Redefining rAAV Vector Identity and Quality Control using Orthogonal Long-Read DNA Sequencing

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Introduction

Reliable assessment of recombinant adeno-associated virus (rAAV) identity and integrity remains a critical bottleneck in gene-therapy manufacturing. Conventional assays and short-read DNA sequencing approaches fail to comprehensively capture vector genome heterogeneity or truncations and contaminating DNA species, limiting their value for reliable product quality assessment. Here, we present an integrated rAAV analytical and quality-control platform that combines orthogonal long-read sequencing technologies with a standardized, “bias-aware” bioinformatic pipeline.

Methods

Viral vector-encapsidated DNA is extracted and processed under rigorously controlled conditions to minimize technical variability. Sequencing is performed using both Single-Molecule Real-Time (SMRT; PacBio) and Oxford Nanopore Technologies (ONT), enabling complementary and cross-validated characterization of rAAV genomic DNA.

Results

The two platforms were systematically benchmarked for sequencing yield, read quality, vector mappability, detection of intact versus partial genomes, and sensitivity for identifying host-cell and plasmid-derived DNA. Duplex digital PCR was used as an orthogonal reference method to validate vector genome integrity. We further identified and quantified platform-specific biases introduced during library preparation and sequencing. SMRT sequencing showed preferential recovery of longer DNA fragments and sensitivity to second-strand synthesis conditions, whereas ONT enabled direct single-stranded DNA analysis with reduced length bias but lower per-read accuracy. To address these limitations, we implemented internal process controls that allow correction of platform-specific biases during downstream analysis. A dedicated bioinformatic pipeline was developed to quantify full-length ITR-to-ITR genomes and to sensitively detect rep-cap, host-cell, and plasmid DNA contaminants. By integrating raw sequence data with experimentally derived bias-correction parameters, the pipeline delivers a more accurate and realistic representation of rAAV products quality.

Conclusion

In conclusion, this control-driven, orthogonal long-read sequencing framework establishes a robust and scalable approach for defining more reliable critical quality attributes (CQAs) of rAAV vectors, supporting improved process understanding and more confident decision-making in preclinical and non-clinical development.