effective high-throughput method of screening for AAV production enhancers. Not only did we optimize all the parameters to do a successful drug screen targeting compounds boosting AAV production and tested the impact of various compounds on AAV production yield, but we also optimized the parameters for the validation stage. The developed pipeline can be used for:

- High-throughput screening for AAV production and transduction enhancers.
- Testing the impact of compounds when treated at various times relative to the transfection.
- Novel means of drug treatment using lower doses than required for standard drug screen, and for extended periods of time over the course of virus production.
- Determination of the impact of drug combinations on AAV production and transduction.
- Validation of potential drug carryover into the transduction.
- Determination of the cytotoxicity of chemical compounds via Alamar blue assay.
- Provide throughput capabilities for process development using DoE approaches.

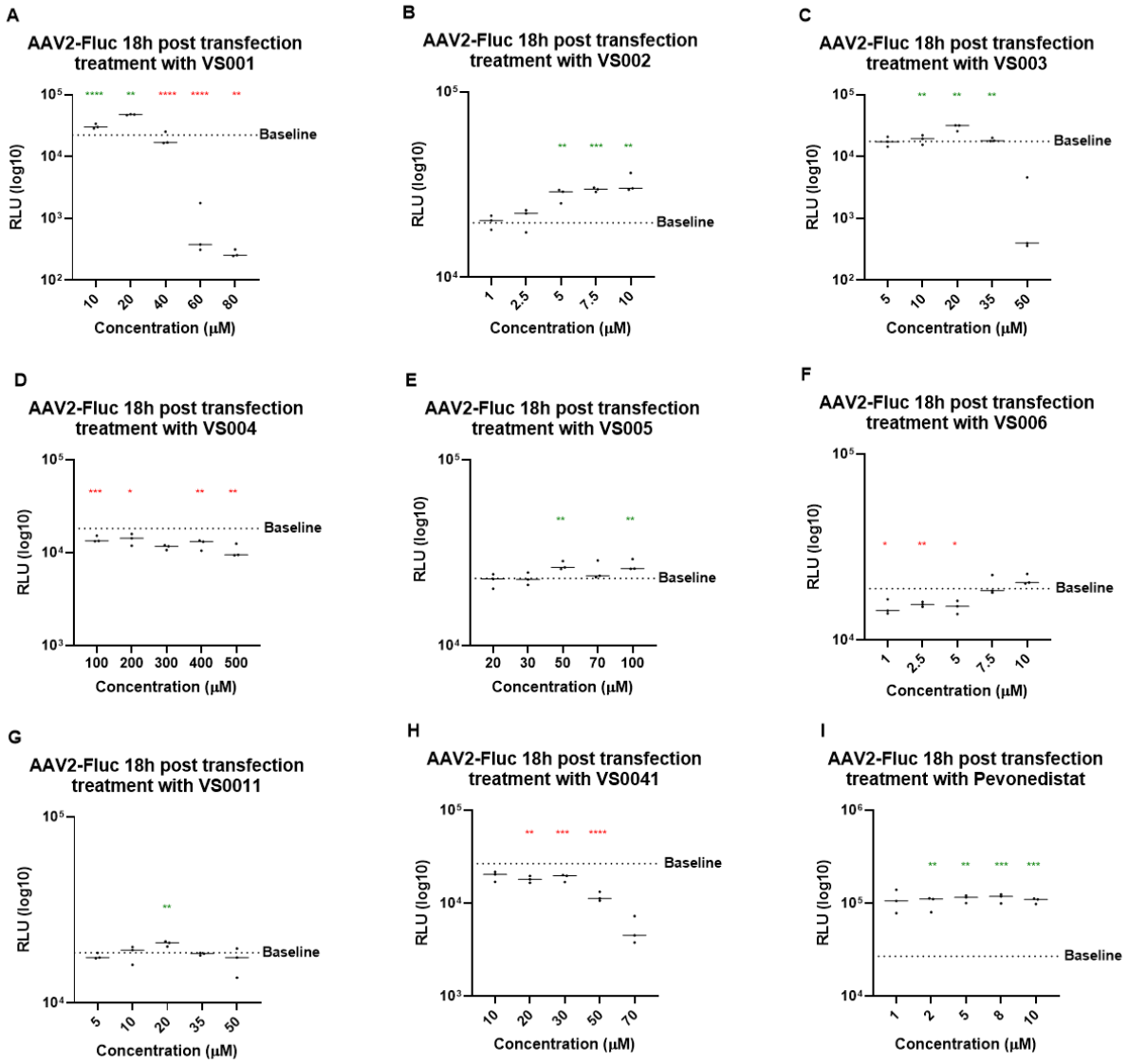
Expectedly, a high-throughput drug screen will be an organic continuation for this project. Hopefully, by identifying and testing compounds boosting production of AAV, AAV-mediated gene therapy would become more available and cost-effective.

Supplementary figures



Supplementary Figure 1. 4-hour pre-transfection drug treatment does not enhance AAV2 production.

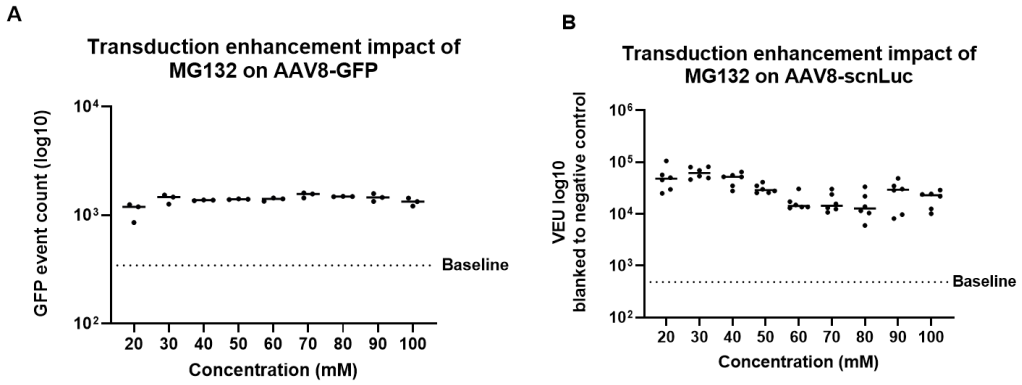
HEK293 were 4 h pre-transfection treated with various compounds and triple transfected with AAV2-Fluc. 72 h post transfection the samples were harvested via three freeze-thaws. HEK293T were transduced with 1:10 diluted samples and the transgene signal was quantified 72 h post transduction. (A-I) Transduction quantification of HEK293 4 h pre-transfection treated with chemicals looking for their ability to enhance AAV production of AAV2-Fluc. Cells were treated with VS001, VS002, VS003, VS004, VS005, VS006, VS0011, VS0041 and Pevonedistat, transfected with AAV2-Fluc and the virus was harvested 72 h post transfection. HEK293T were transduced with 1:10 dilute crude samples. The baseline represents the transduction of HEK293T with samples harvested from transfected untreated HEK293. Dots represent 3 biological replicates (n= 12 technical replicates).



Supplementary Figure 2. 18-hour post transfection drug treatment identifies potential production enhancers for AAV2-Fluc.

HEK293 were triple transfected with AAV2-Fluc and 18 h post transfection treated with various compounds. 72 h post transfection the samples were harvested via three freeze-thaws. HEK293T were transduced with 1:10 diluted samples and the transgene signal was quantified 72 h post transduction. (A-I) Transduction quantification of HEK293 transfected with AAV2-Fluc and 18 h post-transfection treated with chemicals looking for their ability to enhance AAV production. Cells were transfected, treated 18 h post transfection with VS001, VS002, VS003, VS004, VS005, VS006, VS0011, VS0041 and Pevonedistat and the virus was harvested 72 h post transfection. HEK293T were transduced with 1:10 dilute crude samples. The baseline represents the

transduction of HEK293T with samples harvested from transfected untreated HEK293. Dots represent 3 biological replicates (n= 12 technical replicates).



Supplementary Figure 3. Determination of the impact of MG132 on transduction of AAV8.

HEK293T were 4 h pre-transduction treated with MG132 at various concentrations. The culture media was replaced 4 h after the treatment and the cells were transduced with AAV8-GFP and AAV8-scnLuc. The transgene signal was quantified 96 h post transduction. (A) HEK293t cells were 4 h pre-transduction treated with MG132 at various concentrations. 4 h post treatment, the drug containing media was aspirated, new media was added, and cells were transduced with AAV8-GFP. The transgene signal was measured 96 h post transduction, n=3 (technical replicates). (B) HEK293T cells were 4 h pre-transduction treated with MG132 at various concentrations. 4 h post treatment, the drug containing media was aspirated, new media was added, and cells were transduced with AAV8-scNluc. The transgene signal was measured 96 h post transduction, n=6 (technical replicates).

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