Dual-Platform NGS for Comprehensive Characterization of Engineered rAAV Vector Integrity

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Introduction

Recombinant AAV (rAAV) vectors are central to gene therapy, but their complexity presents ongoing analytical challenges. As engineered capsids with enhanced tissue targeting and improved safety profiles enter clinical development, precise characterization of vector genomes and packaged DNA becomes increasingly important.

Traditional assays such as **ddPCR**, **analytical ultracentrifugation (AUC)**, and gel electrophoresis provide critical information on quantity and size but fall short in resolving sequence-level details like ITR heterogeneity, truncation patterns, and structural variants. While **short-read sequencing (SRS)** offers high-throughput quantification of genomic heterogeneity, it lacks the ability to assess full-length genomes and structural integrity. Conversely**, long-read sequencing (LRS)** platforms reveal structural features but with limited quantitative precision.

To overcome these limitations, we developed a **dual-platform** NGS approach that combines the strengths of Illumina and **Oxford Nanopore Technologies (ONT)** sequencing. This integrated strategy enables comprehensive assessment of vector genome integrity and encapsidated DNA species with orthogonal validation across multiple assays—and was instrumental in supporting our first **IND approval**.

Methods and Materials

Production of rAAV and Dual-Platform Sequencing Workflow

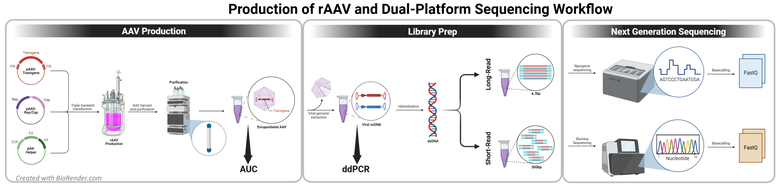

Figure 1. rAAV vectors were produced in HEK293 cells using standard triple transient transfection with pRepCap, pAd helper, and pGOI plasmids. Post purification, viral genomes were extracted, allowing sense and antisense strands to hybridize into double-stranded DNA. Samples were then split into two groups for platform-specific library preparation. Illumina and ONT sequencing were conducted to enable complementary quantification and sequence analysis.

Bioinformatics Analysis Pipeline

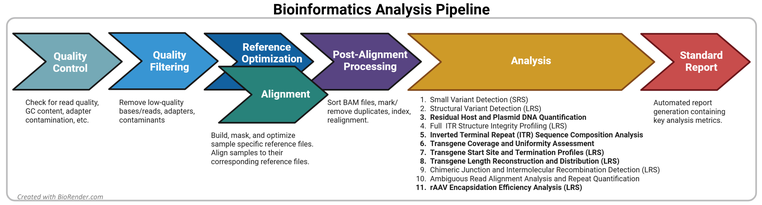

Figure 2. Raw sequencing data undergo quality control and filtering, followed by reference optimization. Reads are then aligned to the optimized reference, with subsequent platform-specific post-alignment processing and downstream analysis.

Results and Discussion

Benchmarking NGS Against ddPCR and AUC for Quantitative Vector Assessment

Correlation of AUC and LRS-Based Packaging Efficiency of Manufactured Drug Product

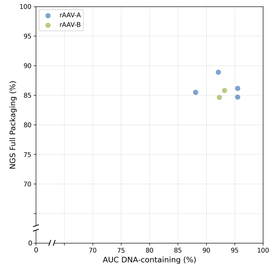

Figure 3. AUC and ONT analysis reveal strong correlation and high packaging efficiency (>80%) across vectors. Scatter plot compares DNA-containing capsid percentages measured by AUC and full-length read quantification by ONT sequencing.

Visualizing Residual Quantification via ddPCR, ONT-LRS, and Illumina-SRS

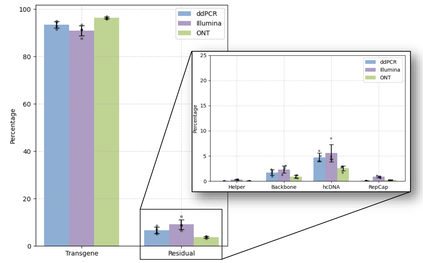

Figure 4. Cross-platform residual quantification from orthogonal methods—ddPCR (blue), Illumina (purple), and ONT (green)—supports concordant quantifications with increased variance at low-frequency residuals due to platform-specific limits of quantification (LoQ).

Compositional and Structural Insights from NGS-Based rAAV Characterization

Drug Product Inverted Terminal Repeat (ITR) Sequence Characterization by LRS

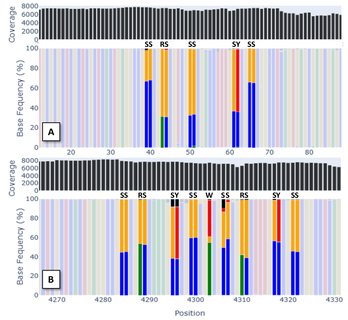

Figure 5. ONT analysis of ITR sequence composition and structural orientations shows expected characteristics. Windowed base composition and coverage analysis across the (A) left and (B) right ITRs reveals positions of significance (*divergence ≥ 15%*) at sites consistent with flip/flop conformations, quantifying known AAV ITR dynamics.

Limitations of SRS NGS: Sequencing Coverage and Sequence Complexity Evaluation

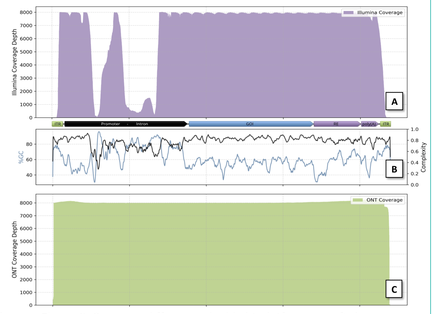

Figure 6. Coverage differences highlight platform-specific bias in rAAV sequencing. (A) Illumina and (C) ONT transgene coverage profiles shown with (B) GC content and sequence complexity. Illumina coverage drops in high-GC, low-complexity regions, while ONT remains stable.

Results and Discussion

Detection of Fragmentation Patterns and Packaging Consistency in rAAV Genomes using LRS

High-Sensitivity Scatter Plot of Drug Product Transgene LRS Alignment Coordinates

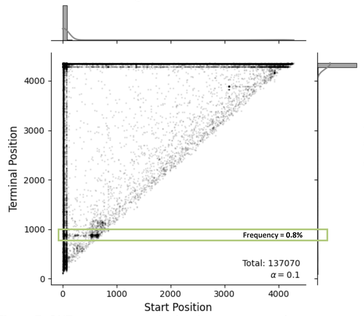

Figure 7. ONT mapped read distributions reveal consistent start and stop sites, indicating successful capture of full-length rAAV genomes. High-sensitivity ($\alpha = 0.1$) allows capture of low-frequency patterns, highlighting potential structural irregularities (e.g., fragmentation bias, structural variants, sequencing artifacts, and alignment artifacts).

LRS Read Length Reconstruction and Distribution of Manufactured Drug Product Transgene Alignments

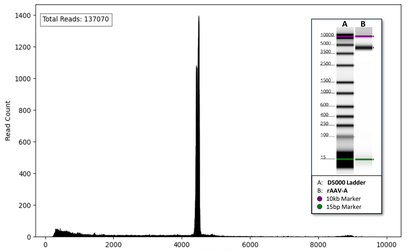

Figure 8. ONT alignment profiles confirm full-length genome predominance in rAAV drug product preparations. LRS reveals a dominant peak at the expected length, consistent with TapeStation and AUC results; deviations can suggest truncated genomes, sequencing artifacts, or alignment artifacts, informing product integrity.

Conclusions

The **dual-platform** sequencing strategy enhances analytical resolution across key quality attributes of rAAV vectors, enabling more complete and confident product characterization.

- **Structural clarity**: LRS resolves genome size, ITR configurations, truncation patterns, and structural variants.
- **Quantitative precision**: SRS accurately quantifies low-abundance species, including residual plasmid DNA and packaging byproducts.
- **Cross-platform validation**: NGS results correlate well with ddPCR, AUC, and gel-based assays, while providing deeper insight into genome integrity.
- **Process insight**: Identifies fragmentation hotspots, dropout regions, and structural artifacts that inform upstream and downstream process improvements.
- **Regulatory alignment**: Enhances assay robustness and reproducibility to meet evolving expectations for gene therapy product release and comparability.

Together, these capabilities directly support **CMC frameworks** by enabling robust, high-resolution characterization of rAAV drug products—improving quality control, accelerating development, and de-risking regulatory submissions, with this analysis serving as a standard component supporting our recent **IND approval**.

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Check out Capsida's other ASGCT presentations!

Presented on TUESDAY
- rAAV Manufacturing Solutions: Strategic Designs of Engineered rAAV Two Plasmid Systems for Cost Effective Scaling and Product Safety, 5:30 – 7:00 PM CT, Abstract Number: 962, Presenter: Jenna Rodden, Senior Research Associate, Capsida

Presented on WEDNESDAY
- Systemic AAV Gene Therapy with Next Generation Engineered Capsid Demonstrates Expression Levels Supporting Potential Therapeutic Benefit for CNS, Cardiac, and Sensory Symptoms in Friedreich's Ataxia, 1:45-2:00 PM CT, Abstract Number: 75, Presenter: Celeste Stephany, Ph.D., Director of CNS and Ophthalmology Preclinical Research, Capsida
- Identification of Multiple Novel Blood-Brain-Barrier Receptors for CNS Gene Therapy and Other Drug Modalities via an Integrated AAV Capsid Engineering Platform, 2:45 – 3:00 PM CT, Abstract Number: 93, Presenter: Nick Goeden, Ph.D., Founder, Chief Technology Officer, Capsida
- Systemic Gene Therapy CAP-002 Demonstrates Potential for Disease-Modifying Treatment of Seizures and Motor and Cognitive Deficits of STXBP1-DEE Using an Engineered, CNS-Targeted AAV, 3:45-4:00 PM CT, Abstract Number: 123, Presenter: Nick Flytzanis, Ph.D., Founder, Chief Research and Innovation Officer, Capsida
- CAP-003, a CNS-Targeted IV-delivered AAV Gene Therapy, Safely Increases Brain GCase in NHPs to Level Supporting Potential Normalization of Activity in PD-GBA Patients, 5:30 – 7:00 PM CT, Abstract Number: 1435, Presenter: Kim McDowell, Ph.D., Director, Preclinical Research, Capsida

Presented on THURSDAY
- Development of a Novel Automated Loading Approach Which Significantly Reduces Processing Time for Enriching Full AAV Capsids Using Ultracentrifugation, 5:30 – 7:00 PM CT, Abstract Number: 1833, Presenter: Varun Gejji, Ph.D., Senior Scientist, Capsida