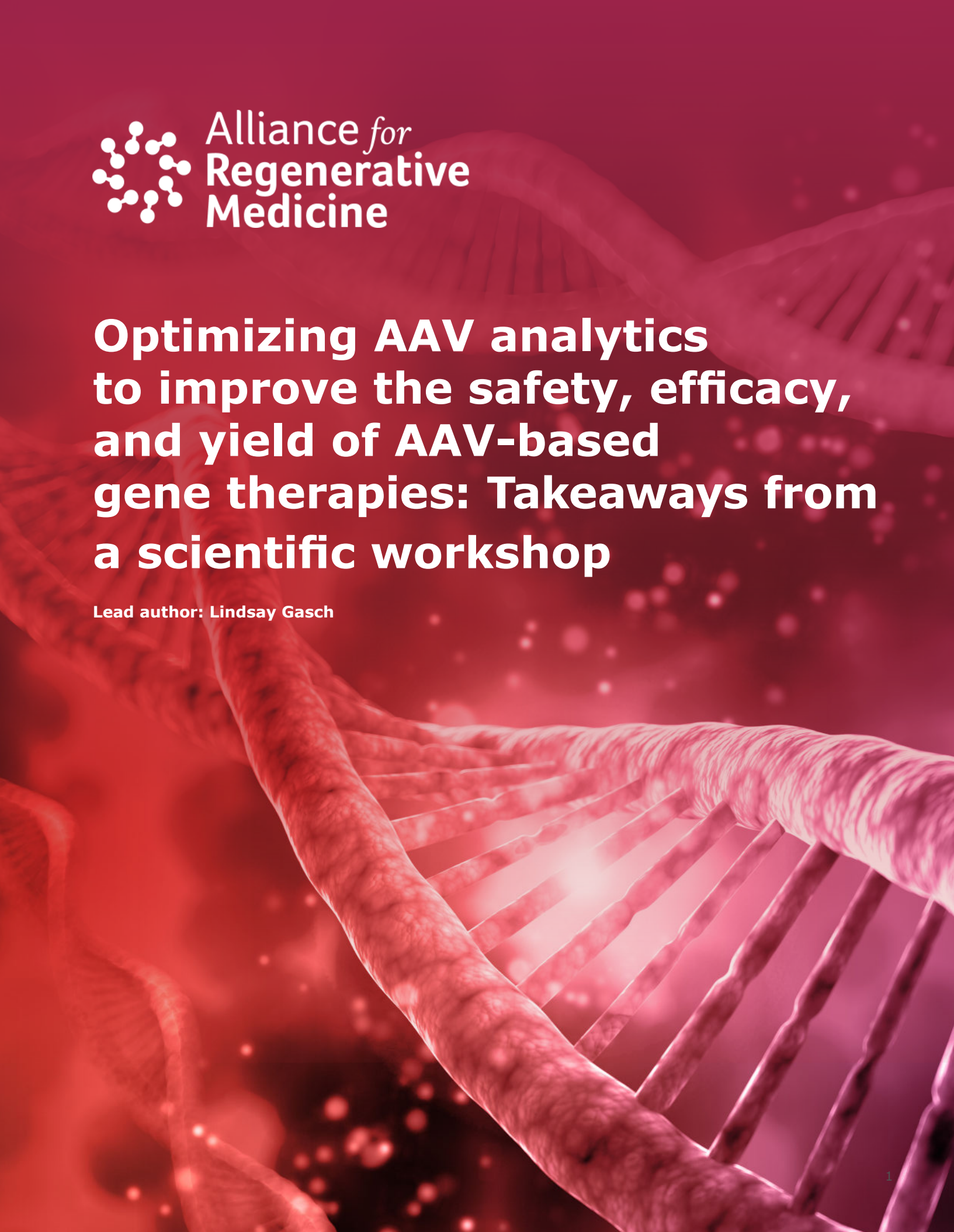
Alliance for Regenerative Medicine

Optimizing AAV analytics to improve the safety, efficacy, and yield of AAV-based gene therapies: Takeaways from a scientific workshop

Lead author: Lindsay Gasch



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INTRODUCTION

Adeno-associated virus-based gene therapies have demonstrated significant promise for the treatment of numerous diseases, including rare conditions with substantial unmet needs. In order to address safety concerns and meet increasing demand for these critical therapies, enhanced drug-product understanding is required. From March 8th-9th, 2023, ARM and USP held a 1.5-day workshop on AAV Analytical Characterization to cover various topics related to AAV quality. The workshop brought together developers, regulators, and other key stakeholders from across the industry (see the Appendix for a full list of presenters and panelists) to review best practices for analytical methodologies, discuss the promise of emerging methodologies, and share learnings on approaches for different products. The workshop goals were to advance the field towards harmonized characterization methods and provide updated regulatory expectations for AAV analytics.

For definition of abbreviations used in this whitepaper, please see the appendix.

Successes and challenges of AAV-based gene therapies

The last decade has seen substantial growth of the AAV-based gene therapy market, with U.S. and/or EU approvals of 6 products since 2017 and additional regulatory decisions expected in 2023-2024. There are currently >150 active clinical trials of AAV gene therapies being conducted worldwide, spanning a variety of indications ranging from genetic disorders and hematology/oncology to ophthalmologic, metabolic, and central nervous system diseases. Most are investigating rare diseases within these niches, and the majority are Phase 1/2 trials.[1]

Box 1. Marketed AAV products and anticipated regulatory decisions

Approved product	Region	Indication	Sponsor
Elevidys (2023)	US	Duchenne muscular dystrophy (DMD)	Sarepta Therapeutics
Roctavian (2022-2023)	US & EU	Hemophilia A	BioMarin
Hemgenix (2022-2023)	US & EU	Hemophilia B	uniQure & CSL Behring
Upstaza (2022)	US	Aromatic L-amino acid decarboxylase (AADC) deficiency	PTC Therapeutics
Zolgensma (2019-2020)	US & EU	Spinal muscular atrophy (SMA)	Novartis Gene Therapies
Luxturna (2017-2018)	US & EU	Retinal dystrophy	Spark Therapeutics

Upcoming 2024-2025 regulatory decisions:

Upstaza
PTC Therapeutics
AADC deficiency

Fidanacogene elaparvovec
Pfizer (formerly Spark Therapeutics)
Hemophilia B

Though the growth and promise of gene therapies is undeniable, these potentially curative therapies are not without challenges. Patient demand is generally outpacing manufacturing capabilities, high AAV doses stretching the patient benefit:risk ratio are being tested, and regulatory agencies are increasingly scrutinizing AAV-based gene therapies from a safety, product consistency, and efficacy standpoint. These challenges warrant significant and consistent attention from all stakeholders.

In response to observed toxicities in animals and humans following administration of AAV-based gene therapies, the FDA CTGTAC convened a meeting in September 2021 to discuss strategies for minimizing toxicity risks. Main takeaways from the meeting included the need for a risk-based approach to evaluating and monitoring safety, a case-by-case evaluation of vector dose and appropriate immunosuppression protocol, as well as the need for standardized methods for characterization of AAV capsids, industry standards on assay controls, and better analytic approaches for AAV products.

Additionally, ARM, the ASGCT, and the FDA co-hosted an October 2022 roundtable discussion between developers, academics, and regulators with the goal of defining some key tenets around product potency. Key takeaways included a desire for (1) informal, live feedback sessions between industry and the FDA early in the therapeutic development timeline; (2) clarity on when potency should be measured and how to potentially prune the potency assay matrix; and (3) finding pre-competitive aspects of potency assay information sharing to de-risk and accelerate potency assay development across the industry.

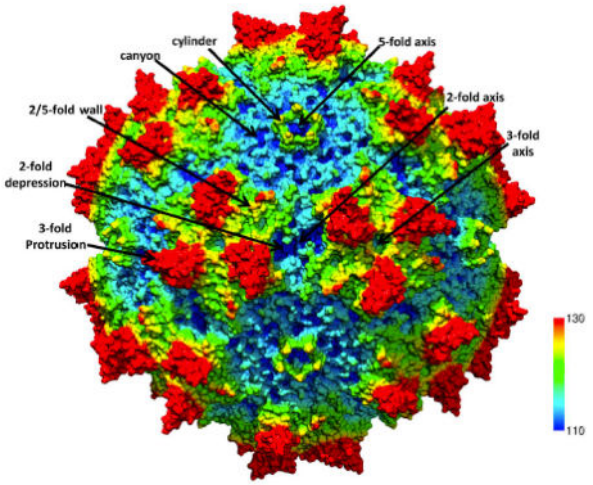
This AAV Analytical Characterization Workshop represents an effort to continue multi-stakeholder engagement and collaboration regarding key challenges facing the AAV-based gene therapy industry. The workshop scope includes the use of AAV vectors to deliver, *in vivo*, new genetic material to human cells. *Ex vivo* AAV use for gene modification of cells and gene editing tools (e.g., CRISPR) are outside the scope of the workshop. The workshop focused on ***three primary topic areas: full/empty capsid characterization, potency assays, and vector genome titer measurement and dosing.***

FULL/EMPTY CAPSID CHARACTERIZATION

Basic AAV structure and principles of AAV-based gene delivery

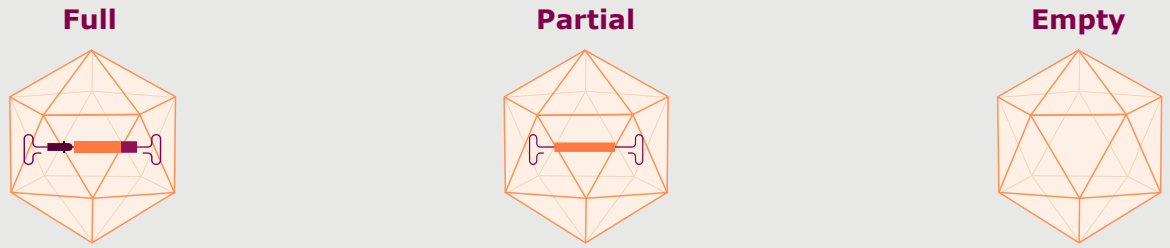
The ~4.7 kb genome of AAV contains three open reading frames (*rep*, *cap*, and *aap*), flanked by two ITRs. The genome is packaged into a capsid roughly 26 nm in diameter and comprised of 60 viral proteins arranged in an icosahedral structure, with an average capsid VP1:VP2:VP3 subunit ratio of approximately 1:1:10. Capsid structures and VP subunit ratios can vary slightly by AAV serotype.[2]

Box 2. AAV1 capsid structure[2]



Recombinant AAVs used for *in vivo* delivery of gene therapies encapsidate genomes engineered to be devoid of *rep*, *cap*, and *aap*, with therapeutic gene expression cassettes inserted in their place. Encapsidation efficiency may be driven by DNA structure and size, but there may be other contributing factors (e.g., grade of raw materials) that are not well understood. Resulting capsids can contain full or partial genomes of interest, can be empty, or can contain other process- or product-related impurities such as host-cell DNA fragments, plasmid DNA fragments, or promoter/enhancer sequences without any coding sequence. Rational design of upstream manufacturing processes may help to maximize the formation of capsids carrying the full-length genome of interest.

Box 3. Illustration of full, empty, and partial AAV capsids



Full capsids may carry the full-length vector genome (i.e., promoter, enhancer, gene of interest, and ITRs) for the given AAV-based product. When this is the case, such capsids will produce both a DNA signal and a potency signal stemming from the expression of the therapeutic protein of interest. In general, full-length genomes are responsible for the clinical benefit of AAV-based gene therapies. In other cases, the full packaging limit of a capsid may be occupied by DNA other than the full-length genome of interest (e.g., multiple partial genomes or a partial genome plus other DNA impurities). If the partial genome contains enough of the coding sequence, a potency signal may still be observed.

Empty capsids are product-related impurities inherent to AAV-based gene therapy manufacturing. They may be devoid of any portion of the vector genome for the given product or could carry an amount of DNA that does not significantly increase the mass of a true empty AAV particle (e.g., small DNAs such as ITR sequences). Empty capsids will not produce a potency signal and, in general, will not produce a DNA signal.

Neither full nor empty, partial capsids may contain partial genomes of interest, promoter/enhancer sequences without any coding sequences, or impurities such as host-cell or plasmid DNA fragments. Hence, a DNA signal is often detected, and a potency signal may be absent or present.

Defining full, empty, and partial capsids

The terminology used to describe full, empty, and partial capsids is not standard throughout the industry of AAV analytics. Capsids may be referred to as "particles", partials may be called "intermediates" or "light" capsids, and capsids may be preferentially described relative to their filling capacity and/or completeness of the "payload" they deliver.

The term "defective-interfering particle" (DIP) was presented as another way to describe any capsid that does not contain the full-length *intended* payload. Though a DIP can be a truly empty capsid with no genomic material, a DIP may also be a capsid containing any size of rearranged payload material or contaminant DNA from plasmid backbones, helper genes, or the packaging cell. In some instances, a DIP may appear to represent a correctly packaged full capsid, which could present challenges for downstream purification.

Though presenters broadly agreed that standard terminology would be desirable, the FDA assured developers that they are open to all types of terminologies. Indeed, payload is the FDA's primary concern, but it may be quite challenging to assess consistently. For the remainder of this whitepaper, the terminology ***"full/partial/empty capsids"*** will be used.

Regulatory expectations related to full/partial/ empty capsids

There are inconsistencies in how empty, partial, and full capsids are reported within regulatory filings. According to the FDA, companies occasionally report empty capsid percentage only, though their methods may be insufficient to fully support that characterization (e.g., using only optical density measurements vs. AUC). In those cases, the FDA is likely to suggest additional characterization studies. In some circumstances, percent empty may become the release criteria, but empty, partial, and full capsids may all be measured as part of the full characterization panel. The FDA noted that robust characterization panels may be helpful when comparability studies are required after manufacturing changes. Because self-complementary AAV vectors encapsidate both single-stranded and double-stranded DNA, partial capsids may be more prevalent with self-complementary than single-stranded AAV vectors,[3] which may be an important consideration for developers.

Moving forward, developers noted that regulatory guidance for establishing meaningful specifications for full/empty/partial capsids may start with standard definitions of these constructs and should be based on aspects specific to AAV gene therapies (rather than to other therapeutic modalities such as cell therapies). In addition, expectations should be based on proven analytical methods (with set reference standards that may also be aided by pre-established definitions) and be compatible with feasible manufacturing methods. Acceptance criteria should also consider inherent differences in gene therapy products, route of administration, and patient dosing.

"Before we can have policy to set specifications, we have to all be speaking the same language in terms of what we mean by 'full' and 'empty' and how we're measuring empty capsids."

– AAV gene therapy developer

To set the best reference standard for capsid populations, developers and regulators agree that better characterization of empty, partial, and full capsids will be required. Notably, the USP conducts a rigorous evaluation and data review from multiple collaborating laboratories when setting a reference standard. Along these lines, the USP encouraged developers to continue their characterization efforts and weigh-in on the most desirable type of standard (e.g., a standard for full, partial, or empty capsids vs. a standard for each).

Box 4. USP efforts to support AAV-based gene therapy development

Background: The USP develops a variety of public standards in order to support marketed therapeutics. Monographs detail specifications for pharmaceuticals in commerce, whereas physical reference standards can help developers demonstrate acceptability of their methodologies. General chapters may be procedural in nature (numbered <1000), with validated methods and associated reference standards, or may be informational (numbered 1000-1999), detailing best practices and considerations.

Key AAV-related efforts

Documentary standards – General Chapters

- **<1040> Quality Considerations of Plasmid DNA as a Starting Material for Advanced Therapies:** A USP Expert Panel is developing this general chapter focused on plasmid DNA suitable for the manufacturing of cell and gene therapies. This chapter may be available as early as late 2023.
- **<1047> Gene Therapy Products:** Currently under revision.
- **<XXXX> Best practice for AAV:** An AAV Gene Therapy Expert Panel established by the USP is developing an informational chapter detailing best practices for AAV vector design, manufacturing, quality control, and regulatory considerations. Panelists initiated work in June 2022 and a first draft may be available for public comment in early 2024.

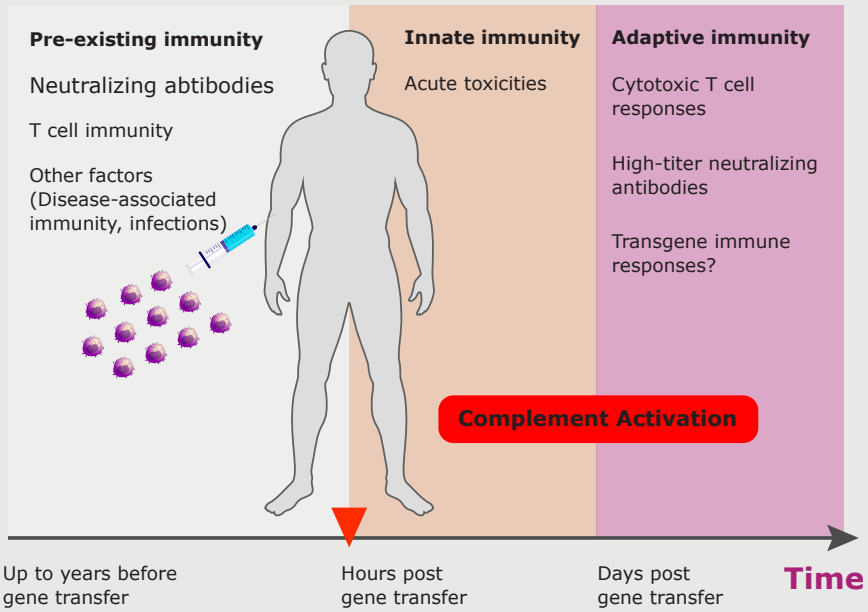
Reference standards

- AAV empty capsid standards
- AAV plasmid standards with multiple AAV-specific targets as a broad PCR standard
- Raw materials (e.g., enzymes, plasmid)
- Collaboration with NIST and NIIMBL to evaluate different analytic methods for the quantification of full:empty capsids (publication expected as early as summer 2023)
- Residual genomic DNA (USP/ATCC collaboration)

Why do empty and partial capsids matter?

Due to the protein composition of the capsid and/or encapsidated DNA, any AAV capsid construct contributes to antigenic load and may impact patient immune responses and other toxicities. Humans are exposed to wild-type AAVs that exist in nature and, therefore, can have pre-existing antibodies and T-cells specific to the AAV capsid. Immediately following vector administration, a local and/or systemic innate immune response can be triggered. In subsequent days, innate and adaptive immune responses develop. Particularly in the setting of high AAV doses (>1E14 vg/kg), synergy between the innate and humoral adaptive immune responses can lead to complement activation, which can induce severe toxicity.[4] A significant driver of the FDA's focus on empty capsids relates to concern for toxicities, including T-cell responses to transduced cells in the liver and thrombotic microangiopathy, which may be related to total capsid burden (i.e., empty, partial, and full capsids).

Box 5. Immune responses to AAV and the contribution of empty capsids



Cytotoxic T-cell responses:
In humans, T-cell responses to AAV9 capsid can be induced[5]

In nonhuman primates, T-cell responses to transgene can eliminate gene expression of transduced cells in the liver[6]

Neutralizing antibodies:
Data in humans and nonhuman primates show that even low levels of neutralizing Abs can block gene transfer[7]

Complement activation:
Data in humans but not nonhuman primates show that IV administration of AAV can cause thrombotic microangiopathy and renal impairment accompanied by activation of the complement system[8,9]

Adapted from: Costa Verdera et al. AAV Vector Immunogenicity in Humans: A Long Journey to Successful Gene Transfer. *Molecular Therapy* 2020; 28(3):723-746.[10]

Do empty/partial capsids induce an immune response in humans?

01

In healthy volunteers negative for pre-existing AAV8 neutralizing Abs, empty capsids were found to induce CD4+ T-cell and B-cell responses associated with transient liver toxicity[11]

02

Empty/partial capsids can mask the detection of neutralizing Abs, which can have implications for clinical trial screening and enrollment

03

Particularly at high doses, empty/partial capsids may increase the risk of immunocomplex formation and complement activation

The extent to which empty and partial capsids contribute to the immunogenic or toxic potential of a given product may increase as total vector load rises with higher product doses. Capsid effects may also vary depending on their serotype and biodistribution as well as the disease state, tissue target, route of administration, and the age and immune status of the patient. Combined with the lack of standardized terminology, differences in these variables make it difficult to understand how empty or partial capsids contribute to immune responses. Contrary to historical belief, the CNS is not an "immune-privileged" environment, particularly when it comes to AAV. Even when administered intrathecally or directly into the brain, a significant amount of AAV migrates into systemic distribution. Similarly, neutralizing antibodies can be found in the serum and CSF after local AAV administration in the CNS. At present, there is no standardized regimen to modulate the immune response to these products. Investigators are experimenting with different immunosuppressants (e.g., prednisone, sirolimus, tacrolimus, monoclonal antibodies) and plasmapheresis, with varied results. It's possible that lower doses of drug product or better capsid design (lower liver tropism) may help to mitigate their immunogenic potential.

Higher proportions of empty capsids have been shown to result in lower *in vitro* potency and reduced transduction in mouse liver, perhaps due to competition with full capsids for cellular binding sites and internalization.[12] Importantly, the effect of empty capsids on potency in non-human primates or humans remains unclear, and learnings from *in vitro* or murine models should not be overinterpreted. With high doses of an AAV-based drug product, panelists theorized that a saturation point could be reached *in vivo*, at which point empty capsids may out-compete full capsids for binding to surface epitopes. If true, the dose threshold required for such competition to impact the efficacy of the drug product would need to be determined.

Capsid heterogeneity

Capsid heterogeneity may also impact the efficacy and immunogenicity of AAV-based gene therapy products. Heterogeneity can stem from differences in VP1:VP2:VP3 capsid subunit ratios, post-translational modifications to AAV capsid proteins or their three-dimensional orientation, and to the formation of aggregates.

Post-translational modifications to AAV capsid proteins can be impacted by the expression system, bioreactor conditions, downstream processing, and exposure to unfavorable conditions during handling or storage. PTMs such as oxidation, deamidation, glycosylation, acetylation, and phosphorylation are common. Additionally, SUMOylation and ubiquitination have been observed, and may be particularly problematic because they tag AAVs for degradation.[13] Acetylation, phosphorylation, and glycosylation are enzymatic PTMs that occur in the cell co- or post-translationally and will not spontaneously appear after drug product synthesis. However, oxidation and deamidation are non-enzymatic modifications that can occur at any point during upstream processing, downstream processing, formulation, and storage.

Interestingly, reducing deamidation has been reported to improve vector function.[14] One developer has found that empty capsids possess higher levels of VP1 deamidation compared with full capsids, adding that VP1 plays an important role in endosomal trafficking. However, the FDA cautioned that this finding may not be generalizable to all empty capsids, as PTMs tend to be very product-, serotype-, or even process-specific.

Several methods exist for examining PTMs to capsid proteins. Reverse-phase HPLC-MS intact mass, which allows for the screening of major viral protein species, can be used to evaluate lot-to-lot consistency. LC-MS peptide mapping is a higher-resolution characterization method for identifying and quantifying PTMs, though it remains relatively low-throughput and yields complex datasets. As such, data gleaned from LC-MS peptide mapping could be used to develop higher-throughput methods to screen for and quantify any PTMs that are determined critical to the quality of the vector product. In determining whether a PTM is an actual CQA, each must be individually assessed for its impact factor and uncertainty.

Forced-degradation studies can be used to help understand why PTMs occur and how they may impact process or product. Inducing oxidative stress enables the monitoring of its impact on product loss and empty/full ratios, whereas exposing AAV to acidic conditions or high-temperatures can increase understanding gleaned from accelerated aggregation and fragmentation. High-temperature stress may provide the most useful information about potential degradation over time at the intended storage condition.

Ultimately, a better understanding of PTMs may pave the way for improving AAV production, transduction, stability, and safety. The FDA is currently studying how PTMs may impact T-cell responses. Should PTMs ultimately be designated as CQAs, a means of ensuring their control could eventually be integrated into a manufacturing control strategy.

Methods to characterize AAV capsids

Considerations for AAV capsid characterization include total particle titer (e.g., all proteinaceous content arising from AAV capsids), assessment of empty vs. partial vs. full capsid content, genomic DNA content and identity of partial and full capsids, AAV serotype identity, and/or PTMs of the AAV capsid subunits. A variety of methods and new technologies are emerging and may be utilized to characterize capsid proteins or their genomic content.

Box 6. Characterization methods for AAV capsids

Methods to characterize capsid proteins

1. **IEX**
   content ratio of empty/ partial/ full capsids
2. **AUC**
   content ratio of empty/ partial/ full capsids, aggregation
3. **CDMS**
   content ratio of empty/ partial/ full capsids
4. **ELISA**
   Capsid titer
5. **SEC-MALS**
   capsid titer, content ratio of empty/partial/ full capsids, aggregation
6. **TEM-Cryo-EM**
   content ratio of empty/ partial/ full capsids

Methods to characterize genomic content

1. **Gel Electrophoresis**
   size
2. **NGS**
   genomic content
3. **PCR**
   titer

To support AAV capsid characterization, the USP has acquired full, empty, and 50% full/50% empty samples for multiple AAV serotypes (2, 5, 6, 8, and 9). Through both in-house efforts and external collaborations, they have examined various methodologies for quantifying AAV5 and AAV8 capsid ratios, including dPCR and ELISA, SEC-MALS, CD-MS, as well as CE-Immunoassay and cIEF. Empty/full ratios determined from these methods generally align well, particularly for AAV5. Planned USP efforts include the analysis of AUC as a characterization method, and future efforts may include the characterization of samples containing partially full AAV capsids.

Next-generation sequencing (NGS) was proposed as a robust method to characterize the genomic content of capsid populations. Though traditional gel electrophoresis can provide important information on the size of viral DNA populations, it does not provide sequence identity. Short-read NGS processes fragment genomic DNA and offer a high-throughput and accurate means of quantifying and confirming sequences. Short-read sequencing provides minimal structural payload and/or contaminant information but can provide insight into where contaminant DNA originated (e.g., plasmid backbone, *rep, cap*) and is an efficient method to examine multiple vector populations simultaneously.

Long-read NGS enables direct DNA sequencing and provides structural information about individual genome sequence configurations. Long-read sequencing has revealed that capsids deemed "empty" may contain small ITR sequence fragments,[15] and that some reads deemed "payload-matching" from short-read NGS actually represent chimeric genomes that will not produce the therapeutic protein of interest. It remains to be determined whether these small ITR sequences or chimeric genomes, which often configure as concatemers that completely fill capsid space, can impact therapeutic outcomes.

Overall, short- and long-read sequencing represent complementary approaches for additional characterization of the genomic content of full and partial capsids. Notably, the FDA is in the process of growing their bioinformatics expertise to better understand how NGS data fits into empty/partial/full capsid characterization. Using complex, emergent, and not yet well-controlled methods such as NGS to characterize an impurity that is poorly understood (e.g., partial capsids) generally involves significant interpretation, which was noted as an undesirable trait for an assay required for lot release. In addition, the feasibility of these NGS methods for characterization is unknown, and their findings may or may not materialize as CQAs of AAV gene therapy products.

Given the volume of drug substance required for multiple characterization studies, a better understanding of CQAs will be essential for determining the best-fit analytical methods as well as how to implement them in a phase-appropriate manner. As an alternative to performing characterization studies on drug substance, developers may consider performing such studies in their research labs to gather information that promotes future understanding.

Downstream purification methods to minimize empty capsids

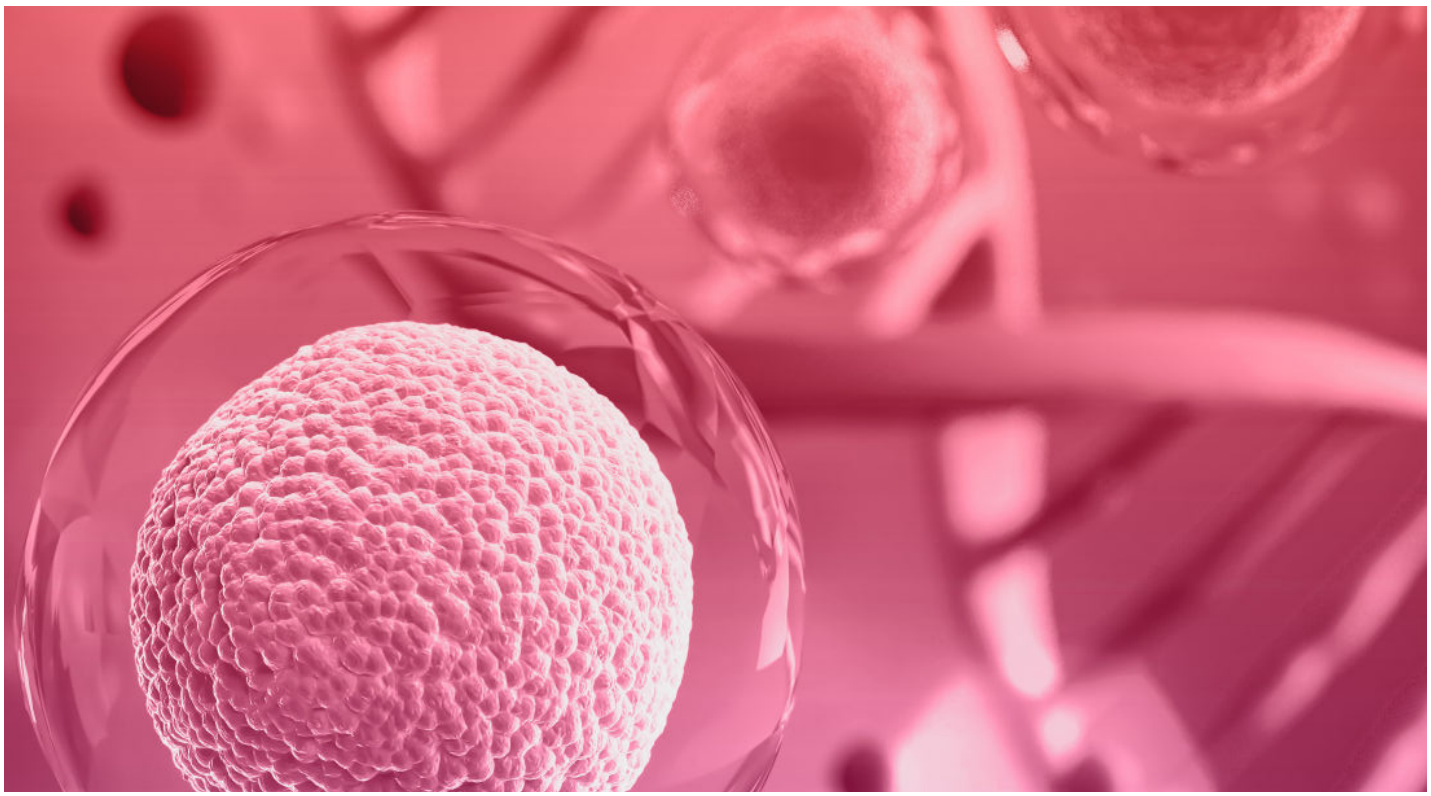
Because much remains unknown about empty capsids' impact on the efficacy and safety of AAV-based gene therapies, developers should seek avenues to minimize empty capsids. However, the surface properties of empty and full capsids are similar, making co-elution common and the removal of empty capsids a significant purification challenge. Developers speculated that PTMs causing a change in charge (e.g., phosphorylation) or a change in capsid protein orientation may cause heterogenous species to co-elute with the full population during chromatography steps.

In AAV downstream processing, affinity chromatography may be used as the first purification step, serving to remove the bulk of host-cell impurities. This step is often followed by AEX, which helps to separate empty and full capsids into "peaks" based on differences in charge due to DNA content. Full-peak material is generally processed to become the final drug product. AEX also helps to remove residual host-cell and chemical impurities as well as AAV aggregates. For developers using AEX as their final purification step, determining the lot-to-lot variability of AEX resins is of utmost importance, as resin differences can impact separation

ability. The AAV serotype and buffers used can also impact how effective AEX is at separating empty and full capsids.

Indeed, AUC has revealed that heterogenous capsid species may remain present after AEX. As such, techniques such as ZUC may be used to further separate empty and full capsids via density differences. This represents an orthogonal mechanism of separation compared with AEX and further reduces AAV aggregates and higher-molecular-weight species. Compared with chromatography, ZUC is more challenging to scale-up; maximizing the loading of AAVs into the ultracentrifuge is important for scalability. A well-optimized ZUC process may not significantly affect yield.

Using orthogonal methods for purification, consistent and progressive removal of empty capsids and other impurities can result in undesirable components of drug substance being reduced to low or undetectable levels. These methods should be generally applicable to multiple AAV serotypes, but will need to be optimized for each serotype given that differences in charge and density characteristics of empty and full capsids would be expected across serotypes. Optimal purification methodologies and their sequencing remain to be determined.



POTENCY ASSAYS

Defining Potency

Potency refers to the specific ability of a substance to produce a defined biological effect. As such, potency represents a key CQA of any AAV-based gene therapy product. Definitions of potency vary somewhat across agencies, but common themes emerge: potency must be linked to the product's relevant biological properties, should be reported as a quantitative measure of its biological activity, and should be measured via appropriate laboratory tests.

Box 7. Potency-related definitions from health authorities

FDA (CFRs):

FDA defines **potency** as "the specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to effect a given result." (21 CFR 600.3(s)).[16]

Strength is defined as "the potency, that is, the therapeutic activity of the drug product as indicated by appropriate laboratory tests or by adequately developed and controlled clinical data (expressed, for example, in terms of units by a reference to a standard)" (21 CFR 210.3(b)(16)).[17]

ICH (Q6B):

The biological activity describes the specific ability or capacity of a product to achieve a defined biological effect. Potency (expressed in units) is the **quantitative measure of biological activity** based on the attribute of the product which is linked to the relevant biological properties.[18]

EMA (2001/83/EC):

In order to give the **'quantitative composition'** of the active substance(s) of the finished medicinal products, it is necessary, depending on the pharmaceutical form concerned, to specify the mass, or the number of units of biological activity, either per dosage unit or, per unit of mass or volume of each active substance.[19]

Goals and requirements of an AAV potency assay

A robust potency assay demonstrates a fundamental understanding of the drug product and is crucial for product release testing, stability testing, and comparability studies. Defining a product's potency requires specific knowledge of how it exerts its effect in the body, and the transgenes inserted into AAV-based gene therapies often possess complex or unclear mechanisms of action. Early on, developers should strive to gain as much understanding of their transgene product as possible. When determining the biological activity that will guide potency-assay design, developers should consider preclinical data, the use of animal models that are as representative of the human condition as possible, available historical experience, and available reference materials and controls.[20]

In 2011, the FDA published a Guidance for Industry entitled "Potency Tests for Cellular and Gene Therapy Products."[20] Though not specific to AAV-based gene therapy products, developers can utilize this and other guidance from the EMA and FDA to implement practical approaches to potency assay development. Many of these guidance documents combine cell and gene therapy topics. Developers noted that future guidance should address these topics separately to increase their specificity.

Box 8. Regulatory guidance documents applicable to AAV-based gene therapies

Health Authority	Guidance Document	Publication Year
European Medicines Agency	Note for guidance on the quality, preclinical and clinical aspects of gene transfer medicinal products[21]	2001
	Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products[22]	2018
	Guideline on quality, non-clinical and clinical requirements for investigational advanced therapy medicinal products in clinical trials (DRAFT)[23]	2019
	Guideline on quality, non-clinical and clinical aspects of medicinal products containing genetically modified cells[24]	2020
U.S. Food and Drug Administration	Potency Tests for Cellular and Gene Therapy Products[20]	2011
	Expedited Programs for Regenerative Medicine Therapies for Serious Conditions[25]	2019
	Rare Diseases: Common issues in Drug Development[26]	2019
	Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs)[27]	2020

Potency assay(s) may reflect multiple points in the biological cascade (e.g., transduction of the AAV particle into cells, transgene expression, and functional activity). During early development, it may suffice to establish the ability of the AAV particle to transduce cells and for the DNA to be translated into mRNA and protein (see *Establishing a potency assay matrix*). Later in development, it is necessary to establish a potency method that demonstrates the biological function of the intended gene product. Once such an assay has been established and demonstrates sufficient robustness and control, it may be possible to reduce the number of matrix assays (e.g., the transduction and gene expression assays could potentially be eliminated).

During product development, assay validation efforts are directed at demonstrating that the method(s) are robust and provide acceptable linearity, range, specificity, precision, and accuracy. Linearity and range are often established via a product-specific standard or reference material (when one is available), whereas specificity may be determined by comparing the drug product's activity to a negative control. Precision can be demonstrated by employing replicates and looking for variability in runs performed by different operators, on different days, using different equipment, etc. Accuracy is often reported relative to an accepted reference standard.

Box 9. Definitions of validation characteristics

Validation characteristic	Definition[28]
Linearity	The ability, within a given range, to obtain test results that are directly proportional to the concentration or amount of analyte in a sample
Range	The interval between the upper and lower concentration or amount of analyte for which the analytical procedure has been demonstrated to determine with a suitable level of precision, accuracy, and linearity
Specificity	The ability to assess the target analyte in the presence of components (e.g., impurities, degradants, matrix) that may be expected to be present
Precision	The degree of agreement between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions
Accuracy	The closeness of agreement between the test result found and the value accepted as the true value or an accepted reference value
Robustness	The measure of an analytical procedure's capacity to remain unaffected by small but deliberate variations in method parameters that might be expected during normal usage

Potency as part of the drug product lifecycle

Conceptualization and development of a potency assay or assay matrix starts early in the drug product lifecycle and matures as the product moves towards commercialization. Aligning with the FDA on potency assay expectations is essential during each stage of product development. Potency assays used during the pre-IND stage are generally not yet qualified and are primarily used to screen and characterize the drug candidate. These methods may be limited to *in vitro* gene expression assays or expression and biodistribution assays in animal models. At the IND stage, developers are expected to provide information to the FDA on their quality control strategy for drug substance and drug product, including components of that strategy relating to potency. For early phase studies, potency assays are expected to be qualified and suitable for lot release of the drug product, but specifications may be wide at this point. By pivotal clinical trial initiation, a validated strength (vg/mL) assay is expected so that the developer can demonstrate analytical readiness for commercialization. Potency specifications are tightened as developers move toward BLA filing; regulators ultimately expect a specification range to be narrow enough to detect a meaningful difference in potency (e.g., a subpotent lot), and the validation report should be included in the BLA. Post-approval, validated potency assay(s) can be used to qualify new reference standards, demonstrate process and site comparability as production expands, and address the "assay drift" that may develop when old reagents or equipment are phased out and newer ones are introduced.

Box 10. Potency assay development across the drug-product lifecycle

	Early product development			Late product development	Biologics license
	Preclinical	Phase 1	Phase 2	Phase 3 (pivotal)	
Potency assay focus	Patient safety the main concern and focus fo testing Establish broad acceptance criteria evaluated with physicochemical and biochemical testing data as the basis for futher testing and assay development			Establish appropriate limits for potency to ensure that product lots are well-defined, biologically active and consistently manufactured Establish stability to inform expiry dating for licensure	Describe and justify a validated potency assay within defined acceptance criteria Use for lot release, product stability and product comparability

Adapted from LabCorp: ATMP Potency Assay Strategies: Adding Value to Your Asset and Avoiding Delays in Development. 2020.[29]

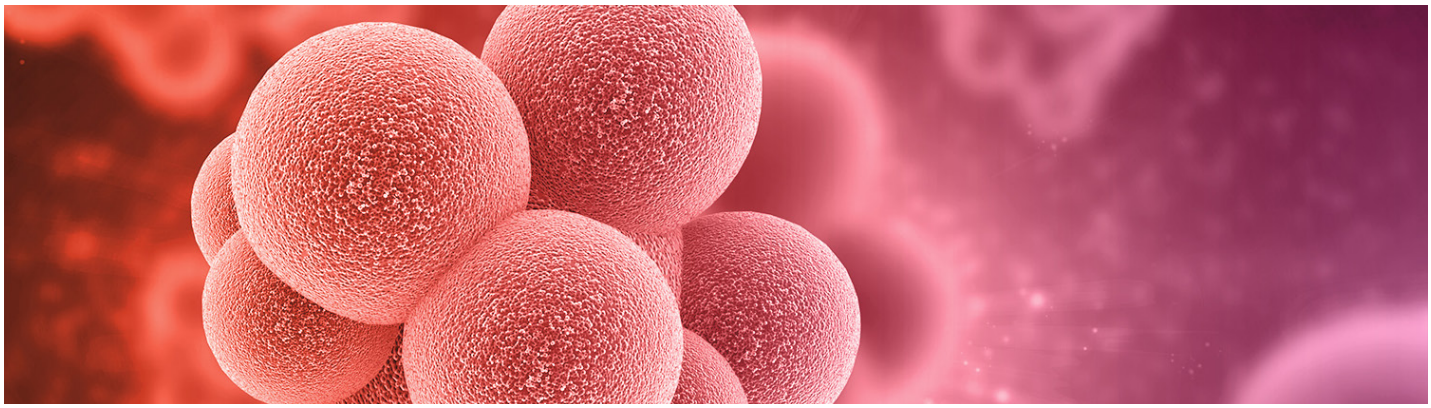
Potency assays require time and money to develop and often represent a rate-limiting step in drug development. Some sponsors choose to invest in functional biological potency assay development early, whereas others may wait until a drug candidate produces significant clinical efficacy signals. Though either strategy may work, developers and regulators generally agree that starting early is the best approach. A qualified potency assay may help with determining drug candidates at the proof-of-concept stage and may eliminate candidates that are unlikely to be effective (e.g., by showing that a promoter is too weak to generate sufficient expression to affect a disease course). Similarly, a functional test could indicate that the protein design may not be appropriate for restoring function (e.g., cDNA truncated to fit into an AAV capsid may eliminate critical functions of the protein). Early investment in potency assays may be particularly important if a developer has been granted an expedited approval pathway such as RMAT designation or breakthrough therapy designation.

Approaches to measuring potency change over the product development timeline. The next several sections will discuss some of these potential changes: moving from *in vivo* to *in vitro* assays, establishing a potency assay matrix, and pruning that matrix to increase efficiency.

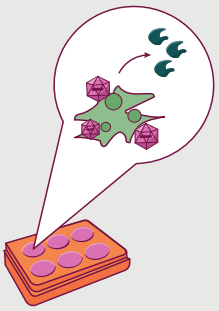
In vivo versus in *vitro* potency assays

Early in product development, *in vivo* potency assays may be used to measure activity or gene expression. Such assays provide a qualitative measurement of potency but become impractical during later stages of development due to their high requirement for labor, cost, and time and their reliance on animal models.

Quantitative *in vitro* potency assays are generally preferred as the drug product moves closer to commercialization. *In vitro* assays allow the measuring of potency relative to a well-characterized lot and may simplify the potency assay used for lot release. In addition, they are generally faster and more reliable than *in vivo* assays and minimize the use of animals for testing, which is in concordance with the EU "3R" directive to replace, reduce, or refine the use of animals.[30]



Box 11. Advantages and disadvantages of *in vivo* and *in vitro* potency assays

In vivo potency assay	*In vitro* potency assay
	

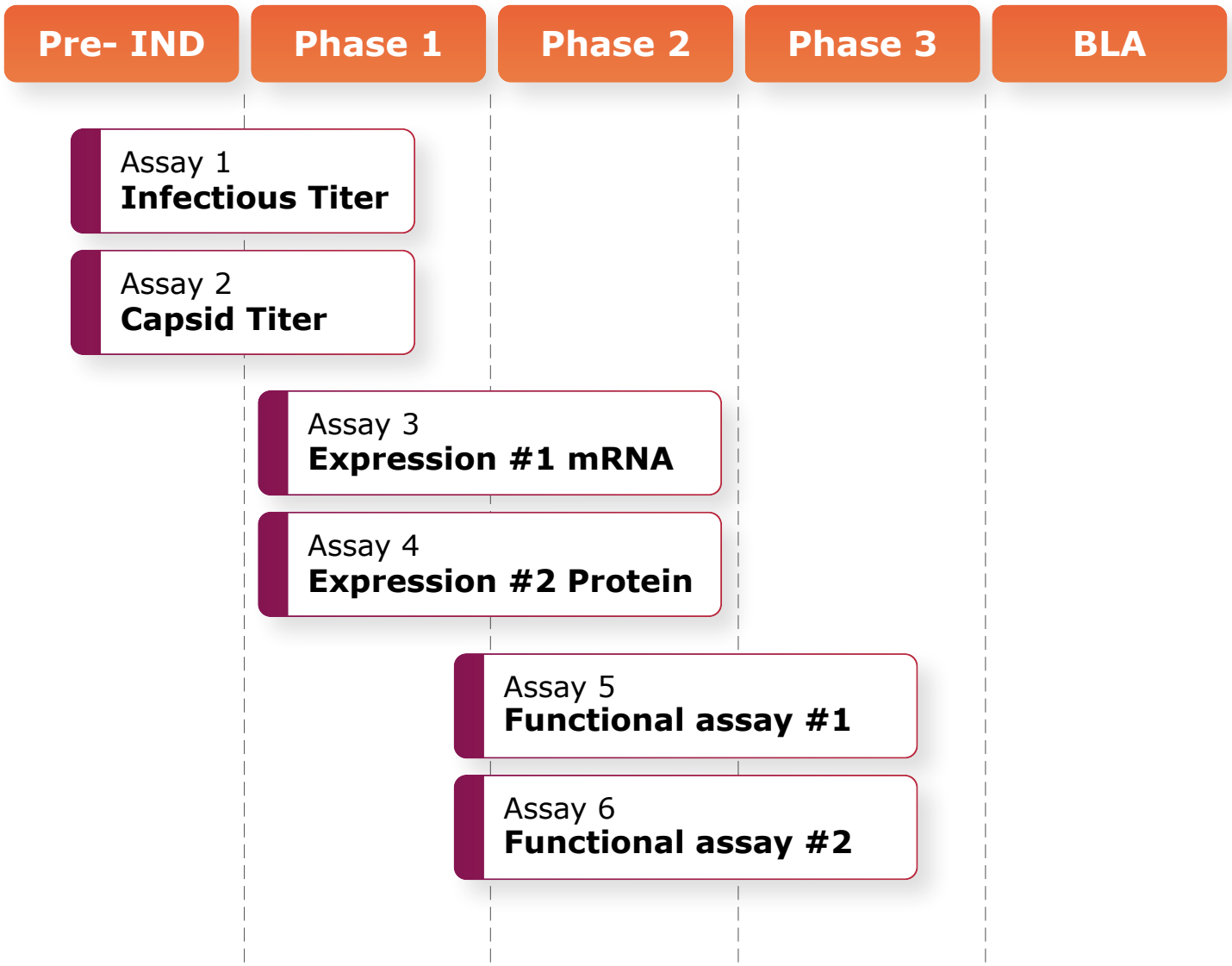
+ **Physiologically relevant**
+ **Allow evaluation of safety/ toxicity**
+ **Can indicate durability of treatment**
– **Complex, highly variable**
– **Long assay time**
– **Labor-and cost-intensive**
– **Animal-based**
  – Fewer laboratories available
  – Ethical concerns
– **Qualitative**
	+ **Cell-based (vs animal-based)**
	  + More laboratories with capabilities
	  + Aligns with EU "3R" directive
	+ **Shorter assay time**
	+ **Quantitative**
	+ **Decreased amount of vector required**
	+ **Reproducible, less variable**
	– **Less physiologically relevant**
	– **Optimization and understanding require time**

Establishing a potency assay matrix

A fit-for-purpose potency assay matrix is often employed in early stages of AAV-based gene therapy development to assess the multiple attributes contributing to its mechanism of action. Assays employed in a traditional matrix approach may provide a measure of AAV transduction (as a measure of DNA delivered to the cell; e.g., $TCID_{50}$); mRNA expression (e.g., by RT-PCR); or protein expression. Protein expression methods could include, but are not limited to, Western blotting, ELISA, flow cytometry, or capillary electrophoresis.

In addition, an orthogonal custom assay to measure protein function is generally developed based on the expected biological activity of the transgene. These custom assays are often time-consuming to develop, qualify, and validate, but may be the most meaningful measure of true biologic relevance.

Box 12. A fit-for-purpose matrix approach to measuring potency



In some developers' and regulators' minds, demonstrating the functional activity of a product should be sufficient to show that protein expression was accomplished. Notably, FDA requirements have often been misinterpreted by developers, mistaking the requirement to demonstrate control of the full biological cascade with a requirement for a full potency assay matrix. FDA representatives reiterated that the agency does not require a potency assay matrix if a quantitative functional assay has been established that demonstrates sufficient robustness and control. Nevertheless, developers have noted that certain international regulators may still expect the use of orthogonal assays to demonstrate the full biological cascade, from transduction to expression to function. When this is the case, it may lead to an assay matrix with a number of methods, each with potentially different acceptance criteria, which together require significant time, labor, resources, and materials investment.

Pruning the potency assay matrix

In general, developers strive to simplify or "prune" the potency assay matrix into a smaller number of assays required for lot release. Some developers expressed a desire to eliminate $TCID_{50}$ as an assay required for lot release due to its highly variable nature and the fact that it does not reflect the true MOA of AAV. They speculated that this could eventually be made possible by showing that other potency assays correlate with $TCID_{50}$.

One sponsor shared an example of how a high-content imaging, multi-attribute *in vitro* assay measuring both infectivity and expression was successfully used to eliminate $TCID_{50}$ as a lot release assay. Development and qualification of this multi-attribute assay was initiated early in product development (pre-IND) alongside development of functional potency assays. In the end, this assay provided a higher throughput, less variable measure of infectivity (and one more reflective of AAV mechanism of action) than $TCID_{50}$ at no additional cost. It directly correlated with relative potency and doubled as a more sensitive stability-indicating assay than $TCID_{50}$ across a wide range of temperatures. Ideally, an assay that measures infectivity, expression, and function in a single assay could be developed as the one "golden" potency assay.

Regulatory considerations for measuring potency

Because potency should reflect individual product attributes, the FDA has stressed that potency assay requirements will be determined on a case-by-case basis. Regulators expect that developers control for all biological activities needed for the function of the drug product. Justification for specific potency assays must include data demonstrating the assay(s) is well-controlled. If sufficient control is not evident, the FDA will seek additional testing and likely request orthogonal assays. However, if sufficient control of the functional test is demonstrated, it will also be judged as demonstrating control of upstream product attributes that are related to bioactivity.

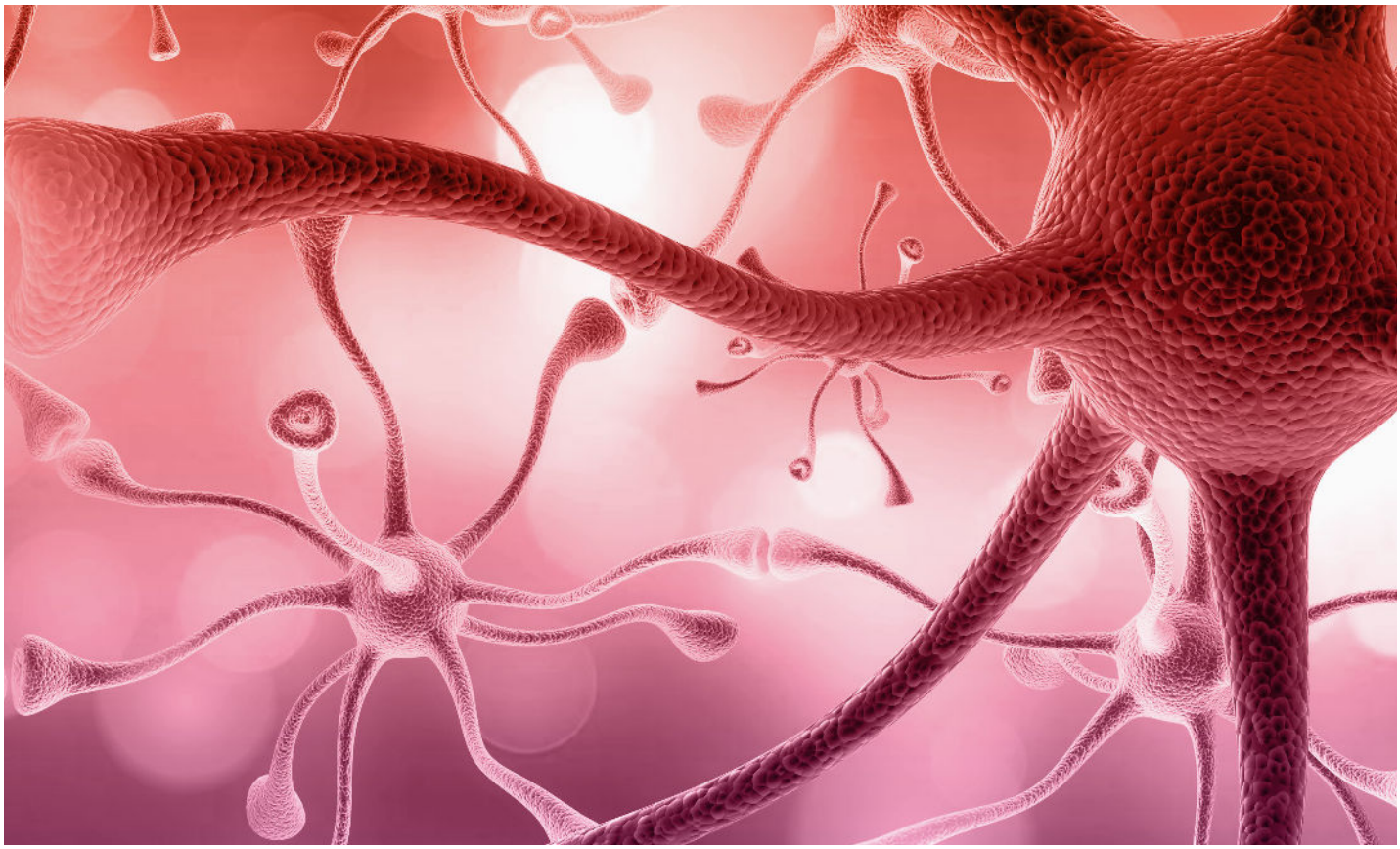
FDA acceptance of potency assays is dependent on data provided by the sponsor. Measuring relative potency (i.e., relative to a well-characterized standard or reference lot to control variability) appears most common in the industry and has been a consistent ask from the FDA, but the agency would consider an assay measuring absolute potency if it was sufficiently supported by data. Essentially, the onus lies on the developer to convince the FDA (or any health authority) how their AAV-based gene therapy product should be characterized.

The FDA realizes that developing and optimizing potency assays takes time. In addition, new analytical techniques for assessing potency are being developed, and regulators may be evolving in both their understanding of AAV and their potency expectations at different paces. In some countries, regulators continue to require $TCID_{50}$ as part of the potency matrix. As such, early engagement with regulators about potency assay expectations is key, regardless of when significant efforts toward this end may commence. To receive focused feedback from the FDA (and likely other health authorities), submitted packages should clearly define the assay and controls, provide available data, be as concise as possible, and ask relevant questions.

Ultimately, potency assay expectations should be driven not only by regulators, but also by developers, through a careful assessment of whether what's being administered to patients is able to deliver its intended biological effect.

"(As a developer,) can you convince yourself that you have potential for clinical benefit? Then you present this case, or matrix, to regulators."

– AAV gene therapy developer



VECTOR GENOME TITER MEASUREMENT AND DOSING

What is vector genome titer?

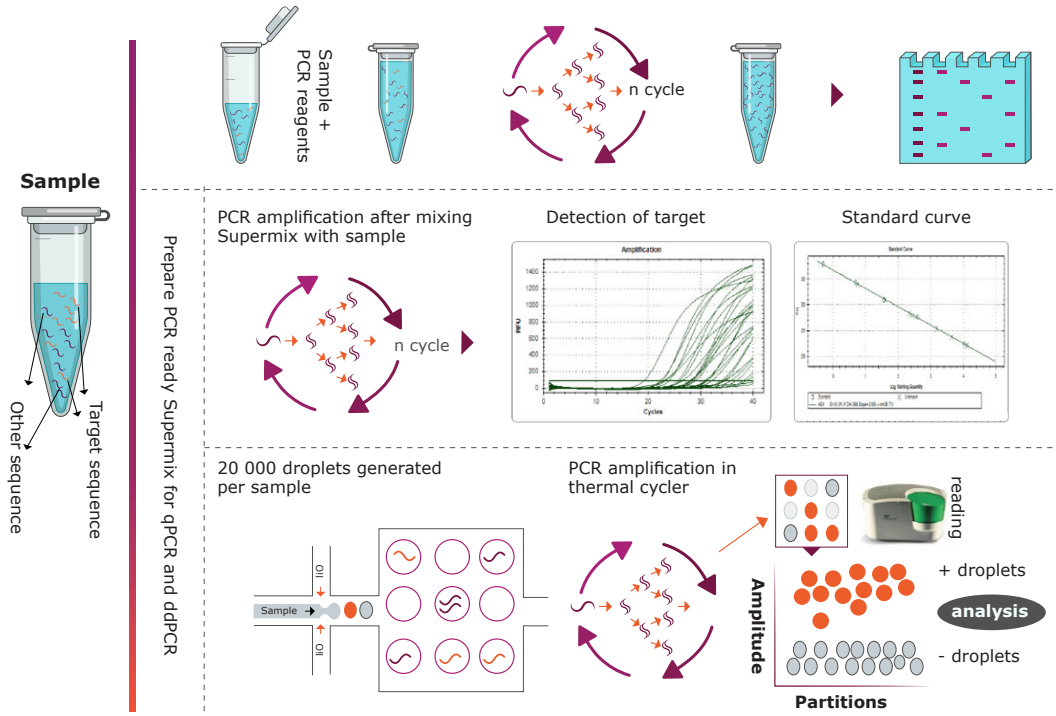
Vector genome titer, often reported as vg/mL, is generally used to determine the clinical dose of an AAV-based gene therapy product. Vector genome titer represents the physical concentration of purified vector particles containing the genome of interest, per mL of product. An accurate and precise AAV genome titer serves as the basis for dose determination in both preclinical and clinical studies.

Common methods for determining vector genome titer: qPCR and ddPCR

The gene therapy industry has evolved past the early days of PCR that used electrophoresis and agarose gels to visualize results. Today, qPCR and ddPCR can be used to determine viral vector genome titer after fit-for-purpose assessments. These quantitative techniques have unique workflows and advantages/disadvantages, often making the choice between them program-specific and/or phase-specific.

One CDMO executive shared an example of changing from a qPCR to ddPCR strategy to align with a client's program. In comparability studies, AAV2 and AAV8 vector genome titer findings were comparable between qPCR and ddPCR. In addition, ddPCR showed good intermediate precision after multiple replicate analyses (<6% CV). The close comparability seen in this example may not be demonstrated with all gene-of-interest–specific primer/probe sets. Indeed, another presenter shared an example where vector genome titer determined from qPCR was consistently ~2.0-fold higher than that determined from ddPCR. This average conversion factor will be included in FDA submissions and used for transition to clinical studies.

Box 13. Example workflows for different PCR platforms used for titer assessment



A) PCR

- End-point detection
- Assay is qualitative or quantitative

B) qPCR

- Real-time detection
- Relative quantification
- Absolute quantification needs a standard curve
- Less sensitive and less specific

C) ddPCR

- Detection at end-point
- Droplets read as either positive or negative
- Absolute quantification
- Quantitative assay
- More sensitive and more specific

Adapted from: Nyaruaba R et al. Droplet digital PCR applications in the tuberculosis world. *Tuberculosis*. 2019; 117:85-92.[31]

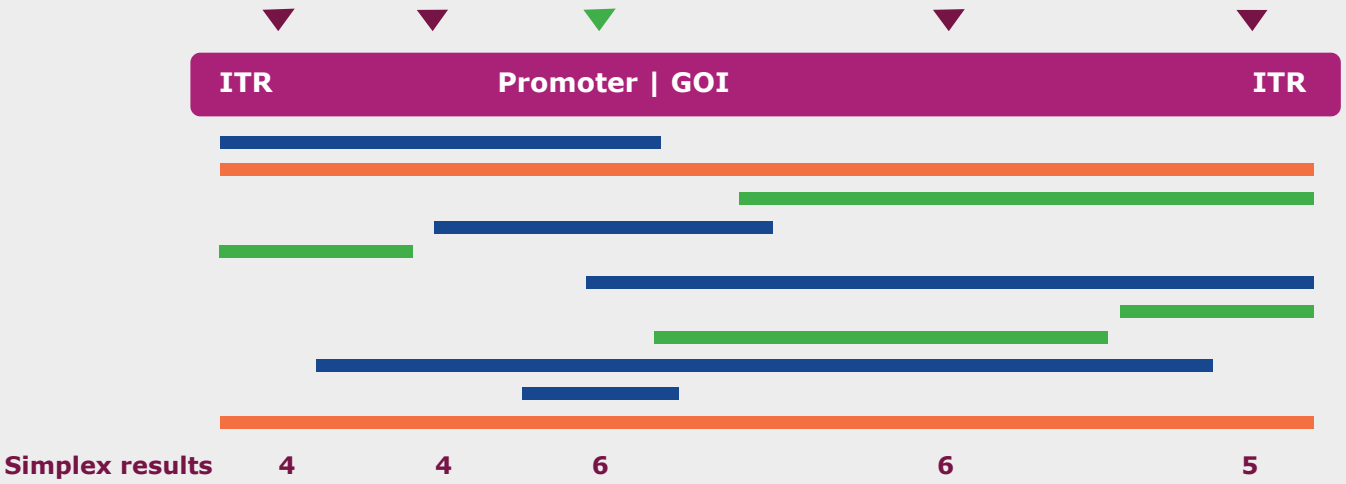
Box 14. Advantages (+) and disadvantages (-) of qPCR and ddPCR

qPCR	ddPCR
+ Relatively quick, easy, and inexpensive	+ Absolute quantification via Poisson statistics without need for standard curve
+ Flexibility to utilize ready-to-use primers/probes/standards or design program-specific probes	+ Better signal-to-noise ratio than qPCR
+ Most labs have equipment	+ More precise quantification than qPCR
+ Real-time detection	+ More sensitive and specific than qPCR
+ Large dynamic range	+ More tolerant to PCR inhibitors than qPCR
– Requires standard curve (cloning)	– Time and money required to develop gene-of-interest-specific primer/probe set
– Quantification is relative to the standard curve	– Detection at end-point
– Possible background noise/interference	– Less sensitive to template secondary structure (e.g., ITR hairpins) than qPCR
– Must understand how standards are diluted, stored, and their stability; standard source could change (potential introduction of error)	– Operator expertise required

Moving from vector genome titer to quantification of vector genome integrity

Simplex PCR methods (using one target per reaction) such as qPCR and ddPCR provide limited information on genomic integrity. Indeed, the titer determined by qPCR or ddPCR may represent an overestimation of full-length genomes due to the inherent amplification of small fragments by PCR. This is problematic, as full-length genomes constitute the payload of interest.

Box 15. Simplex PCR may overestimate full-length genomes

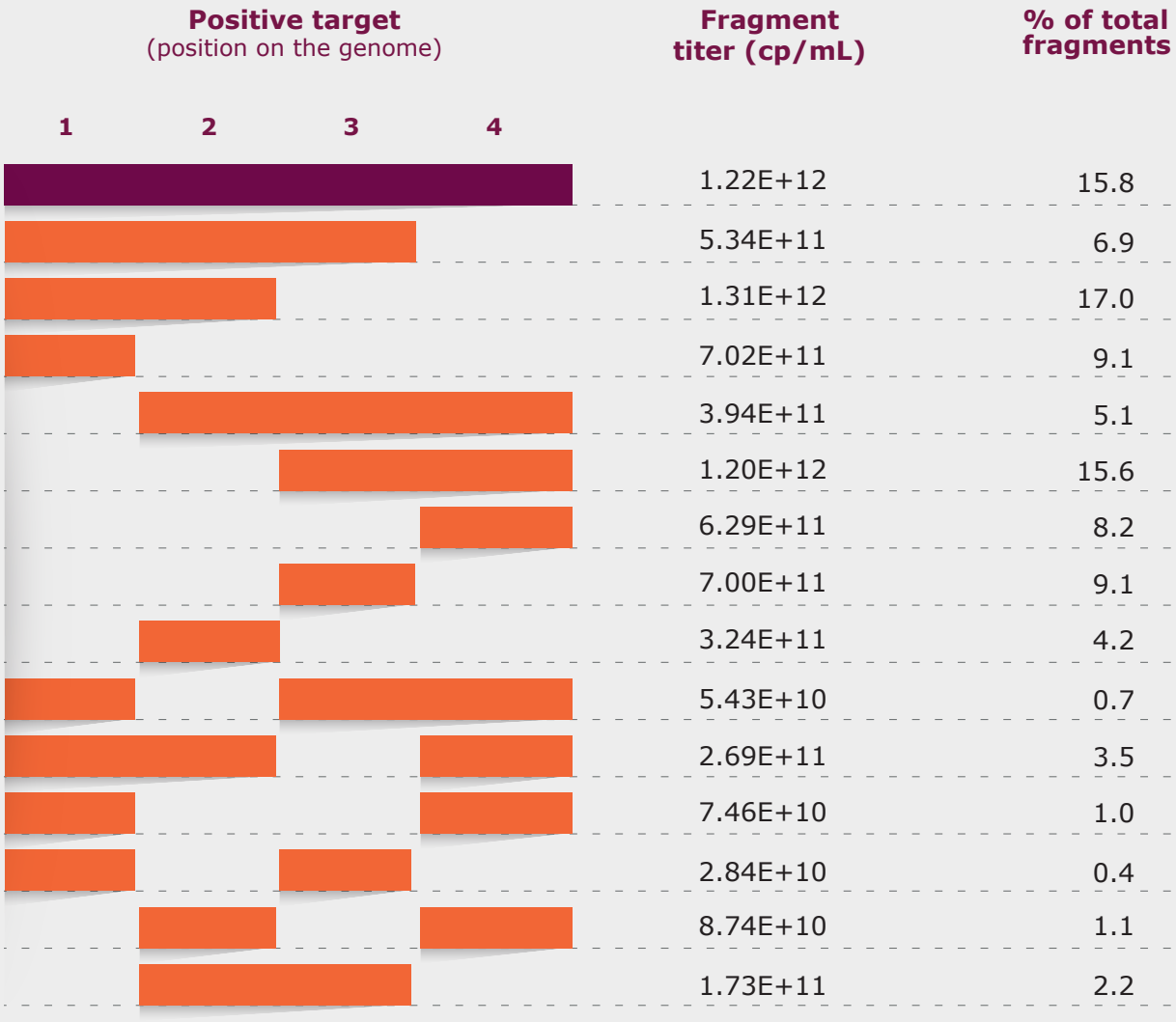


In this sample containing only 2 full-length genomes, the target chosen for simplex PCR may lead to as many as 6 "hits", thereby consistently overestimating the number of full vector genomes.

Adapted from: Hayes DB, Dobnik D. Commentary: Multiplex dPCR and SV-AUC are promising assays to robustly monitor the critical quality attribute of AAV drug product integrity. *J Pharm Sci.* 2022;111(8):2143-2148.[32]

One developer proposed that vector genome integrity may become an important CQA, though he noted this remains controversial. A multiplex dPCR assay, with more targets per reaction, allows for a closer look at genome integrity. A duplex assay, with one target at the beginning of the vector genome and one at the end, is the simplest example of multiplex dPCR. However, duplex assays remain likely to detect individual fragments, highlighting the need for a good understanding of target distribution within the sample. Triplex and 4-plex assays, though increasingly complicated to develop, qualify, and validate, could provide a more detailed view of target sequence integrity.

Box 16. Gaining insight into vector genome integrity through 4-plex dPCR



In this example of 4-plex dPCR, full genomes represented 15.8% of the sample. Fragments containing either end of the genome are very common.

Adapted from: American Pharmaceutical Review; Dobnik D. Moving Towards Genome Integrity Evaluation of Gene Therapy Viral Vectors. 2023.[33]

Indeed, this developer described a 4-plex assay that has been developed to quantify the AAV vector genome, with the possibility to add additional targets. This assay has been qualified, showing good repeatability, precision, and linearity through the tested range of possible full-length percentages. He suggested that results of genome integrity quantification may be used to guide process development, perhaps even identifying different steps within upstream or downstream processing that enrich for a certain fragment population.

In summary, multiplex dPCR assays enable better product characterization and may allow for process optimization that leads to a higher yield of full-length viral genomes within capsids. Multiplex assays generally reduce costs, as combination assays preclude needing to run individual assays for each potential target. Enriching for full-length encapsidated genomes could result in higher potency, thereby allowing for a lower administered dose and improved patient safety. More detailed studies are needed to establish any true correlation between genome integrity and potency.

Determining particle titer via SEC-MALS

One developer described a fair amount of variability seen when validating a ddPCR method for vector genome titer. This led one's company to examine SEC-MALS as a robust, sensitive, and automated method for genome titer. SEC-MALS, which combines size-exclusion chromatography with multi-angle light scattering and online concentration detectors, allows examination of multiple AAV characteristics (e.g., aggregation profile, impurity profile, empty:full capsid ratio, and DNA content) as well as a means of genomic titer analysis.

Box 17. Advantages (+) and disadvantages (-) of ddPCR and SEC-MALS

ddPCR	SEC-MALS
+ Suitable for all in-process samples (can get titer information from crude, upstream samples)	+ High-throughput
– Requires precise pipetting	+ Automated
– Provides only vector genome titer	+ Provides information on total particles in one assay
– Requires multiple assays to produce an accurate titer	+ Fast time to result
– Lengthy time to result	– Does not distinguish residual DNA (e.g., host-cell, plasmid)
	– Upstream samples require sample purification

In the case of this developer, SEC-MALS is not being viewed as a tool that can completely replace ddPCR, but rather as one that can help to characterize batches in quicker succession and provide better feedback to process developers on how manufacturing runs are going.

Regulatory considerations for vector genome titer measurement

The FDA is not proscriptive on the specific assay chosen to determine AAV genome titer, but the assay should be validated prior to initiating clinical studies. This requires demonstration of assay linearity, accuracy, range, precision, sensitivity, and specificity. General regulatory expectations for early-phase studies include (1) a spike recovery of ~80-120% of the expected value; (2) an intermediate precision of 15% CV or less; and (3) use of an unrelated AAV vector as a control (rather than just a buffer). Additionally, the FDA recommends that developers spend significant time understanding sources of variability (e.g., reagents, personnel, equipment) for their AAV genome titer assay.

For PCR-based methods, use of product-specific primers/probes (e.g., specific to the transgene or junction) may allow the AAV genome titer (strength-determining) assay to double as an identity assay for product release. Complex PCR targets such as ITR or SV40 regions should generally be avoided. Other strength-determining assay recommendations from the FDA include (1) establishing a suitable reference material (i.e., a well-characterized lot of the product) early on; (2) using the same assay in both preclinical and clinical studies (this gives the FDA confidence that dose ranges and effects seen in preclinical studies are relevant to those anticipated during human trials); and (3) using a bespoke assay for the product being studied (a platform approach is not appropriate in this case).

Box 18. Regulatory advice for vector genome titer provided at a pre-IND meeting

Vg Titer Considerations (For Phase 1)

- The assay must be **qualified prior to Phase 1** clinical studies. Failure to submit adequate information supporting assay suitability will result in your IND being placed on clinical hold.
- Please be aware that the qualification **data should be collected for the product under study** and should include appropriate reference standards of consistent quality and nature, including product-specific controls.
- Please provide a detailed protocol for the qualification study and the SOP for the assay, including **information about the reference standards, controls,** and assay optimization.
- Please provide the study report with data documenting assay qualification, including accuracy, precision, specificity, range, and linearity. **The intermediate precision of the assay should be ≤ 15% coefficient of variation (CV).**
- Please describe any deviations that occurred during the qualification study.
- To ensure consistent dosing between clinical and preclinical studies, **we recommend using the same qualified assay** for calculating the vector genome titer of the **preclinical and clinical lots.**
- Please plan to **validate the assay prior to the conduct of clinical studies that will assess product efficacy for licensure.**

Like potency assay development, AAV genome titer (strength-determining) assay development follows a lifecycle approach. Clinical development and CMC development should essentially move forward at the same pace. If a developer anticipates efficacy data from a Phase 2 trial (e.g., in conjunction with an expedited product development program), then the expectation for CMC will be as if the Phase 2 trial is the pivotal study. In such cases, validation of the strength-determining assay would be required prior to initiating the Phase 2 trial.

For any AAV-based product entering interstate commerce, the expectation is that dosing will be based on a nominal titer, which reduces the likelihood of dose calculation errors. During Phase 1, dosing is often determined by measuring the vector genome titer of each specific lot (i.e., a "measured titer"). This may not account for the inherent variability of the strength-determining assay, and it requires the physician or pharmacist to prepare a dose volume based on the lot-specific titer and patient weight (when applicable). With "nominal titer", AAV-based drug product vials are labeled with the *target* vector genome concentration, meaning the same strength is printed on every vial label of every released lot. The pharmacist or physician will prepare the dose volume based on the target titer and patient weight (if applicable), perhaps using a dose worksheet or dosing table. The nominal titer presented in a BLA filing must be supported by clinical data. Hence, between Phase 1 and BLA submission, safety and efficacy data should be obtained using nominal dosing. This will require substantial communication between preclinical personnel, clinicians, manufacturing operations, and quality-control team members.

Box 19. An example of dosing calculations using measured vs. nominal titer methods

Example dose of 8E+13 vg/kg

Using measured titer (complicated calculation)*:

DP vial label of 3.78E+13 vg/mL

- **For 80 kg subject, total dose = 80kg x 8E+13 vg/kg**
  - 640E+13 vg (or 6.4E+15 vg)
- **Volume of DP Needed = 6.4E+15 vg / 3.78E+13 vg/mL**
  - Administer 169 mL of DP

Using nominal titer (simplified):

Dose worksheet

DP vial label of 2.00E+13 vg/mL

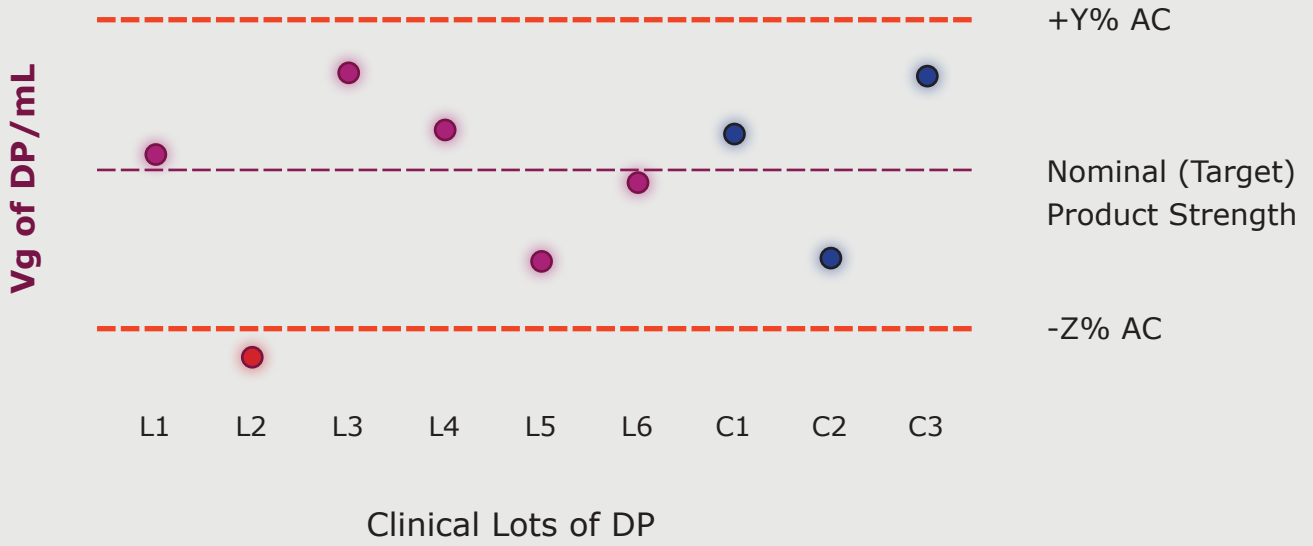
To determine volume in mL:

Multiply body weight in kg by 4

* May be further complicated if 2 lots with different measured titers are required for a dose

Ensuring appropriate dosing via nominal titer methods requires very tight acceptance criteria (i.e., X vg/mL plus Y% or minus Z%) for product strength. These acceptance criteria need to be established and justified based on how much variation is acceptable in the final clinical dose, which may be informed by the dose ceiling prior to seeing adverse events and the basement dose required to see bioactivity in preclinical trials. Ultimately, achieving tight acceptance criteria requires robust manufacturing and both an accurate and precise strength-determining assay, but serves to decrease the chance of failing lot release.

Box 20. Acceptance criteria for a nominal titer assay



Notably, total capsid titer assays are not generally appropriate for strength determination. The only exceptions are situations with negligible (<1-2%) and very consistent percentages of empty capsids from lot-to-lot. However, total capsid titer determination can provide information on the antigenic load delivered to patients, which the FDA considers important. A direct measure of capsid titer (e.g., ELISA) may be more appropriate and accurate than back-calculating from AUC data, but any assay qualified as fit-for-purpose will be considered. In addition to vector genome titer and capsid titer determination, the FDA recommends that the drug product is tested with a direct measure (e.g., IEX, AUC, EM) of full:empty capsid ratio. This informs the robustness of downstream processing for removing empty capsid impurities and allows developers to track lot-to-lot consistency.

A platform approach to AAV-based gene therapy analytics

Gene therapy products may cost as much (or more) to develop than other biologics but result in a one-time dose to patients rather than chronic administration. Hence, developers must try to recoup their development costs through a single dose, resulting in high costs to patients and/or the healthcare system. Ultimately, the industry is working towards finding manufacturing methods that make gene therapy products at a cost that is reimbursable and readily accessible to patients, but also financially attractive enough that companies continue to invest in the field.

The path to AAV-based gene therapy product development tends to be iterative rather than linear, and presenters agree that growth and demand within the industry necessitate a move toward industrialization and operationalization. A platform approach, in which learnings and assays from previous product development are leveraged for new drug candidates, may help companies improve their level of product supply (first priority) while minimizing costs through increasing yield (via process improvement and/or automation) and minimizing depreciation (through wise choice of manufacturing equipment).

One developer discussed how his company is moving from a pioneering/custom-made mindset toward a platform approach (i.e., a structured sequence of activities) to AAV product development. Ideas for this platform approach were gleaned from more mature industries, and ultimately resulted in a modular system that is consistent across products, manufacturing sites, and different scales of production. Importantly, these modules were developed to accommodate slight adjustments to account for inherent differences between products. The platform incorporates not only manufacturing operations, but also supporting infrastructure such as information technology and logistics. Despite using an insect-cell, baculovirus-based platform to AAV development (which is generally slower than platforms using HEK-293 cells), his company has reduced their CMC development timeline to 18 months, resulting in a time-to-clinic that is as fast as large CMO timelines for HEK-293 platforms. Through multiple iterations of their platform, they have reduced cost of goods by 100-fold. He noted cost of goods as a primary advantage of insect (e.g., baculovirus) vs. mammalian (e.g., HEK-293) production systems, adding that therapies with high market demand in Phase 3 trials tend to use the insect-cell system.

Box 21. Potential generational platform approach to AAV development

Generation 1		Generation 2		Generation 3		Future	
							
Bioreactor	8x25L	Bioreactor	500L STR	Bioreactor	500L STR	Bioreactor	500L STR
Speed to IND	Years	Speed to IND	~24 month	Speed to IND	~18 month	Speed to IND	~12 month
Yield/batch	benchmark	Yield/batch	x10	Yield/batch	x50-70	Yield/batch	x100
% Full capsids	benchmark	% Full capsids	DSP opt.	% Full capsids	USP/DSP	% Full capsids	USP/DSP
#batches/year	22	#batches/year	22	#batches/year	44	#batches/year	88
GC/year	benchmark	GC/year	x10	GC/year	x100-140	GC/year	x400

100x cheaper

3x faster

3x purer

CONCLUSIONS

This workshop brought together experts from across the AAV-based gene therapy industry to better-align on analytical advancements and regulatory requirements within the field. Overall, presenters and attendees agreed that workshops like this one are beneficial for the whole industry and all key stakeholders, providing an opportunity to collaborate and share information learned from past experiences. The hope is that the industry can strike the appropriate balance between the promise of AAV-based gene therapies and their inherent risks, while meeting increasing patient demand and still remaining attractive to developers and manufacturers.

Several clear needs, expectations, and opportunities arose from the workshop. Relating to full/partial/empty capsids, there was a call for standard terminology, appropriate reference standards, regulatory guidance for setting meaningful specifications, and for further understanding of how different capsid types elicit immune responses and impact toxicity and efficacy. In terms of potency and vector genome titer assays, there was recognition that such assays evolve throughout the drug product lifecycle, require time and money to develop, and that efforts to these ends should start early in the development process to prevent bottle-necks at the clinical trial stage. Any analytical methodology presented to the FDA must be backed by data. Interacting with regulators early and often is recommended, and, in order to receive actionable feedback, all packages submitted to regulators should be clear, concise, and ask relevant questions.

APPENDIX:

Workshop Presenters and Panelists

- **Josephine Lembong,** Senior Manager, Science and Industry Affairs, Alliance for Regenerative Medicine
- **Diane McCarthy,** Senior Director, Biologics, US Pharmacopeia
- **Khandan Baradaran,** Vice President, Regulatory CMC, Ultragenyx Pharmaceutical
- **Jerome Jacques,** Director, Biologics Portfolio Management, US Pharmacopeia
- **Rahul Sheth,** Senior Scientist 2, Purification Process Development, BioMarin Pharmaceutical
- **Lauriel Earley,** Principal Scientist, Shape Therapeutics
- **Adriana Kita,** Associate Director, Analytical Development, Ultragenyx Pharmaceutical
- **Andrew Byrnes,** Chief, Gene Transfer and Immunogenicity Branch, U.S. Food and Drug Administration
- **Christopher Miyake,** Senior Director, Regulatory Affairs CMC, Sarepta Therapeutics
- **Erandi De Silva,** Co-founder and Senior Vice President, Product Development, Forge Biologics
- **Connie Tsai,** Associate Director, Analytical Development Bioassay, Novartis Gene Therapies
- **Ravindra Kumar,** Director, Bioassay Lead, Analytical Sciences and Quality Control, Spark Therapeutics
- **Denise Gavin,** Director, Office of Gene Therapy, Center for Biologics Evaluation and Research, U.S. Food and Drug Administration
- **Pierre Caloz,** Chief Operating Officer, uniQure
- **Roberto Calcedo,** Vice President, Preclinical and Immunology, Affinia Therapeutics
- **Matthew Hewitt,** Vice President, Technical Officer CGT and Biologics, Charles River
- **Adam Davis**, Vice President, Analytical Development, Forge Biologics
- **David Dobnik,** Co-founder and Chief Scientific Officer, Niba Labs
- **Andrea Hamilton,** Associate Director, Quality Control, Gyroscope Therapeutics
- **Andrew Harmon,** Team Leader, Gene Therapy Branch 1, Division of Gene Therapy 1, Center for Biologics Evaluation and Research, U.S. Food and Drug Administration

Term key

AAV: adeno-associated virus

Ab: antibody

AC: acceptance criteria

AEX: anion exchange

ARM: Alliance for Regenerative Medicine

ASGCT: American Society of Gene & Cell Therapy

ATCC: American Type Culture Collection

AUC: analytical centrifugation

BLA: Biologics License Application

CD: cluster of differentiation

CDMO: contract development and manufacturing organization

CD-MS: charge detection mass spectrometry

cDNA: complementary DNA

CE-Immunoassay: capillary electrophoresis immunoassay

CFR: Code of Federal Regulations

cIEF: capillary isoelectric focusing

CMC: Chemistry Manufacturing and Controls

CMO: contract manufacturing organization

CNS: central nervous system

CQA: critical quality attribute

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

Cryo-EM: cryogenic electron microscopy

CSF: cerebrospinal fluid

CTGTAC: Cellular, Tissue, and Gene Therapies Advisory Committee

CV: coefficient of variation

ddPCR: digital droplet polymerase chain reaction

DIP: defective-interfering particle

DNA: deoxyribonucleic acid

DP: drug product

dPCR: digital polymerase chain reaction

ELISA: enzyme-linked immunosorbent assay

EM: electron microscopy

EMA: European Medicines Agency

EU: European Union

FDA: United States Food and Drug Administration

GC: genome copy

HEK-293: human embryonic kidney 293 cells

HPLC-MS: high performance liquid chromatography- mass spectrometry

ICH: International Council for Harmonisation

IEX: ion exchange chromatography

IND: Investigational New Drug

ITR: inverted terminal repeat

LC-MS: liquid chromatography-mass spectrometry

MOA: mechanism of action

mRNA: messenger ribonucleic acid

NGS: next-generation sequencing

NIIMBL: National Institute for Innovation in Manufacturing Biopharmaceuticals

NIST: National Institute of Standards and Technology

PCR: polymerase chain reaction

PTM: post-translational modifications

qPCR: quantitative polymerase chain reaction

RMAT: Regenerative Medicine Advanced Therapy

RT-PCR: reverse transcription-polymerase chain reaction

SEC-MALS: size exclusion chromatography-multi-angle light scattering

SOP: standard operating procedure

SV40: simian virus 40

$TCID_{50}$: median tissue culture infectious dose

TEM: transmission electron microscopy

US: United States

USP: United States Pharmacopeia

vg: vector genome

VP: viral protein

ZUC: zonal ultracentrifugation

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