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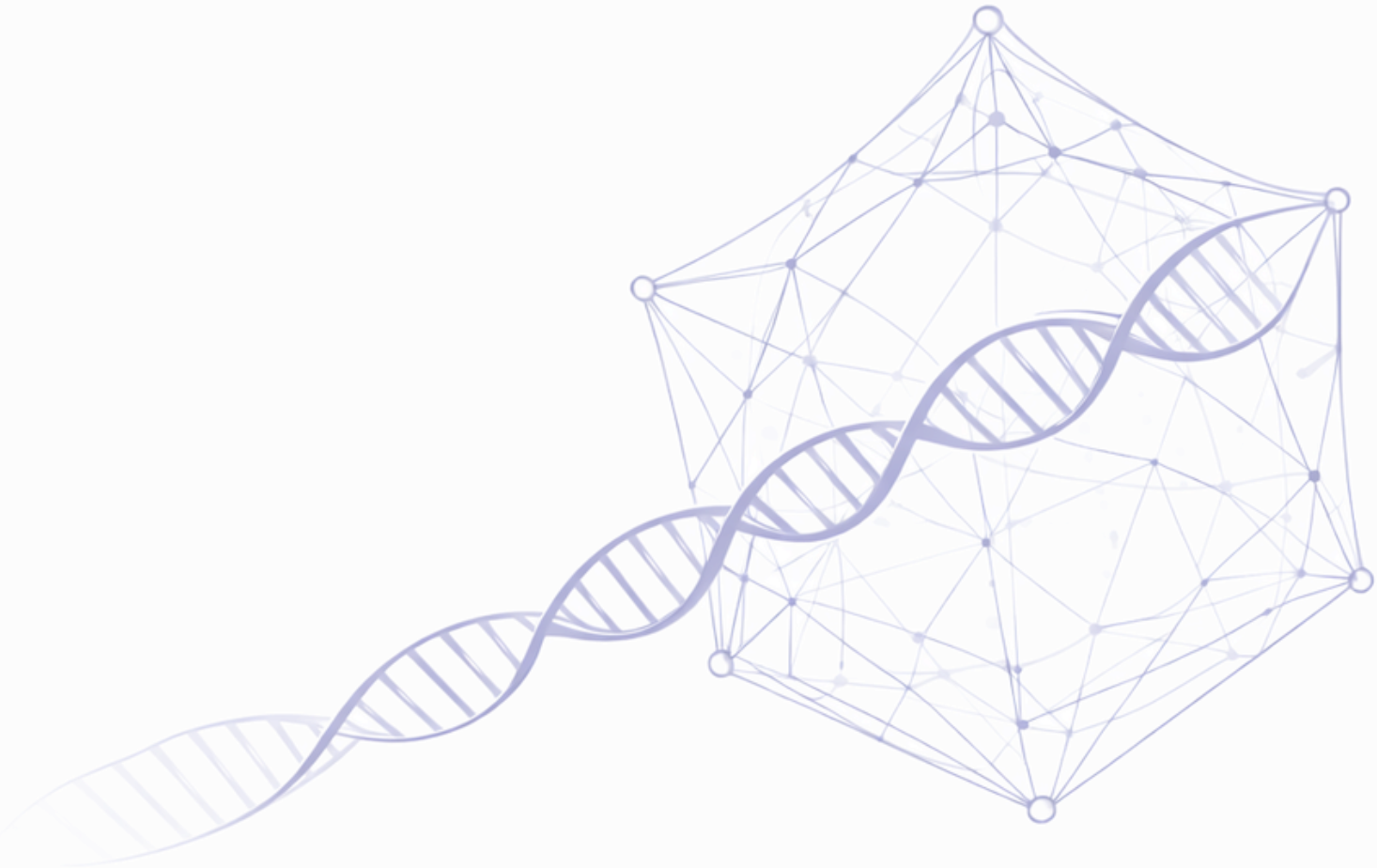
Viral Vector Genome Integrity: The Role of Complete Genomes in Gene Therapy

As gene therapy continues to advance, ensuring the integrity of the packaged genome within the capsid will be just as important as the capsid itself.



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Abstract

Viral vector genome integrity is emerging as a key focus and a potential critical quality attribute (CQA) that could better inform development of processes and quality of gene therapy products. There is growing recognition of its importance, along with increasing support for digital PCR (dPCR) as a robust analytical method for its precise determination.

Vector genome integrity refers to verifying whether viral vector genomes remain intact – with a complete and functional sequence – throughout production, purification, storage, and administration. For recombinant adeno-associated virus (rAAV) vectors, heterogeneity of packaged genomes presents a particular challenge, which includes the presence of full, partial, and empty capsids.

While vector genome (vg) titer remains the standard dosing metric, it does not differentiate between functional and non-functional genomes. Emerging evidence suggests that defining dose solely based on vg titer is insufficient, as two products with identical vg values per dose may result in different therapeutic effects due to differences in genome integrity.

In this context, dosing can be more meaningfully interpreted by considering genome integrity, distinguishing between dose quantity, defined as vg titer, and dose quality, reflecting the fraction of intact, functional genomes.

Moreover, improving genome integrity could have direct implications for patient safety. Ensuring the completeness of the therapeutic genetic material is critical to achieving the intended biological effect. High doses of rAAV vectors, often necessary to achieve therapeutic effect, carry increased risk of immune activation, especially when the product contains a high proportion of empty or partially filled capsids. These impurities can contribute to T-cell and complement activation, reducing therapeutic durability and potentially leading to adverse events.

Genome integrity may be a critical determinant of safer gene therapy applications. Orthogonal analytical strategies, integrating complementary physical, structural, and molecular assays, are essential to ensure accurate genome integrity assessment, product consistency, and ultimately, patient safety.

The multiplex dPCR assay development platform, NibaPlex®, supports duplex, 3-plex, 4-plex, and higher-order assay configurations, enabling simultaneous quantification of multiple distinct viral vector genome regions within a single reaction. These assays improve genome coverage, detecting truncated genomes (i.e., incomplete genomes) that simplex vg titer assays miss, thereby providing better insight into genome integrity.

Introduction to CGT therapy

Cell and gene therapies (CGTs) represent a rapidly advancing area in medicine, offering targeted treatments and the potential for curing complex diseases through genetic modification. These therapies utilize two primary approaches (Figure 1) to deliver therapeutic genes to patients:

In vivo gene therapy

In this method, therapeutic genes are delivered directly into the blood, target tissues, or cells of patients. This is most commonly achieved using viral vectors, which facilitate the transfer of genetic material into the patient's body, enabling precise modification of diseased or dysfunctional cells.

Ex vivo gene therapy

Here, cells are first extracted from the patient or a donor. These cells are then cultured and genetically modified outside the body. Once the desired genetic changes have been introduced, the transgene-expressing cells are reintroduced into the patient, providing a targeted therapeutic effect.

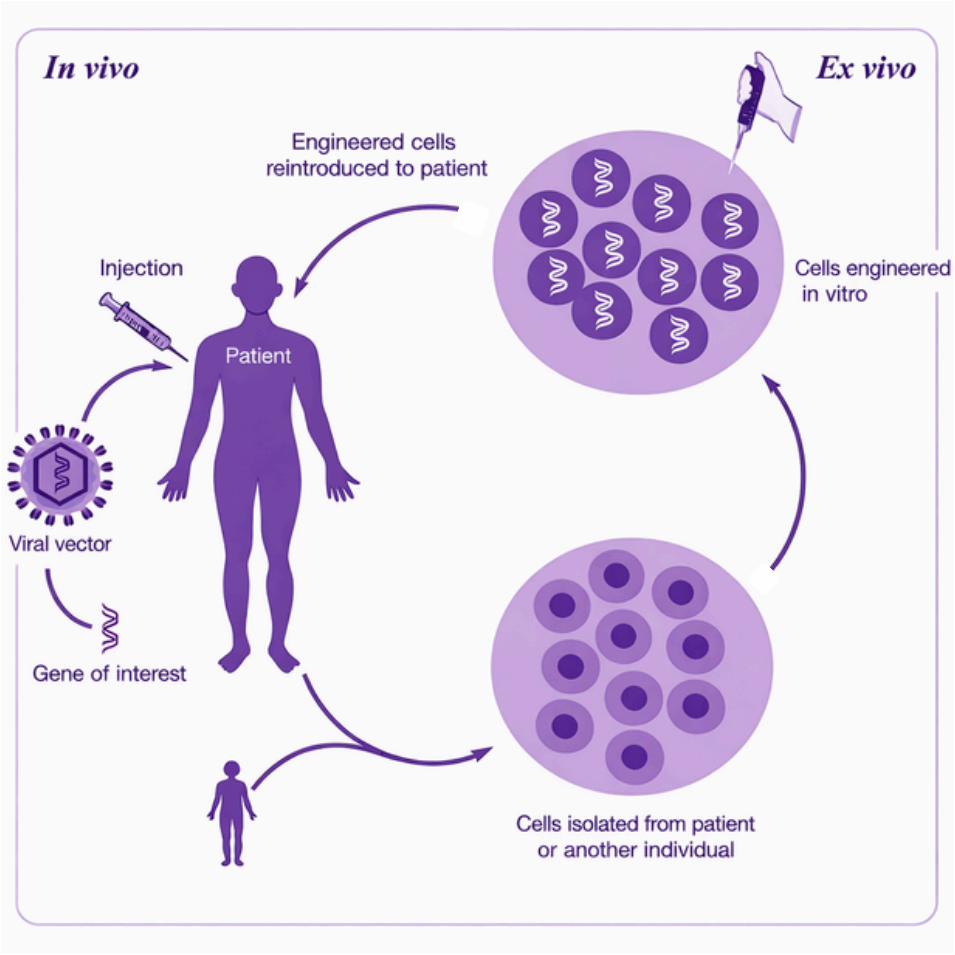


Figure 1: In vivo and ex vivo gene therapy (Ebrahimi et al., 2024).

Both strategies aim to address underlying genetic causes of disease by introducing, correcting, or silencing specific genes, thereby transforming the therapeutic landscape for a wide range of medical conditions (Geng et al., 2025).

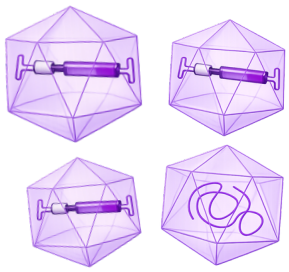
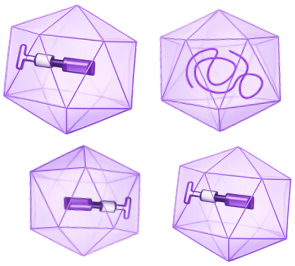
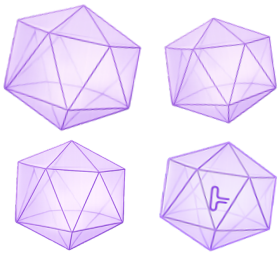
Viral and non-viral delivery platforms

These advanced therapeutics rely heavily on viral vectors – such as adeno-associated virus (AAV) preferred for *in vivo* applications, and lentivirus (LV) preferred for *ex vivo* use – to deliver therapeutic genes into patient cells. In addition to viral vectors, other delivery platforms such as lipid nanoparticles (LNPs) are increasingly used, especially for nucleic acid-based therapies, broadening the options for gene delivery in CGT development (Taghdiri & Mussolino, 2024; Volodina & Smirnikhina, 2025).

Heterogeneity of packaged rAAV genomes

During the production of recombinant AAV (rAAV) vectors, a heterogeneous population of viral particles that differ in their genome content is generated. The variability in genome packaging is the basis for classifying rAAV particles into categories of full, partial, and empty capsids (Table 1), reflecting the degree to which each capsid contains the intended therapeutic DNA payload. However, as genome packaging into capsids is not a uniform process, the distinction between full, partial, and empty capsids in terms of genome content is not always clear-cut and should be interpreted as an operational simplification rather than discrete populations. The relative abundance of these capsid species may be correlated to vector potency, dose consistency, and patient safety (ARM, 2023).

Table 1: Summary of capsid composition and therapeutic function

Graphic view of capsids			
Description	Full Capsids	Partial or partially filled or intermediate capsids	Empty capsids
Contents	DNA payload of intended length (complete vector genome construct* or impurities).	DNA payload shorter than intended length of complete genome (incomplete vector genome or impurities).	Without any DNA payload or including short, non-functional nucleic acid fragments.
Therapeutic Effect	*Functional gene delivery	Non-functional, increases risk of immunotoxicity, reduces transduction efficiency	

Adapted from: https://www.genengnews.com/topics/bioprocessing/single-assay-evaluation-of-aav-capsid-quality-novel-digital-pcr-approaches-advance-gene-therapy-research-and-production/

Recombinant AAV-based therapeutics are inherently heterogeneous, meaning that the production process yields a mixture of:

- **Full capsids:** Containing the intended length of DNA payload (complete vector genome of interest intended for delivering therapeutic effect).
- **Partial capsids:** Containing DNA payload that is shorter than intended length of complete genome.
- **Empty capsids:** These may be entirely void of DNA or include short, non-functional nucleic acid fragments.

During rAAV vector production, only about 10% to 50% of viral particles harvested from cells contain the expected complete genome of interest (Blahetek et al., 2025). This was reported in several studies (Adamson-Small et al., 2017; Wang et al., 2017; Gao et al., 2014; Eisenhut et al., 2024), indicating substantial heterogeneity in viral genome packaging. Many viral particles may contain partial genomes, or can be empty, not contributing to therapeutic efficacy.

The central role of genome integrity in CGT products

Recombinant AAV vectors are leading vehicles for gene delivery in advanced *in vivo* gene therapies. Maintaining genome integrity is critical to ensuring both safety and efficacy of rAAV vectors. Only capsids containing intact, complete genomes, carrying the full-length sequence for the therapeutic gene, together with the appropriate promoter and regulatory elements can thereby mediate effective therapeutic gene expression in target cells, thereby enabling synthesis of the desired therapeutic protein and ultimately driving clinical efficacy. (Blahetek et al., 2025).

Partial and empty capsids, as well as capsids containing unintended DNA, are classified as product-related impurities. Although they originate from the same manufacturing process as the intended product, they lack a complete functional genome and therefore do not contribute to the desired therapeutic activity (Figure 2). They reduce bioactivity and act as impurities that dilute effective dose and may trigger immune responses and can also pose significant safety concerns (Verdera et al., 2020).

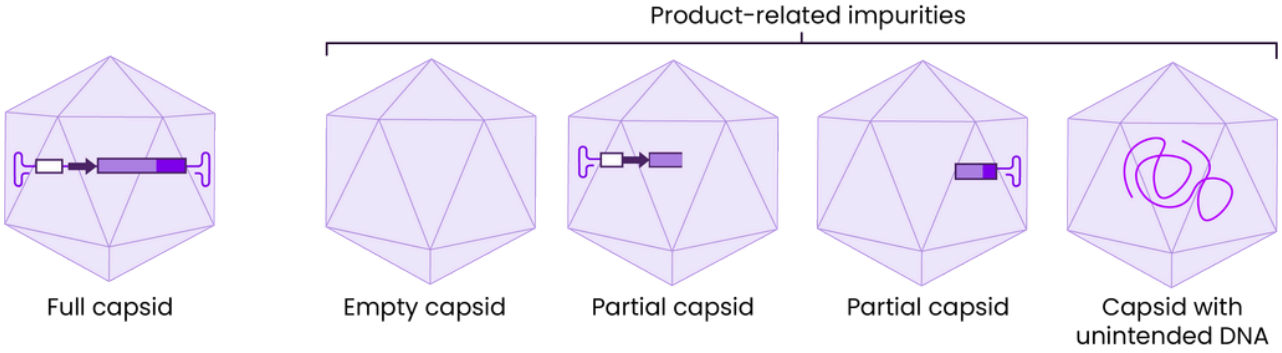


Figure 2: Product related impurities

Potential impact of genome integrity on therapeutic potency

Recent studies including those by McColl-Carboni et al. (2024) and Wang et al. (2024) have unequivocally demonstrated that the therapeutic potency of rAAV gene therapy vectors could be closely tied to genome integrity, when vg titer is used for dosing.

Wang et al. (2026) emphasize that vg titer does not accurately reflect biologically effective dose. While vg titer is widely used as a standard metric across discovery, manufacturing, and clinical studies, enabling dose normalization and lot-to-lot comparison, it does not capture the structural or functional integrity of packaged genomes. Specifically, vg titer quantifies all DNA-containing particles, regardless of whether they represent full-length, complete genomes capable of driving therapeutic gene expression (Wang et al., 2026).

Capsid populations enriched in complete genomes exhibit markedly higher *in vivo* efficacy than equally sized populations containing a higher proportion of incomplete genomes. While incomplete genomes contribute to measured viral genome titers, they mostly fail to translate into functional gene expression. When comparing two gene therapy products with the same viral titer, differences may arise because they do not contain the same number of complete genomes, potentially highlighting the importance of genome integrity in assessing product potency.

All of the above factors underscore the critical importance of minimizing the content of incomplete genomes throughout the production process to ensure the highest quality and safety of rAAV gene therapy products. Controlling incomplete genomes early on could reduce potential risks such as unpredictable biological effects and compromised therapeutic efficacy.

Although rigorous quality control testing is ultimately performed at the end of production, proactive minimization of partial genomes during manufacturing optimization (upstream and downstream processing) could be critical for achieving consistent quality of the products.

Analytical approaches to full, empty, and partial rAAV capsid determination

The analysis of full, empty, and partially filled capsids, respectively, has traditionally relied on biophysical methods, particularly analytical ultracentrifugation (AUC) and mass photometry (MP).

AUC allows for the separation of rAAV particles based on their sedimentation coefficients, which vary depending on whether the capsid is full (i.e., contains a complete genome), empty (i.e., contains no genome), or partial (i.e., contains a truncated - incomplete genome) (Burnham et al., 2015). Similarly, MP measures the mass of rAAV particles, directly correlated to the presence and size of its genomes, by quantifying light scattering.

Other complementary techniques capable of distinguishing between full, empty, and partial rAAV capsids (i.e., full/empty (F/E) analysis) include transmission electron microscopy (TEM) and ion exchange chromatography (IEX). TEM offers visual confirmation of full versus empty capsids but is severely limited by resolution and throughput, while IEX enables the separation of capsid populations based on surface charge differences, which correlate with DNA content (presence and length of genomes in capsids) (Colomb-Delsuc et al., 2022).

These methods are valuable for assessing the gross distribution of capsid content; however, they only differentiate rAAV capsids based on physical properties, such as mass and charge, and do not provide sequence-specific information about the packaged genome. Importantly, a particle classified as 'full' based on physical methods does not necessarily contain the correct, complete, or therapeutically functional genome sequence. Full, in this context, simply means the capsid has packaged some DNA content of intended length.

Because each analytical method has its own advantages and limitations, no single approach is sufficient for the comprehensive evaluation and characterization of rAAV preparations. Methods for assessing vector genome integrity should be combined with orthogonal analytical strategies to enable a more complete evaluation (Wang et al., 2026).

Methods for targeted detection of specific genomic regions

Traditional quantification methods such as quantitative PCR (qPCR) and simplex dPCR enable quantitative determination of vector genome copy numbers by measuring the total number of specific short fragments of viral vector genomes; however, they are unable to distinguish between complete and incomplete genomes, even if more individual targets across the genome are considered.

The titer determined by a simplex assay might not be the same as the titer of complete vector genomes. As a result, simplex assays can substantially overestimate the number of complete, functional vector genomes (Figure 3).

This limitation creates critical gaps impacting accurate dosing, bioactivity evaluation, and ultimately patient safety. Dobnik et al. (2019) noted that this can lead to substantial overestimation of functional genome titers, since even fragments of the genome containing the primer binding sites will be amplified, falsely inflating viral vector genome counts (Dobnik et al., 2019). Hayes and Dobnik (2022) further emphasized that this methodological gap limits the predictive value of viral genome titers in relation to actual biological potency, especially in clinical or manufacturing settings where high accuracy is essential (Hayes & Dobnik, 2022).

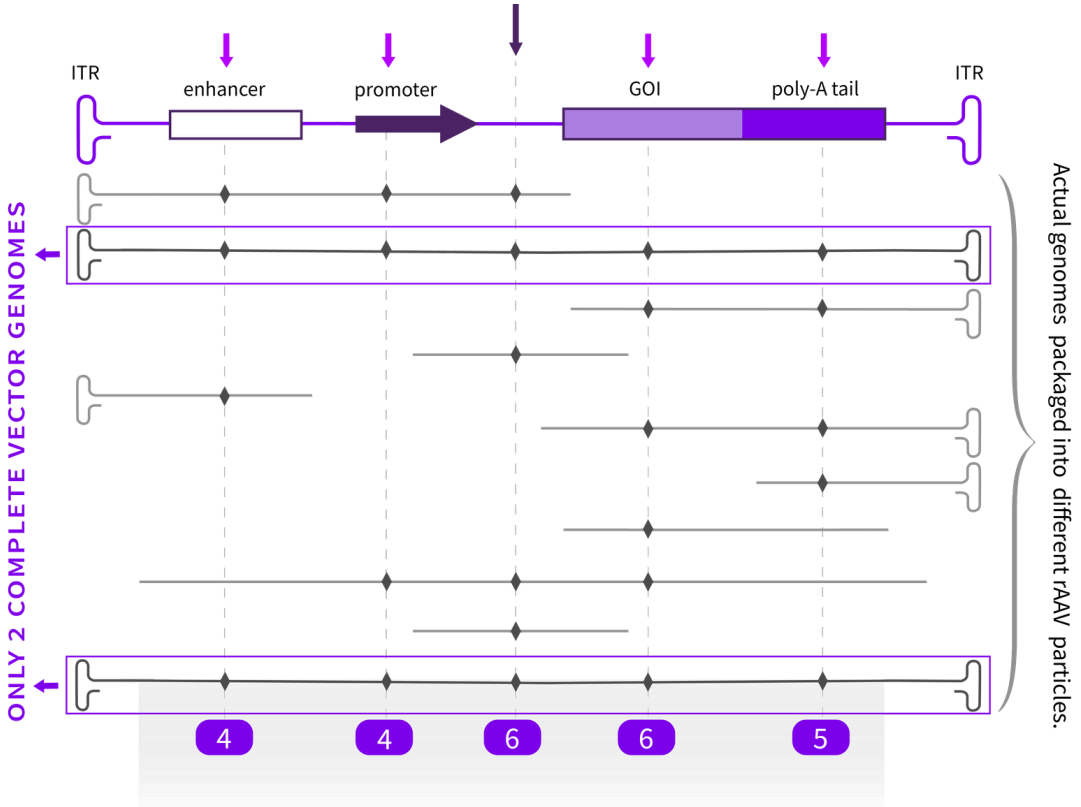


Figure 3: Simplex dPCR results: a linear map of the rAAV vector genome is shown, including key elements such as the ITRs, enhancer, promoter, gene of interest (GOI), and poly-A tail. This schematic provides a reference for the positions of the dPCR assay targets, indicated by the vertical dashed lines. Beneath the genome map, the horizontal lines represent the actual genomes packaged into different rAAV particles - only two of these are complete, spanning from ITR to ITR, while the others are partial. Diamonds mark the assay target sites detected within each genome. The numbers 4, 4, 6, 6, and 5 at the bottom of Figure 3 correspond to the copy numbers measured by simplex dPCR assays targeting each region. These values reflect all genomes – both complete and incomplete – that contain the respective target sequence.

In contrast, multiplex assays interrogate multiple regions of the viral vector genome simultaneously within a single reaction (Figure 4), enabling more accurate quantification of complete rAAV genomes and thereby improving product characterization. Multiplex assay formats include duplex, 3-plex, 4-plex, and higher-order assay configurations designed to target multiple distinct genomic regions.

Genome integrity of rAAV vectors is a critical quality attribute for ensuring safety, efficacy, and accurate dose determination in gene therapy products. Multiplex dPCR assays provide a reliable and scalable approach for assessing rAAV genome integrity by simultaneously targeting multiple regions of the vector genome, enabling accurate discrimination between complete and incomplete genomes.

While multiplex dPCR substantially improves multi-locus genome assessment, it does not replace full-length sequencing-based characterization methods when detailed sequence fidelity analysis is required. Nevertheless, thanks to their high precision, robustness, simplified data analysis, and independence from standard curves, multiplex dPCR assays are well suited for comprehensive genome integrity assessment across both process development and quality control environments.

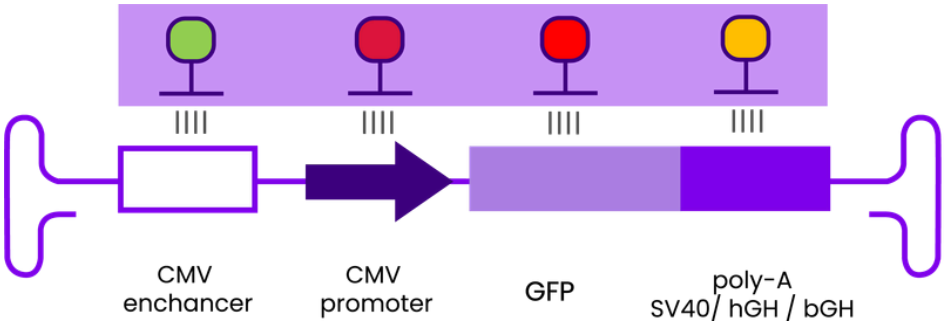


*Figure 4: 4-plex multiplex dPCR assay (NibaPlex®) case study designed to simultaneously target four key viral vector genome elements essential for the therapeutic efficacy of CGT products: the enhancer region, promoter region, gene of interest (GOI) (*represented in our case study by GFP) and the poly(A) tail.*

Multiplex dPCR assay – quantification of complete and incomplete vector genomes present in rAAV capsids

The current viral vector genome titer quantification method using traditional simplex dPCR assays, targets only one genomic site per reaction. Consequently, this limited genomic coverage restricts simplex assay's ability to assess overall genome integrity. Since each assay provides information about only one specific region, structural variations, deletions, or rearrangements outside the targeted site may go undetected, limiting the assay's utility for comprehensive genome characterization (Maccani et al., 2025).

However, by simultaneously analyzing multiple genomic sites by dPCR, multiplex assays offer substantially broader coverage and more informative views of the genome within a single reaction, thereby enhancing genome characterization.

Responding to this need, the NibaPlex® dPCR multiplex assay development platform offers a transformative advancement in vector genome integrity analysis. Multiplex assays enable direct assessment of genome integrity by simultaneously detecting multiple regions of the genome. It distinguishes complete genomes, which are positive for all targets (Figure 5), from incomplete genomes in a single reaction. Unlike simplex assays that measure a single region, multiplex assays can provide much better insight into the completeness of the genomes. By analyzing the patterns of positive signals, it is possible to identify which regions are usually missing in incomplete genomes, allowing for precise quality control of rAAV vectors.

While simplex dPCR remains valuable for precise quantification of individual targets, multiplex dPCR's ability to assess multiple genomic sites simultaneously makes it a superior approach for robust and comprehensive monitoring of genome integrity. By providing a clearer picture of the actual number of complete genomes, NibaPlex® multiplex assay development platform supports improved process development, product characterization, and represents a key step toward safer and more effective gene therapies.

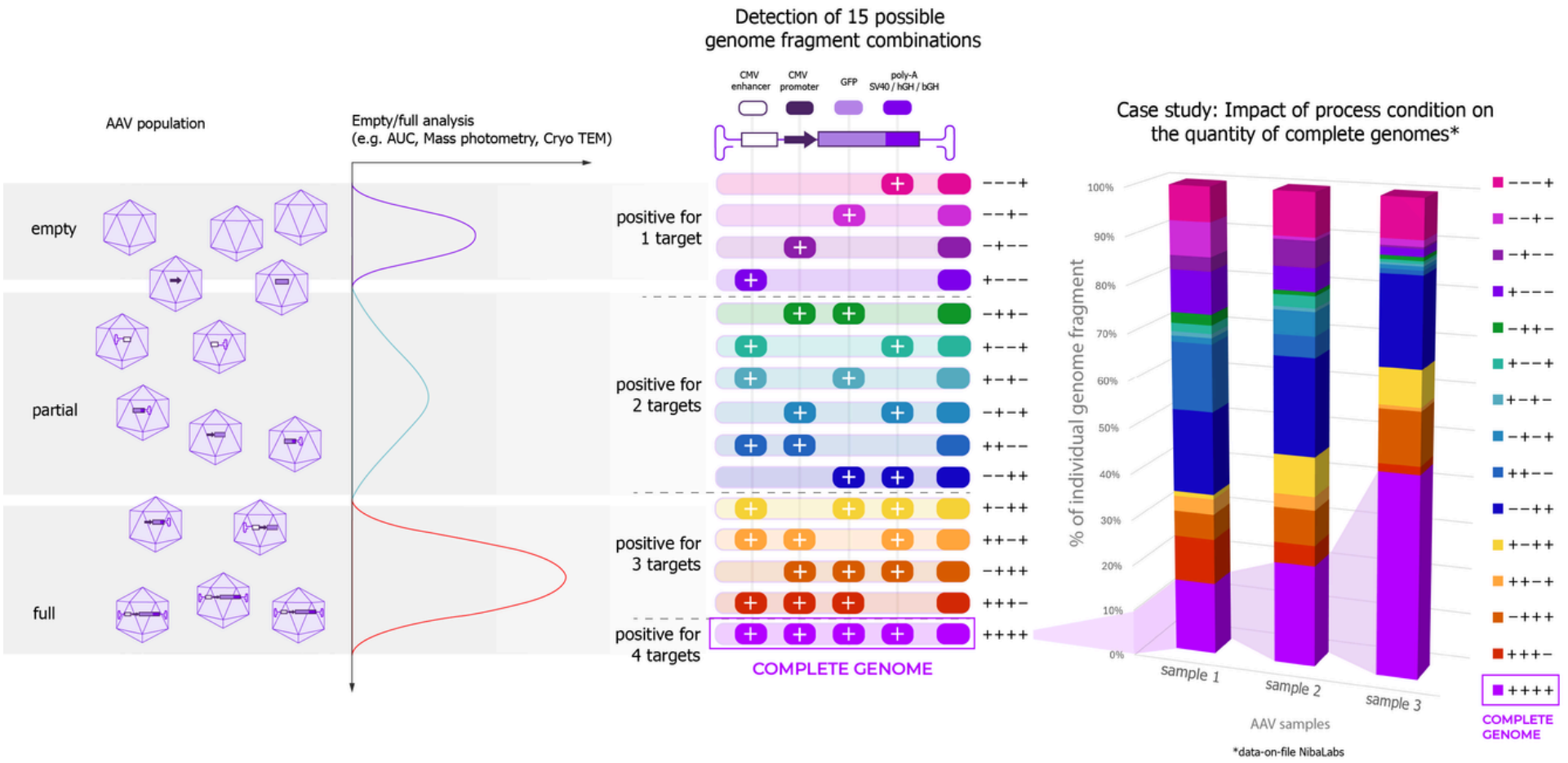


Figure 5: Multiplex dPCR assay for quantification of complete and incomplete vector genomes within rAAV capsids. This example illustrates the 4-plex assay developed using our assay development platform NibaPlex®.

Why is multiplex dPCR assay better for genome integrity than multiple individual simplex dPCR assays?

Multiplex assay enables direct assessment of genome integrity, distinguishing between complete and incomplete genomes. For example, to target four different genome regions in a simplex format with four assays, four separate dPCR reactions are required (Figure 6), while our case study 4-plex dPCR assay simultaneously quantifies four independent regions of the rAAV genome within a single reaction in all-in-one reaction mix (Figure 7).

Simplex: 4 separate reactions

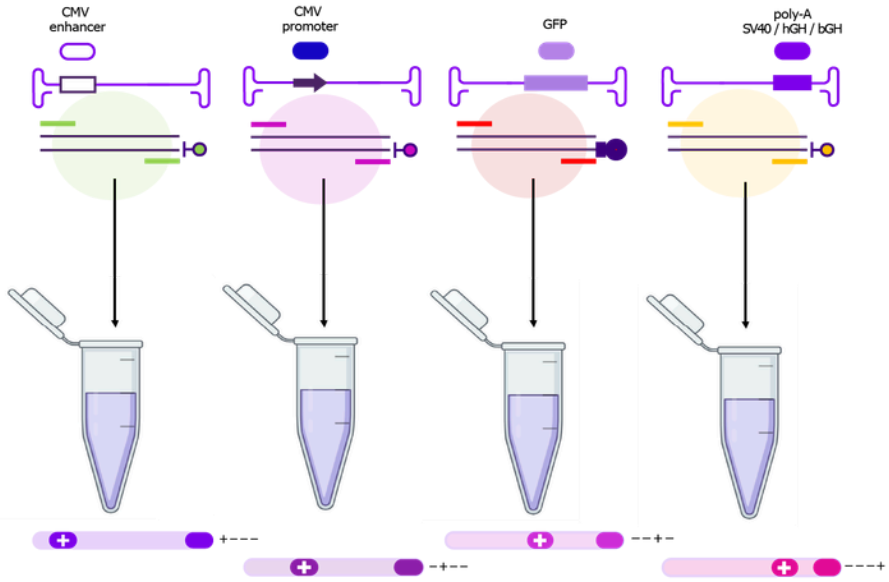


Figure 6: Targeting four genome regions with four independent simplex assays.

4-plex: single reaction mix

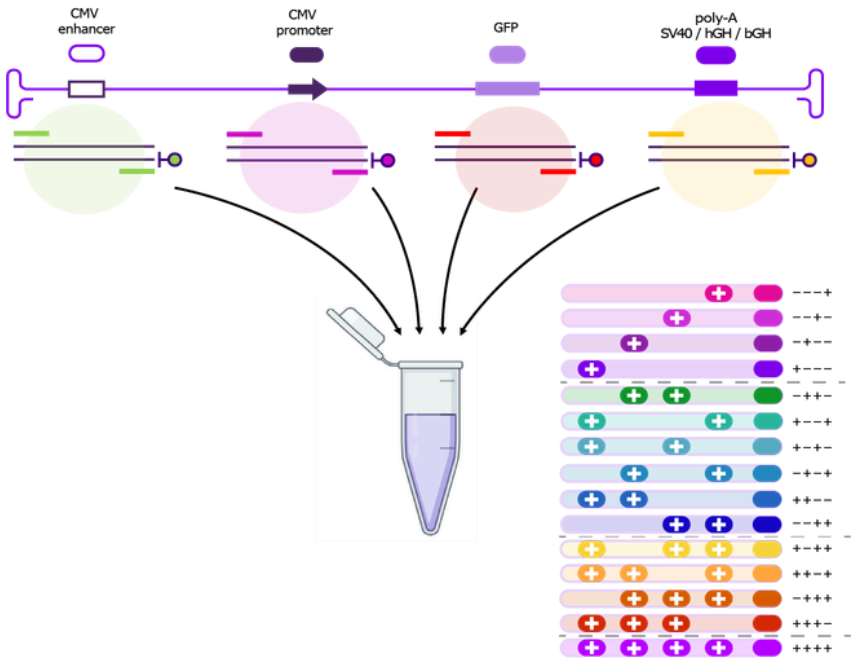


Figure 7: Example of our case study 4-plex dPCR assay, developed by NibaLabs using the NibaPlex® multiplex assay development platform.

Table 2 compares the analytical capabilities of simplex and multiplex dPCR assays. While individual simplex dPCR enables precise quantification of a single target in one reaction, results of multiple simplex assays cannot be combined to infer genome integrity, as positive signals may originate from different partial genomes.

On the other hand, multiplex dPCR assays allow simultaneous detection of multiple genomic regions within one reaction, significantly saving on sample volume, reagents, and analysis time while increasing throughput and data richness, by providing broader genomic coverage.

Table 2: Comparison of main features between simplex and multiplex dPCR.

Feature	Simplex dPCR	Multiplex dPCR (NibaPlex® Platform)
Number of Targets	One per reaction	Multiple per reaction (2,3, 4 or more target regions)
Genomic Coverage	Limited	Broad (multiple mapping points across the genome)
Assay Complexity	Low	High (requires careful design and optimization)
Data Richness	Single data point	Multiparametric data
Use Cases	Precise quantification of one target	Comprehensive genome integrity

While multiplex dPCR assays enable the simultaneous detection and quantification of multiple genomic targets, the summed or individual titers they generate do not inherently reflect genome integrity, as signals from distinct targets may originate from different genome fragments rather than from a single, complete viral vector genome.

Obtaining integrity information requires shifting the analysis from normal target quantification to the examination of multiple occupancy at the partition level. By interrogating which combination of target signals co-localizes within individual partitions (and considering probabilities of co-localization of fragments), one can distinguish intact genomes in which all targets are present together, from fragmented ones, where targets are detected independently. This partition-level signal interpretation thus transforms a standard multiplexing readout into a direct measure of genome integrity.

Although multiplex assay designs are more complex, they offer enhanced analytical power for assessing vector genome integrity and may ultimately contribute to improved assessment of therapeutic efficacy and safety – making them a more comprehensive solution for gene therapy applications.

Key benefits of multiplex dPCR assays for genome integrity assessment

The benefits of multiplex assays such as NibaPlex® are both scientific and operational.

Scientific benefits

Multiplex assays may enable more accurate prediction of biological potency by linking complete genome titers with gene expression after transduction. As dosing is typically based on dPCR-derived titers, multiplex testing can further clarify how many of these measured genomes are truly intact and potentially biologically active, providing a more precise estimate of the effective dose delivered. While gene expression is an initial indicator, it is essential to show that the produced protein is functionally active and capable of generating activity levels that lead to the desired therapeutic outcome, such as disease correction. When viewing a dose from the perspective of numbers of complete genomes, a more informative correlation between the dose and therapeutic potency could be obtained.

Operational and manufacturing benefits

From an operational perspective, multiplex assays may give better insight into batch-to-batch consistency, support biologically meaningful release specifications, and provide a robust analytical foundation for comparability and change management. Multi-locus genome integrity assessment aligns with FDA and EMA expectations for comprehensive product characterization and risk-based quality management.

Beyond clinical implications, use of multiplex assays also offers significant advantages during development and manufacturing. Integrating genome integrity analysis early in process development enables:

- Rapid feedback and optimization of transfection conditions, plasmid design, and purification strategies.
- Support for a Quality by Design (QbD) approach
- Proactive control of critical process parameters (CPPs) that influence genome integrity.

The demonstrated robustness of NibaPlex® multiplex assays across commercial dPCR platforms confirms its suitability for ongoing genome integrity monitoring, that may ensure consistent therapeutic dosing and product quality control throughout the gene therapy manufacturing lifecycle.

Accurate characterization of the viral genome is critical and includes assessment of:

- Quantity (vector genome titer)
- Quality (complete versus incomplete genomes)
- Construct identity

These factors play a pivotal role in determining the success of gene therapy using rAAV, as the viral genome titer provides essential information for establishing both preclinical and clinical dosages required for an effective rAAV therapeutic product (Duong et al., 2025).

Comparability studies supporting viral vector manufacturing with genome integrity monitoring

Comparability studies are essential in viral vector manufacturing to ensure that production changes – including site transfers, scale-up, or batch-to-batch variability – do not compromise viral vector product quality and safety.

Using advanced multiplex dPCR assays for reliable quantification of complete vector genomes, genome integrity can be robustly monitored. Overall, combining detailed analytical testing and genome integrity assessment supports consistent product quality and regulatory acceptance during manufacturing changes. This approach provides actionable insights for comparability and change management.

By thoroughly assessing physicochemical and biological attributes, including potency, comparability studies offer regulators the necessary evidence that manufacturing changes do not compromise patient safety. Incorporating genome integrity evaluation into comparability frameworks facilitates smooth change management during clinical development and commercialization. This comprehensive approach helps maintain consistent quality of viral vectors throughout the manufacturing lifecycle.

Conclusion

Multiple studies suggest that rAAV gene therapy potency may be influenced by the proportion of capsids containing complete vector genomes. Only complete genomes support functional therapeutic gene expression, making genome integrity an important prerequisite for biological activity. Truncated genomes may be present despite high viral capsid titers but do not contribute to effective transgene expression. In both viral vector and cell-based therapies, genome integrity directly affects functional performance and safety. Therefore, genome integrity analysis can support potency evaluation, although functional assays are still required to confirm biological activity (ARM, 2023).

Empty and partially filled capsids act as product-related impurities, diluting the effective dose, competing for cellular uptake, and potentially increasing immunogenicity. In addition, viral vector genome size and construct design influence rAAV vector packaging efficiency.

Accurate genome integrity assessment is therefore essential for defining complete genome titers, establishing dose-response relationships and supporting prediction of clinical performance.

The recently released draft guideline from the British Pharmacopoeia, Characterisation of the Capsid Particle Population in rAAV Products: Determination of Vector Genome Identity, Integrity and Encapsidated DNA Impurities (BP Draft, 2026), further underscores the importance of standardized approaches to vector genome identity and integrity assessment. Such regulatory developments reinforce the need for analytical methods capable of reliably distinguishing complete and incomplete genomes.

In this context, multiplex dPCR assays, including platforms such as NibaPlex®, enable comprehensive, customized and high-resolution assessment of genome completeness. They provide a robust and scalable solution for multi-region genome integrity evaluation. By enabling discrimination between complete and structurally incomplete genomes within a single reaction, multiplex dPCR strengthens product characterization, supports comparability assessments, and enhances process understanding throughout development and manufacturing (Dobnik, 2023).

In conclusion, genome integrity represents a defining quality attribute for rAAV gene therapy products, with direct implications for safety and regulatory acceptance. A higher proportion of complete genomes may allow administration of a lower dose while still achieving the intended therapeutic potency. Comprehensive and standardized assessment of vector genome integrity is therefore essential for maintaining consistent product performance and ensuring patient safety.

Maximizing the proportion of complete genomes within viral capsids could improve efficacy, lower dosage, and minimize immune toxicity risks.

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