Beyond Empty and Full: Understanding Heterogeneity in rAAV Products and Impurities

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Abstract

Several recombinant adeno-associated virus (rAAV)-based gene therapies are now approved in the US and abroad, with many others soon to follow. Advancements in the field, including the development of novel and improved analytical techniques, have enabled an increased understanding of these complex biological products. As the field matures, so should characterization of rAAV products to ensure consistent quality, safety, and efficacy. Empty, full, and partial capsid ratios, and the inherent product heterogeneity that occurs in rAAV products is one area receiving increased attention. This document describes topics around heterogeneity and product-related impurities associated with rAAV products, and how these attributes can contribute to the product's overall quality profile. Additionally, this document summarizes several leading analytical techniques available to characterize heterogeneity and provides recommendations to sponsors for advanced rAAV characterization.

Heterogeneity: The quality or state of consisting of dissimilar or diverse elements (1).

Introduction

In recent years, several instances of treatment-emergent serious adverse events (TESAEs) have occurred following treatment with high dose, systemic rAAV products administered *in vivo*, with some resulting in patient fatalities (2). This led to an FDA Cellular, Tissue, and Gene Therapies Advisory Committee Meeting held in September 2021 to discuss Toxicity Risks of Adeno-associated Virus (AAV) Vectors for Gene Therapy (3). No single root cause could be attributed across all reported TESAEs, suggesting a multi-faceted approach is likely needed to reduce the risk of immunogenicity. These observations have highlighted the need for industry-wide advancements in product and manufacturing understanding, such as defining additional quality attributes which may be associated with rAAV TESAEs. Control of additional product attributes may help mitigate certain product risks in future studies resulting in improved rAAV safety and efficacy profiles. Notably, the presence of high levels of empty

capsids was identified as a potential contributor to TESAEs across rAAV studies since they contribute to the total capsid dose without providing a direct therapeutic benefit. Empty capsids, however, are just one type of product-related impurity that could contribute to rAAV associated toxicity.

A key factor for consideration as the field advances its understanding of rAAV safety and efficacy in clinical studies is the complexity of both the impurities within the product, and the product itself. Put simply, rAAV Drug Products are complex mixtures of DNA, proteins, and chemicals that each play an important role in the safety and efficacy of the final product. As a complex biological product, both the product and product-related impurities are expected to have an innate level of heterogeneity. As such, it is critical that sponsors clearly define the degree of heterogeneity in both the product and product-related impurities throughout development. Sponsors should clearly define and justify attributes categorized as product heterogeneity versus product-related impurities. We refer to product heterogeneity as molecular variants of the desired product that can have properties comparable to those of the desired product with respect to activity, efficacy, and safety. Product-related impurities include molecular variants with properties that are not comparable with those of the desired product formed during manufacturing and/or storage.

To date, there has been limited regulatory guidance specific to the control of heterogeneity in rAAV products. One guidance document that describes general principles for setting specifications for biological products, ICH Q6B, states “it is expected that from biosynthetic processes, an inherent degree of structural heterogeneity will occur and may impact the activity, efficacy, and safety of the Drug Product” (4). Structural heterogeneity in rAAV products can include variants of different vector genome lengths and composition relative to the designed expression cassette, or variants in capsid protein integrity, stoichiometry, or modification. Characterization of product heterogeneity should be performed to improve product understanding and identify potential product design or manufacturing controls to maximize product variants that yield the intended product. Furthermore, characterization of product heterogeneity pre- and post-manufacturing changes can be used to detect possible shifts in a product profile.

Heterogeneity in product-related impurities also exist in rAAV products. Identifying and eliminating product-related impurities in products with inherent heterogeneity can be challenging in some instances due to the technological challenges of detecting small quantities of impurities and/or due to absence of specific test methods.

This document describes how both rAAV product heterogeneity and product-related impurities can contribute to overall rAAV product quality. Also discussed are current best practices in analytical techniques to characterize and evaluate rAAV heterogeneity and product-related impurities. We recommend that the sources of heterogeneity described in this document, and defined in **Appendix I**, be increasingly monitored by sponsors throughout product development as the industry gains deeper understanding and greater ability to reproducibly quantify the impact of rAAV product and impurity heterogeneity on clinical safety and efficacy.

While it is inconclusive which source(s) of product hetereogeneity pose the greatest risk to rAAV product quality including safety (e.g., immunogenicity), it is likely that multiple sources could contribute to the overall product quality. Therefore, we recommend characterizing and controlling potential sources of rAAV heterogeneity in product and product-related impurities, which are categorized in this document as follows:

- *Heterogeneity in encapsidated rAAV DNA sequence lengths*
- *Heterogeneity in rAAV DNA sequence identity*
- *Heterogeneity in rAAV capsid protein integrity, stoichiometry, and post-translational modifications (PTMs)*

Background

rAAV Gene Therapy Landscape

Gene therapies have proven to be a promising approach to alleviate symptoms, halt disease progression, or even potentially cure a patient of disease in which limited treatment options exist. rAAV is a common viral vector modality used for *in vivo* gene therapy applications due to its persistent expression profile in non-dividing cells and its ability to target a broad range of cell and tissue types based on the capsid variant (i.e., serotype) used (5). As of December 2022, there are three US Food and Drug Administration (FDA) approved rAAV products; Luxturna, for treatment of patients with biallelic RPE65 mutation-associated retinal dystrophy, Zolgensma, for treatment of spinal muscular atrophy (SMA), and Hemgenix, for the treatment of adults with hemophilia B. In Europe, the European Medicines Agency (EMA) has granted approval for 5 rAAV gene therapies including Luxturna and Zolgensma, as well as Upstaza, Roctavian (conditional marketing authorization (6)), and Glybera (conditional marketing authorization, removed from market by sponsor). The number of approved rAAV products is expected to rise given the increasing number of ongoing clinical studies using rAAV. For example, the number of rAAV clinical studies in the US rose from 5 in 2010 to 26 studies in 2017 (7). As of June 2022, there were 255 US clinical studies using rAAV targeting a range of predominantly genetic disorders and infectious disease indications (8).

The recent increase in rAAV gene therapy clinical studies has led to a similar increase in cGMP manufacturing demand for rAAV. Demand is driven not only by the overall increasing number of preclinical and ongoing clinical studies, but also by the need to generate larger quantities of vector for systemic administration and/or treating diseases with large patient populations. To meet demand, sponsors and Contract Development and Manufacturing Organizations (CDMOs) alike have been increasing manufacturing capacity, as demonstrated by the nearly $8 billion in viral-vector manufacturing deals since 2019 (9). As the rAAV field matures and gains deeper experience and understanding of rAAV manufacturing, it is crucial that the efficiency, quality, and scale of manufacture is improved to meet the current and future clinical and commercial demands of the industry. Critical to the success of these efforts is the need for consistent, robust, and efficient manufacturing processes to increase throughput, and analytical techniques to ensure product consistency across the industry.

A major challenge to the rapid advancement of rAAV manufacturing and controls is the lack of standardized manufacturing processes and analytical methods. Standardization of rAAV manufacturing processes and controls has not been achieved yet for several reasons. First, multiple, distinct production and purification platforms exist, each having their own advantages and disadvantages. Second, the desired product quality attributes for an rAAV product may differ based on the capsid variant, payload, route of administration, and clinical indication. Two leading production systems are typically used for rAAV manufacturing efforts: the human-derived HEK293 cell line and the insect-derived Sf9 cell line. These two production systems result in a distinct impurity profile due to differences in the host-cell species and production-related factors utilized. As a result, each platform may require different analytical test methods. Differences in

purification methods can further lead to divergence in product quality and impurity profiles across platforms. Of note, both Sf9 and HEK293 rAAV production approaches have been used for manufacture of licensed rAAV products in the US and EU, demonstrating that a favorable safety and efficacy profile can be achieved from multiple platforms (8). Therefore, different approaches to rAAV manufacturing may be used so long as associated risks are addressed, and appropriate safety and efficacy profiles are obtained for the indication of interest.

rAAV Heterogeneity Quality and Regulatory Considerations

While there are challenges to achieving standardization of rAAV manufacturing and controls, several relevant guidance documents have been published by regulatory agencies such as the FDA and EMA, describing recommendations and requirements for Biological Products that can be applied to rAAV products where appropriate. Additionally, several guidance documents specific to rAAV products have also been published by regulatory agencies. Harmonized documents are also published through various international organizations such as the International Pharmaceutical Regulators Programme (IPRP) and the International Council for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). While each agency may have its own specific requirements, FDA and EMA both adopt common guidelines described in ICH documents. Common themes are also prevalent across agencies, such as the recommendation to implement a systematic approach that follows quality-by-design (QbD) principles, where possible. A QbD approach can help gene therapy sponsors understand the design space, critical process parameters, and control strategies that can be used to conduct complete and successful gene therapy product development.

To ensure that the final product consistently achieves appropriate product quality, Critical Quality Attributes (CQAs) should be identified early in product development (4). As product understanding and manufacturing experience increases and CQAs are established, appropriate acceptance criteria should be set to assure product quality and consistency (10). Additional characterization methodologies may also be useful to gain deeper understanding throughout the product development lifecycle to support process characterization, manufacturing changes, and process improvements.

ICH Q6B describes details for setting specifications for biological products and includes recommendations on characterizing and defining product heterogeneity; “since the heterogeneity of these products defines their quality, the degree and profile of this heterogeneity should be characterized to assure lot-to-lot consistency.” In the context of rAAV products, sources of heterogeneity should be investigated and controlled if they are found to contribute to product quality and consistency. Additionally, ICH Q6B defines the use and implementation of novel analytical techniques that can improve the understanding of product quality. To align with these guidelines, new analytical methods that can improve the understanding of rAAV heterogeneity and other quality attributes should be implemented and utilized as appropriate.

Classification of rAAV Product-related Impurities and Product Heterogeneity

During the FDA Advisory Committee meeting in 2021 (11), it was clear that additional guidance for sponsors is needed to improve the field’s understanding of rAAV-related immunogenicity. As a response to the conclusions of this meeting, DHC published a proposed draft guidance for FDA consideration (submitted to FDA 15May2022), which included a list of known rAAV vector impurities and their

classifications (12). The document focused on empty/full rAAV characterization, and as additional questions arose surrounding partial capsids, non-functional rAAV, and heterogeneity in rAAV products, a deeper discussion into these topics was needed. This document describes these categories of rAAV attributes in detail and summarizes common methodologies for sponsors to assess heterogeneity in rAAV products and impurities.

Product heterogeneity here refers to molecular variants of rAAV which can be considered 1) part of the product (i.e., while not the intended molecular form of the vector, nevertheless can contribute to gene transfer and therapeutic efficacy), or 2) vector-related impurities that do not contribute to efficacy. **Table 1** describes these attributes within rAAV preparations that may contribute to heterogeneity.

Table 1. Proposed classifications of rAAV attributes related to heterogeneity.

Identified rAAV Vector Attributes	**Classification**
Empty capsids	Product-related Impurity
Capsid protein integrity (e.g., degraded capsids)	Product-related Impurity
Non-functional rAAV	Product-related Impurity
Aggregated rAAV	Product-related Impurity
Replication-competent rAAV	Product-related Impurity
Encapsidated non-payload DNA (e.g., host-cell, plasmid, helper virus DNA, etc.)	Product-related Impurity
Non-encapsidated residual DNA	Process-related Impurity
Residual host-cell protein	Process-related Impurity
Residual manufacturing reagents (e.g., nuclease, antifoam, etc.)	Process-related Impurity
Residual helper components (e.g., baculovirus, adenovirus, herpesvirus, or plasmid)	Process-related Impurity
Encapsidated partial payload sequences*	Product Heterogeneity
Encapsidated hypomethylated CpG DNA*	Product Heterogeneity
Encapsidated payload sequence mutations*	Product Heterogeneity
Capsid protein stoichiometry*	Product Heterogeneity
Capsid post-translational modifications (e.g., glycosylation, methylation, etc.) *	Product Heterogeneity

**Generally considered a source of Product Heterogeneity if the particular variant has the potential to have properties comparable to the desired product. However, in some instances these molecular variants can have a negative impact to product quality and would then be considered a product-related impurity.*

While the profile of product heterogeneity in rAAV products has the potential to impact safety and efficacy, there are notable examples in which a heterogeneous product results in a safe and efficacious product. Roctavian, for example, utilizes an expression cassette that exceeds the maximum packaging capacity of the AAV5 serotype (13). During analysis of patient liver biopsy samples, it was concluded that "30-40% of those containing the central portion of the transgene were full length or ITR fused (13). This indicates that some circular episomes were incomplete, missing either the 5' or 3' ITR, and suggests that adjacent promotor or polyA regions might also be truncated…" (13). In this example, these truncated species can be considered attributes of *Product Heterogeneity*, as it has been concluded through successful clinical studies that the safety and efficacy profile of this product is appropriate to support marketing authorization, despite the high percentage of truncated species.

To align with ICH guidelines, the degree of rAAV product heterogeneity should be characterized. However, the above example demonstrates that a heterogeneous product profile can yield a product with an overall favorable safety and efficacy profile in certain scenarios. While this may be true in the above circumstance, each product should be characterized individually, as additional factors (e.g., total capsid dose, route of administration, etc.) could impact the overall effect of product heterogeneity. Sponsors should consider the combined effect of multiple factors when assessing the impact of product heterogeneity. As rAAV is a complex biological product, sources of heterogeneity can arise from multiple components within the product, as outlined in subsequent sections below.

Heterogeneity in Encapsidated rAAV DNA Sequence Lengths

Historically, rAAV capsid content has been defined as "empty" or "full" and analyzed by biophysical characterization methods such as AUC, CDMS, cryo-TEM, etc. In reality, rAAV preparations may contain capsids with a broad range of sequence lengths that are less than, equal to, or greater than the intended rAAV product payload sequence. To improve the consistency of the final product, a major goal in rAAV process development is to identify parameters which consistently enrich for the full, desired payload length, while removing rAAV capsids that are empty or do not contain the correct sequence length. A table of proposed classifications and definitions of the various encapsidated DNA sequence lengths is provided in **Table 2**.

It should also be considered that while characterization methods may be able to determine the relative ratios of Empty/Partial/Full/Oversized, these methods are not capable of simultaneously determining the unique identity of encapsidated sequences. Therefore, characterization of encapsidated sequence lengths should be paired with appropriate methods for analyzing encapsidated sequence identity, as described in subsequent sections.

The ability to distinguish empty, partial, full, and oversized capsids will depend on the analytical method(s) used and the rAAV payload design. For example, if a sponsor designs a 2.0 kilobase (kb) payload, then the analytics should reflect the 2.0 kb payload as a "full" capsid rather than that of the maximum payload capacity (4.7 kb). Alternatively, for a product utilizing a 4.7 kb payload, a 2.0 kb band may be considered product-related impurity or product heterogeneity and should be characterized and controlled. Additionally, the analytical method chosen, and input parameters selected for a given method, may impact how species within a product are categorized as empty, partial, or full, which may not be consistent across manufacturers and testing sites. For example, analytical ultracentrifugation relies on user-defined sedimentation coefficients to define

empty and full capsids and thus is susceptible to site-to-site variability. In addition, several techniques may require post-processing analysis to generate a result. Regardless of the analytical method used, it is critical to define the parameters chosen and demonstrate that the assay is suitable for its intended purpose and appropriate for the stage of product development.

Empty Capsids

Empty capsids containing no encapsidated DNA are regarded as a product-related impurity and should be controlled and removed to the greatest extent possible. DHC previously proposed recommendations to ensure that the total amount of empty capsids administered is < 30% of total capsids unless otherwise justified. This value should also be assessed in the context of route of administration and total capsid dose, as high dose, systemic indications typically result in greater total capsid dose level exposure as compared to localized, direct-injected products.

Table 2. Classifications of encapsidated rAAV sequence lengths

AAV Sequence Length Classification	Encapsidated DNA Length	Description
Empty	rAAV capsid with no encapsidated DNA	Contributes to the total capsid dosed to patients without providing a direct therapeutic impact in absence of payload sequence. Poses a safety risk
Partial	Encapsidated DNA is smaller than the full expression cassette length	Partial capsids that contain truncated versions of the intended payload may anneal and recombine post-transduction to generate an intact and functional expression cassette. Inefficiency may require higher dose and associated risk.
Full	Encapsidated DNA corresponds to one copy of the full expression cassette	Capsids containing DNA corresponding to the designed expression cassette length and contribute towards the intended mechanism of action
Oversized	Encapsidated DNA is larger than one copy of the expression cassette	If payloads have been designed smaller than the maximum packaging capacity of rAAV capsids, additional DNA may be packaged, which can provide more than one expression cassette per capsid

While a statistical analysis is challenging, a correlation has emerged between the total viral capsid dose and safety profiles in clinical programs, consistent with standard immunology concepts suggest that a greater viral load will result in a greater immune response. As such, the presence of empty capsids contributes to the total viral capsid load delivered in a rAAV drug product dose and could have an impact on product safety and efficacy.

Due to limitations in bioseparation techniques and analytical method sensitivity, capsid particles containing small fragments of DNA may be classified as "empty" (14). In reality they can contain ITR-resembling sequences which impact the total amount of CpG content delivered, since ITRs are naturally high in CpG content. An immune response can be triggered by activation of monocytes and neutrophils reacting to pathogen-associated molecular patterns (PAMPs) such as CpGs. These findings suggest that the empty capsid content in final rAAV preparations could impact the total CpG content delivered in a patient dose which may play a role in the PAMP-initiated TLR9 immunogenicity pathway (15), potentially impacting safety and efficacy of the rAAV products with different ratios of empty and full particles.

DHC previously proposed recommendations to ensure that the total number of empty capsids administered is <30% unless otherwise justified. Methodologies for this analysis should be scientifically sound and can be based on techniques including but not limited to Analytical Ultracentrifugation (AUC) or Charge Detection Mass Spectrometry (CDMS) which provide accurate and precise measurements of capsid content in purified samples (16). Several common analytical methods for quantifying the percentage of empty particles are listed in our proposed draft guidance for FDA consideration and involve biophysical characterization using size, shape, and/or charge of capsids.

The methodology used for quantification of empty capsids should be clearly defined and consistently applied throughout the development lifecycle. Early in product development, several methods to assesses percentage of empty capsids may be explored. At the time of licensure, a validated release assay along with justified acceptance criterion should be used for rAAV product release. Quantification of empty capsids should be utilized to inform a specification for total capsid dose relative to genome-containing particles.

Capsids Containing Partial Length Expression Cassettes

Partially filled capsids are defined as capsids containing a portion of the expression cassette sequence length that is less than one complete expression cassette – the full desired payload length. As such, a wide range of sequence lengths may be considered "partials".

Current empty/full quantification techniques generally utilize physiochemical characteristics to distinguish between empty and full particles, with a pre-defined threshold for each bin of "empty", "partial", "full", or "oversized". Sponsors should justify the analytical method and the thresholds used to define full capsids from empty, partial, or oversized.

As wildtype (*wt)* AAV for serotypes 1-5 contains an expression cassette of 4.7kb in length including ITRs (17), rAAV packaging machinery tends to preferentially package sequence lengths similar to *wt* sequences (18). Payloads should be designed to align closely to the *wt* sequence length to avoid a reduction in productivity and product yield and increases in packaged impurities. Genomes slightly larger than the *wt* 4.7kb can still be packaged, but at much lower efficiencies and with the risk of packaging more truncated species (17).

Not all analytical methods are sensitive enough to resolve partial genome capsids, resulting in their categorization as either an

empty or full capsid instead. Furthermore, partials become more challenging to resolve from empty and full capsids as the payload sequence length of a product is reduced. This is because the differences in a physiochemical property such as the density of an empty versus a full capsid becomes smaller. Because of the differences in partial capsid quantification across analytical methods and rAAV payload design, it is challenging to standardize the acceptance criterion for percentage of partial capsids. Therefore, we recommend that rAAV products be characterized using a combination of biophysical and sequence-based methodologies and that the acceptance criterion for partial capsids be initially based on historical batches, including development runs, pre-clinical toxicology batches, and refined through clinical manufacturing experience. Additionally, the percentage of empty and partially filled capsids should be evaluated to support manufacturing changes.

Capsids Containing Oversized Lengths of DNA

Oversized capsids (i.e., capsids containing a greater amount of DNA than the intended payload size) should be characterized and minimized in the final drug product. Several payload design strategies can be employed to reduce the risk of oversized species being present. Strategies include designing the payload to align closely to the *wt*AAV genome sequence length (4.7 kb) or in the case of small payloads less than half the length of *wt*AAV (<2.4 kb), implementation of a self-complementary genome configuration. Alternatively, non-coding sequences can be added as "stuffer" sequences to reduce the risk of packaging foreign DNA or chimeric species. Stuffer sequences should be selected carefully to not impact product safety or efficacy. Use of these sequences should also be justified.

Analytical Techniques for Characterization of Heterogeneity in Packaged Sequence Lengths

Several analytical techniques exist for characterization of empty/full ratios, but only a subset of these methods reliably distinguishes partial capsids from empty and full capsids. For example, AUC can report the proportion of intermediates (i.e., partial genome capsids), but quantification using ddPCR/SEC-MALS ratio can only report the percentage of genome-containing particles. A table summarizing the advantages and disadvantages of several analytical techniques is provided in the proposed draft guidance for FDA consideration (12).

Additionally, manual steps and user-defined conditions may impact the consistency of the results. For example, AUC relies on user-defined sedimentation coefficient ranges to quantify the ratio of empty, partial, and full capsids. These user-defined parameters may vary between test sites, operators, and products, and can result in different results for comparable products. Regardless of the method, it is critical to define the method parameters and demonstrate that the assay is suitable for its intended purpose before implementing it in routine testing.

Finally, many empty/full methods analyze the sample based on the assumption that the sample is homogeneous and report the test result as a single value (e.g., 70% full). As it is expected that there will be an inherent degree of heterogeneity in the packaged sequence lengths, methods that quantify individual capsids and/or describe the distribution of sequence lengths are necessary for determining packaged sequence length heterogeneity.

Heterogeneity in Encapsidated DNA Sequence Identity

While encapsidated sequence length quantification should be utilized to assess the rAAV product purity, it is also important to understand the identity of the encapsidated

sequences. Full capsids, containing encapsidated DNA sequences of the appropriate length, may be composed of impurity sequences, or the desired payload with mutations or hypomethylation patterns that impact the expression of the therapeutic protein.

ITR integrity and identity testing by Sanger sequencing are commonly performed as an rAAV release specification criterion (19). However, this method cannot detect hypomethylation sites and is not sensitive enough to detect minority-fractions of mutated, truncated, chimeric, or non-payload sequences, allowing the opportunity for large fractions of heterogeneous populations to go undetected.

Table 3 identifies the common sources of rAAV packaged sequence impurities that are often present in rAAV products.

Table 3. Identity of commonly packaged DNA sequences during rAAV production

Sequence Identity	**Classification**	**Description**	**Potential Impact to Product Safety and Efficacy**
Non-payload sequences	Product-related Impurity	Payload sequence is not present in a portion of the encapsidated DNA population. Generally, non-payload DNAs are composed of impurity sequences such as host-cell DNA, helper sequences, or plasmid backbone sequences. May fall into the range of partial, full, or oversized	Risk to product safety if not limited to a small fraction of total packaged DNA content. Minimal expected impact to product efficacy
Payload sequences (includes partials)	Product Heterogeneity*	The expression cassette is truncated. May be detected as a partial or full capsid, Truncated species typically fall in the size range of partials	Risk to product safety if truncated species exacerbates disease progression. Potential risk to product efficacy if truncations impact transgene function. Decreased efficiency and potential requirement of higher doses to generate a full-length expression cassette post-transduction from partial genomes (20; 21)
Hypo- or hyper-methylated CpG dinucleotides in expression cassette	Product Heterogeneity*	The expression cassette is hypo-methylated on CpG dinucleotides, providing pathogen associated molecular patterns (PAMPs). Methylation sensitive promoters are hypermethylated and therefore attenuated in strength	Risk to product safety and efficacy due to activation of innate immune responses via TLR9 and amplification of adaptive immune responses. Higher doses of vector required, reducing product potency, and increasing immunogenicity risks

Sequence Identity	Classification	Description	Potential Impact to Product Safety and Efficacy
Mutated expression cassette sequences	Product Heterogeneity*	The desired payload is mutated (nucleotide insertion, deletion, or substitution) which can repress transcription of a gene or result in a non-functional payload. Likely detected as a full capsid if insertion/ deletion is small.	Risk to product safety if mutations exacerbate disease progression or if mutations in ORF of the expression cassette trigger a host immune response to the therapeutic payload product. Risk to product efficacy if mutations impact expression or function.

**Generally considered a source of Product Heterogeneity if the particular variant has the potential to have properties comparable to the desired product. However, in some instances these molecular variants can have a negative impact to product quality and would then be considered a product-related impurity.*

Non-Payload Sequences

During production and packaging, sequences contained within the ITRs are packaged into the rAAV capsid. However, non-payload genomes can also be packaged. The identity of packaged DNA may be derived from any source of residual DNA used throughout the process prior to packaging. These sequences may be inherently immunogenic due to their often hypo-methylated status. Regardless, any residual impurities should be identified and controlled (22). Currently, Next-Generation Sequencing (NGS) is an effective analytical methodology for identifying non-payload genomes packaged in rAAV capsids. Common examples of known non-payload genomes include those in **Table 4** but may vary depending on the process and history of materials used during production. Non-payload sequences should be identified and controlled using a risk-based assessment.

Table 4. Examples of known non-payload sequence sources.

Non-Payload Sequence Identity	Description	Potential Impact to Product Safety and Efficacy
Antibiotic resistance gene	Sequences typically present in the plasmid backbone for plasmid production	Antibiotic resistance proteins could result in antibiotic resistance in patients
Host-cell DNA (non-oncogenic)	Host-cell DNA comprised of regions not known to pose an oncogenicity risk to humans	Low likelihood of immunogenicity to human host-cell DNA sequences. Immunogenicity risk is greater from non-mammalian host-cell DNA
Host-cell DNA (oncogenic regions)	Host-cell DNA comprised of regions known to pose an increased oncogenicity risk to humans	Safety risk of oncogenesis in patients

Non-Payload Sequence Identity	Description	Potential Impact to Product Safety and Efficacy
Plasmid backbone(s)	Sequences from plasmid backbones used in transient transfection, not encoding full-length genes	Bacterial sequences generally have increased unmethylated CpG content and may pose greater risk of immunogenicity
Helper virus or shuttle vector DNA (not impacting rAAV replication)	Residual DNA from helper viruses or vectors used during manufacture of rAAV such as Baculovirus, Herpes Simplex Virus (HSV), and Adenovirus (Ad), not mapping to any known oncogenes or regions impacting AAV replication	Viral DNA may pose immunogenicity risks
Helper sequence DNA (with potential to impact rAAV replication or integration)	Residual DNA from helper viruses or vectors used during rAAV manufacture, mapping to regions involved in AAV replication or integration (e.g., E1A, E1B, E3, etc.)	Increases risk of replication competent AAV
Rep/cap sequences	Rep/cap sequences used during manufacture of rAAV.	Increases risk of replication competent AAV

Encapsidated Truncated Payload Sequences

Packaged DNA can be truncated during production, potentially packaging DNA containing disrupted promotors or transgene sequences. The impact of these truncations to product safety depends on the location of the truncation and the impact to expression of the therapeutic protein. Truncations in the transgene can cause therapeutic protein misfolds, potentially resulting in either non-functional or immunogenic forms of the protein. In rare cases, the formation of dominant-negative species could occur.

Alternatively, it is possible that truncated species have no negative net effect on the safety and quality of the final product, as overlapping truncated species may recombine to form a full-length product post-transduction (20). Dual vector payload delivery is a strategy implemented where recombination of two genomes is desired to achieve the full-length payload that otherwise exceeds the capacity of a single payload. As such, payload truncations may not necessarily pose a safety or efficacy risk but should be assessed in the context of pre-clinical and/or clinical experience.

Methylation of CpG Dinucleotides

Sequences that contain an accurate payload sequence can be hypomethylated or hypermethylated which may impact gene expression and rAAV potency post-transduction depending on where the mis-methylation occurs. Methylation occurs primarily at CpG sites (23) where cytosine bases can be methylated to form 5-methylcytosine. When located in promotor regions this could result in downregulation of the associated genes; however, when located in

intergenic regions it can promote enhanced expression of that gene.

In contrast to concerns of promoter inactivation by CpG methylation, expression cassettes of rAAV vectors have been shown to be markedly hypomethylated at CpG dinucleotides, an epigenetic feature that appears to be independent of vector generation / production method. Hypomethylated DNA is an epigenetic feature of microbial DNA and provides innate pathogen-associated molecular patterns (PAMPs) that activate the Toll-like receptor (TLR)9-MyD88 signaling pathway (24). This pathway has been implicated in CTL responses to AAV vectors in non-clinical models (25) and shown to correlate with increased immunotoxicity and reduced efficacy in clinical studies (26) (27). The methylation state of packaged payloads, including CpG sequences could provide insight to rAAV product quality, safety, and efficacy, but publicly available data on CpG content for clinical and commercial rAAV products is limited, making it challenging to compare across multiple serotypes, indications, and production methods.

Chimeric Sequences

Analysis by single molecule, real-time (SMRT) sequencing often reveals that packaged sequences may match a combination of multiple sources, (e.g., transgene, host cell DNA, backbone, etc.). These chimeric sequences may form due to homologous recombination during production and can result in packaged sequences spanning a variety of sequence lengths. In addition to chimeras with non-payload DNA, payloads can also form self-chimeras, resulting in more than one payload being packaged into the capsid if the designed payload is small. It is unknown if packaging multiple payloads into a single capsid has an impact on product safety and efficacy, but this probability should be assessed for each product. Depending on the length of chimeric sequences, capsids containing chimeric sequences will generally fall under the categories of either partial, full, or oversized.

While the presence of chimeric sequences may not negatively impact product quality, the impact should be assessed and compared to lots that have provided demonstrations of safety and efficacy. The presence and quantity of chimeric sequences should also be characterized as part of a comparability assessment following a manufacturing process change.

Mutated Payload Sequences

Payload sequence mutations are common in rAAV preparations and can include Single Nucleotide Variants/Polymorphisms (SNV/SNPs) and Sequence Mutations spanning multiple base pairs (e.g., insertions, deletions, substitutions, etc.).

It is important to understand the impact of mutations in the payload and determine whether these mutated sequences contribute to the total vg titer based on the PCR primer-robe set used. Furthermore, Sanger sequencing methods do not detect mutations unless present in most of the population. Small percentages of polymorphic mutations should be analyzed using sufficiently sensitive methods, as described in subsequent sections. For example, if it is discovered that a large portion of the rAAV product contains a mutation within the transgene, the population of rAAV particles carrying this mutation may not result in a therapeutic benefit upon delivery.

Payload mutations, if they arise, should be compared against the designed payload and any impact to product quality and rAAV function should be assessed.

Analytical Methods for Identifying and/or Quantifying Encapsidated Sequence Impurities

As encapsidated impurity sequences can be composed of a variety of known and unknown sequences, targeted PCR-based methodologies

may not detect all the impurities present. Additionally, electrophoresis-based methodologies may provide an insight into sequence length and distribution, but do not allow for exact sequence identity. Therefore, an approach that implements sequence-agnostic identification and/or quantification of packaged sequences has become the preferred method for identification of impurity sequences.

Novel NGS methods have been developed which enable complete analysis of packaged sequences in rAAV. Common technologies can be grouped into categories of Short-Read NGS and Long-Read NGS, although each manufacturer and technology will have differences that are more nuanced than the two categories. Both technologies have proven to be useful in the analysis of packaged sequences, and general advantages and disadvantages pertaining to analysis of rAAV packaged DNA are described in **Table 5**.

Table 5. Summary of short-read and long-read NGS technologies.

	Short-Read NGS	**Long-Read NGS**
Method Advantages	• High number of reads and low error rates allows for deep coverage of sequences and a lower threshold of detection for minor variants and impurities present • Greater accuracy for analysis of low-representative molecules • Large numbers of samples can be multiplexed via barcoding, reducing the per-sample cost significantly • Minimal length-bias results in a greater potential for accurate quantification • Ability to analyze methylation	• Long read length allows for single-read coverage across the entire packaged genome length (~5kb) reducing the complexity of bioinformatic analysis, and allowing for the identification of DNA truncation sites, chimeras, and other rearrangements • Ability to generate large number of reads allowing for deep coverage and identification of minor sequence variants • Ability to analyze methylation
Method Disadvantages	• Short reads do not allow for coverage of entire genome length (<800bp per read) from a single read and instead relies on post-processing bioinformatics for assembly • Poor sequencing efficiency in areas of high secondary structure (e.g., ITRs) • Computationally challenging to assemble and analyze • Inability to directionally phase repeat sequences (e.g., ITRs)	• Lower throughput and higher error rate • Read bias towards shorter reads reduces quantification accuracy for reads of varying lengths
Recommended Use Cases	• Allows for the identification and accurate quantification of single nucleotide polymorphisms (SNPs) and short indels • Quantification of non-payload DNA sequences (e.g., plasmid, host-cell, contaminant sequences)	• Identification of truncation hotspots, chimeras, overpackaging, and large rearrangements • Identification of non-payload DNA sequences (e.g., plasmid, host-cell, and other impurity sequences)

We recommend characterization of rAAV products using the appropriate NGS method(s) to detect and quantify the following characteristics:

- Payload sequence truncation hotspots
- Packaged impurity sequence profiles (identity, size distributions, and relative abundance)
- Profile of payload sequence lengths and integrity
- ITR rearrangements and integrity
- CpG methylation patterns

Challenges for NGS Implementation

Current NGS methods face challenges with respect to assay qualification and validation per ICH Q2(R2) (28) due to the variability and subjectivity encountered in sample preparation and sequencing procedures, as well as the lack of consistent consensus bioinformatics analysis methods and associated data interpretation. Therefore, it may not be feasible to set quantitative limits on payload sequence mutations as a validated release assay. Currently, the major utility of NGS resides in its use as a characterization approach to confirm the presence, length, and identity of packaged sequences. However, as NGS methodologies improve, assay validation for rAAV sequencing may become more common. This in turn could allow for implementation of NGS-based sequencing as a release assay for setting limits on mutated or methylated payload sequences or non-payload sequences in the future.

Heterogeneity in rAAV capsid protein integrity, stoichiometry, and PTMs

rAAV Capsid Protein Integrity and Stoichiometry

Additional sources of rAAV product heterogeneity can be attributed to variance in the integrity of capsid proteins and protein ratios. An impact to product safety and efficacy could arise due to this variability, as the ability of rAAV to target and transduce specific cell and tissue types is largely dictated by receptor binding sites found on the outer capsid structure (12). Cellular attachment occurs most often through binding a primary receptor and then subsequent endocytotic entry prompted by binding a coreceptor (12). Differential recognition of cell surface glycan or tissue-specific protein or lipid coreceptors are believed to dictate differences observed in cellular tropism, transduction efficiency, and antigenicity between capsid variants (29). Given the role capsid structure plays in delivery and tropism, it is important to characterize products for capsid integrity and stoichiometry, and any potential impacts to product infectivity, potency, and immunogenicity compared to batches demonstrated to be safe and effective.

AAV capsids are comprised of a 60-mer protein structure containing viral protein 1 (VP1), viral protein 2 (VP2), and viral protein 3 (VP3). Each of these viral proteins are encoded by a single Cap gene. The entire VP3 sequence, termed the VP3 common region, is shared across the VP1 and VP2 sequences. The VP2 sequence contains the VP3 common region and an additional ~57 amino acids. The additional ~57 amino acid sequence relative to VP3 is termed the VP1/VP2 common region. The VP1 sequence is comprised of the VP3 common region, VP1/VP2 common region, and an additional 137 amino acids which is termed VP1u. The VP3 common region forms the receptor binding sites on the capsid structure, VP1/VP2 common region contain nuclear localization sequences, and the VP1u region contains a phospholipase A2 enzyme. The presence of each of these viral proteins is important for successful endosomal trafficking and nuclear entry for genome release.

The assembly of *wt*AAV is generally recognized as a VP1:VP2:VP3 ratio of 1:1:10. The ratio of VP1:VP2:VP3 of rAAV, however, can vary based on the production platform. A given product may also contain a heterogenous population of capsids at different ratios (30).

Furthermore, the conditions for purification, formulation, and storage of the product can impact capsid integrity. Products should be characterized to confirm the presence of all VP proteins, to understand the heterogeneity in VP ratios, and assess any degradation of VP proteins that may occur throughout production or storage. The outputs of this analysis can provide a deeper understanding of the product quality attributes and how changes in a manufacturing process may impact capsid structure and integrity.

Capsid Post-Translational Modifications (PTMs)

The structural proteins that make up the rAAV capsid can undergo PTMs at various stages during viral production in the host cell (31). PTM is a broad term to describe covalent modifications of a protein which can change its properties. Changes can include the addition of functional groups, proteolytic cleavage of regulatory subunits, or degradation of the protein. Factors such as storage conditions, buffer composition during purification, and final formulation, can impact the stability of these modifications over time.

A statistically significant correlation between capsid PTM profiles and product efficacy or safety has not been established. However, PTMs of proteins in general are known to affect the biological properties of expressed proteins *in viv*o. As such, the FDA recommends that recombinant therapeutic protein biotechnology products analyze PTMs using comparative analytical characterization studies aiming to demonstrate biosimilarity of a candidate therapeutic protein product to a reference product (32). Characterization of rAAV capsid PTMs is complicated by the fact that rAAV is comprised of a 60-mer protein structure, adding significant complexity compared to most therapeutic protein modalities. Accurate characterization of these PTMs is also technologically challenging and highly dependent on the sample preparation and analytical methods. Further, site localization and accurate quantification of PTMs with mass spectrometry requires highly trained personnel and expert manual read validation at this time. If an accurate and reliable method for sample preparation and analytical characterization can be achieved, capsid PTM characterization can be informative in providing a deeper understanding of the product, especially for assessment of comparability pre- and post-process changes.

Several studies have been carried out to characterize rAAV capsid PTMs across production and purification platforms (33; 34). Several identified PTMs are shared across platforms while others are distinct between human and insect-based platforms. **Table 6** provides a non-exhaustive list of several PTMs identified in rAAV products.

Analytical Methods to Detect and Quantify Heterogeneity in Capsid Composition and Stoichiometry

*wt*AAV has been described in the literature to contain a stoichiometric 1:1:10 VP1:VP2:VP3 ratio (35). However, different production systems and capsid serotypes may produce rAAV preparations that contain variable ratios of capsid proteins.

Common techniques for protein characterization in AAV products include sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver stain or western blot to calculate a VP ratio and detect VP degradation (36). Recent advancements have improved the accuracy of SDS-PAGE by implementing Capillary-gel Electrophoresis (CE-SDS) using automated systems that substantially reduce user-bias and variability. These methods provide a single output that measures the entire capsid population and are unable to discriminatingly assess heterogeneous populations of VP ratios in individual capsids.

Table 6. Common PTMs identified in rAAV products.

Post-Translational Modification	Description
Acetylation	Addition of an acetyl group
Deamidation	Removal or conversion of asparagine or glutamine residue. Asparagine can be converted to aspartic acid or isoaspartic acid. Glutamine can be converted to glutamic acid or pyroglutamic acid
Glycosylation	Addition of oligosaccharide, glycan, to the nitrogen in the amide group of asparagine or the oxygen atom of serine or threonine
Methylation	Addition of methyl group to lysine or arginine residues
Phosphorylation	Addition of a phosphate group on serine, threonine, or tyrosine residues
Ubiquitination	Addition of ubiquitin protein to lysine residue. A single or multiple ubiquitin molecules can be attached. Presence influences proteolysis, degradation, or endocytosis

The study of AAV proteomics and post-translational modifications is still in its infancy. However, recent advancements in instrumentation and method development have made it possible to perform non-native and native mass spectrometry on AAV preparations to gain insight into the heterogeneity of both individual capsid proteins and vector assemblies (37; 38; 39). Mass spectrometry (MS) offers enhanced sensitivity when paired with an appropriate separation technique such as liquid chromatography (LC), enabling detection of heterogeneous VP stoichiometries in products as well as identification and quantification of capsid PTMs. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) can be used to identify post- translational modifications and uses proteases to cleave rAAV capsid proteins into smaller subunits prior to separation by LC and tandem MS (40). Common methods used for analysis of capsid proteomics are described in **Table 7.** These advancements in protein analysis have led to an enhanced understanding of rAAV capsid protein heterogeneity, and recent findings have demonstrated that VP ratios can vary drastically between batches, platforms, and serotypes, and even within individual capsids of the same preparation (30).

Table 7. Common methods for the analysis of capsid proteins.

Method	Description	Common Use Case(s)
LC/MS	Liquid Chromatography followed by mass spectrometry	Quantification of VP ratios
LC-MS/MS	Liquid Chromatography followed by tandem mass spectrometry in series	Peptide mapping, PTM analysis, quantification of VP ratios
MALDI-TOF	Matrix assisted laser desorption/ionization – time-of-flight	Peptide mapping and PTM analysis
Isoelectric focusing	Separation of proteins based on pI and size	Verification of presence of VP/1/2/3, simple PTM detection
Western Blot	Separation of proteins based on size, followed by identification by fluorophore-labelled antibodies	Verification of presence of VP/1/2/3

Impact of Capsid Post-Translational Modifications on rAAV Product Heterogeneity

Capsid PTMs could either enhance or negatively impact capsid function, and PTM profiles can vary between production systems, purification methods, and storage conditions. Additionally, PTM profiles can be transitory, as storage conditions and stability can impact profiles. As such, analysis of PTM profiles can be affected by process changes. Therefore, when making significant process changes, analysis of PTM profiles should be assessed during comparability to determine if the change has impacted PTM profiles.

Collecting retain samples across multiple product lots used for preclinical and clinical studies is highly recommended. These retains can be used for testing once a suitable analytical method has been identified. Additionally, if a future manufacturing change is made, a baseline for PTM profiles can be used to support the comparability assessment. However, as stability and storage conditions impact capsid integrity and PTMs, any potential impact of degradation on PTM analysis should be considered when analyzing samples at multiple time points. Finally, as analysis of PTM heterogeneity is a relatively recent advancement in the rAAV field, it is still unknown if there is an impact to product safety and/or efficacy. As such, analysis of PTM profiles can be useful to determine the overall impact that each PTM could play across multiple products, indications, and platforms, but should be assessed within the context of additional product quality analysis methods.

Limitations for Assessment of PTM and Capsid Heterogeneity

The study of rAAV proteomics and post-translational modifications is still in its infancy, but the technology and understanding in the field is rapidly advancing. Adapting from FDA guidance and recommendations on protein therapeutics (41), AAV capsid PTMs and protein heterogeneity may be assessed for products during development and/or after manufacturing changes. However, current technology and industry understanding of the impact of PTMs and capsid heterogeneity requires further advancement before specific limits and/or specifications should be set.

No standardized methods or reference standards exist in the field for interrogating these characteristics, making it challenging to consistently test different products across platforms and type. As such, presently we recommend sponsors consider implementation of PTM and capsid heterogeneity analysis to provide for deeper product characterization that could lead to greater understanding regarding key structural features and quality attributes. Finally, it is also critical to define sample preparation as this can alter the capsid structure in a way that impacts results of PTM analysis.

Summary of Recommendations for Characterization of rAAV Product Heterogeneity

Analytical technologies and methodologies for sequence and capsid protein-related product heterogeneity in rAAV preparations have greatly advanced in recent years, enabling a more comprehensive characterization of rAAV products. NGS can be utilized to characterize packaged sequence lengths, assess identity, and delineate heterogeneous populations of rAAV sequence variants. Recent advances in proteomics technologies have allowed for identification of PTMs and advanced characterization of capsid protein heterogeneity. The combination of these technological advances in rAAV analytics have enabled characterization of the intrinsic heterogeneity in rAAV products that has historically been out of reach.

While no single root cause can be attributed across all reported TESAEs observed in high-dose, systemic administration of rAAV products, it is likely that a combination of effects due to both product-related impurities and product heterogeneity plays a role in the safety and efficacy of these products. As the gene therapy industry begins to tackle a wider range of indications, such as those requiring high dose and systemic delivery, it is important that sponsors augment their understanding of product quality related to heterogeneity, which may require a deeper analytical characterization panel.

As technologies rapidly advance, it is important that sponsors also understand the limitations of each analytical method employed and utilize orthogonal methods where available, while keeping sufficient sample retains for future analysis as warranted. We encourage analyzing rAAV product heterogeneity early in the product development lifecycle to establish a benchmark for heterogeneity-related product quality, which can be used to accelerate product and process development.

While these advances provide the possibility of characterizing product heterogeneity and impurities to a higher standard, they have yet to be widely adopted throughout the industry. As the rAAV field advances and matures, sponsors may be held to a higher standard that aligns with harmonized guidelines, such as those described in ICH Q6B around product heterogeneity. This provides an opportunity for sponsors to gain a greater understanding of the rAAV product, product heterogeneity, and product-related impurities that will allow for better product quality understanding across the gene therapy industry, paving the way for the development of safer and more efficacious rAAV products.

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If you wish to reach out to us with comments or questions regarding this paper, please direct all correspondence to rAAV@darkhorseconsultinggroup.com until April 15, 2023. If you have ideas or requests concerning DHC guidance or though leadership after that time, please write to us at thoughtleadership@darkhorseconsultinggroup.com

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Appendix I

Term	Definition
Empty Capsid	Recombinant adeno-associated virus with no encapsidated DNA
Expression Cassette (or Payload)	ITR to ITR inclusive sequence containing the designed transgene and associated regulatory elements (e.g., promoter, polyA)
Product Heterogeneity	Molecular variants of the desired product which have the potential to have properties comparable to those of the desired product with respect to activity, efficacy, and safety
Partial Capsid	Recombinant adeno-associated virus with encapsidated DNA that is smaller than the full expression cassette
Full Capsid	Recombinant adeno-associated virus with encapsidated DNA corresponding to one copy of the full expression cassette
Oversized Capsid	Recombinant adeno-associated virus with encapsidated DNA that is larger than one copy of the full expression cassette
Process-related impurity	"Impurities that are derived from the manufacturing process. They may be derived from cell substrates (e.g., host cell proteins, host cell DNA), cell culture (e.g., inducers, antibiotics, or media components), or downstream processing (e.g., processing reagents or column leachables)." (ICH Q6B)
Product-related impurity	"Molecular variants of the desired product (e.g., precursors, certain degradation products arising during manufacture and/or storage) which do not have properties comparable to those of the desired product with respect to activity, efficacy, and safety" (ICH Q6B)
Recombinant adeno-associated virus (rAAV)	A viral vector able to transduce dividing and non-dividing cells. Multiple naturally occurring capsid variants (i.e., serotypes) exist with preferential targeting of specific tissues. The transgene of interest replaces the rep and cap gene present in wildtype AAV. Used primarily for gene replacement, augmentation, or editing *in vivo*.