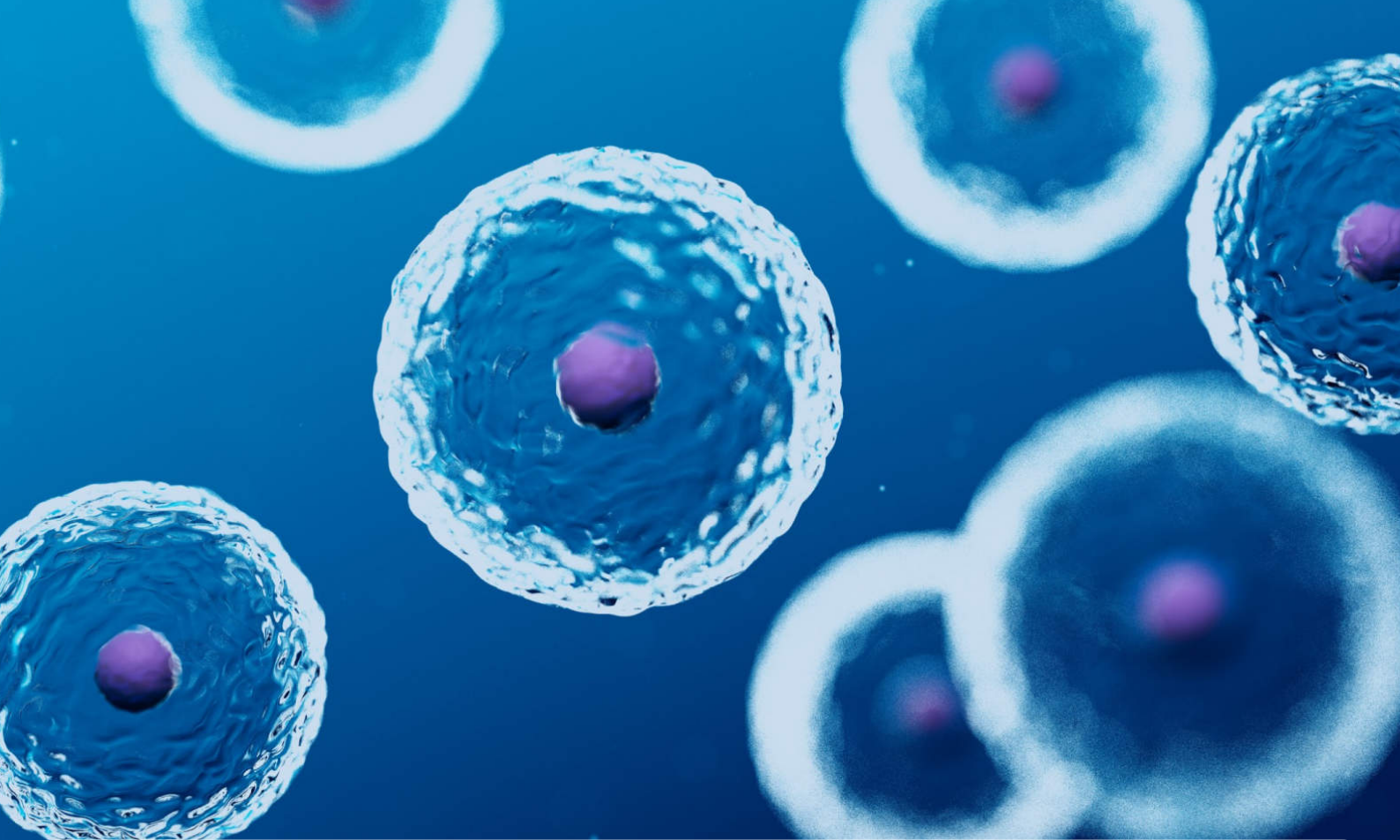




A Q&A on Viral Vector Integrity Analysis: Advancements in Gene Therapy Development

Challenges of AAV integrity assessment: Key takeaways from a roundtable discussion with leading experts in the cell and gene therapy field

Gene therapy is reshaping how we approach genetic disorders, turning what was once considered untreatable into areas of possibility. With over 30 FDA-approved treatments and more than 2,000 therapies currently in development worldwide, the field is advancing at a remarkable pace.

A key component of many gene therapies is the use of recombinant Adeno-associated virus (AAV) vectors, which serve as delivery vehicles to introduce therapeutic genes into patients. However, ensuring the safety, efficacy and precise dosing of these therapies requires robust and reproducible characterization of AAV vectors. Genome integrity plays a crucial role in vector performance. If genomes are truncated or incomplete, the vector may lose its effectiveness, making careful assessment an essential part of the process.

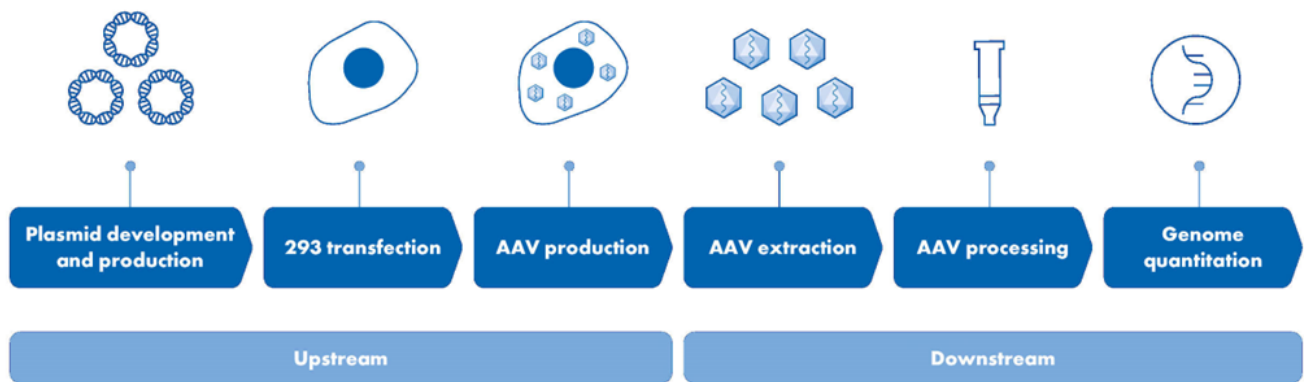


Figure 1. AAV production workflow: From plasmid development to genome quantitation.

Digital PCR (dPCR) is a precise and sensitive tool for assessing AAV genome integrity. It accurately distinguishes full from fragmented genomes, ensuring reliable data for regulatory compliance and therapeutic consistency.

QIAGEN has recently organized a [roundtable discussion](#) with leading experts, exploring the advancements in vector integrity analysis. They talked about the advantages of dPCR-based solutions, the challenges of multiplex assay design and the role of reference standards and assay qualification in enhancing the precision and efficiency of gene therapy development.

Speakers:

- **David Dobnik, PhD, Co-founder and Chief Scientific Officer, Niba Labs**
With extensive experience in gene therapy, David has been at the forefront of developing precise quantification methods for viral vectors, including digital PCR and genome integrity evaluations. His work supports pharmaceutical companies in process development and viral vector characterization.
- **Andrzej Noyszewski, PhD, MScEng, Senior R&D Scientist, Agathos Biologics**
Andrzej specializes in synthetic gene constructs, analytical methods, and product development. His expertise in developing and validating analytical methods positions him as a leader in genome integrity research and AAV characterization.
- **Alex Deiulio, B.S., Scientist, Agathos Biologics**
A molecular biologist with six years of experience, Alex leads the analytical testing team at Agathos. His focus on digital PCR and Agathos' Universal AAV Standard development demonstrates his commitment to advancing vector analysis methods.
- **Robert L. Hills, PhD, Director of Molecular Cell and Gene Therapies, SK pharmteco**
A biochemist with a rich history in academia and industry, Robert has played a key role in developing innovative molecular technologies. His expertise spans small and large molecule development, contributing to cell and gene therapy advancements.

Moderator:

- **David Eckert, PhD, Business Development Manager dPCR, QIAGEN**
David has a background in biochemistry and molecular biology. His career has focused on leveraging digital PCR technologies to enhance precision in molecular analyses. His current role at QIAGEN involves driving advancements in dPCR applications for gene therapy.

Introduction

Moderator: David Eckert

Welcome to our roundtable discussion on advancements in genome integrity assessment for vector-based gene therapies. Over the last five to ten years, we've seen a huge acceleration in gene therapy development, culminating in several FDA-approved treatments. This progress has increased the need for improved analytics in AAV production, including upstream processes like plasmid development and downstream stages like vector extraction and processing.

Today, we'll focus on genome quantification and its role in ensuring safety and efficacy. Proper quantification of the AAV genome, which mediates gene transfer, is essential for accurate dosing. Although DNA quantification through PCR seems straightforward in theory, it involves complexity due to full genomes, partial genomes, and impurities.

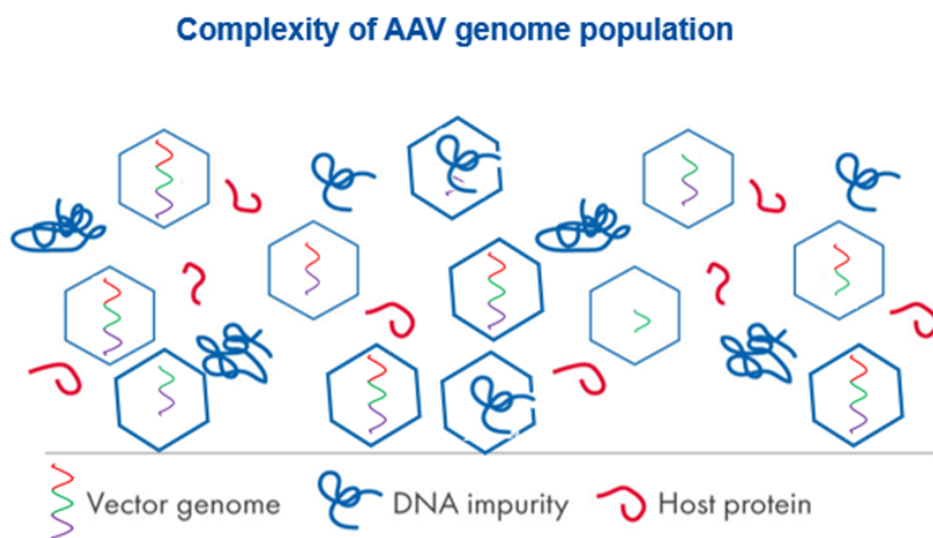


Figure 2.
Heterogeneity in AAV genome population: Vector genome, DNA impurities, and host proteins.

Importance of vector integrity and quantification

Moderator: Why are vector integrity and accurate quantification of different vector fragments essential?

Rob Hills: The primary reason is the efficacy of the drug product. It is critical to distinguish between full and partial AAV genomes and to understand the role of partial sequences—whether they are fragments, truncations, or splice variants. Comprehensive characterization of the product enables us to provide

valuable insights to research and development teams for potential sequence optimization. Recent studies have highlighted concerns such as splice variants and non-homologous end-joining, particularly within promoter regions. A thorough understanding of these issues allows for the design of more optimized AAV constructs.

Available solutions for genome integrity analysis

Moderator: Niba Labs places significant emphasis on genome integrity analytics. What current solutions exist for assessing genome integrity, and how does digital PCR (dPCR) compare?

David Dobnik: Various methods are available. Traditional approaches, such as agarose gel electrophoresis and capillary electrophoresis, can detect nucleic acids but do not provide quantification. Long-read sequencing, while highly precise and capable of delivering detailed sequence data, has low throughput and cannot accurately quantify copy numbers. Digital PCR (dPCR), in contrast, enables absolute quantification of both complete genomes and fragments, offering higher throughput and faster results. For example, a 4-plex assay allows for genome completeness assessment while providing absolute copy number quantification. This shift toward absolute quantification has the potential to enhance correlations with other key metrics, such as potency.

Andrzej Noyszewski: While sequencing provides detailed information, it is both time-consuming and costly. Digital PCR offers a faster, cost-effective alternative, particularly in AAV production, where targeted dPCR assays can be employed instead of full sequencing. Both methods are complementary, but dPCR provides rapid and reliable insights.

Alex Deulio: Another advantage of digital PCR is its high sensitivity. Traditional approaches, such as agarose gel electrophoresis, lack the sensitivity required to detect distinct species within the AAV genome population. Digital PCR, however, excels in this area.

Designing multiplex assays for genome integrity

Moderator: What considerations and challenges arise when designing a multiplex assay for genome integrity analysis?

David Dobnik: The first step is selecting target regions that comprehensively cover the genome. This can be achieved by targeting specific regions, such as promoters and poly-A tails, or by distributing assays across the genome at regular intervals, such as every 1,000 base pairs. It is crucial to include both the 5' and 3' ends to ensure a complete assessment of genome integrity.

Another important aspect is oligonucleotide design. Even minor variations in base pairing can significantly impact amplification efficiency, and oligo interactions can be difficult to predict. Although in silico

evaluations are useful, real-world testing often reveals unforeseen interactions. Once the assay is optimized, data analysis becomes critical. Initially, digital PCR platforms lacked built-in integrity analysis features, requiring the development of custom analysis methods.

Alex Deulio: The introduction of multiplexing support and the intactness percentage feature in QIAGEN's QIAcuity system has significantly improved integrity measurements, making them more streamlined and efficient.

Challenges in integrity analysis

Moderator: What is the most significant challenge in conducting integrity analysis?

Alex Deulio: Data analysis and managing assay cross-talk present major challenges. In silico predictions often fail to account for real-world sample variability, leading to signal bleed-through between detection channels. However, recent advancements, including QIAcuity Software v3.1 and cross-talk compensation matrix, have significantly mitigated this issue, improving data clarity.

Andrzej Noyszewski: Precisely. Without appropriate color compensation, cross-talk can lead to misinterpretation of data, particularly for users unfamiliar with the nuances of the analysis. Recent improvements now allow for the design of more sophisticated multiplex assays, extending beyond traditional 3-plex or 4-plex formats.

User expectations for integrity analysis

Moderator: From a user perspective, what are the key expectations for a multiplex assay assessing genome integrity?

Rob Hills: Simplicity and robustness are of utmost importance. Excessive steps introduce variability and compromise precision. When implementing a new assay, we qualify it based on parameters such as precision, accuracy, linearity, and specificity. The latest data analysis package from QIAGEN has greatly streamlined our workflow. Previously, integrity calculations were performed manually; now, the instrument automates this process, enhancing efficiency.

Factors affecting genome integrity in production

Moderator: What factors influence genome integrity during production, and what should be monitored to ensure an intact product?

Rob Hills: Many integrity issues originate from the sequence itself. Promoter regions, which are GC-rich, can form secondary structures, such as short hairpins, that hinder replication. Nicking during replication may also result in non-homologous end-joining. Additionally, the method used during the capsid lysis step—whether employing temperature or Proteinase K—can impact integrity values. Optimization of these factors is essential.

Andrzej Noyszewski: We have observed differences in packaging efficiency between the 5' and 3' ends of the genome, which can result in fragmented genomes and impact overall integrity.

Moderator: Agathos has developed a universal AAV standard. How is it designed to enhance utility in integrity analysis testing?

Andrzej Noyszewski: In developing assays for AAV quantification, I sought a reliable approach to assess various assay targets, such as ITR, PolyA, and Promoter. Initially, I explored existing positive controls for AAV but found that none adequately represented the specific assays I was working with. It became clear that having a flexible tool capable of accommodating different assays for a gene of interest would be highly beneficial.

QIAGEN addressed this need by introducing the Cell and Gene Therapy Assays for dPCR, designed for these specific targets. By incorporating these assays, I was able to compare titers across different targets and evaluate their performance under varying conditions, such as differences in AAV lysis or sample preparation methods. The ability to apply multiple assays to a single template allowed for comparative analysis across different products within our laboratory, making the tool highly versatile.

Over time, this tool became invaluable not only for titer quantification but also for assessing genomic integrity. By utilizing well-characterized assays across the template, we no longer had to rely solely on the longest fragment for analysis. Instead, we could obtain a more comprehensive and detailed representation of the genome's integrity.

Additionally, the process of titering can be complex. This standard provides a reliable control for genomic integrity, allowing researchers to validate their calculations with greater confidence. The design also incorporates additional space, enabling future expansion by incorporating other commonly used fragments, thereby increasing its applicability. We have been pleased with its performance, as it provides consistent amplification across the genome while remaining easy to use and adaptable to various analytical needs.

Orthogonal methods for integrity verification

Moderator: What orthogonal methods do you use to validate dPCR results?

David Dobnik: When we initially conducted dPCR integrity assays, we found that only 20% to 40% of genomes were fully intact, despite analytical ultracentrifugation (AUC) measurements indicating that 95% of capsids were full. Through collaboration with AskBio, next-generation sequencing (NGS) data confirmed a strong correlation between genome integrity and potency.

Moderator: Have you observed any correlation between genome integrity and efficacy?

Rob Hills: Yes. When our team presented these findings internally, researchers working on potency assays for the same construct observed similar potency effects. Our results indicated approximately 40% genome integrity, and they noted that this explained much of the potency data observed for the construct. We plan to release a white paper next year, examining different AAV constructs and correlating integrity data with potency and other key metrics.

Measuring full-to-empty capsid ratios

Moderator: How do you measure full-to-empty capsid ratios?

Rob Hills: SK pharmteco is utilizing several methods to evaluate empty to full populations, as well as partials. Depending on the purpose of the measurements, we utilize orthogonal analytical techniques such as analytical ultracentrifugation (AUC), Mass Photometry and SEC-MALS, all of which demonstrate strong correlation.

Alex Deiulio: In addition to digital PCR and ELISA for routine assessments, we have recently incorporated UV spectroscopy and dynamic light scattering (DLS) to evaluate additional parameters such as aggregation.

Moderator: Is there a specific DNase you recommend for digesting host cell DNA?

Alex Deiulio: We utilize the QIAGEN CGT Viral Vector lysis kit, which includes a highly effective DNase that has produced reliable results. To clarify, when I previously referred to evaluating DNase options, I was speaking about bulk production applications rather than routine assay workflows.

Rob Hills: In previous programs, we have successfully used Turbo DNase, which has provided consistent and effective results in our workflows.

Moderator: What part of the dynamic range can be utilized for integrity analysis?

David Dobnik: The usable dynamic range for integrity analysis depends on the platform. QIAGEN's digital PCR system, for instance, offers a broad dynamic range due to its specialized analysis features, which account for co-localization in multiplex assays. In contrast, other platforms may have a reduced dynamic range unless specific adjustments are made to correct for co-localization effects.

Learn more about performing vector integrity analysis.



Explore this application note on "Analysis of DNA integrity and stability using digital PCR".
Request a demo of our QIAcuity dPCR instruments and meet your cell and gene therapy needs.

More about the speaker companies:

Agathos Biologics:

Agathos Biologics is a biotechnology company pursuing transformational science in biomanufacturing, biologic payload delivery, and cell and gene therapy. Discoveries in bioprocessing and genetic characterization and control have created an abundance of scientific possibilities that can help us all lead better lives. Our mission as The Good Science Company is to create breakthrough products and services within a strong ethical and moral framework that benefits everyone. We believe in Science that Serves and have a relentless focus on serving our clients, employees, and society.

Contact info@agathos.bio for more information.

NIBA Labs

Niba Labs is well known for quantitative assays development, targeting specific regions of any kinds of nucleic acid constructs (i.e. digital PCR assays for viral vector genome quantification). Additionally, we can also develop custom advanced multiplex approaches that can help investigate sequence integrity. Our mission is to advance analytical approaches for genome integrity evaluations.

Contact info@niba-labs.com or david.dobnik@niba-labs.com for more information, or explore www.niba-labs.com to learn more.

SK pharmteco

SK pharmteco is a global contract development and manufacturing organization specializing in the production of active pharmaceutical ingredients (APIs), advanced intermediates, and cell and gene therapy for the pharmaceutical industry. Built on 80 years of experience, our global operations have the capability and capacity to support client needs from development through commercial production.

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