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White Paper

Navigating Next-Generation Quality Control Strategies for AAV Testing

Adeno-Associated Viruses (AAVs), first identified in the 1960s as a contaminant in adenovirus preparations, have since emerged as a cornerstone of gene therapy, owing to their non-pathogenic nature and simple genome. In the 1970s, AAV began to see increased research interest as a promising vector for delivering genetic material, gaining prominence in the growing cell and gene therapy sector. As of 2024, AAV-based therapies have achieved remarkable commercial milestones, with seven new and supplemental regulatory approvals, reflecting their transformative potential for treating genetic diseases.

However, the path to these successes has required, and will continue to require, overcoming stringent regulatory and quality control challenges. Developers must establish robust process and product controls to ensure the safety and efficacy of these products in clinical applications. As the regulatory landscape for gene therapies continues to evolve, quality control testing strategies for AAV products play a critical role in meeting industry expectations for defining and confirming Critical Quality Attributes (CQAs).

This whitepaper explores the essential considerations in quality control testing for AAV-based gene therapies, highlighting key methodologies and emerging technologies that enable precise characterization and validation of these innovative products. By incorporating these aspects, manufacturers can confidently navigate regulatory requirements and deliver life-changing therapies to patients worldwide.

Measuring Infectivity: Adapting the $TCID_{50}$ Assay for AAV Infectivity and Consistency in Gene Therapy

Adequately analyzing AAV infectivity is critical for gene therapy applications, as it directly correlates to the therapeutic efficacy of AAV-based treatments. Infectious titers determine the number of viral particles capable of delivering a functional genetic payload to target cells, ensuring consistent dosing. Proper analysis also helps identify suboptimal vector preparations, ensuring product quality and safety. Moreover, regulatory agencies require detailed characterization of infectivity as part of quality control, emphasizing its importance in meeting safety and efficacy standards for clinical use.

The $TCID_{50}$ assay, originally developed in the 1950s, has undergone significant advancements to become a cornerstone for measuring viral infectivity in gene therapy, particularly for adeno-associated virus. This assay determines the dilution at which 50% of cell cultures show cytopathic effects, a method adapted over time to specifically assess AAV vectors.

As AAV has gained prominence for gene therapy applications, the need for precise and consistent characterization methods has become paramount. Infectious titer, a CQA representing the number of viruses capable of delivering genetic material, requires robust quantification. In the early days, methods

for AAV infectivity were rudimentary, reflecting the complexity of AAV's dependency on helper viruses like adenovirus. Early adaptations involved permissive cell lines, such as modified HeLa cells expressing AAV-specific replication and capsid genes, to enhance the reliability of infectious titer measurements. However, advances in molecular biology, such as quantitative PCR (qPCR), have significantly improved sensitivity and specificity, transforming this paradigm and enabling deeper characterization.

Our validated $TCID_{50}$ platform, introduced in 2014, exemplifies this evolution. Designed with adaptability in mind, the assay incorporates a molecular endpoint targeting vector-specific promoters, enabling selective infectious titer quantification even in the presence of helper viruses. This flexibility allows customization for emerging viral vectors while maintaining regulatory compliance.

Over decades, We refined our $TCID_{50}$ platform to balance innovative technology with evolving regulatory standards. Automation and molecular advancements reduced assay variability, achieving positive control consistency with less than 0.3 log variability — well below the 1.0 log industry standard. These innovations set a benchmark for quality and reliability in AAV testing. Additionally, our semi-custom working model leverages its validated platform to expedite validation timelines for tailored assay components (**Figure 1**). By integrating custom elements into a pre-validated framework, this approach meets phase-appropriate validation requirements, streamlining development processes while maintaining the rigorous standards demanded for gene therapy applications.

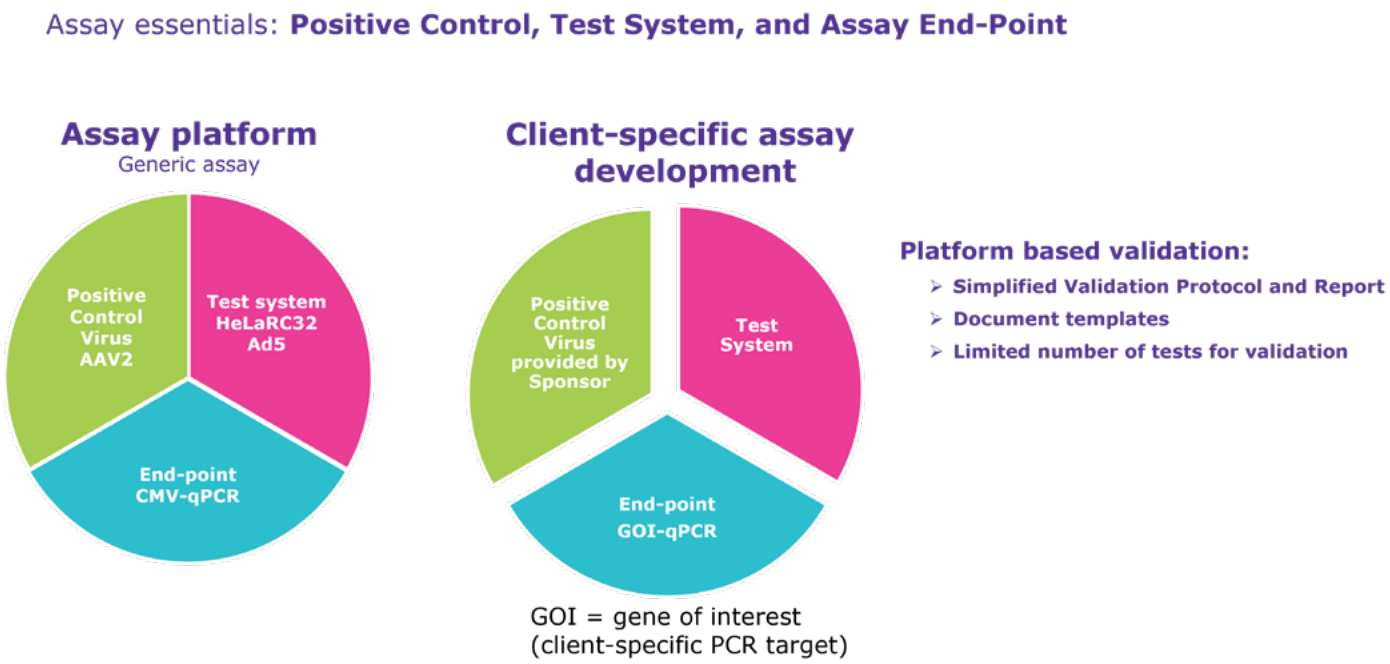
The $TCID_{50}$ assay's transformation from a basic virology tool to a sophisticated, adaptable platform highlights its critical role in AAV-based gene therapy. Its ability to deliver accurate, reproducible, and flexible results ensures it remains indispensable for the development and quality control of life-changing therapies.

Optimizing AAV Identification and Risk Mitigation With Next-Generation Sequencing

The precision and capability of Next-Generation Sequencing (NGS) make it a critical tool for ensuring the quality, identity, and safety of Adeno-Associated Virus gene therapy products. As AAV deliverables rely on the integrity of a gene sequence, thorough characterization using NGS allows for the detection of low-level variants and process inefficiencies early in development, ultimately safeguarding therapeutic outcomes. Some of the key benefits of leveraging NGS for this testing include:

Dual-Stage Testing for Risk Mitigation: The risk of low-level variants compromising AAV products underscores the importance of dual-stage testing — first at the plasmid stage and later during the final product release. By employing NGS early in the process, variants in the plasmid starting materials used in triple transfection production of AAV can be identified and corrected before they propagate into the final product, potentially saving millions in manufacturing costs. For example, there have been manufacturing scenarios wherein unidentified plasmid variants later surfaced as low-level gene mutations in the final AAV

Figure 1: AAV Infectivity: From generic to sponsor-specific assay



vector, a situation that could have been avoided with early NGS analysis. This approach also aligns with Quality by Design (QbD) principles, ensuring high-quality inputs lead to high-quality outputs.

Regulatory and Quality Assurance with NGS: Regulatory requirements increasingly emphasize sequencing of AAV vectors across multiple lots to confirm product identity and purity. Full-vector sequencing is essential to identify any subpopulations, and NGS is uniquely capable of unbiased, high-resolution analysis of entire AAV genomes. NGS can detect rare variants at frequencies as low as 1%, meeting regulatory expectations while providing deeper insights into the composition and quality of AAV vector preparations.

Long-Read NGS for Comprehensive Characterization: Long-read NGS technology enables a detailed characterization of encapsidated nucleic acids within AAV preparations, addressing packaging inefficiencies inherent to the manufacturing process. This includes assessing the content of full, partial, and empty capsids, as well as identifying impurities such as residual host cell DNA or plasmid sequences. By analyzing length distributions and verifying the integrity of Gene of Interest (GOI) sequences and Inverted Terminal Repeats (ITRs), long-read NGS supports process optimization and regulatory compliance. This detailed profiling not only ensures a high-quality final product but also improves process understanding, leading to safer and more effective gene therapies.

Advancing Process Development: NGS analysis informs manufacturing processes by identifying inefficiencies and ensuring robust encapsidation of target sequences. Such advancements enhance product consistency and safety, ultimately benefiting patients. By integrating NGS into the quality control pipeline, manufacturers can achieve the highest standards of AAV product quality, fulfilling both regulatory expectations and therapeutic goals. NGS serves as an indispensable tool in AAV gene therapy development, offering unmatched precision in identifying variants, ensuring regulatory compliance, and advancing manufacturing processes. Its integration at multiple stages of production reduces risk, ensures high-quality outputs, and supports the development of safer, more effective therapies.

The Importance Of Viral Clearance And The Value of Expertise

Viral Clearance (VC) is a critical aspect of ensuring the safety of AAV gene therapy products, guided by the updated ICH Q5A(R2) regulatory guidance. VC studies evaluate a downstream purification process's capacity to remove or inactivate potential viral contaminants, ensuring a sufficient level of viral safety in AAV-based therapeutics.

The nature of AAV as a non-enveloped viral vector presents unique challenges in viral safety. Unlike other biologics, such as monoclonal antibodies, AAV production involves fewer downstream purification steps, heightening the need for robust VC studies.

Regulatory agencies, as outlined in the ICH Q5A(R2) guidance, require VC studies for commercialized products and for certain manufacturing processes recommend them for early-phase AAV products to support Investigational New Drug (IND) or Investigational Medicinal Product (IMP) applications. These studies address safety risks associated with expression systems and production viruses, such as baculovirus or helper viruses.

A VC study involves spiking potential contaminants into downstream purification steps and evaluating the removal or inactivation efficiency. The viruses included in the study panel are selected based on the AAV expression system and associated risks of viral contamination. Each downstream process step, from chromatography to filtration, is independently tested to determine its specific contribution to viral clearance. The benefits of VC studies include:

- **Risk Mitigation:** Early-phase VC studies can identify and address contamination risks before commercialization, reducing the likelihood of safety concerns during clinical trials.
- **Regulatory Compliance:** Meeting the expectations of ICH Q5A(R2) ensures alignment with evolving global standards, facilitating regulatory approval.
- **Process Optimization:** Insights from VC studies inform the design of robust downstream purification steps, enhancing product safety and consistency.

As leaders in viral safety, we offer expertise in navigating ICH Q5A(R2) requirements. From assessing the need for VC studies to designing and executing them, we guide clients through regulatory complexities, ensuring their AAV products meet the highest safety and quality standards. Integrating viral clearance studies into AAV manufacturing is essential for regulatory compliance and patient safety, enabling the development of reliable and effective gene therapies.

Potency Assay Development and Testing for AAV-Based Gene Therapy

Potency assays are a critical component of AAV drug product (DP) and, in some cases, drug substance (DS) characterization. These quantitative measurements ensure consistency, quality, and biological activity, key indicators for regulatory approval. Below are the three primary types of potency assays:

Expression assays measure the levels of therapeutic proteins encoded by the AAV vector. After transduction into a suitable cell line, the analyte (protein of interest) is quantified to ensure a dose-dependent response. Detection methods include ELISA for secreted or intracellular proteins and flow cytometry for surface or intracellular staining. These assays provide precise analyte levels, crucial for determining expression potency.

Functional bioassays assess the biological activity of the therapeutic protein, reflecting its intended mode of action. These assays require a detailed understanding of the protein's function and signaling pathways. Common readouts include cell viability, enzymatic activity, or engineered luminescence signals for simpler quantification. Functional bioassays are typically developed later in the drug's lifecycle for in-depth characterization.

Relative potency assays address the variability of functional bioassays by using a dose-response curve to compare new batches against a reference standard. This approach quantifies potency as a percentage relative to the reference material (e.g., EC50-based calculation to assess the effective drug concentration) while ensuring curve similarity (parallelism). Such assays enable robust batch-to-batch comparisons, improving reliability over time.

By integrating expression, functional, and relative potency assays, a comprehensive framework for evaluating AAV potency is established, meeting regulatory requirements and ensuring high-quality therapeutics.

Conclusion

AAV-based therapies have transformed the landscape of gene therapy, offering groundbreaking potential to treat genetic diseases. However, their development and manufacturing require precise quality control strategies to ensure safety, efficacy, and regulatory compliance. Critical methodologies, including infectivity analysis, next-generation sequencing for risk mitigation, viral clearance studies, and potency assays, form the backbone of a robust AAV testing framework. By integrating these advanced tools and approaches, manufacturers can confidently meet regulatory expectations, optimize processes, and deliver high-quality, life-changing therapies to patients worldwide.

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