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Optimization of rAAV Manufacturing at the Triple Transfection Step

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A Thesis in the Field of Biotechnology

for the Degree of Master of Liberal Arts in Extension Studies

Harvard University

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Abstract

The recombinant adeno-associated virus (rAAV) is the vector of choice for the delivery of gene therapies for a wide range of monogenic disorders because of its track safety record in clinical trials and prolonged gene expression with relatively low immunogenicity. More recently, rAAVs have also gained attention as vaccine carriers against infectious agents because the rAAV particle is thermodynamically stable and durable under the right conditions at room temperature, a feature that facilitates vaccine distribution where it is most needed without expensive preservation measures.

rAAVs are biological drugs, and therefore, their manufacturing relies on highly complex biological processes that are not completely understood. While several rAAV-based drugs are available in the market, the production of high-titer, high-quality vector remains a challenge despite the many chemical, biological and engineering advances implemented to date. Several studies show, for instance, that rAAV preparations are heterogenous in character, comprised of filled, partially filled or empty capsids of which the desired product may be represented only as a small sub-population of particles.

This heterogeneity is not understood in all its potential implications. It is compounded by the observation that packaged recombinant genomes often contain deletions or insertions or are recombined via mechanisms that are not firmly established but that are being actively investigated. The nucleic acid material incorporated into viral particles may itself be derived from alternative sources arising from the biological reagents used in production, namely host cell DNA, RNA, or backbone plasmid

sequences, the latter via a process called reverse packaging. The illegitimate virions arising from these processes are often accompanied by protein impurities that escape purification procedures and may elicit an immune response when administered. In addition, depending on the manufacturing platform chosen for vector production, the proteins making up the viral capsid and the non-structural factors responsible for their assembly may differ due to post translational modifications which may impact rAAV transduction efficiency, particle stability or vector tropism.

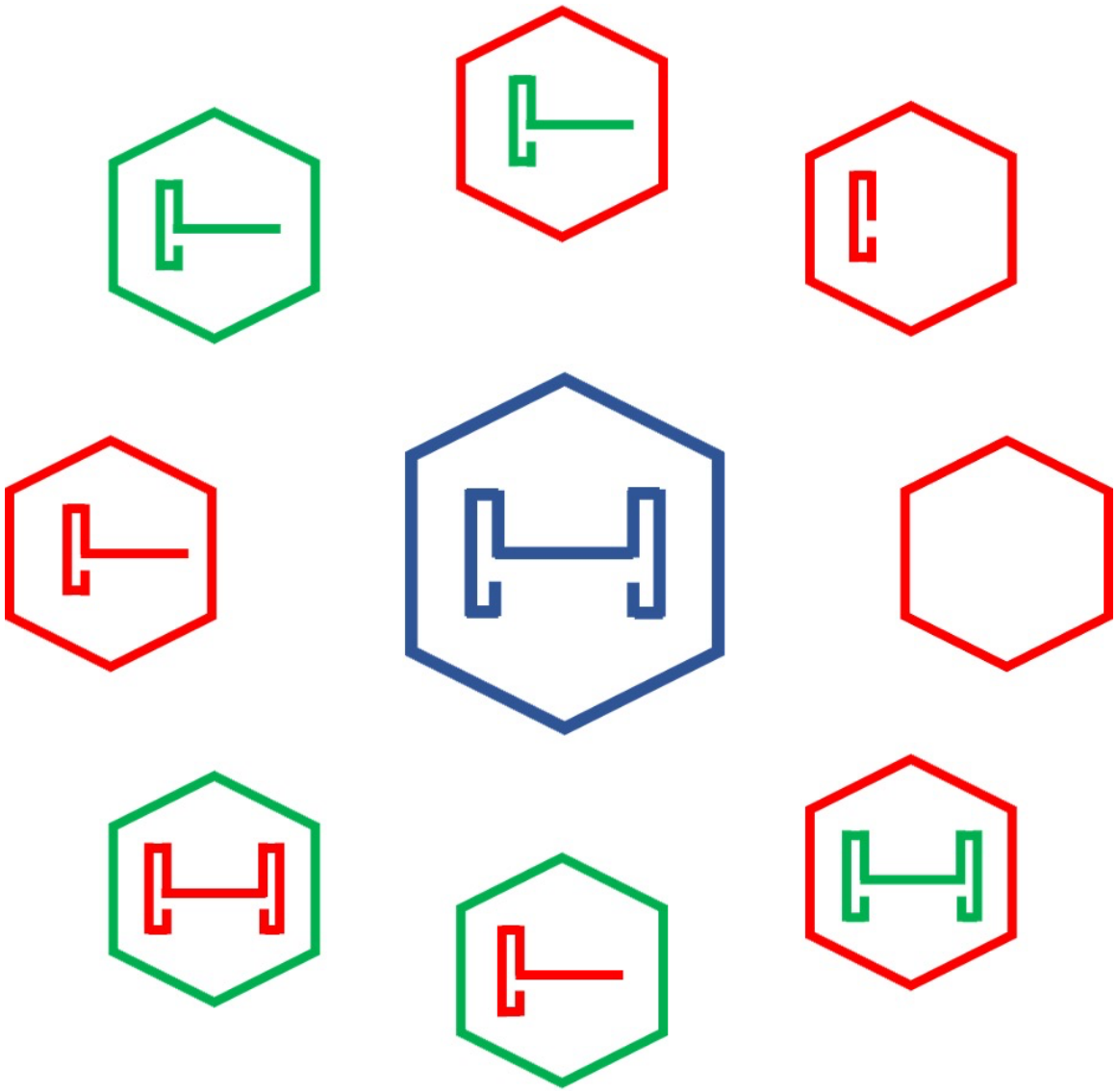
Thus, heterogeneity and purity issues raise questions about the potency and safety of the final products, add uncertainty to experimental work from all stages of development to clinical testing, limits reproducibility, and complicates translational medicine where the clinical vector likely differs from the pre-clinical one. Considering recently documented adverse reactions and fatalities in gene therapy trials at very high doses, research studies aimed improving vector titer and quality are pressing.

The main goal of this study was to optimize the manufacturing of rAAV by altering the ratio of segregated adenovirus helper genes provided during production. Adenovirus helper genes are involved in rAAV genome transcription, translation and packaging. For this, molar equivalents of the known canonical adenovirus helper genes were delivered separately or in combination to study their contribution to rAAV vector titers in a manner that matched current production conditions.

It was found that the adenovirus DNA binding protein (DBP) mRNA is underexpressed when it is under the control of the early E2a gene promoter compared to DBP mRNA that is expressed from the more complex pΔF6 genome, the intact helper plasmid. The difference in DBP expression was not enough to explain lower rAAV

production levels because rescue of early DBP mRNA expression by modulation of the early E2a plasmid input at transfection did not result in a comparable rescue of rAAV production. An error was discovered.

Frontispiece



Dedication

To Mélida, Julio and my dogs

Acknowledgments

I have a lot to be thankful for. There was darkness along the way, but fortunately also people who lighted my path with their advice and example, especially in the United States. I found kindness and learned kindness. I work on it every day. Staying here was the best decision I made.

I want to thank Dr. Luk H. Vandenberghe, Ph.D. and Dr. Nerea Zabaleta Lasarte, Ph.D. for their mentorship and patience during the preparation of this report, but especially to Luk who gave me a valuable opportunity to work in his lab and for providing one of the best examples of leadership anyone could hope to obtain in this highly competitive field.

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Chapter I.

Introduction

The adeno-associated virus (AAV) is a small DNA virus (genus Dependoparvovirus) with a T=1 icosahedral, non-enveloped protein shell about 26nm in diameter that protects a single-stranded 4.7kb genome that is equally of 'plus' or 'minus' polarity (Mietzsch et al., 2019; Wang, Tai & Gao, 2019; Le Mercier et al, 2023). The name derives from the observation that the first AAVs were discovered in human and simian adenovirus preparations in the mid 1960's (Atchison et al., 1965; Hoggan et al., 1966) and are dependent upon adenovirus' genetic input for their propagation and life cycle. They were found to be serologically distinct from each other and their co-infecting adenovirus.

In the span of 35 years after their discovery, five of the initially identified serotypes (AAV1-6) were found in human and non-human primate adenovirus samples, but only AAV4 in African green monkeys exclusively (Blacklow et. al, 1968, Chiorini et al, 1997). AAV5 is the only early AAV that was discovered in a lesion (Bantel-Schaal and zur Hausen,1984). It was determined that the capsids for AAV1 and AAV6 are 99.2% identical to each other and have very similar serological profiles, but distinct tropism (Rutledge et al, 1998); they belong to the same clade and, therefore, share a common ancestor (Gao et al, 2004). AAV2 is by far the most extensively studied AAV and the variant upon which the majority of recombinant AAV vectors are based (Hermonat and Muzyczka, 1984; Carter, 2004).

Wild-type AAVs regularly infect humans without known clinical consequences (Gao et al., 2004), a fact that did not initially attract virologists to the study of AAV (Carter, 2004). Before the first clinical trial to treat cystic fibrosis patients in the mid 1990s (Mueller & Flotte, 2008), the study of AAV focused on its life cycle and genome structure, its latency and value as a potential vector, along with understanding the role of the adenovirus helper genes in priming AAV's genome transcription and, ultimately, viral expansion. Because AAV is dependent on a helper virus, it was at first nearly impossible to purify and study it in isolation until the advent of infectious molecular clones in the early 1980s when the first mutagenesis and gene transfer studies were carried out in earnest (Hermonat and Muzyczka, 1984).

Consequently, the AAV genome was determined to be divided into two open reading frames (ORFs) under the control of three promoters whose numerical designation p5, p19, p40 corresponds to their relative location within the genome sequence. The left half codes for the non-structural proteins Rep78/68 under the control of the p5 promoter and Rep52/40 under the control of the p19 promoter together which have important functions in the AAV life cycle including genome replication, packaging and integration. In the absence of helper virus, transcription of Rep is largely repressed by the host cell as AAV enters its latent phase of infection and the genome is integrated at a specific location in chromosome 19 known as AAVS1 (Samulski et al, 1991).

The right half of the genome codes for the AAV capsid proteins VP1, VP2, and VP3 under the control of the p40 promoter and arise via alternative splicing of the primary transcript. The three proteins share the same carboxy-terminal region but differ in their amino-terminal sequence. VP1, for instance, includes a phospholipase A2 domain

which plays a vital role in capsid trafficking through the cell at the endosomal escape step (Stahnke et al, 2011); specialized serine-rich motifs in both VP1 and VP2 at their N-terminus may have an impact in AAV genome transduction efficiency (Robinson et al., 2020).

VP3, on the other hand, represents the bulk of the capsid enclosure and confers sequence identity to AAV serotypes; its hypervariable regions are primarily responsible for a serotype's tropism and immunogenic profile (Govindasamy et al., 2013; Raupp et al., 2012). A total of 60 capsid subunits form a complete virion where VP1, VP2 and VP3 are represented at a ratio of 1:1:10. An assembly-activating protein (AAP) is also expressed from the right ORF using an alternative start site (Sonntag et al., 2011); it is a co-factor that serves to promote VP monomer assembly and virion stability while providing the capsid more freedom to evolve along some lineages (Maurer et al., 2018). Thus, AAP is not required by all AAV serotypes (Earley et al., 2017).

Importantly, at the extreme ends of the genome, flanking the two ORFs, are palindromic sequences 145bp long that fold upon themselves to form a "T"-shaped structure called an inverted terminal repeat (ITR). The ITRs are promoters, origins of replication, and transcription initiation sites and play an essential role in genome packaging and integration**.** They carry specialized sequences, a Rep binding region and a terminal resolution site (trs), that are indispensable for segregating the single-stranded forms of the replicated AAV genome prior to packaging. Consequently, the ITRs are the only elements of the wild-type virus that are obligate features of the recombinant genome because they direct the packaging of the gene of interest (Xiao et al, 1997).

The helper functions that the *Adenoviridae* provide in the context of AAV's discovery are incidental; they may be supplied by other viruses, as well, such as herpes simplex, vaccinia, or human papilloma viruses (Schlehofer et al., 1986; Ogston, Raj & Beard, 2000) but not necessarily in a 1:1 equivalent manner. What adenovirus and herpes simplex appear to have in common in this regard is that they render the host cell permissive for AAV growth in the face of a massive anti-viral response (Chung et al, 2023). This is supported by the observation that AAV may propagate *in vitro* in the absence of outside help when cells are exposed to genotoxic agents, carcinogens, or UV light leading to cell cycle arrest (Yacobson et al., 1989), or if the cell density is sufficiently high (Yalkinoglu et al., 1991; Ni et al., 1998).

More specifically, the molecular basis for rAAV production is modeled after the lytic phase of the wild-type AAV life cycle from the perspective of proviral genome rescue, expansion and packaging, events catalyzed by the presence of helper virus. In this case, the recombinant genome, bounded by AAV ITRs, is rescued from the plasmid in the cell nucleus in a manner analogous to the rescue of the wild-type genome integrated into the host chromosome. The early rAAV manufacturing efforts proceeded in an identical manner: transfection of two plasmids, one carrying the recombinant genome bounded by ITRs and the other providing the AAV rep and cap genes *in trans* (or combinations thereof), along with co-infection with a human adenovirus to provide helper functions in a mammalian cell line. Because the co-infecting adenovirus would also propagate under these conditions, it would need to be heat-inactivated and removed prior to rAAV purification, a process that nevertheless left contaminating adenovirus

proteins in vector preparations but paved the way for future innovations in vector manufacturing.

rAAV is the preferred vehicle for gene therapy applications today and recently gained interest as vaccine carriers, as well. The wild-type virus transduces dividing and non-dividing cells and is widely disseminated in human tissues which makes it attractive for gene transfer applications. Currently used AAV vectors typically derive from AAV2 non-structural proteins expressed from a plasmid, AAV2 inverted terminal repeats (ITRs) flanking the recombinant genome and capsid proteins expressed from other serotypes, a strategy called pseudotyping (Mueller & Flotte, 2008). Vectors based on AAV have proven safe in numerous clinical trials. The recombinant genomes they carry are often expressed and tolerated for long periods of time, attesting to their therapeutic potential against debilitating genetic diseases that may last a lifetime.

Progress made in pre-clinical work and gene therapy trials worldwide has resulted in the first approved rAAV drugs, first by regulatory agencies in Europe: Glybera® in 2012 for lipoprotein lipase deficiency (withdrawn in 2017), Upstaza™ against AADC deficiency and Roctavian® against hemophilia A in 2022; and by the FDA in the United States: Luxturna® for Leber congenital amaurosis in 2017, Zolgensma® in 2019 for spinal muscular atrophy and Hemgenix® in 2022 for the treatment of hemophilia B (Commissioner, *U.S. Food and Drug Administration,* 2023). As of this writing, there are 171 clinical trials worldwide using rAAV as the vector of choice that are either active, recruiting, in progress or completed covering 14 disease categories targeting the nervous system, heart and blood disorders, cancers, the respiratory tract, among others (*clinicaltrials.gov* 2023).

The use of rAAV vectors for vaccination purposes has been under consideration since the late 1990's when they were first tested against HSV (Manning et al, 1997), and then against other infectious and non-infectious diseases and in ex-vivo cell therapy applications. While it has become clear that AAV can induce strong humoral immune responses which include antigen neutralization, the responses are hampered by pre-existing immunity against the capsid and wane overtime (Nieto and Salvetti, 2014). Only some AAV serotypes, however, can induce strong cytotoxic T cell responses that can be recalled upon re-exposure to the antigen they originally expressed, a strong indication of their vaccine potential (Lin et al., 2009; Mays et al., 2009, Nieto and Salvetti, 2014).

Consideration of rAAV for a vaccine reached its apotheosis at the outset of the COVID-19 pandemic. Recombinant AAV genomes expressing a codon-optimized version of the SARS-CoV2 spike gene were packaged in the hybrid capsid AAV.rh32.33 and administered via intra-muscular injection in mice and non-human primates and demonstrated to be an effective and durable platform against this emerging pathogen (Zabaleta et al, 2021; Zabaleta et al, 2022, Maciorowski et al, 2022). As vaccine carriers, AAV vectors have the advantage that they do not readily degrade at room temperature and can remain effective for months in the right formulation or may be lyophilized which are attractive features that align well with global distribution goals at relatively low cost.

The consensus from all these studies combined is that there is an unmet need for mass quantities of high-quality vector despite advances in vector production to this day. A small high-dose study, for instance, may administer as many as 3x10E14 vector genomes per kilogram per patient which translates to 1.5E16 vector genomes for a 50kg individual. Although high-dose studies such as these have raised safety concerns after the

deaths of several patients (Paulk, 2020; Wilson and Flotte, 2020) and may never be repeated, the demand for high titer, high quality vector is a pressing reality. These estimates do not consider annual live birth incidences of genetic disease in the population for which there will be increasing need for vector; the actual numbers run into the hundreds of thousands of liters at harvest (Nguyen et al, 2021). Similarly, a low-dose vaccine project (1xE10- 1xE11 vector genomes per subject) purporting to immunize large swaths of the population also requires large manufacturing capabilities.

Consequently, contract research organizations (CROs) have sprung up to meet demand for vector leading to the growth of the biotechnology sector (Vuksanaj, 2021; Sylvestri and Conti-Permanne, 2022). This in turn requires accurate quantification methods, precise vector characterization tools, and high throughput potency assays. Within this framework, the analytical tools needed for assessing AAV vector composition have evolved to meet the needs for speed, resolution and precision, work that continues to this day.

For example, the genome quantification methods based on the polymerase chain reaction (PCR) are complex and difficult to standardize within and across laboratories, even when the same starting material is quantified (Lock et al, 2010; Ayuso et al, 2014). This is especially true of this technology where the slightest change must be justified and validated, where every loss or gain must be accounted for. While it is very easy to generate a real-time PCR signal, it is not immediately evident what these signals mean, much less whether they are correct and can be trusted (Johnson et al, 2014). Even an excellent R^2 value for a real-time PCR standard curve is potentially misleading if an error of judgement about plasmid purification is implemented, a critical reagent is omitted

from the dilution buffer or the comparison between standard and test sample is not equivalent. Because the technology is exquisitely sensitive and specific, a real-time or digital PCR operator must always be alert concerning the slightest discrepancy in the data generated (Lock et al, 2014; Sanmiguel et al, 2019).

Likewise, analytical ultracentrifugation (AUC), the gold standard for measuring the genome content of rAAV samples is laborious, low throughput and requires a relatively large volume of high titer vector for analysis (Burnham et al, 2015); alternatives to AUC are now available that rely on capillary gel electrophoresis and isoelectric focusing that consume very small amounts of starting material and purport to provide equivalent information about rAAV preps (Hutanu et al, 2022; Rumachik et al 2020).

On the other hand, potency assays which traditionally require test sample administration to live animals in large groups and lengthy incubation times before rendering a measurement are being successfully upgraded to high throughput cell-based assays that can provide equivalent answers in record time (Verch et al, 2018; Aronson et al, 2020). For instance, it is now possible to measure the thermal stress response of rAAV vectors in a high-throughput manner with a cell-based assay which will allow comprehensive evaluations of formulation choices (Prashant et al., 2023). Similarly, developments in light scattering technologies, next generation sequencing (NGS), and mass spectrometry are driving efforts to improve rAAV vectors (Gimpel et al, 2021). NGS studies, for example, have provided quantitative and detailed descriptions of the packaged nucleic acid material in rAAV preps which will pave the way for vector improvements (Tai et al, 2018; Namkung et al, 2022).

Equally important and on a broader scale, these developments are tied in with efforts to understand the seroprevalence of the wild-type virus. To date, twelve naturally occurring AAV variants have been identified in humans and hundreds more in non-human primates and other species (Gao et al, 2002) with a glimpse at their distribution and epidemiology (Blacklow et. al, 1968; Calcedo et al, 2009; Calcedo et al, 2011). Such studies, based predominantly on the frequency of neutralizing antibodies, are essential because they inform on the chances of success of intended therapies and clinical trials, the choice of AAV serotypes better suited for specific applications and the direction of future technological innovation. The elucidation of the three-dimensional structures corresponding to the phylogenetic tree of naturally occurring AAVs complements epidemiologic studies inasmuch as they suggest the molecular basis for the rational design of new variants in a modular, combinatorial fashion (Mietzsch et al, 2021).

For instance, this knowledge has been harnessed to humanize the AAV8 serotype by reconstructing antigen-antibody interactions on the 3D AAV8 surface using cryo-electron microscopy, mapping critical amino-acid residues in the variable regions of AAV8 contacting these antibodies (the antigenic footprint), and mutating these residues in a stepwise manner *in vitro* using capsid libraries in a directed evolution approach by transducing human hepatocytes with a co-infecting adenovirus. Under these conditions the library members for a variable region most suited to expand in human hepatocytes are selected via several rounds of amplification. The process is repeated for the remaining variable regions using selected mutants from the previous rounds until the best adapted capsids are chosen from the pool. Because the antigenic footprint of AAV sits at the intersection of several biological attributes of the virus, such as tropism, immunogenicity,

genome packaging, and transduction efficiency, the selected mutants represent hypothetical enhancements that can be tested. Vectors based on AAVhum8, for instance, coincided with high production titers, improved tropism and evasion of neutralizing antibodies *in vitro* (Havlik et al, 2020).

Alternatively, a step back in time is also a possibility using *in silico* ancestral sequence reconstruction. Inferring from the probabilistic spaces at the evolutionary nodes leading to the characterized, extant, wild-type AAV isolates informed the prediction of potential ancestors of the dependoparvovirus lineage culminating with the discovery of Anc80, a potent gene therapy vector (Zinn et al, 2015). A more in-depth look at Anc80 and its putative descendants using advanced cloning strategies, next generation sequencing technology (NGS) and powerful bioinformatics tools to identify and catalog thousands of new ancestral variants *in vitro* and *in vivo* resulted in the discovery of a critical amino acid toggle capable of avoiding the liver by more than 500-fold, a significant milestone in capsid design (Zinn et al, 2022).

The drive to identify or engineer new AAV variants comes from a desire to target specific organs or cell types where therapeutic recombinant genomes will likely deliver the most benefit while minimizing adverse effects or the activation of host immune responses (Zolotukhin and Vandenberghe, 2022). These efforts are leveraged against improvements in the design of recombinant genomes that seek to maximize expression of therapeutic genes at specific locations with the least amount of vector, the proverbial doing more with less (Shitik et al, 2023). This raises the bar for vector engineers and manufacturing facilities alike to generate high quality vector while pushing titer boundaries.

Exactly what a high-quality vector is requires proper definition to guide the work ahead. From a purification standpoint, it means the elimination of host cell impurities, helper virus proteins, and plasmid expression byproducts from the final sample at minimum, along with the use of appropriate vector formulations which may vary with the nature of the rAAV capsid. Adequate vector formulations prevent aggregation and foster virion capsid integrity, thermostability, and genome retention and minimize discomfort at the site of administration (Wright et al, 2003; Bennett et al, 2017). These goals are currently manageable and appear within reach given the current of state of technological development. There is extensive experience in the field of protein purification and enough understanding of rAAV vector production systems to suggest ways to minimizing these impurities (Penaud-Budloo et al, 2018; Su et al, 2022).

On the other hand, a high-quality prep also means a homogeneous prep where one virion is identical to the one next to it, an all or nothing undertaking. This is an idealized scenario considering the complexities involved, but nevertheless it informs the direction of future work to improve these valuable and expensive reagents. For all their successes as therapeutic gene carriers and now vaccines, it is known that rAAV preparations are patently heterogenous in character. The implications of this observation are not completely understood, but they are a source of uncertainty and concern regarding safety and efficacy for issues that may not be immediately apparent with the technology available or that may arise in the future after vector administration, if a causation chain can be established that is statistically significant. For now, the relative safety of the rAAV vectors that have been used to date is a blessing. There is hope.

The heterogeneity found in current rAAV preps is two-fold. First, it relates to the platform used to make recombinant vector. No two platforms yield the same amounts of vector or vector with the same attributes. It was reported, for instance, that double transfection using PEI as the transfection reagent yielded lower titers than one based on herpex simplex virus (HSV) infection in the same HEK293-based suspension cell line. HSV-based rAAV manufacturing also resulted in more filled capsids and less host cell and helper DNA impurities, but the same potency (Trivedi et al, 2020). Along this line, the use of next generation transfection reagents has been reported to yield higher vector titers in both adherent and suspension cell lines with purported improvements in batch-to-batch reproducibility, and scalability (Wosnitzka et al., 2021). Supplementing the cell culture medium with select agents has also been reported to improve vector titers and stabilize the host cell line during vector assembly (Shiina et al, 2022), but questions remain regarding vector quality and potency with these reagents.

Alternatively, the baculovirus expression system (BEV) method to make rAAV in suspension *Spodoptera frugiperda Sf9* cells, a fall armyworm moth cell line, was initially touted as a superior method to scale up vector production. Indeed, filled vector particle totals recovered from 1000mL Sf9-infected cells (about 40-50 175cm^2 adherent cells) at 5x10E4 particles/cell were estimated to be equivalent to the amount of vector recovered from 500-1000 175cm^2, 10-20 times more than the currently available adherent cell-based systems, an impressive feat, due in part to the high density of Sf9 cells that can be BEV-infected at a time. These rAAV2 vectors were initially reported to be indistinguishable from those generated by other platforms based on their physical properties and biological attributes (Urabe et al, 2002; Smith et al, 2009), but were later

found to have different capsid protein stoichiometry and required genetic recalibration of capsid gene expression for improved performance (Bosma et al, 2018).

Moreover, it was revealed that there are significant differences between vectors made in BEV-infected Sf9 cells versus those made using an HSV-infection system in a human cell line. There were differential post-translational modifications in the AAV capsids on vectors from the same serotype which translated to different patterns of stability by 2D-gel electrophoresis as a response to changes in pH and temperature. Recombinant genomes of the same sequence were also found to be differentially methylated which likely inform the differences in vector potencies between the two systems. Human-cell-line-manufactured vectors were more potent and had a higher proportion of filled capsids than the Sf9-based counterparts. Protein impurities in the two systems were found to be differentially post-translationally modified with an increase proportion of potential allergens in the baculovirus based vectors (Rumachik et al, 2020).

Second, and most importantly, from a vector assembly perspective, a high-quality rAAV prep also means maximizing empty capsid utilization and ensuring that only the intended recombinant genome is packaged, the transgene cassette, with little or no damage to it. This type of heterogeneity rests primarily on a genome packaging challenge that requires immediate attention and is the more difficult task to solve. A mathematical model of rAAV production by the triple transfection method in HEK293 cells suggests that there is a time difference between empty capsid accumulation and rAAV genome amplification in the packaging cell line. The model suggests that the lag of genome replication is the result of excess Rep gene expression which represses it (Nguyen et al, 2021).

rAAV preps are often composed of three kinds of capsids: empty, full, and partially full, each of which is identifiable by a peak corresponding to a unique sedimentation coefficient in an AUC plot (Burnham el al, 2015). The genome content of these particles has been measured and determined to be of mixed composition limited by the packaging capacity of the capsid (Wu et al, 2010). They may include the desired transgene cassette in various states of integrity, including deletions and insertions. But a small percentage of the virions also package prokaryotic sequences and host cell DNA attributed to recombination events and DNA polymerase mispriming (Tai et al, 2018). The deliberate inclusion of secondary structures in the transgene, such as those that express short hairpin RNA (shRNAs), promote transgene cassette truncations that are packaged. This in turn suggests new ways of constructing a double-stranded transgene cassettes, with the secondary structures serving as alternative terminal repeats (Xie et al, 2017). But, at the same time, they inform of transgene secondary structures to avoid in the cassette sequence to promote efficient packaging or serve as useful markers for exploring the mutagenesis and evolution of key proteins of the packaging machinery, the AAV Rep proteins to start. Understanding Rep means unraveling the role of the helper proteins that activate it (Geoffroy and Salvetti, 2005) and integrating this knowledge with the activities of the host cell where the rAAV particles are assembled (Chung et al, 2023).

This short study is a small attempt toward improving rAAV vector sample titers and quality. The triple-transfection method in which three plasmids are delivered to HEK293 cells to make rAAV was used as a reference to compare the role played by the canonical adenovirus helper genes in vector manufacturing. As mentioned earlier, the three plasmids deliver the AAV rep and cap genes without ITRs, the recombinant AAV

genome bounded by ITRs (in this case, the firefly luciferase reporter gene under the control of the cytomegalovirus promoter and simian virus 40 poly adenylation signal), and the adenovirus helper genes (pΔF6 or pALD-X80). The latter is a fragmented adenovirus 5 genome incapable of making mature adenovirus under the triple-transfection method, hence resulting in cleaner rAAV vector samples that are easier to purify (Xiao, 1998).

The canonical adenovirus helper gene sequences and their native regulatory regions known to be essential to making rAAV (Geoffroy and Salvetti, 2005) were extracted from the literature and available databases and subsequently cloned. These are the virus associated RNA (VARNA I/II), the E2a DNA binding protein (E2a DBP), and the E4 orf6 genes. These were delivered in different molar equivalent amounts to study their contribution to vector production. The adenovirus E1a/b genes, which also provide vital helper functions, are constitutively expressed by the packaging cell line HEK293 where they played an important role in cellular transformation (Graham et al, 1977).

The E1a gene product is a powerful transactivator that promotes AAV genome transcription via the rep p5 promoter. It relieves repression at this site by interacting with the cellular transcription factors YY1 and MLTF in a manner that remains to be fully understood (Chang et al, 1989). Likewise, the E2a DNA binding protein was also found to activate transcription of a reporter gene via the AAV p5 promoter and support activation of both the E1a and its own promoter but found to repress the E4 gene (Chang & Shenk, 1990), as well as delivering support for AAV mRNA translation and genome replication (Geoffroy and Salvetti, 2005). The E2a DBP role in translation is not well

defined and may overlap with the activity of other factors at play, possibly via the VARNA transcripts or other cellular genes.

VARNA genes I/II, on the other hand, code for small non-coding RNAs that are transcribed by RNA polymerase III (the only adenovirus genes so processed) and become rapidly abundant during a lytic adenovirus infection. Generally, the genes are believed to help with mRNA transport to the cytosol and provide stability during translation. More specifically, however, VARNA transcripts are known to be processed by the RNA interference pathway of the cell to yield miRNAs that block the activity of Dicer and disrupt gene expression of some cellular genes. In this context, VARNAs are functionally within the scope of an immune mechanism that render the cell permissive for adenovirus growth and, by extension, AAV's but work remains to be done in this area for better understanding of these activities (Andersson et al., 2005; Aparicio et al., 2010; Belluti et al., 2015). Incidentally, AAV is known to produce its own small non-coding RNAs whose activity does not appear to result in cellular gene regulation, a finding that agrees with the virus being non-pathogenic (Stutika et al, 2016).

The E4 orf6 gene appears to be pleiotropic in nature, as well. Like its E2a DBP counterpart, it has been implicated in AAV genome replication, but also plays a role in transcription initiation and Rep protein synthesis (Geoffroy and Salvetti, 2005). The E4 orf6 product is known to form a complex with the adenovirus 55Kda E1b protein that enhances AAV mRNA accumulation in the cytosol. It enhances transduction by participating in the conversion of the single-stranded genome form to linear double stranded intermediates, a role differentially shared with the E2a gene (Duan et al., 1999). All four genes, E1a/b, E2a, E4orf6 and VARNAs are sufficient for virus assembly, but

there are unknowns, especially within the packaging cell, that remain to be identified and may be exploited to drive the path to better and more abundant vector production.

Chapter II.

Materials and Methods

Plasmids

Intact helper plasmids

The helper plasmids pΔF6 and pALD-X80 are commercially available, the first one through Addgene and the latter through Aldevron. pALD-X80 is about 3.5kb longer than pΔF6. The extra sequence is additional adenovirus sequence. Both are based on the human Adenovirus Type 5 genome.

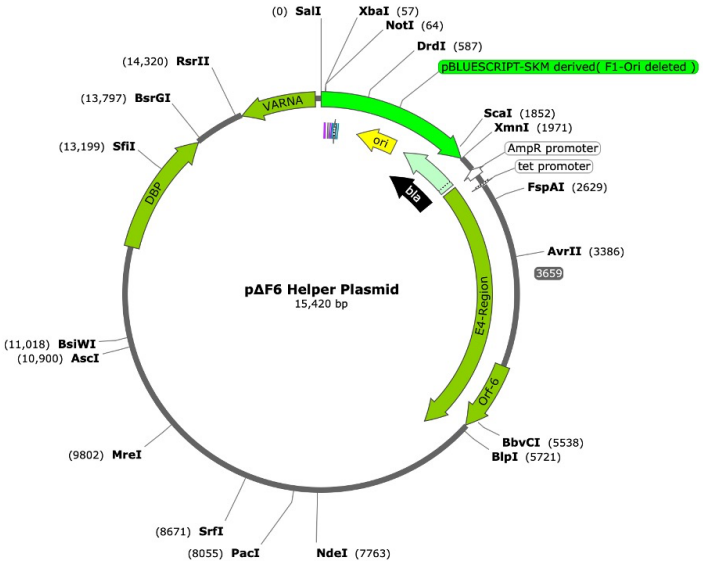


Figure 1. pΔF6 Helper Plasmid map. The canonical helper genes are shown in green. The regulatory regions for the E2a DBP coding sequence are hidden.

Canonical adenovirus helper plasmids: pAd2.VARNA, pAd2.E4orf6, pAd2.E2a.

The scientific literature was consulted for the selection of the adenovirus helper genes that would be needed for this work, and the human adenovirus 2 genome (GenBank Accession# J0917.1) served as the reference sequence. After ascertaining the

right coordinates for early promoters, coding sequences, and poly-A regions in the Ad2 genome, a golden gate assembly approach was followed to extract the desired regions for cloning (*NEB® Golden Gate Assembly Kit (BsaI-HF®V2) Instruction Manual*, n.d.). Short modifications were carried out at the end of genes of interest, or internally as needed, to add the needed Type IIS restriction sites that would generate the correct sticky ends for cloning in the correct reading frame. Subsequently, gene fragments were either ordered as double stranded blocks and amplified by Phusion® PCR, or synthesized as single-stranded oligonucleotides, annealed, and phosphorylated and finally combined in a single reaction with the appropriate Type IIS restriction enzyme and T4 DNA ligase and assembled in an ampicillin resistant plasmid backbone. For pAd2.VARNA cloning, both the Ad2 VARNA I and II genes were chosen and a BsaI-directed golden gate strategy was followed (588 bp gene block and Phusion® PCR only). For, pAd2.E4orf6, a BbsI-directed golden gate strategy was used (1182 bp promoter and cds and Phusion® PCR, plus two annealed, phosphorylated oligos for the 3'-end of the gene); for pAd2.E2a, BsaI-directed strategy was used, as well (two gene fragments, 356 and 1596bp, representing the beginning and the middle of the gene including regulatory sequences, plus an annealed, phosphorylated oligonucleotide for the 3'-end and UTR).

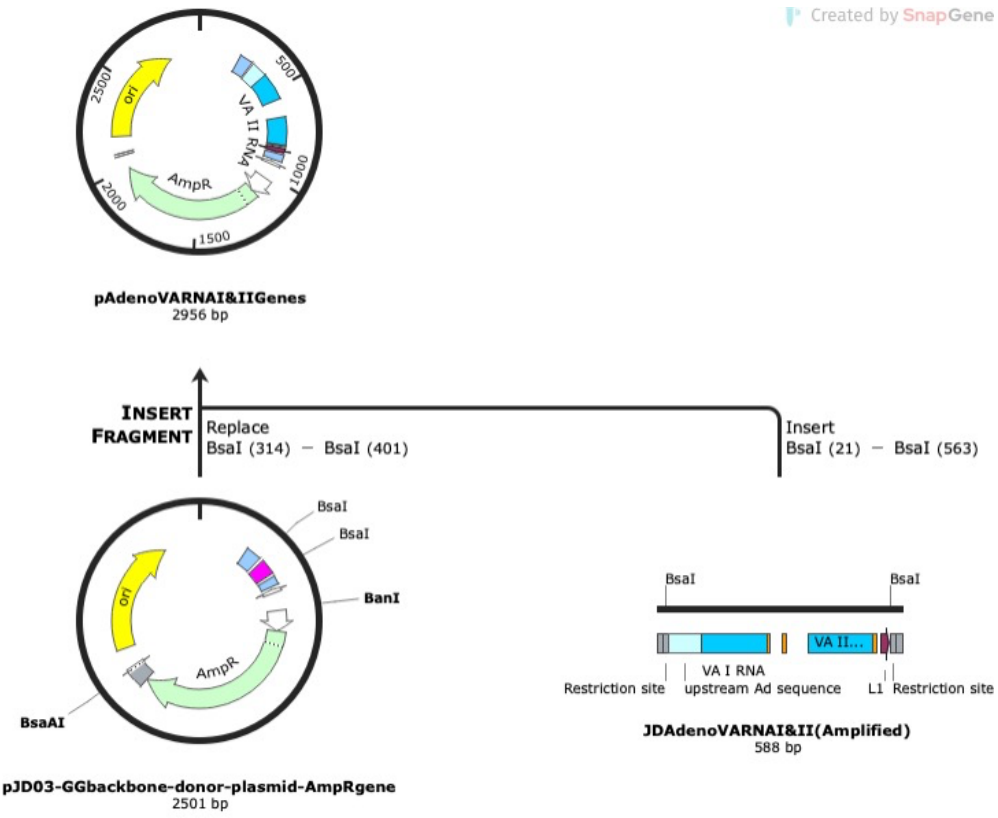


Figure 2. Cloning of the adenovirus VARNA I & II genes based on the Adenovirus 2 genome. The golden gate assembly method was used.

Packaging plasmid.

Locally sourced AAV serotype 2 (AAV2) packaging plasmid was used for all experiments. It codes for AAV2 rep and cap genes under the control of native AAV p5, p19 and p40 promoters which deliver non-structural factors necessary for recombinant genome expansion and packaging, and structural proteins VP1, VP2 and VP3 for virion assembly. The AAVrh32/33 plasmid which delivers the AAV2 rep gene and the hybrid rh32/33 capsid was also used for comparison. These plasmids do not carry AAV's inverted terminal repeats (ITRs).

cis-plasmid (recombinant AAV genome)

Locally sourced 3.07 kb rAAV genome coding for the firefly luciferase gene under the control of constitutive CMV promoter and carries the SV40 polyA sequence. In the presence of magnesium and ATP, Luciferase catalyzes the bioluminescent oxidation of d-luciferin which will serve to report production titers via a transduction assay.

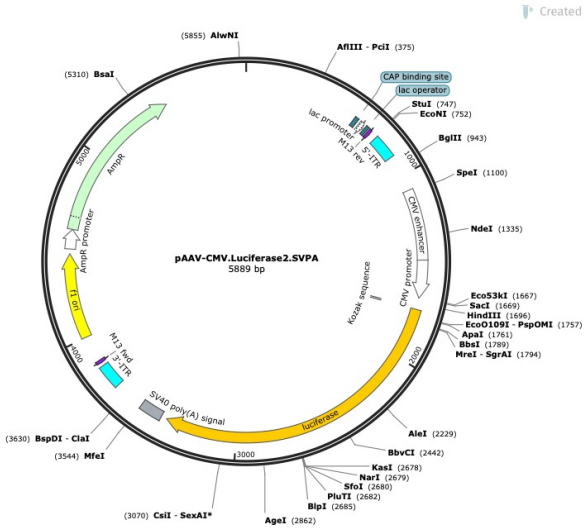


Figure 3. Map of pAAV.CMV.Luciferase.SVPA. The recombinant AAV genome used for all measurements in this study.

Transfection

On Day 0, 80-95% confluent HEK293 cell monolayers on 15cm plates were carefully washed with 1X PBS (room temp, no Ca^{2+} or Mg^{2+}), disrupted with trypsin (room temp, 3.5mL/plate, carefully aspirated), incubated at 37°C for 5 minutes, tapped for dislodging and resuspended in 10mL complete media (1X Dulbecco Modified Eagle Medium [DMEM], 10% FBS, 1% Penn/Strep) in a 50mL conical tube. Resuspended, viable cells were manually counted in a hemocytometer via the trypan blue dye exclusion

(0.4%) method, diluted in complete media in a new 50mL conical tube, and seeded at a concentration of 1E+6 cells/well in 6-well plates (2mL per plate) and allowed to settle, spread, and grow for up to 24hr in an incubator set at 37°C with 5% CO_2 and sufficient ambient humidity.

On Day 1, small scale transfections for rAAV production were performed according to an experimental table (example below, 1x = 2.081x10E-13 mol per plasmid) using FectoVIR®-AAV as the transfection reagent in a biosafety cabinet (1.375µg FectoVIR®-AAV per 1µg of plasmid). FectoVIR®-AAV and plasmid serum-free DMEM fractions ("1" and "2" on the table, "250µL" each) were combined by pipetting (FectoVIR®-AAV added to plasmid to minimize plasmid loss) and incubated at room temperature for 15 minutes. At the end of the incubation period, the 500µL emulsion was combined with 4.5mL serum-free DMEM in a 15mL conical and briefly mixed by inversion. The media supporting overnight growth of HEK293 cells in 6-well plates was carefully aspirated in preparation for FectoVIR®-AAV:plasmid delivery and exactly 2mL of FectoVIR®-AAV:plasmid emulsion were added per well. Each 5mL FectoVIR®-AAV:plasmid emulsion supported a duplicate transfection (1 sample or condition tested). The transfected 6-well plates were returned to a BSL-2 incubator (37°C, 5% CO_2) for rAAV production.

On Day 4 (72hrs post-transfection), the production experiments were collected for both gene expression and luciferase assays. Two 1.5mL microcentrifuge tubes with 0.75mL of TriZOL reagent each were used to collect gene expression samples per condition tested, while two 15mL conical tubes and corresponding 2.0mL microcentrifuge tubes were used for luciferase assay sample collections. Using a 1mL

manual pipette and sterile tip, the contents of each well were vigorously disrupted by pipetting without overflowing neighboring wells, changing tips in-between. After the material became sufficiently homogeneous, 250µL was transferred to the 0.75mL TriZOL tube per replicate, the tip rinsed in the mixture by pipetting, and the tube mixed again by inversion and placed on dry ice; the remaining homogenate was collected in a corresponding 15mL conical tube for all replicates and conditions in a manner to avoid contamination (negative samples first), freeze-thawed 3x, centrifuged (pelleted) at 4700rpm for 15 minutes and the supernatant transferred to a 2mL microcentrifuge tube and stored at -80C for luciferase assay analysis.

	Cells Only	No Helper	No pLuc	1X pHelper	10X pHelper	1X Seg pHelper	10x pE4	10x pE2a	10X pVARNA
pHelper	-	-	2.124µg	2.124µg	21.24µg	-	-	-	-
pAAV2/2	-	1.025µg	1.025µg	1.025µg	1.025µg	1.025µg	1.025µg	1.025µg	1.025µg
pLuc	-	0.8184µg	-	0.8184µg	0.8184µg	0.8184µg	0.8184µg	0.8184µg	0.8184µg
pHelper_ E4	-	-	-	-	-	TBD	TBD	TBD	TBD
pHelper_ E2a	-	-	-	-	-	TBD	TBD	TBD	TBD
pHelper_ VARNA	-	-	-	-	-	TBD	TBD	TBD	TBD
Fectovir	5.45µg (13.64µL)	2.54µg (6.34µL)	4.33µg (10.83µL)	5.455µg (13.64µL)	31.74µg (79.35µL)	TBD	TBD	TBD	TBD

Figure 4. Example transfection scheme table. Fectovir®-AAV is the transfection reagent. All other reagents are plasmids referred at the left of the table. Molar equivalents were calculated based on an established protocol and used for subsequent transfection experiments.

Luciferase Assay

On Day 0, black 96-well flat-bottom assay plates (Corning, #3904) are coated with 100µL room temperature poly-L-Lysine from a reagent reservoir in a biosafety cabinet using a multichannel pipette and incubated at 37°C for 1 hour. Following incubation, the poly-L-Lysine is completely removed and recycled, and the plates are allowed to dry, lids off, at room temperature for 30min inside the biosafety cabinet and stored at 4°C if not immediately seeded. Then, 200µL of room temperature 1X PBS (without Ca^{2+} or Mg^{2+}) in a reagent reservoir is added to wells in the periphery of the plate using a multichannel pipette. Subsequently, 80-95% confluent HEK293 cells in a 15cm dish were washed, trypsinized, and counted as described above, resuspended and diluted in complete media (100 cells/µL), transferred to a reagent reservoir and delivered to the inner wells of the 96-well assay plates (200µL or 20,000 cells/well) and incubated overnight at 37°C, 5% CO_2,

On Day 1, the complete media supporting the growth of cells in the inner wells of the assay plate(s) are carefully aspirated in a checkerboard pattern without disturbing the PBS in the periphery or the monolayers. This set up allows for six rows for light measurements, 5 wells each: two rows per condition and one per replicate. The 5 cleared wells are inoculated with 25-50µL of freeze-thawed lysate containing virus [25µL is easier to deliver via an electronic pipette]. Skipped wells retain 200µL of complete media from the day before. The inoculated plate(s) are incubated for 1hr at 37°C, 5% CO_2 in a BSL2 incubator. After 1 hour, 175-150µL of complete media is added to the wells that received virus to make up 200µL final volume and the plate(s) are returned to the BSL2 incubator.

On Day 3 (48 hrs post-infection), all the inner wells of the assay plate(s) are carefully aspirated, changing tips between infected and uninfected wells, conditions, replicates, and controls and leaving the perimeter wells alone. The inner wells are then disrupted with 1X lysis buffer (20µL/well; Promega, Cat# E397A) and the plate(s) are incubated at -80°C for 30 minutes or until frozen, followed by incubation at 37°C for 15 minutes or until completely thawed. A microplate reader is purged and primed in advance of a readout, first with ultra clean MilliQ water, then with the Luciferin substrate solution (200mM Tris, pH 7.8, 10mM MgCl2, 0.3mM ATP, 0.05mM D-Luciferin, 1X Signal Enhancer [1x]), the plate(s) are transferred to the instrument and the inner wells are sequentially exposed to 100µL Luciferin subtrate solution and immediately read at 613nM and the relative light units data is collected and analyzed.

Gene Expression Assay

Trizol® samples were thawed, equilibrated at room temperature, processed with ultrapure, sterile RNA-dedicated reagents, and centrifuged in the cold. First, they were extracted with 200µL of chloroform in a chemical hood, mixed by inversion and pulse-vortexed, incubated 2-3 minutes at room temperature and centrifuged for 15 minutes at 12,000 *x g*. The clear top aqueous layer of RNA was carefully transferred to a new 1.5mL microcentrifuge tube, avoiding the DNA interphase or protein layer below. Setting up a 1mL pipette at 500µL and refraining from collecting more material generally avoids this sort of contamination. An equal volume of isopropanol was added to the RNA, pulse-vortexed, and incubated at room temperature for 10 minutes. The precipitated RNA was centrifuged for 10 minutes at 12,000 *x g* and the excess volume mostly removed without

disturbing the pellet at the bottom. The pellets were washed, dislodged with 1mL room temperature 75% ethanol, and centrifuged for 5 minutes at 7500 *x g*. Excess volume was carefully removed this time, leaving only the pellet(s) behind. The pellets were air-dried for 5 minutes inside a biohazard cabinet, resuspended with 50µL of nuclease-free water, vigorously vortexed to aid resuspension, pulse-centrifuged, incubated for 15 minutes at 37°C, and quantified via Qubit fluorescence.

A fraction of the total RNA collected (5µg) was DNase-treated at 37°C in a 50µL reaction using the Turbo DNA-*free*™ kit (ThermoFisher), and 10µL of the treated RNA was used for reverse transcription via random primers of the High-Capacity cDNA Reverse Transcription Kit. Negative control reactions (-RT) were also set up with the same kit for all samples tested and 2µL of the reactions were used for real-time PCR per gene tested. Quantitative real-time PCR was carried out in a QuantStudio 7 Flex instrument in 10µL reactions. The three adenovirus genes tested (E2a-DBP, VARNAI, and E4 orf6) and the reference 18S rRNA gene from the host HEK293 cells were measured separately from the same reverse transcription reactions to allow maximum sensitivity of detection and no interference. The oligonucleotides used were as follow:

AdE2a Assay:

Fwd Primer: 5'- CCAAGTGGAAGCTCTGG -3' Tm= 59.5°C, 0.7µM

Probe: 6FAM- CGGCCGTATGCAAGA -MGBNFQ Tm= 68.0°C, 0.25µM

Rev Primer: 5'- GCGGTGCTCCTCGTT -3' Tm= 59.5°C, 0.12µM

Amplicon size: 55bp

AdE4orf6 Assay:

Fwd primer: GGTCTTCCCTGCAGTG Tm = 59.5°C, 0.7µM

Probe: 6FAM- TACGCTGATTCAGGAATG -MGBNFQ Tm = 70.0°C, 0.25µM

Rev primer: AGAACCATATCCCAGGGA Tm = 59.5°C, 0.6µM

Amplicon size: 65bp

AdVARNA I Assay:

Fwd primer: CTCCCCCGTTGTCTG Tm = 59.5°C, 1.25uM

Probe: 6FAM- ACGTCGCACACCTG -MGBNFQ Tm = 68°C, 0.25µM

Rev primer: GTTACCGCCCGCGTG Tm = 60.7°C, 0.1uM

Amplicon size: 51bp

Homo sapiens 18S rRNA Assay

Fwd primer: GCCGTTCTTAGTTGGTGG Tm = 60.8°C at 0.9µM

Probe: VIC- TTGTCTGGTTAATTCCG -MGBNFQ Tm = 68°C, 0.25µM

Rev primer: CATGCCAGAGTCTCGTTC Tm = 61.1°C, 0.9µM

Amplicon size: 64bp

The reactions conditions were the default set by the instrument minus an initial incubation for which the master mix reagent did not have an enzyme. Briefly, these included an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of a denaturation step at 95°C for 30 seconds and an annealing/extension step at 60°C for 1 minute.

Relative quantification of gene expression was carried out according to the ΔΔCt method against a no transfection control (the calibrator or negative sample) which receives a value of 1.0 in the analysis. Test samples are measured to express x times the calibrator (Livak & Schmittgen, 2001).

Chapter III.

Results

Under or over delivery of adenovirus helper plasmid undermines rAAV production

Vector production where the helper plasmid is delivered at 10-times more or 10-times less than the requested protocol amount resulted in markedly reduced rAAV titers (Figure 5). Excess helper plasmid is likely toxic to the cell and/or the intrinsic machinery of the rAAV manufacturing effort, whereas insufficient helper plasmid likely fails to kick off production. The underlying molecular mechanisms involved in these observations were not explored and are not understood. Also, competency at making vector was demonstrated here because the regular protocol that delivers 1X helper plasmid at transfection yielded the most luciferase output (relative light units), direct evidence that a higher number of transducing particles was present.

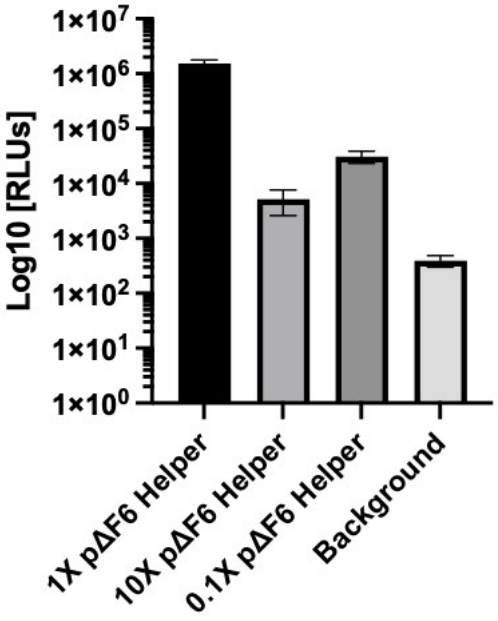


Figure 5. rAAV2.CMV.Luciferase production in HEK293 cells. The mass of input helper plasmid was varied at transfection time as indicated. The amount of helper plasmid delivered to the reaction was solely based on mass.

Delivery of segregated canonical adenovirus helper genes does not result in comparable rAAV production.

Satisfied that there was competency making rAAV to high titer, the ability of the segregated canonical adenovirus helper genes for making rAAV was tested (Figure 6). In this case, the intact helper plasmid used for comparison is pALD-X80 which is routinely used to make vector in the local core facility.

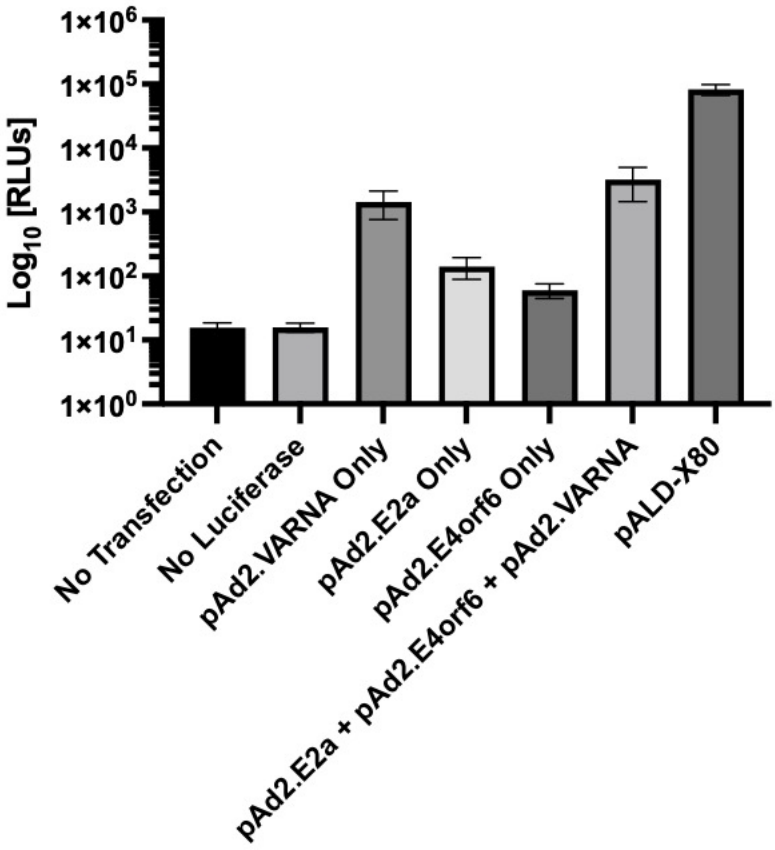


Figure 6. rAAV2/2.CMV.Luciferase production with a single helper plasmid at a time. The delivery of segregated canonical helper plasmids was not equimolar in this experiment, as well, but solely based on mass, one third of the amount of the intact helper.

Individual canonical genes alone failed to produce as much rAAV as the intact helper plasmid, pALD-X80. Similarly, the three canonical helper genes together failed to make as much rAAV as the intact plasmid, as well. The pAd2.VARNA plasmid alone appears better at promoting vector production and is likely synergistic in supporting the action of the other two canonical genes given the light output in the transfection where all three canonical genes were delivered. There was interest in further testing this observation by delivering the canonical helper genes in pairs and complementing the

action of the individual canonical genes by adding a second one and repeating the comparison (Figure 7).

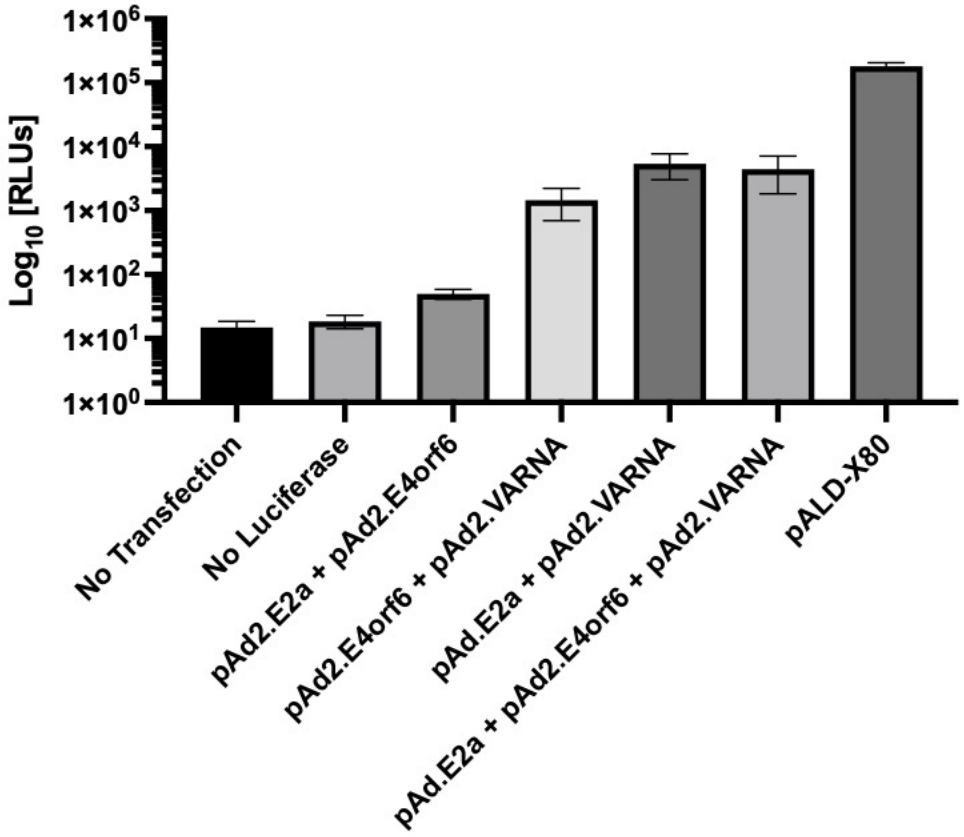


Figure 7. rAAV2/2.CMV.Luciferase production where one of the canonical helper genes is excluded from the transfection. The plasmids used for this experiment were not delivered at equimolar levels but by consideration of the mass of the intact helper plasmid alone (2.6μg). No stuffer plasmid was used to keep the mass of plasmid constant across transfections since this is not the manner vector is made. Exacly one third of the mass of the intact helper plasmid was used for the determination of the input of segregated canonical helper plasmids (0.87μg).

Once again, the segregated genes in pairs failed to produce as much rAAV as the intact helper plasmid and as observed from the single helper gene delivery experiment. The stabilizing effect of the VARNA genes is again. The data also suggests that there is a

negative correlation between the canonical pAd2.E2a and pAd2.E4orf6 genes when they are transfected in the absence of pAd2.VARNA.

To rule out whether these observations were not related to the packaging plasmid used to make recombinant vector, the experiment was repeated with a different packaging plasmid, pAAVrh32.33 (Figure 8). While the rAAV2 transfections yielded slightly higher titers of rAAV than rAAVrh32.33, the trends observed earlier were comparably the same. This suggests that the identity of the capsid does not explain the results.

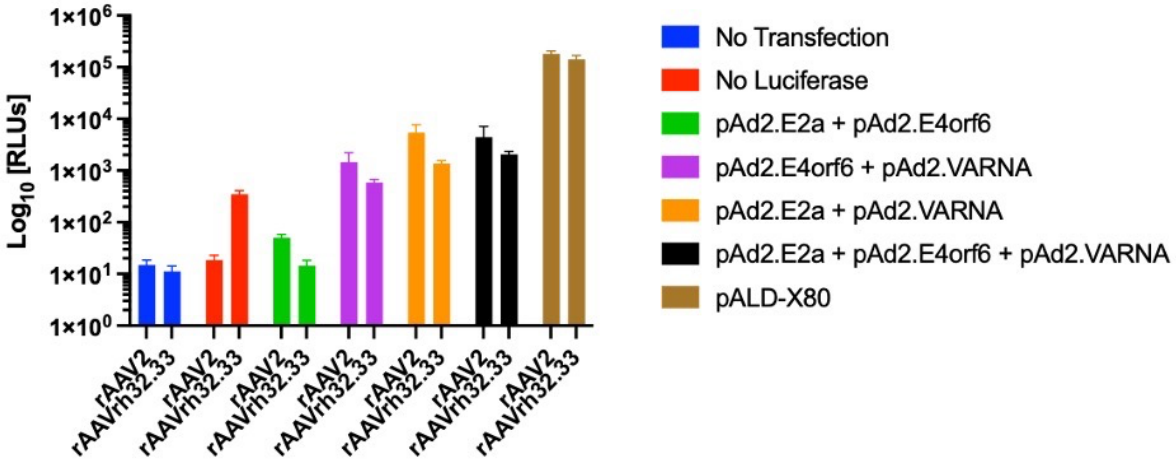


Figure 8. rAAV2 and rAAVrh32.33.CMV.Luciferase production where one of the canonical helper genes is excluded from transfection as in the previous figure. The higher light output of the rAAVrh32/33 "No Luciferase" control is considered a contaminant.

pALD-X80 is a slightly better helper plasmid.

Following these experiments, attention was re-directed to delivering equimolar quantities of plasmid (or multiples thereof) per transfection condition. Based on the number of moles of helper plasmid pALD-X80, 18876 bp, a calculation was made of the number of moles delivered per transfection reaction at 2.6μg (the mass of helper plasmid called for in the local core's production protocol) per well in small-scale rAAV production in 6-well plates (Figure 9). 2.6μg of pALD-X80 (2.08x10E-13 mol) appears to be slightly better than the same amount of pΔF6 for making rAAV. Whether this is the result of the extra adenovirus sequence present in pALD-X80 was not explored in this work. The objective, once again, was rather to demonstrate competency at producing rAAV in a more controlled set of conditions and to have a stronger quantitative basis for further experimentation.

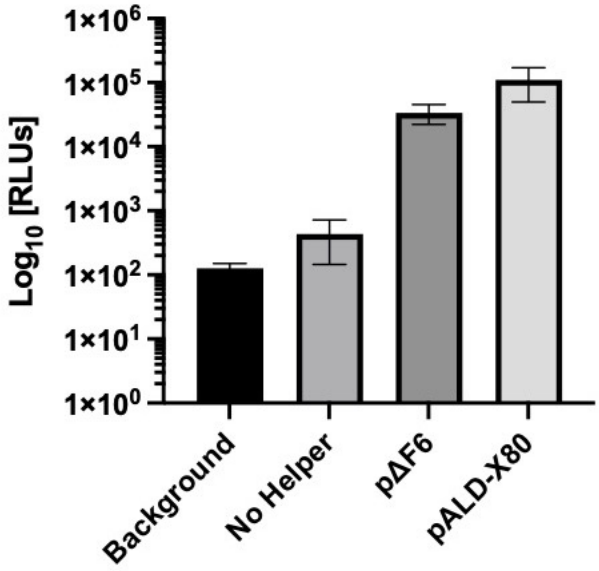


Figure 9. rAAV2.CMV.Luc production by equimolar delivery of manufacturing plasmids during triple transfection (2.08x10E-13 mol per plasmid).

The early E2a gene is under-expressed in the segregated adenoviral helper gene system of rAAV production.

Rather than exploiting the pAd2.VARNA effect at this point, the focus shifted to understanding the effect pAd2.E2a had on pAd2.E4orf6 and viceversa (Figure 10). It was found that there is a positive correlation to rAAV2.CMV.Luc production by multiplying the number of moles of pAd2.E2a relative to pAd2.E4orf6, and a negative correlation to rAAV2.CMV.Luc production by increasing the number of moles of pAd2.E4orf6 relative to pAd2.E2a. Nevertheless, increasing the number of moles of pAd2.E2a did not improve rAAV2.CMV.Luc production beyond the levels achieved by the intact helper plasmid. The potential role of pAd2.VARNA in improving production under these conditions is also suggested by the data.

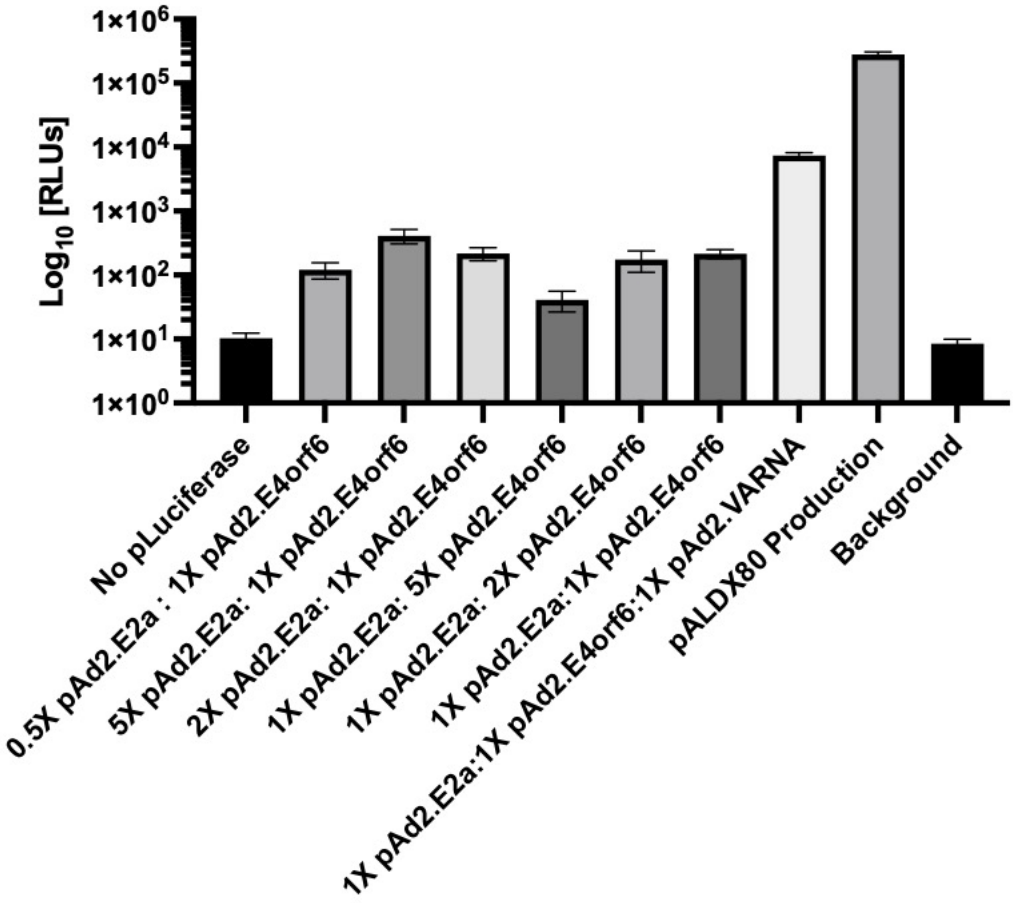
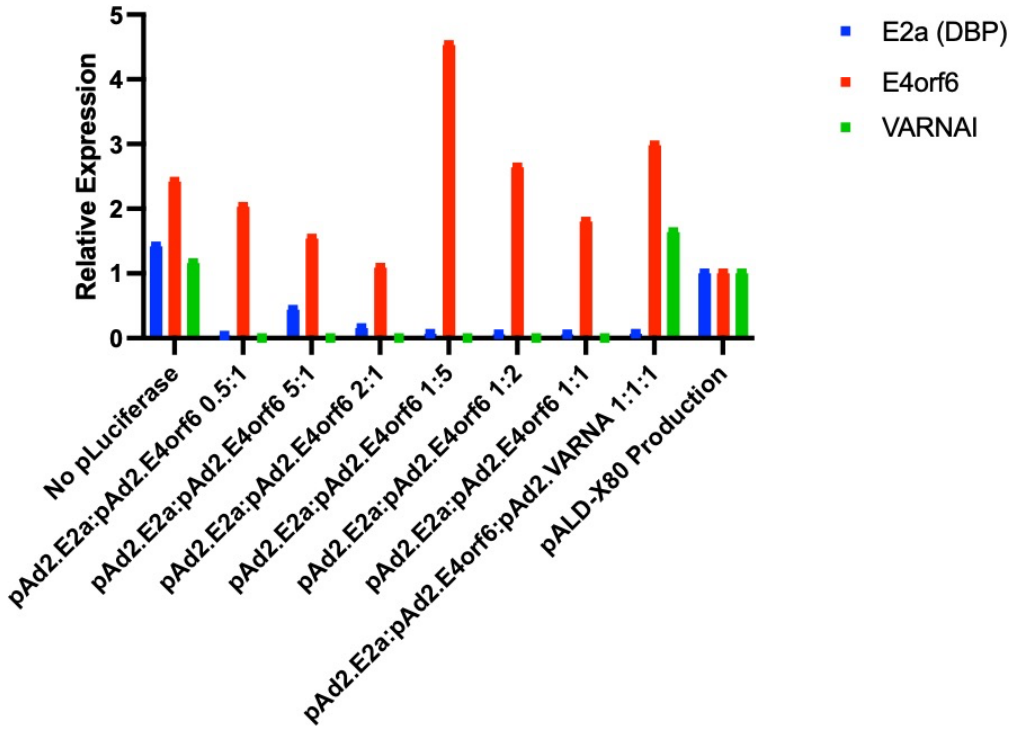


Figure 10. rAAV2.CMV.Luc manufacturing by luciferase output. A comparison of the effect of E2a and E4orf6 on each other. It appears that E4orf6 has an inhibitory effect on production over pAd2.E2a. The VARNA genes substantially improve rAAV2 production.

To understand these observations better, attention shifted to measuring the RNA expression levels of these genes (Figure 11). In the absence of VARNA, an inverse correlation was found between the level of E2a DBP and E4orf6 gene expression. More E2a DBP mRNA resulted in less E4 orf6 expression even though the input pAd2.E4orf6 plasmid was kept constant. This suggests that E2a DBP transcription and possibly translation is driving the repression of E4orf6.

A.



B.

Sample ID	AdE2a	AdE4orf6	VARNAI
No pLuciferase	1.42E+00	2.42E+00	1.16E+00
E2a:E4orf6 0.5:1	4.13E-02	2.03E+00	2.88E-05
E2a:E4orf6 5:1	4.41E-01	1.54E+00	9.14E-05
E2a:E4orf6 2:1	1.55E-01	1.09E+00	5.35E-05
E2a:E4orf6 1:5	6.84E-02	4.53E+00	7.03E-05
E2a:E4orf6 1:2	6.24E-02	2.64E+00	4.04E-05
E2a:E4orf6 1:1	5.91E-02	1.80E+00	4.95E-05
E2a:E4orf6:VARNAI 1:1:1	6.75E-02	2.98E+00	1.64E+00
pALD-X80 Production	1.00E+00	1.00E+00	1.00E+00

Figure 11. A. Graphical representation of gene expression for the experiment in Figure 10. **B.** The raw relative expression data for the same experiment.

The opposite was not true to the same extent, however. Increasing the E4orf6 mRNA levels by modulating the pAd2.E4orf6 plasmid input up to 5-fold has a positive, but very small effect on E2a DBP RNA levels (and a negative effect on rAAV

production) which varied between 16.9-fold to 14.6-fold less than the DBP expression of the intact helper plasmid or in the presence of pAd2.VARNA in the segregated helper plasmid transfections.

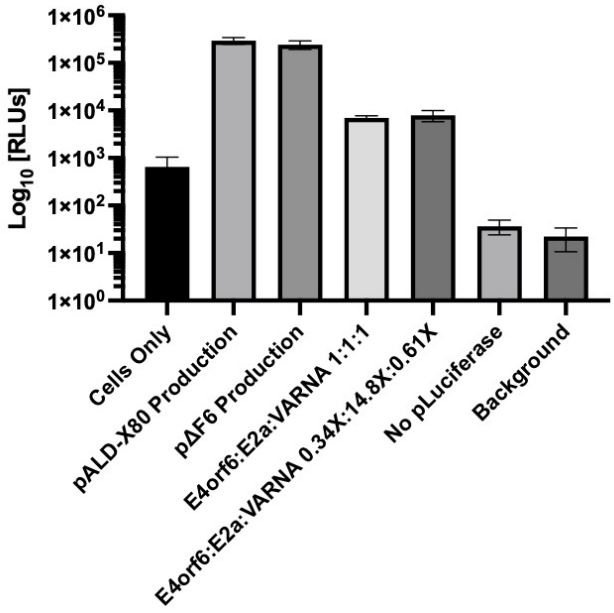
Interestingly, a 4-fold increase in pAd2.E2a input at transfection resulted in a 3.75-fold increase in E2a DBP expression, and about 2-fold decrease in E4orf6 expression (and a modest increase in rAAV production). Yet, when the pAd2.E2a input was increased from 2-times to 5-times the molar input of the intact helper plasmid (a 2.5-fold difference), E4orf6 expression increased along with a greater, but still modest, increase in rAAV production. These observations point to a possible threshold effect between these two genes that has already been noted in the scientific literature (please see the Discussion). Finally, only the pAd2.VARNA input significantly boosted rAAV manufacturing by more than 30-fold compared to the 1:1 pAd2.E2aDBP: pAd2.E4orf6 molar equivalent transfection reaction but was still nearly 40-fold below production levels of the intact helper plasmid.

Rescue of early E2a DBP gene expression does not improve rAAV production but increases VARNA expression.

The most salient features of the last experiment were the nearly 15-fold lower expression level of the early E2a DBP gene driven by the pAd2.E2a plasmid compared to the E2a DBP expression levels driven by the intact helper plasmid, and the comparatively more modest, but elevated expression of the two other genes. Thus, the next steps were focused on addressing these differences by modulating the expression of these genes via

increasing or decreasing the input of the segregated canonical helper plasmids designed for this study (Figure 12).

A.



B.

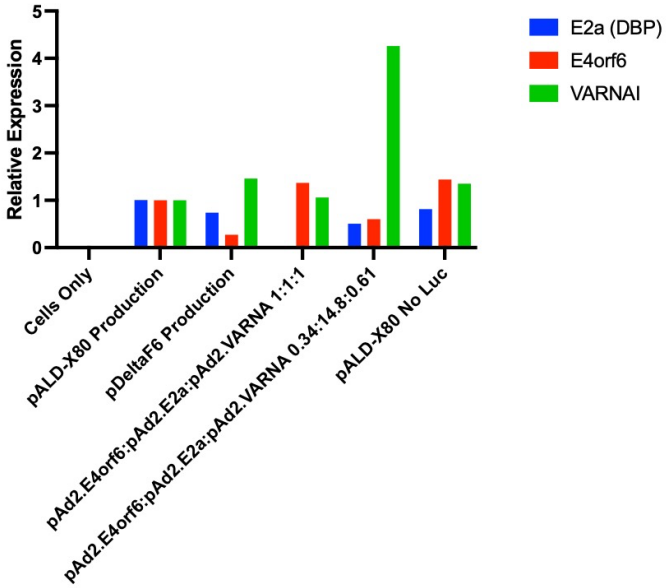


Figure 12. A. rAAV2.CMV.Luciferase production data via transduction and light output of modifed transfection. **B.** Gene expression data for the same experiment.

The new test rAAV2.CMV.Luciferase production transfection carried 0.34-times less pAd2.E4orf6 plasmid, 14.8-times more pAd2.E2a and 0.61-times less pAd2.VARNAI. In addition, a transfection condition using the more traditional pΔF6 helper plasmid was included as a control. Despite the adjustment made to the segregated helper vector inputs to match previously obtained expression data, the amount of rAAV manufactured did not improve compared to production with either intact helper plasmid but remained at similar levels to the transfection mediated by the equimolar delivery of the three segregated canonical helper plasmids.

RNA expression was measured for the experiment and several features were noted. First, E2a DBP expression in the test condition increased, but was not yet as high as DBP expression in the intact helper plasmid transfection mediated by pALD-X80. Despite this, DBP expression in the helper plasmid transfection mediated by pΔF6 was not as high as in the pALD-X80 transfection either, so this observation could not account for the low rAAV production level in the test condition.

Second, the expression level of the E4orf6 gene decreased in the test condition as expected but was lower than anticipated. Yet, once again, the E4orf6 expression in the transfection mediated by pΔF6 was even lower, so this small discrepancy cannot account for the differences in rAAV production levels, as well.

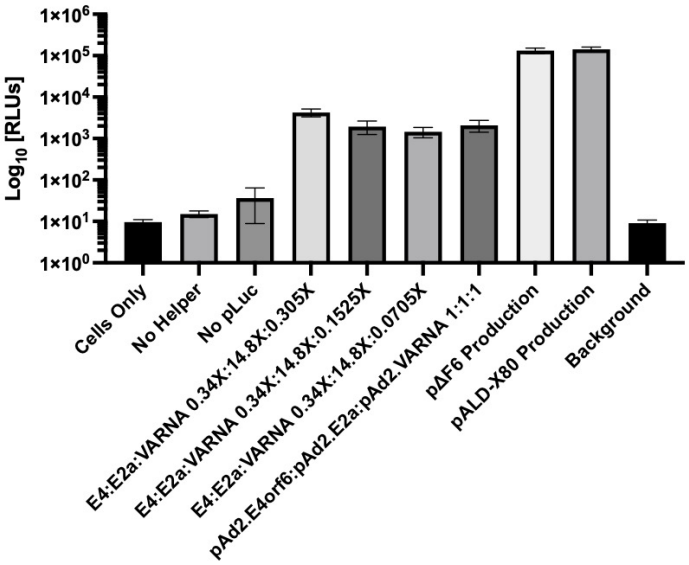
Third, rather than lowering the expression of the VARNAI gene due to lower pAd2.VARNA input levels at transfection, as expected, the test conditions unexpectedly increased VARNAI gene levels 4-fold above VARNAI expression in the intact helper plasmid pALD-X80 control transfection, and slightly lower than in the pΔF6 control reaction. Despite noticeably improving rAAV production in other experiments in this

study, increased expression of the VARNAI gene did not improve rAAV production in the test condition.

Modulating VARNA gene expression while rescuing early E2a DBP gene expression does not improve rAAV production, either.

Utilizing the same means to reduce expression of the VARNA gene while boosting E2a DBP expression in a new experiment, no improvements in rAAV production were achieved (Figure 13). An unexpected consequence of lowering VARNA expression was the increased expression of the E2a DBP gene from the pAd2.E2a plasmid in a dose-dependent manner, contrary to previous observations. There was also a concomitant, but more modest boost to the expression of the E4orf6 gene through the reduction of VARNA expression which was also unexpected and the opposite of previous observations. While increasing E2a.DBP and E4orf6 expression and reducing VARNAI levels in the test conditions, the expression of the three genes closely resembled that in the two intact helper plasmid transfections. However, this did not translate to improvements in rAAV production for reasons that remain unknown.

A.



B.

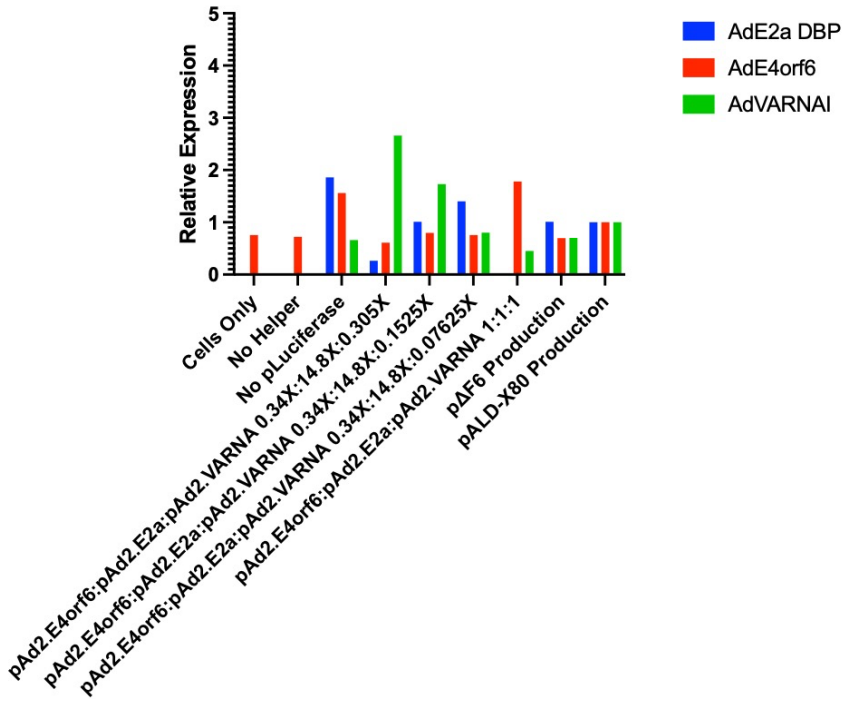


Figure 13. A. rAAV2.CMV.Luciferase production after modulating the input of the pAd2.VARNA plasmid at transfection. To deliver such small quantities of vector, the surfactant Pluronic® F-68 was used to minimize plasmid loss

Chapter IV

Discussion

The initial aim of the study was simple: establish competency at making recombinant vector using the local core facility's protocol. For small-scale transfections, the local protocol called for 2.6μg of helper plasmid, and 1.3μg each of packaging plasmid and rAAV genome per well in a 6-well plate, a 5.2μg total mass of plasmid DNA input which yields relatively abundant rAAV on a regular basis. This is not a strictly equimolar transfection considering that it has been used to make hundreds of different rAAVs with no adjustment due to plasmid size, but it was not important.

What matters is that the helper plasmid, pΔF6, undermined the ability to produce rAAV when delivered in large excess or at very low levels during transfection. Apart from the bacterial origin of replication, associated sequences, and the ampicillin resistance operon which allows for pΔF6's propagation in selective growth medium, the remainder of the helper plasmid's 15,420 base-pair sequence belongs to the human adenovirus type 5 genome of which the E4, VARNA I, and E2a helper genes are the most conspicuous. Other open reading frames are present in the helper plasmid, as well.

The E1a/b, E4, VARNA I, and E2a genes are considered the canonical helper genes, the minimum essential, that lend helper functions to the propagation of wild-type and recombinant AAVs in imitation of the lytic phase of the virus (Xiao et al., 1998). The polypeptide arising from orf6 of the E4 gene (there are at least 7) and the E2a DNA binding protein (DBP) indispensable for rAAV production. The virus-associated RNA I

gene is non-coding. Its transcript has been implicated in modulating the anti-viral response of the adenovirus infected cell. AAV is an indirect beneficiary of these functions.

The E1a and E1b genes whose products are provided by complementation via the HEK293 host cell line, are also very important. The E1a gene products serve as the primary transactivation factors for the other helper genes, whereas the E1b products in connection with the E4orf6 factor help create a tolerable environment in the cell for viral replication. E1a and E1b are constitutively expressed (Graham et al., 1977; Tan et al. 2021).

The likely reason why an excess of helper plasmid shuts down rAAV production is the dysregulation of specific pathways under their control. The E1a/b genes are presumed to have a fixed level of expression, but this may change upon a higher helper plasmid load from the outside that may positively regulate transcription of the genes under its control. With higher E1a/b expression, it is possible that the AAV's Rep gene becomes overexpressed, leading to host cell shutdown given that Rep is known to be toxic to the cells. Alternatively, dedifferentiation of the HEK293 cell line by overloading the cell with helper plasmid could possibly shut down E1a/b transcription, leading to poor rAAV recovery (Zemke et al, 2019). The expression of these genes, however, was not measured. Similar possibilities are likely with the other genes, but it was not confirmed. Under-delivering the helper plasmid is perhaps easier to explain and may point to insufficient activation of the pathways leading to transgene rescue, replication and packaging.

The helper gene clones that were made for this study were focused primarily on delivering the canonical helper proteins. They were meant to carry minimal native regulatory sequences and the coding region of interest. They were synthesized de novo and cloned, rather than being derived directly from the adenovirus genomic sequence by simple sub-cloning from the helper plasmid or adenovirus DNA. The regulatory sequences of the VARNA I gene are embedded within its coding region and were synthesized directly and cloned.

It follows that delivering the canonical genes together after separating them should yield the same amount of rAAV compared to the intact helper plasmid. This was not observed in these experiments. While the VARNA I gene did show signs of improving the outcome of the transfections tested, the luciferase output was consistently below expectations. When the VARNA I gene is absent, production is barely above backgound and appeared to be consistent with observations that the E2a and E4orf6 genes repress each other.

Expression of the E2a DBP gene under the control of the early promoter was consistently 15-fold below the expression of E2a DBP from the helper plasmid. The attempt to re-balance mRNA expression levels was successful, but it did not improve luciferase assay metrics. There are several explanations for this outcome.

First, it is possible that the intact helper plasmid is providing other factors beyond the three canonical helper genes that are not accounted for. This is a reasonable result because the intact pΔF6 is much more complex than the separated genes that were cloned. There are other ORFs in the plasmid that we did not clone or study.

Second, maybe the expression of DBP from the intact, more complex, helper plasmid is driven by the alternative late E2a promoter, instead of the early one. Both are present in the intact helper plasmid. This would imply that the early DBP transcript, activated by the E1a gene, is shut down sometime during rAAV production by the host cell. This is consistent with the results because evidently the DBP expression level from the segregated canonical gene is very low. It also implies that AAV found a way to take advantage of early E2a gene repression, perhaps because it is the cell that is now driving DBP synthesis in the system rather than the adenovirus E1a gene. This is entirely possible because E1a is known to activate the early E2a promoter and not the late one (Rossini, 1983). Of course, this would need confirmation and it assumes that the segregated early E2a DBP plasmid was cloned correctly with all its regulatory regions present.

Lastly, a closer inspection of the de novo cloned early E2a gene used in this experiment revealed that the plasmid was missing 107bp from the 5'UTR and had an incorrect Kozak sequence (Figure 14). So, very likely, the plasmid was not expressing DBP from the outset because it was missing a transcription factor binding site but needs confirmation. This result is consistent with the data generated.

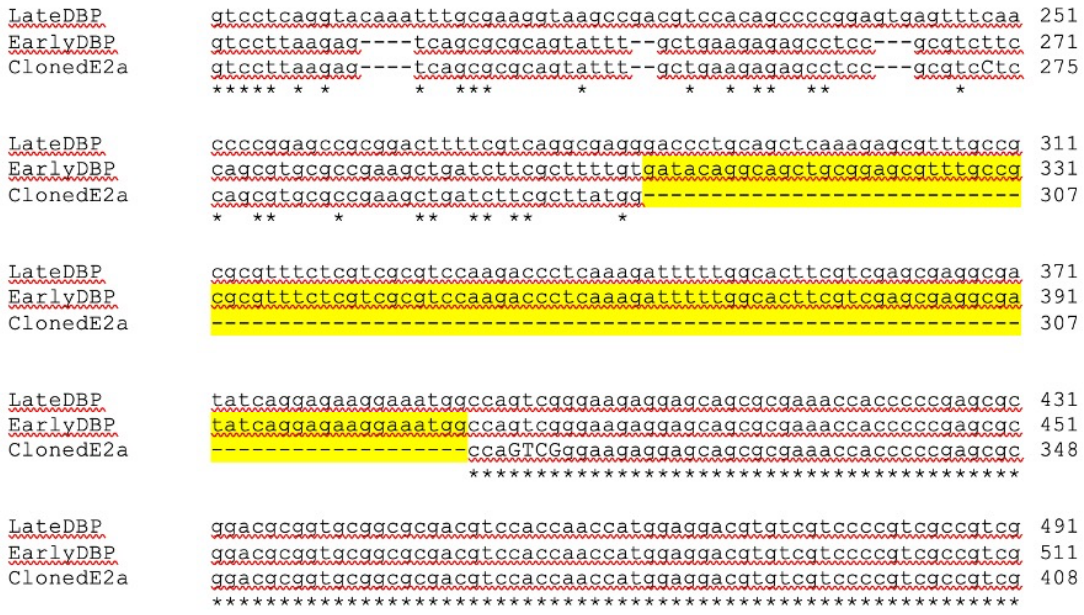


Figure 14. Alignment of Late, Early, and cloned E2a DBP sequence. The missing region is highlighted for convenience.

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