Optimizing rAAV yield and quality using a multi-output DoE approach applied to a fully characterized HEK293 cell line

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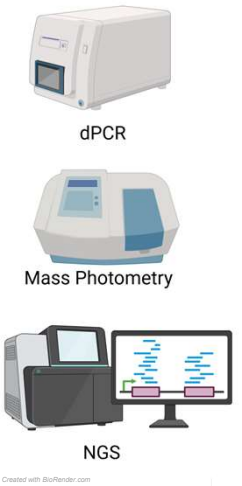
Introduction

- NewBiologix has developed its proprietary Xcell™ Eng-HEK293 cell line, optimized for efficient plasmid transfection and rAAV production.
- Extensive genomic and phenotypic analyses have demonstrated the stability of Xcell™ Eng-HEK293.
- Prior studies have confirmed its superior performance across multiple rAAV capsids and transgenes when compared with other commercially available cell lines.
- Efficient rAAV manufacturing, however, requires careful optimization of multiple parameters, including media composition, choice of transfection reagent, cell density, and plasmid ratios, among others.

Objective

To establish a multi-parametric Design of Experiments (DoE) framework for systematically optimizing rAAV manufacturing conditions in the NewBiologix Xcell™ Eng-HEK293 cell line, with the goal of maximizing **(i) vector titers, (ii) viral genome integrity, and (iii) packaging efficiency**.

Outputs



- Titer: genome copies / mL
- Genome Integrity assessment
- Empty / full / partial / over-full particles
- Particle titers: particles / mL
- Encapsidated DNA
  - ITR-GOI-ITR
  - Rep/Cap/Helper
  - Host Cell
  - Plasmid
  - Other
- Genome Integrity
  - Intact
  - Left / right partials

Figure 1. Outputs used to feed the design of experiment (DoE) algorithms for the optimization of rAAV production in Xcell™ Eng-HEK293. Data from digital PCR, mass photometry and next generation sequencing (NGS) were used to evaluate and optimize rAAV production parameters.

High titers do not ensure high integrity, nor full particles

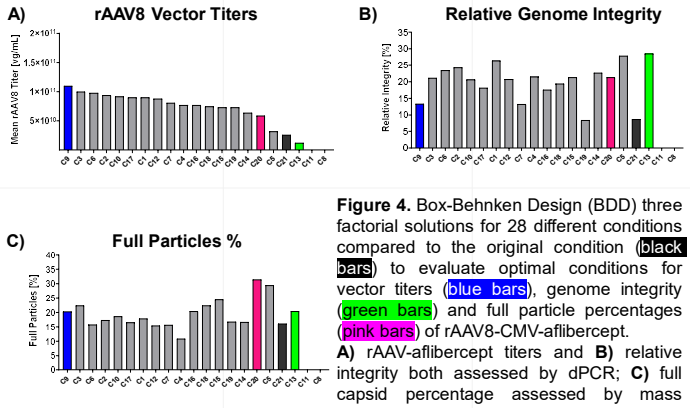


Figure 4. Box-Behnken Design (BDD) three factorial solutions for 28 different conditions compared to the original condition (black bars) to evaluate optimal conditions for vector titers (blue bars), genome integrity (green bars) and full particle percentages (pink bars) of rAAV8-CMV-aflibercept.
A) rAAV-aflibercept titers and **B)** relative integrity both assessed by dPCR; **C)** full capsid percentage assessed by mass photometry.

Finding balance using multi-output approach

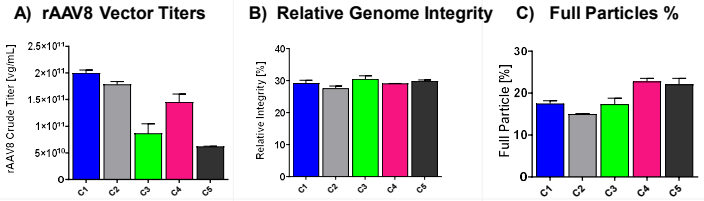


Figure 5. A multi-output approach finds optimal conditions for vector titers (blue bars), genome integrity (green bars) and full particle percentages (pink bars) for rAAV8-CMV-aflibercept production. **A)** rAAV8-CMV-aflibercept titers and **B)** relative integrity both assessed by dPCR; **C)** full capsid percentage assessed by mass photometry.

Table 1. DoE effects on encapsidated DNA species determined by Oxford Nanopore Technologies (ONT) sequencing

	Condition 1	Condition 2	Condition 3	Condition 4	Condition 5
ITR-GOI-ITR	72.5%	75.2%	74.3%	69.0%	80.9%
Rep/Cap/Helper	1.7%	2.2%	2.1%	1.1%	1.9%
Host Cell DNA	13.4%	9.9%	11.0%	18.4%	5.6%
Plasmid backbone	1.4%	1.7%	1.7%	0.9%	1.2%
Other	11.0%	11.0%	10.9%	10.6%	10.4%

Media formulations affect titers and encapsidation

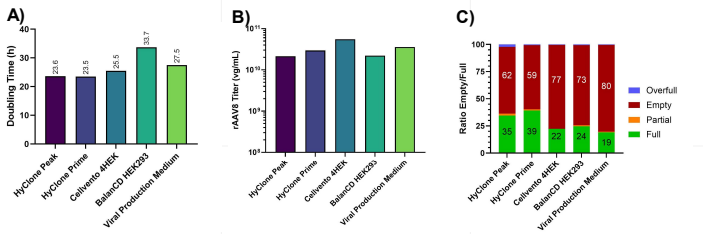


Figure 2. Performance of Eng-HEK293 in different media formulations. **A)** doubling time, **B)** rAAV8-GFP production by dPCR and **C)** ratios of empty/full rAAV by mass photometry were assessed to identify media formulations that result in optimal performance. Transfection conditions and plasmid ratios were maintained constant.

Design of Experiment models

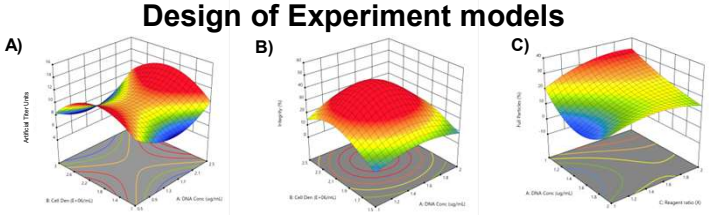


Figure 3. Surface 3D plots showing models for optimal rAAV production conditions. Predicted effects of cell density vs plasmid DNA concentration on: **A)** vector titers and **B)** genome integrity. **C)** Predicted effect of DNA concentration vs plasmid DNA concentration on full capsid percentage. Models were performed in Stat-Ease Design Expert.

Conclusions

- Xcell™ Eng-HEK293 is a fully-characterized, clonal HEK293 cell line specifically for viral vector production.
- Using a multi-parametric Design of Experiment (DoE) approach, we systematically optimized rAAV manufacturing conditions. Key outputs included cell doubling time, vector titers, empty/full capsid ratios, genome integrity, and vector quality as assessed by dPCR and long-read sequencing.
- Through this strategy, vector titers were increased from ~1×10^{10} vg/mL to 2.5×10^{11} vg/mL, while relative genome integrity improved from ~20% to >30% and full capsid percentages increased from ~10% to ~25%.
- Conditions that maximize titers did not necessarily yield the highest-quality vectors.

This multi-output approach **places vector quality at the center of rAAV production**, integrating both the Xcell™ Eng-HEK293 cell line and the NewBiologix rAAV Analytical Platform to advance the development of high-performing rAAV manufacturing systems.