WHITE PAPER

AAV vectors: challenges and solutions for gene therapy manufacturing.

Introduction

Gene therapy is the modification of specific genes in targeted cells in order to address genetic mutations that cause some of the most debilitating human conditions. After a rocky start in the 1990s, the development of successful gene therapies has gained momentum in the last two decades.

The world's first approved gene therapy became available in China in 2003 for the treatment of squamous cell carcinoma in skin cancer. Russia and the EU approved their first gene therapies in 2011 – 2012 for the treatment of peripheral artery disease and lipoprotein lipase deficiency, respectively. In 2017, the US approved its first three gene therapies targeting acute lymphoblastic leukemia, large B-cell lymphoma, and retinal dystrophy. Fast forward to 2022 and there are more than 20 gene therapy products approved for the treatment of a range of conditions.

The backdrop upon which every successful gene therapy is developed is the ability to target specific cells or organs without affecting others. The therapy delivery system – or vector – is a crucial part of this challenge.

Gene therapy vectors

Vectors for the delivery of gene therapies are generally grouped as viral or non-viral vectors. Non-viral vectors currently of interest include chemical disruption, electroporation, polymers, and inorganic nanoparticles. Such vectors have positive attributes including the ability to deliver large genes and having low levels of cytotoxicity, immunogenicity, and mutagenesis. Conversely, non-viral vectors also have limitations such as low gene transfer efficiency, lack of specificity, and short gene expression duration. Researchers continue to investigate ways to overcome these challenges.[1]

In the meantime, viruses remain the most common vectors in gene therapy clinical trials. Viral vectors efficiently invade cells and insert their genetic material into the host genome. Challenges with viral vectors include initiation of acute immune responses, unwanted genome manipulation in the host cell, and potential oncogenic transformation.[1,2]

Some of the most commonly investigated viral vectors and their attributes are described in Table 1. Adeno-associated virus (AAV) vectors provide a highly suitable combination of characteristics that make them the leading viral vector in gene therapy today.

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Table 1: Commonly investigated viral vectors and their attributes[2]

Characteristic	Viral Vector			
	Recombinant retrovirus	Lentivirus	Adenovirus	AAV
Type	RNA	RNA	DNA	DNA
Insert capacity	9 kb	10 kb	30 kb	4.6 kb
Integration of genes into recipient genome?	Yes, in dividing cells	Yes, in dividing and non-dividing cells	No	Rare
Duration of expression	Long	Long	Transient	Long in post-mitotic cells
Immunogenicity in recipient	No	No	Yes	Yes
Adverse effects	Possible insertional mutagenesis	Possible insertional mutagenesis	Inflammatory response	Mild inflammatory response
Other characteristics	Possible germline transmission	Germline transmission	Foreign DNA easily inserted into vector genome	Possible germline transmission

AAV vectors

AAV is one of the smallest non-enveloped viruses. Its genome is small compared to adenoviruses, consisting of a linear single-stranded DNA molecule approximately 4.7 kb in size. It lacks a polymerase gene, relying instead on cellular polymerases for genome replication.

AAV requires minimal genome size for replication. Thus, as a vector, most of its genome can be replaced with the therapeutic DNA insert, enabling the delivery of DNA inserts up to nearly five kb in size. AAV vectors are effective in both actively dividing cells and non-dividing cells where they integrate the therapeutic insert into the host cell's genome without incorporating the viral genome.

AAV is also versatile for targeting different tissues. There are 11 AAV serotypes with more than 100 variants, providing distinct tropisms for gene delivery to specific tissues. AAV's unique features make it the most suitable viral vector for ***in vivo*** gene therapies, especially for conditions that require long-term gene modifications.

In 2017, the US Food and Drug Administration (FDA) approved its first AAV-delivered gene therapy. The therapy is for children and adults with RPE65-associated retinal dystrophy, a mutation-driven inherited form of blindness. The therapy has also been approved by the European Medicines Agency (EMA). The FDA has since approved another AAV-delivered gene therapy for treatment of spinal muscular atrophy and, as of January 2022, there are 264 AAV vector clinical trials underway.

The global gene therapy market was USD 4.1 billion in 2021, and AVV vector therapies made up more than 43% of that market value (Figure 1). North America is the largest regional market, and Asia is the fastest growing regional market. The global market is expected to increase to USD 14.8 billion by 2027, driven primarily by increasing R&D investments, technological advancements (especially in CRISPR and CAR-T gene editing), and the growing prevalence of target diseases, especially cancers.[3,4]

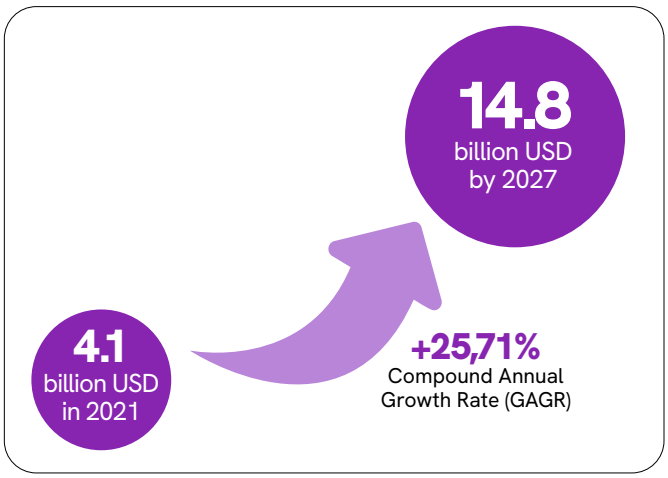

Figure 1: Gene therapy global market growth prevision

AAV vectors and *in vivo* gene transfer

In vivo gene therapy involves the direct infusion of the therapeutic product into the patient's bloodstream or target organs. The viral vector then enters the targeted cells where it replicates and delivers its therapeutic insert. AAV, however, is a helper-dependent virus that cannot replicate on its own. Thus, co-infection with a helper virus, often adenovirus or herpesvirus, enables AAV replication and infection of the target tissue.

The AAV linear DNA genome contains two genes—rep and cap—that encode the replication and capsid proteins, respectively. It also includes inverted terminal repeats (ITRs) that are needed for the replication and packaging of the AAV genome.

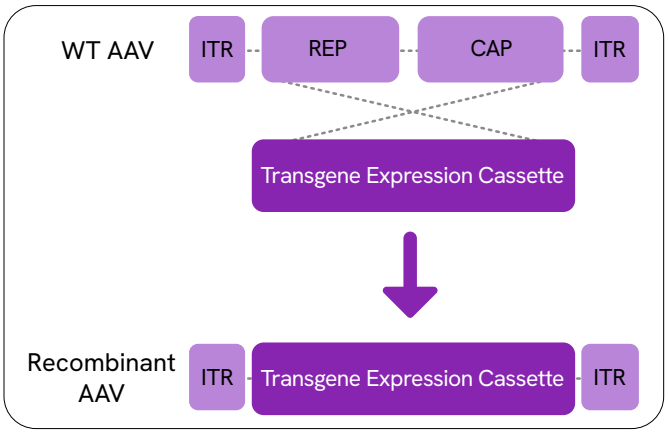

Figure 2: AAV DNA genome

In most AAV vectors the *rep* and *cap* genes are replaced by a transgene expression cassette located between the ITRs. (Figure 2) With the vector's *rep* and *cap* genes gone, the helper virus produces vector particles via a plasmid containing the vector genome that expresses the *rep* and *cap* proteins.

Once inside the host cell, the AAV vector genome enters the cell nucleus and, in the vast majority of cases, is converted to a double-stranded circular episome by second-strand synthesis or complementary strand pairing. The episomes can be converted into high molecular weight, tandem-repeat concatamers that can provide long-term transgene expression (Figure 3).

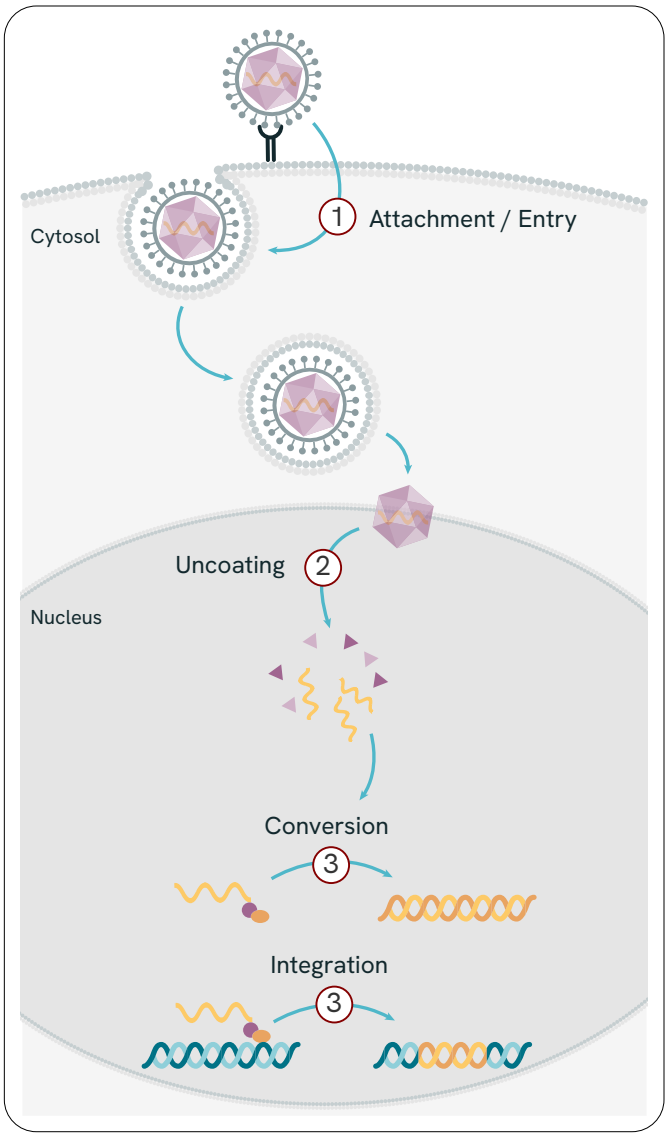

Figure 3: In vivo gene transfer using an AAV vector

AAV vector selection and design

Viral vector designs must have certain characteristics to be considered successful:

- Modifiable, to provide safe handling
- Safe, with low toxicity and immunogenicity, resulting in no effect on recipient physiology
- Stable, without the ability to rearrange the recipient genome or produce new virions
- Quantifiable, for reproducibility in manufacturing batches
- Scalable, in order to be adaptable for large scale production

Design considerations that will help create those vector characteristics include:

- The target cell or tissue type
- The choice of systemic versus local delivery
- The safety profile of the gene to be delivered
- Its potential immunogenicity
- Use of tissue-specific or constitutively active promoters
- AAV-dependent and non-AVV-dependent parameters that influence gene transfer

Two ongoing challenges in AAV vector design are 1) the size of the expression cassette, and 2) potential vector immunogenicity.

As mentioned previously, most AAV vectors contain a transgene expression cassette between the ITRs that replaces the *rep* and *cap* genes (Figure 4). In addition, other regulatory elements – e.g., enhancers, cis-regulatory modules, introns, and post-transcriptional elements – can be included in the cassette to improve the efficiency and specificity of transgene expression. Given the small size of AAV vectors (< 5 kb), the cassette packaging must also remain small, which limits the number and size of additional cassette elements.

In regard to potential immunogenicity, patients who were previously exposed to the AAV virus may have neutralizing antibodies to AAV. Advanced recombinant AAV vector design is seeking new means of improving transduction potency, providing vectors with the ability to evade neutralizing antibodies, and increasing cell and tissue specificity thereby allowing lower dosages that in turn improve therapeutic safety.

Emerging AAV vector design insights and concepts include:

- New insights into the role of AAV DNA structures on vector genome stability, integrity, and functionality
- Novel synthetic enhancers and promoters that greatly increase tissue specificity
- New genetic circuit designs that use biological (microRNAs) and physical (light) triggers to regulate AAV gene expression
- Improvements in capsid engineering

These and other promising innovations will usher in the next generation of AAV vectors for human gene therapy.[5]

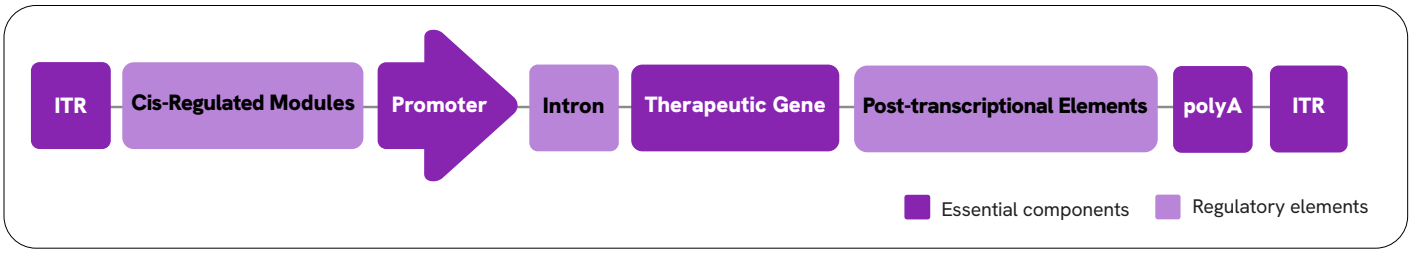

Figure 4: The transgene expression cassette

AAV vector manufacturing

The production of AAV vectors is divided into two primary workflows: upstream vector production and downstream vector purification and enrichment. The scale up of workflows to support clinical trials and commercialization is still often a bottleneck in gene therapy research and application. Many different manufacturing platforms for viral vector production have been developed in recent years, and each has its advantages and challenges.

Upstream workflow and challenges

The upstream viral vector production workflow includes four primary steps (Figure 5):

1. Plasmid development in which three plasmids are designed and produced:
   - A cis-plasmid that encodes the gene of interest between the AAV's ITRs
   - A trans-plasmid that encodes the AAV rep and cap genes
   - A helper plasmid that encodes the adenovirus helper genes E2A, E4, and VA RNA
2. Cell expansion in which E1 transduced cells are expanded to a desired cell density via cell culture
3. Plasmid transfection in which the plasmids are introduced to the cells once the cell culture reaches the required density
4. Vector production in which transiently transfected cells are allowed time to produce the virus

Key challenges that must be addressed in plasmid development are improving yield and purity.6 GMP-grade plasmid DNA must have a purity level of more than 95% and be free of process-related variants and impurities. The substantial lot-to-lot variability in fermentation and plasmid yields must also be addressed in order for manufacturers to meet the growing demand for viral vectors.

Scaling up the cell expansion step has its own challenges. The use of adherent cells increases the risk of contamination during culture manipulation, and it can be difficult to monitor and regulate culture conditions. Suspension-based cell expansion cultures are easier to scale up, but they produce a lower cell density than adherent-based cultures. Additionally, the AAV vector's three-plasmid transfection system is inefficient in suspension cell culture resulting in suboptimal ratios of the three plasmids in expression packages. This plasmid imbalance may also contribute to the variation in empty-to-full capsid ratios between vector batches.

The routine use of animal-based products in cell culture is an ongoing source of contaminants to the workflow. Such products, e.g., serum, often contain viruses that are similar in size and characteristics to the AVV vector, making it extremely difficult to separate the two without affecting product yield and efficacy.

Challenges in plasmid transfection vary by the method used. Calcium phosphate methods have reagent purity and pH sensitivity concerns that often result in significant batch-to-batch variability. Liposome methods use expensive reagents that drive up costs for commercial AAV production. The most widely used transfection method employs polyethyleneimine which is highly pH-sensitive and toxic to producing cells.

These and other challenges in the upstream AAV vector workflow need to be addressed to enable more efficient vector manufacturing.

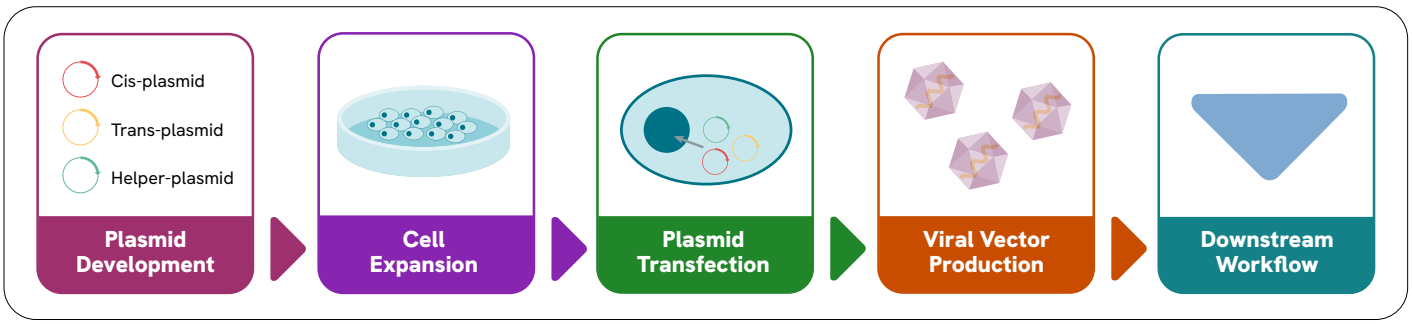

Figure 5: Upstream manufacturing of AAV vectors

Downstream workflow and challenges

Downstream processes are more standardized than upstream workflows, making them easier to scale up and more adaptable to different manufacturing platforms. The typical downstream workflow includes six primary steps:

1. Cell lysis to release viruses
2. Nucleic acid removal where lysates are digested with endonucleases to reduce nucleic acid contaminants
3. Solids removal by centrifugation or microfiltration to remove cell fragments and debris prior to chromatographic purification
4. Affinity chromatography to remove host cell proteins (HCPs) and any serum protein impurities
5. Separation of full gene-containing infectious viruses from empty, non-infectious viruses, either by cesium chloride gradient ultracentrifugation procedures or ion-exchange chromatography
6. Final purification to further reduce HCPs or other low molecular weight contaminants using core-bead adsorbents

Key challenges in the downstream workflow center on improved methods for cell lysis, filtration, purification, and separation.[6] Cell lysis methods include mechanical and chemical techniques such as detergent, mechanical stress, hypertonic shock, and freeze-thaw procedures. Each of these places some type of stress on the viral vector that can impact the safety or efficacy of the final therapeutic product. For instance, vector loss due to shear-stress-induced aggregation and precipitation, and toxicity from a long-used lysing detergent.

Additionally, not all lysing methods are amenable to scale up, such as the commonly used mechanical technique of repeated freezing and thawing of the cells followed by low speed centrifugation. Improved downstream workflows will benefit from new cell lysis techniques that preserve vector integrity and can be easily scaled up for commercial production.

Challenges with filtration methods are primarily related to correct filter size selection. The AAV serotype being used is a key consideration for proper filter size selection. Forcing a lysate through a too-small filter can cause mechanical shear stress to vectors resulting in vector loss and reduced process efficiency. Since filtration is the most expensive process in the downstream workflow, improved filtration methods are necessary to prevent vector loss and maximize the efficiency of scaled up operations. One option that is yet to be widely adopted is continuous filtration methods that reduce filter clogging.

One of the biggest challenges in downstream purification processes is that each AAV serotype requires a specific approach in order to maintain vector integrity, optimize yield, and ensure final product potency. Other needs are the isolation of viral particles and reduction of process- and product-related impurities without lowering yield. A manufacturing platform's downstream purification process needs to be versatile and customizable for a variety of viral vectors.

The presence of empty capsids in the final therapeutic product can reduce its efficacy and safety due to the increased immunogenicity risk. Separation of empty capsids from full capsids is challenging because they are similar in size and electrical charge. Numerous separation methods are currently available, but all have one or more challenges that need to be overcome to ensure high product purity and yield. Those challenges include extreme chemical and physical conditions such as pH and conductivity, lack of specificity for different AAV serotypes, equipment costs, and scale up difficulties. Platform advances are needed to provide separation technology that is customizable for a variety of AAVs and provides high product purity and yields.

Stability and storage challenges

There are several crucial challenges with AAV vector stability during manufacturing and storage: degradation, long-term stability, denaturation, aggregation, oxidation, and purity. Research into formulation changes, modifications to the vectors themselves, and rigorous stability studies are helping to solve some of these challenges.[6]

Degradation

AAV vectors can degrade over time and affect both the efficacy and safety of the final therapeutic product. The top three degradation pathways for AAVs are:

- Freeze/thaw-induced unfolding and activity loss
- Aggregation at low ionic strength
- Shear induced unfolding, aggregation, and precipitation.

Fully characterized excipients that have very low levels of impurities and endotoxins are a promising approach to controlling degradation rates. The use of such deeply scrutinized excipients is especially crucial for applications in which AAV viral vector products are introduced to confined spaces such as the eye or the brain.

Oxidation

Oxidation in viral vectors can impact virus infection efficiency, vector safety, and final product efficacy. Capsid proteins, in particular, readily oxidize during downstream processing and storage. Oxidation can be prevented by using free amino acids such as methionine and histidine, and metal ion scavengers such as ethanol, EDTA, and DTPA, in product formulations.

Denaturation

Denaturation refers to the loss of the native structure (and thereby function) of an AAV via the loss of secondary, tertiary, or quaternary protein folding. In addition to the normal unfolding and refolding during capsid protein expression and production, AVV vectors can denature in response to various chemical and physical parameters such as temperature, pH, ionic strength, protein concentration, and the presence or concentration of any number of chemical agents. Mechanical parameters can also cause denaturation, such as shear stress, adsorption to manufacturing, storage, or injection surfaces, and dilution processes during dose preparation.

Aggregation

There are many mechanisms of protein aggregation, such as the association of unfolded or largely unstructured proteins and the self-association or oligomerization of native proteins. Protein aggregations have a high potential to cause immunogenic responses, making them a key concern for gene therapy products and other biologics. Aggregations can also cause decreased purity levels and disrupt AAV testing. Concentrated AAV stocks are particularly susceptible to aggregation. One promising approach to reducing aggregations is the removal of residual host-cell DNA from the vector surface using nucleases.

There are a number of potentially helpful design considerations for vectors, production processes, and product formulations that may help prevent or minimize denaturation and aggregation:

- Osmolytes (small organic compounds) can stabilize proteins against denaturation and aggregation
- Buffers can be used to modulate and control pH to decrease pH-induced denaturation
- Surfactants can minimize protein contact with the air-water interface
- Salts can help stabilize capsid proteins

Characterization methods for AAV vector production

AAV vector manufacturing platforms must include ongoing characterization of process intermediates and the final product. Doing so allows the monitoring and optimization of production processes and provides important product quality control data.

Three important attributes of the AAV vector production process are the virus titer, genome content ratio, and the level of aggregation formation. Routine characterization of these attributes provides valuable information about the purity, potency, and safety of gene therapy products. The following sections describe these three attributes and identify the analytical methods most commonly used to characterize each of them.[7]

Virus titer

The term “virus titer” encompasses the genome titer and the capsid titer. The genome titer quantifies capsids that contain the complete vector genome, making it a good indicator of the potency of the sample. The capsid titer quantifies all capsids regardless of their vector genome content. The capsid titer is used to evaluate and optimize downstream purification processes that depend on product load rather than potency.

Genome titer is most commonly determined using quantitative polymerase chain reaction (qPCR) and digital droplet PCR (ddPCR) during or after amplification in a thermocycler. These PCR methods are known for their simplicity, specificity, and robustness.

Capsid titer is most commonly determined using an enzyme-linked immunosorbent assay (ELISA). The assay is typically conducted following a purification step but can also be used for analysis of cell lysates and other samples with matrix effects. Capsid titer can also be determined using optical density (OD) methods. OD methods require thorough removal of any other impurities from the sample to ensure accurate analysis of the capsid titer.

Other methods being used or evaluated for virus titer analysis include:

- Bio-layer interferometry (BLI)
- Dye-based binding assays (DyeBA)
- Flow virometry (FV)
- Size-exclusion chromatography (SEC) with multi-angle light scattering (MALS) detectors

Content ratio

The content ratio attribute refers to the ratio of viral capsids that contain no vector genome or only a partial genome. Such capsids are the most common product-related impurity in AAV vector production and can increase the risk of immunotoxicity by eliciting unwanted immune responses.

Transmission electron microscopy (TEM) is the most commonly used method for the determination of content ratios. Different levels of stain uptake produce images in which empty, partially filled, and full capsids are easily distinguishable. TEM requires purified samples that are free of cell debris and proteins.

Other methods being used or evaluated for determination of content ratio include:

- Anion-exchange chromatography (AEC)
- Analytical ultracentrifugation (AUC)
- Charge detection mass spectrometry (CDMS)
- Mass photometry (MassP)
- OD
- SEC-MALS
- Dynamic and static light scattering (DLS/SLS)

Aggregation

Capsid aggregates are impurities that have negative effects on the safety and long-term stability of the AVV vector product even at trace levels. Aggregation typically occurs in downstream processes, making it crucial to monitor those processes for aggregation levels and optimize the process conditions to ensure product safety.

Aggregate content is typically determined using DLS methods. DLS quantifies aggregate content in the sample and determines the average size of the aggregates. SEC is also a commonly used and rapid method for aggregate assessment.

Other methods being used or evaluated for aggregates analysis include AEC and SEC-MALS.

These methodologies and their uses in attribute characterization are summarized in Table 2.

Table 2: Methods used for analysis of quality control attributes

Method	Quality Control Attribute		
	Virus Titer	Content Ratio	Aggregation
AEC		X	
AUC		X	X
BLI	Capsid titer		
CDMS		X	
ddPCR	Genome titer		
DyeBA	Genome titer		
ELISA	Capsid titer		
FV	Capsid titer		
MassP		X	
OD	Capsid titer	X	
qPCR	Genome titer		
SEC-FS			X
SEC-MALS	Capsid titer	X	X
SLS/DLS		X	X
TEM		X	

AAV vector analytics

The many different materials and processes used in AAV vector production require a robust range of analytical capabilities.

As discussed previously, reliable and accurate methods are needed for quantifying AAV viral titers, empty, full, and partially full viral capsids, and impurities such as aggregations and other particles. These parameters need to be held consistent and must be closing monitored throughout the production process to ensure both the safety and efficacy of the therapeutic product.

Some of the methods discussed for vector production monitoring can be tedious and time-consuming. For example, wash-based assays like ELISA are currently the gold standard for measuring viral titer, but they are labor intensive and are not fully amenable to scaling. Innovative no-wash assays that allow higher throughput with scalability have the potential to drastically improve lab efficiency and advance the design and production of AAV vectors.

Electron microscopy also has the limitation of being expensive and having low throughput rates. Novel methods that can provide the same high-quality data in a high-throughput format will aid in development and production processes.

Another emerging analytical need is for the evaluation of new synthetic AAV capsids. A few of the potential analytical and treatment methods being considered include:8

- Mass spectrometry to characterize synthetic capsid integrity
- Differential scanning fluorimetry (DSF) to measure synthetic capsid thermostability
- Methods to reduce bacterial and cellular contaminants in or on synthetic capsids.

Summary

Gene therapy development has come a long way since 2003 when the first treatment was approved and became available. Viruses are the most common vectors in gene therapy development today, and AAV vectors are the leading viral vector.

AAV have certain characteristics that make them effective as vectors. For instance, they require minimal genome size for replication such that most of its genome can be replaced with the therapeutic DNA insert. AAVs are also versatile for targeting different tissues.

Two ongoing challenges in AAV vector design are optimizing the size and content of the transgene expression cassette, and addressing potential immunogenicity in pre-exposed populations. Emerging AAV vector design innovations show promise for overcoming these challenges.

AAV vector manufacturing must have reliable and accurate methods for quantifying AAV viral titers, empty, full, and partially full viral capsids, and impurities such as aggregations and other particles. Traditional analytical methods are often time-consuming and not fully amenable to scaling.

Revvity has developed and manufactured a line of immunoassays that detect and quantify AAV capsid (in Viral Particles per milliliter - VP/mL) utilizing AlphaLISA technology (Figure 6) and HTRF technology.

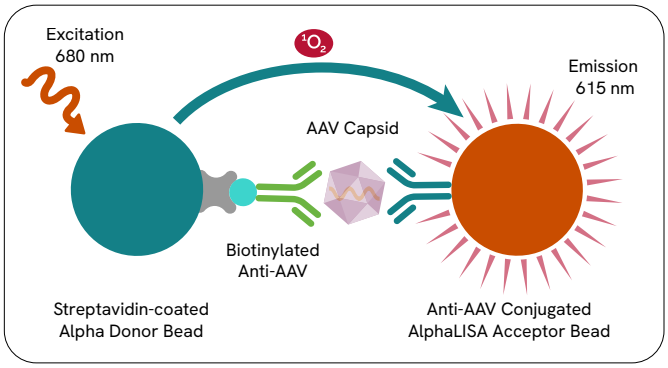

Figure 6: AlphaLISA AAV detection kit

The AlphaLISA AAV Capsid Detection Kits are available for AAV1, AAV2, AAV3B, AAV5, AAV6, AAV8 and AAV9 serotypes, and can measure AAV particles present in cell culture media, lysis buffer and cell lysate. The AlphaLISA AAV1 and AAV6 Capsid Detection Kit Donor Bead chemistry is identical to the other AAV AlphaLISA kits. However, the Acceptor Beads are conjugated to an Anti-HRP antibody, which binds an HRP-conjugated Anti-AAV antibody (Figure 7).

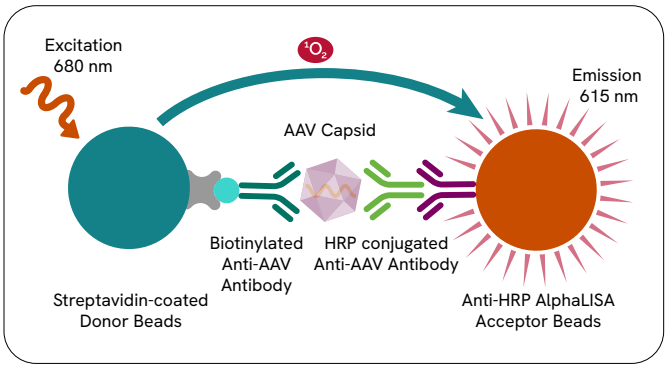

Figure 7: AlphaLISA AAV detection kit for AAV1 and AAV6

The HTRF AAV Capsid Detection Kits are available for AAV1, AAV2, AAV3B, AAV5, AAV6, AAV8 and AAV9 serotypes. For AAV1, AAV2, AAV3B, and AAV6, AAV capsid is detected in a sandwich assay by using a pre-mixture of biotinylated anti-AAV antibody bound to the streptavidin Europium cryptate (donor), and a second pre-mixture of the HRP anti-AAVantibody bound to an anti-HRP labeled with d2 (acceptor) (Figure 8).

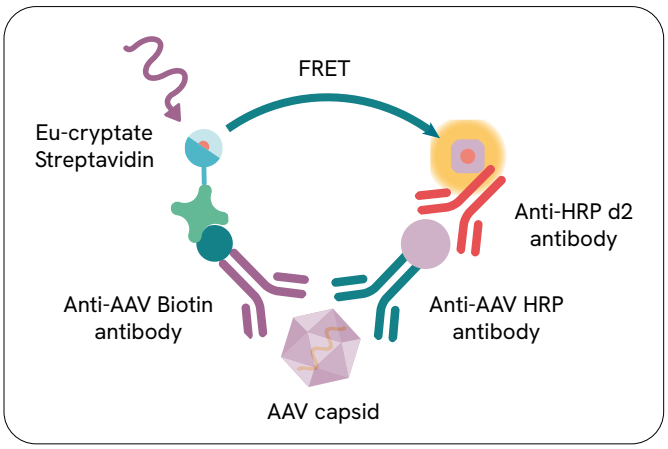

Figure 8: HTRF AAV detection kit for AAV1, AAV2, AAV3B and AAV6

The AAV5 assay uses two anti-AAV5 antibodies: one labelled with d2 (acceptor) and the other coupled to biotin that binds to Streptavidin Eu-cryptate (donor) in a premix.

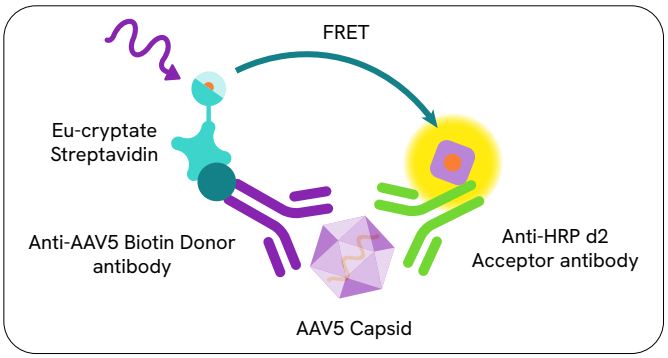

Figure 9: HTRF AAV detection kit for AAV5

In the HTRF AAV Capsid Detections Kits for AAV8 and AAV9, one anti-AAV antibody is directly labeled with Europium cryptate (donor) and the other anti-AAV antibody is directly labeled with d2 (acceptor) (Figure 10).

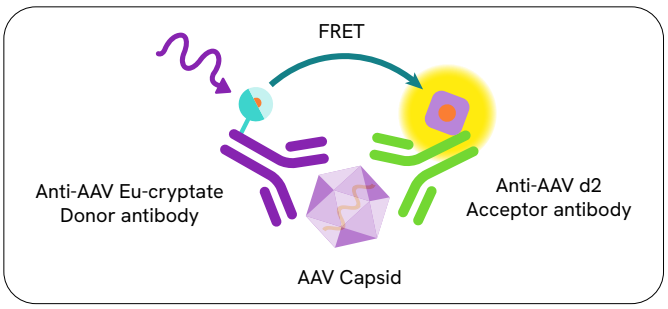

Figure 10: HTRF AAV detection kit for AAV8 and AAV9

The main benefit of these technology over ELISA is ease of use. While AlphaLISA and HTRF can be performed well with minimal hands-on time, ELISA takes much longer due to many incubation and wash steps. AlphaLISA and HTRF are not only time saving but sample saving as well: Alpha and HTRF assays need much less sample than ELISA to give equivalent, or better, results. The range of detection is wider and these assays are easily automatized and miniaturized. These new advanced methods are high- throughput and readily scalable, thereby greatly improving lab and manufacturing efficiency.

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