A Surrogate Assay to Monitor Replication-competent AAV Formation Using Nanopore Long-read Sequencing

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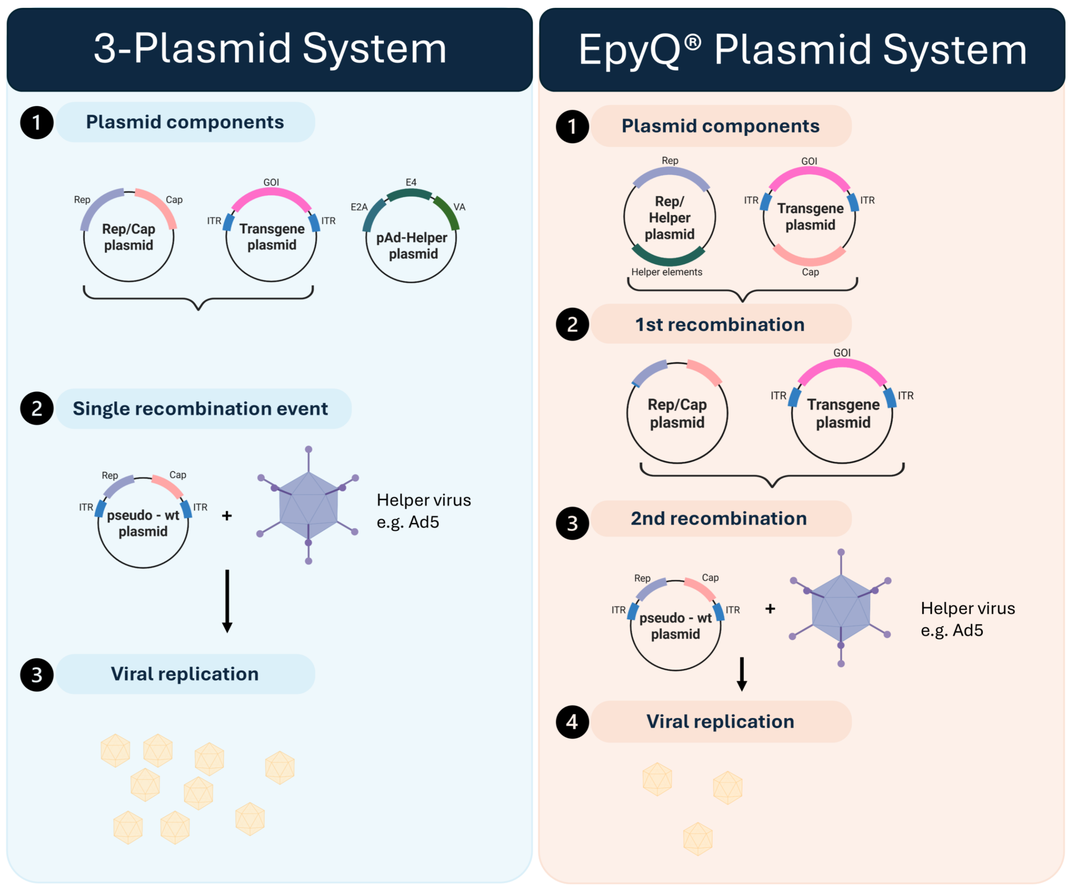
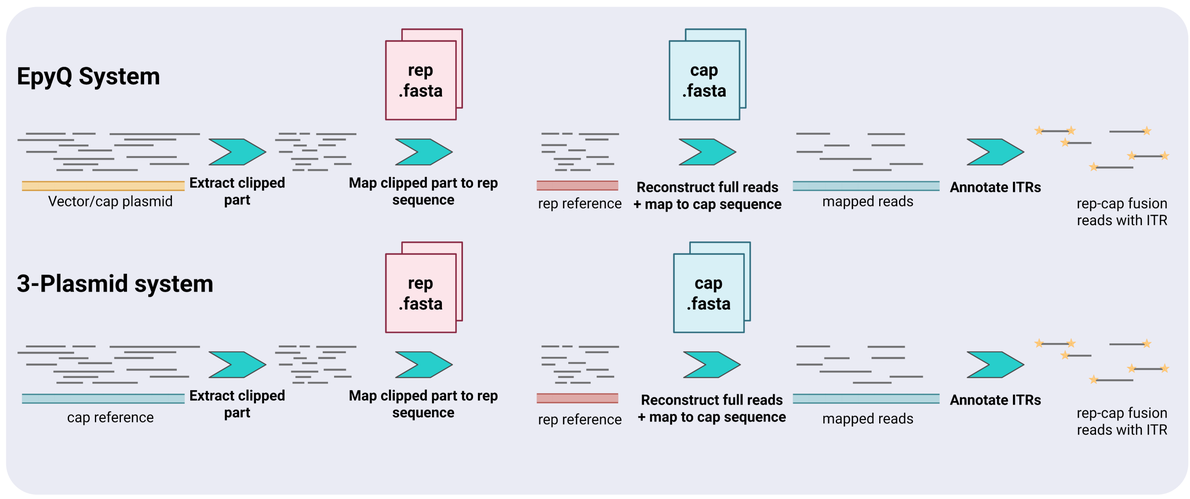
Introduction

Recombinant adeno-associated virus (rAAV) is the most common vector for *in vivo* gene therapy with an overall very favorable safety profile. However, a safety concern is the formation of replication-competent (rc)AAVs during manufacturing by non-homologous recombination of the ITRs with rep and cap sequences leading to a virus with wild-type like genome composition that can be mobilized in presence of helper virus. To this end, regulators demand rigorous analytics to demonstrate that rcAAVs are below a predefined threshold. The current gold standard are cell-based assays, that use several passages of the rAAV vector batch in the presence of a helper virus such as Ad5 to amplify potential traces of rcAAV. The detection of rcAAVs is achieved by ddPCR or qPCR with ITR-rep spanning primers. However, this assay has several limitations as it is time consuming, resource intense and does not allow for a rapid comparison of multiple transgene designs or manufacturing strategies. Here, we present a surrogate assay to estimate the rcAAV formation potential based on nanopore sequencing. In contrast to Illumina short read sequencing, nanopore sequencing can quantify rep/cap and rep/cap/ITR associations within a single read covering the entire rAAV vector genomes. Notably, this assay can be carried out simultaneously with the general rAAV sequencing workflow at Ascend without specific adaptions to the wet lab pipeline and can guide candidate selection and CMC development in high throughput.

Model of rcAAV Formation in 3-Plasmid and EpyQ® System

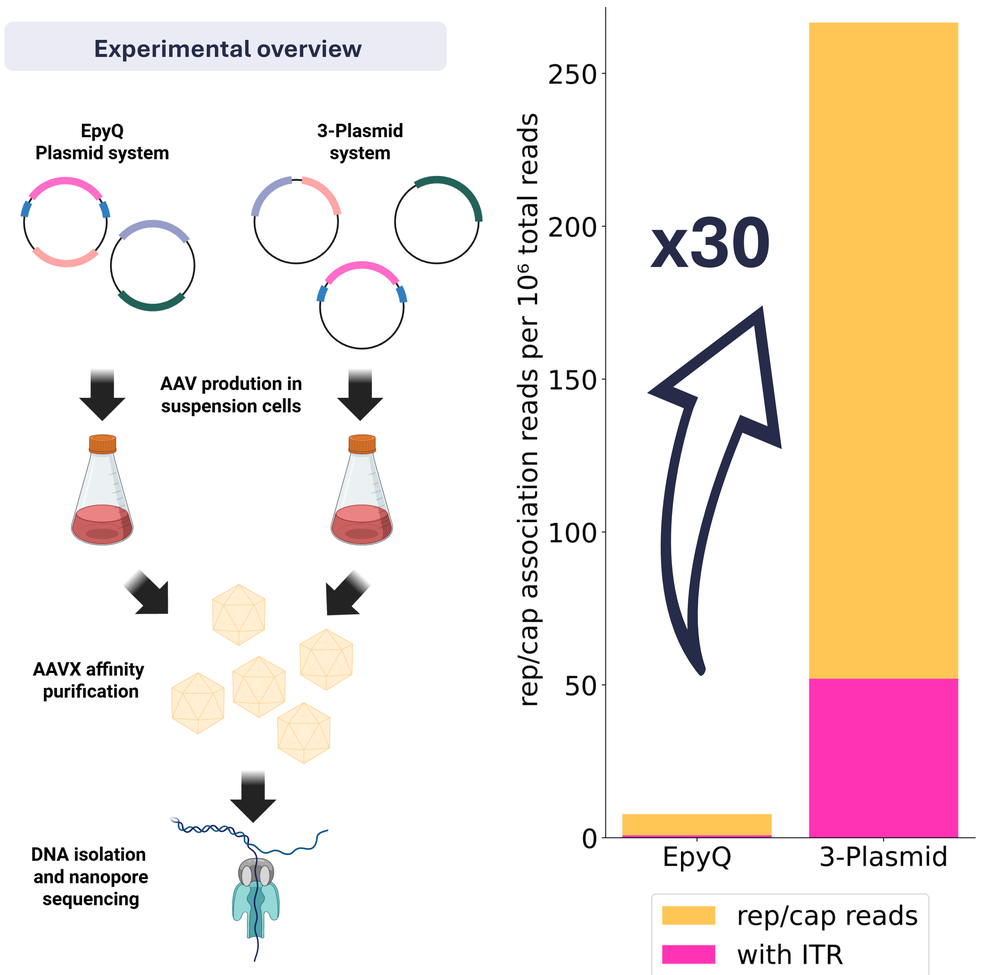


Bioinformatic Pipeline for rep/cap Association Detection



- The pipeline filters out any reads that contain both rep and cap sequences but does neither verify completeness of the genes and ITRs nor their correct order. To this end filtered out reads reveal the recombination potential of the vectors providing the first step for rcAAV formation, not genuine rcAAVs
- The entire pipeline was implemented in a fully automated bash script to run efficiently and consistently

Conventional 3-Plasmid System Demonstrated >30-Fold Greater Potential for rcAAV Generation Compared with EpyQ



Comparison: Cell-based Standard Assay vs Nanopore Sequencing

Feature	Cell-based rcAAV assay	Nanopore surrogate assay
Detected species	Genuine rcAAVs	Rep/cap/ITR associations
Cost	High	Low
Assay time	Very long (≥ 4 weeks)	Very fast (< 3 days)
Effort in lab	Very high	Low
Throughput	Low	High
Quantification	Limited (amplification of virus)	Easy and precise
GMP QC assay	Available	No

The nanopore surrogate rcAAV assay is especially suited for early-stage comparisons between manufacturing platforms or plasmid systems

Summary

Nanopore sequencing offers the opportunity for a quick and easy to implement surrogate assay for the rcAAV formation potential.

- Reveal rep/cap/ITR associations in a single long read
- Cost saving
- Time saving
- Enabling next-generation platform development

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