CELL & GENE THERAPY INSIGHTS

ANALYTICS

Fit-for-purpose analytical considerations to support the clinical readiness of engineered AAV vectors

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VIEWPOINT

"The development of optimized analytical workflows compatible with both natural serotypes and engineered capsids ensures robust characterization of AAV vectors across different programs."

Engineered AAV capsids are designed to improve tropism, enhance immune evasion, and increase manufacturability, yet these modifications can introduce unique analytical challenges that, if unaddressed, may limit the safe and efficacious use of engineered capsids in AAV gene therapies. Compared to natural AAV serotypes, engineered capsids may exhibit altered physicochemical properties, including reduced thermostability and changes in binding affinity to antibodies used for purification or detection. These differences necessitate the development of fit-for-purpose analytical methods to ensure accurate quantification and

characterization of key quality attributes for AAV gene therapies. Here, we outline which commonly used assays are expected to be robust across capsids and which may require adaptation, and propose a fit-for-purpose, orthogonal analytical strategy to reliably characterize engineered AAV vectors, a critical step towards their safe and efficacious use in gene therapy products.

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INTRODUCTION

AAV vectors are composed of a protein capsid that packages a single-stranded DNA genome; the mature AAV capsid consists of a total of 60 monomers of three distinct structural viral proteins (VPs) encoded by a given capsid's *cap* gene, known as VP1, VP2, and VP3, which assemble to form an icosahedral shell [1]. The amino-acid sequence of these proteins determines a capsid's structure and its physicochemical and biological properties, which in turn influence key gene delivery properties including receptor engagement, tropism, immunogenicity, and stability [2]. Differences in properties can be observed across AAV natural serotypes as a result of their distinct amino-acid sequences [3]. Engineered AAV capsids, which may incorporate modifications such as peptide insertions, site-specific mutations, chimeric sequences, or chemical conjugations [4], may differ sufficiently in their properties to necessitate capsid-specific, fit-for-purpose analytical method development. Establishing such methods is essential to ensure that engineered capsids can be appropriately characterized and supported for safe and efficacious use in gene therapy products.

ANALYTICAL CONSIDERATIONS FOR ENGINEERED AAV CAPSIDS

Table 1 presents a list of quality attributes that are commonly examined as part of the analytical release of AAV vector preparations [5], as well as examples of corresponding analytical assays. Each analytical method relies on specific physicochemical or biological properties intrinsic to each AAV vector. Certain properties are expected to vary only modestly across individual capsids (i.e., capsids that are not conjugated to other molecules); these include total capsid protein content, capsid molecular mass, vector particle density as a function of packaged genome, and hydrodynamic size. Consequently, corresponding analytical methods such as bicinchoninic acid assay, Bradford assay, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), mass photometry, cryogenic transmission electron microscopy, and dynamic light scattering are expected to require minimal (if any) refinements to become suitable for the analysis of natural AAV serotypes and engineered capsids alike, provided sample preparation conditions (e.g., buffer, surfactant, concentration) are appropriate for the capsid in question.

Other properties may vary more significantly when working with engineered AAV capsids. Capsid modifications can impact solution stability, leading to increased material losses while handling or diluting virus material when buffer conditions are suboptimal [6]. This underscores the importance of using a suitable virus formulation buffer that maintains an appropriate pH and contains suitable salts and surfactant as a diluent during all dilution and sample preparation stages for analytical testing.

Due to their modifications, engineered capsids can have lower thermostability compared to natural AAV serotypes or their parental capsid [7]. As a result, engineered capsids may be incompatible with

TABLE 1

Capsid-specific quality attributes and associated assays requiring fit-for-purpose method-suitability confirmation to support clinical readiness.

Attribute category	Quality attribute	Assay	Key analyte properties	Recommendation
Strength	Total protein concentration	BCA, Bradford, UV280	Presence of amino acid residues	No expected capsid-dependent issues; confirm suitability under routine conditions
Strength	Genomic titer (viral genomes per unit volume)	ddPCR, qPCR	Thermostability	Confirm suitability of genome extraction step; chemically inhibit nuclease prior to heating
Strength	Potency	Cell-based transduction assay	Transduction efficiency	Confirm transduction of cell line; adjust MOIs if needed
Strength	Capsid titer (capsids per unit volume)	Capsid ELISA	Protein sequence; binding affinity to ELISA ligand	Confirm binding to commercially available reagents; confirm suitability of available empty vectors as reference material
Strength	Infectious particle titer (TCID50)	Transduction of permissive cell line + genomic titer assay	Transduction efficiency; thermostability (genomic titer assay)	Confirm transduction of cell line; adjust MOIs if needed; follow recommendations for genomic titer assay
Safety	Presence of replication competent AAV	Transduction of permissive cell line + genomic titer assay	Transduction efficiency; thermostability (genomic titer assay)	Confirm transduction of cell line; adjust MOIs if needed; follow recommendations for genomic titer assay
Purity	General purity	SDS-PAGE	Presence of amino acid residues; protein size	No expected capsid-dependent issues; confirm suitability under routine conditions
Purity	Full/empty capsid ratio	Mass photometry	Particle molecular mass	No expected capsid-dependent issues; confirm suitability under routine conditions
Purity	Full/empty capsid ratio	Cryo-Transmission Electron Microscopy (cryoTEM)	Particle density	No expected capsid-dependent issues; confirm suitability under routine conditions
Purity	Full/empty capsid ratio	Genomic titer + capsid ELISA	Particle density	Follow recommendations for genomic titer assay and capsid ELISA assay
Purity	Aggregation	Dynamic light scattering	Hydrodynamic size distribution	No expected capsid-dependent issues; confirm suitability under routine conditions
Identity	Capsid protein identity	Western blot	Binding affinity to detection antibody	Confirm binding to commercially available antibody; confirm suitability of routine conditions for appropriate detection
Identity	Genomic identity	Sequencing	Thermostability (viral genome extraction step)	Confirm suitability of genome extraction step; chemically inhibit nuclease prior to heating
Identity	Genome integrity	Deep sequencing methods; CE-LIF	Thermostability (viral genome extraction step)	Confirm suitability of genome extraction step; chemically inhibit nuclease prior to heating

certain analytical method processing steps involving the heating of a vector sample above 37°C, as is done in viral genome extraction methods employed for genomic titer determination, genome sequencing, and genome integrity analysis. For example, some viral genome extraction methods in genomic titer assays heat-inactivate DNase I before capsid digestion/denaturing: they may proceed from a 37°C incubation step to a 65+°C enzyme inactivation step before continuing with capsid digestion/denaturing. It is critical that DNase I (or the alternate nuclease being employed) be fully inactivated before the sample is heated above 37 °C. One practical approach is to add sufficient ethylenediaminetetraacetic acid to fully chelate the divalent cations required for nuclease activity before the heating step. Otherwise, residual enzyme activity can digest prematurely released viral genomes during the heating step, leading to an under-counting of viral titer [6,7]. This may happen regardless of whether a proteinase-based method or a heat-based method is being used for viral genome release. Consequently, viral genome extraction steps may need to be adapted in assays for genomic titer, genome sequencing, and genome integrity.

Surface-exposed modifications on engineered capsids may reduce or ablate binding to antibodies and other affinity reagents; this can impact downstream processing (when using affinity resin for capture chromatography) as well as Western blot and capsid ELISA assays. Routinely used anti-AAV Western blot antibodies or capsid ELISA kits may not be compatible with engineered capsids despite working with their parental capsid [6], which may motivate the development of assays using alternative antibodies or affinity ligands with binding epitopes that are preserved in the engineered capsid, respectively.

Engineered capsids may exhibit altered transduction properties, which are often improved under conditions where their target receptor is sufficiently expressed, including cells cultured *in vitro*. This may enable more facile development of potency assays with lower multiplicities of infection compared to the parental capsid. Accordingly, cell-based potency assays, infectious particle titer assays, and replication-competent AAV assays may require MOI and readout adjustments for each engineered capsid relative to its parental serotype.

RECOMMENDATIONS

Confirming the suitability of analytical methods to characterize different AAV vectors is integral to the development of manufacturing processes and the advancement of gene therapy programs. Such analytical readiness is critical for identifying capsid-specific risks and ensuring the safe and efficacious use of engineered capsids in gene therapy products. When evaluating a new engineered capsid, existing qualified assays cannot be assumed to be directly transferable; instead, assessments are required to determine whether each method remains appropriate given the capsid's physicochemical properties. A structured method qualification extension process, guided by the specific features that differ from the parental or historical capsids, can be undertaken to confirm assay suitability. An effective first step is to assess how the engineered capsid differs in sequence, structure, and predicted physicochemical properties. These differences can then guide suitability assessments across analytical methods.

Generating a reference standard for the target AAV capsid can further support analytical development and the confirmation of the suitability of each individual analytical method. Such a standard can ideally be produced using manufacturing processes and qualified analytical assays that have been confirmed to be compatible with other engineered capsids that have been generated using similar modification

strategies. In the absence of such a standard, AAV vector preparations should be carefully analyzed by combining assays whose readouts may differ across capsids with orthogonal methods expected to behave consistently across capsids. For example, complementing genomic titer data with results from bicinchoninic acid assay, SDS-PAGE, and mass photometry assays can provide a more complete analysis of the virus material and facilitate comparisons to historical data from the manufacturing of other capsids.

CONCLUSIONS

The development of optimized analytical workflows compatible with both natural serotypes and engineered capsids ensures robust characterization of AAV vectors across different programs. This is important for therapeutic developers, academic vector cores, and CDMOs, which routinely produce a diversity of AAV capsids. Analytical methods traditionally used for characterizing natural AAV serotypes must be evaluated to confirm their suitability and applicability for analyzing engineered AAV capsids. Evaluating a new engineered capsid may therefore require reassessing analytical methods in the context of the capsid's specific physicochemical properties rather than assuming direct assay transferability. The capsid-specific quality attributes summarized in Table 1 can further support a structured, risk-based assessment of which assays warrant the most immediate investigation, consistent with Quality-by-Design principles [8]. Methods that rely on properties that vary minimally as a result of engineered capsids' modifications, such as SDS-PAGE or mass photometry, may often be employed across capsid types after verifying that sample preparation and assay conditions are appropriate for the specific capsid. Other methods, like genomic titer and capsid ELISA assays, may require refinements to address engineered capsids' physicochemical properties. These refinements enhance the reliability of AAV drug product characterization, supporting clinical translation and manufacturability.

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AUTHORSHIP & CONFLICT OF INTEREST

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