Design-of-experiment (DoE) approach suggests transfection viable-cell density as key parameter for optimized rAAV productivity and total DNA amount for rAAV packaging

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Abstract

Transient triple-plasmid transfection of HEK293 cells is one of the main production methods employed for clinical and commercial recombinant adeno-associated virus (rAAV) manufacturing[1,2]. There is currently a challenge to meet the rAAV demand for clinical trials, making it essential to enhance the yield per cell while obtaining the maximum amount of genome-containing capsids. We believe transfection efficiency is critical for increased rAAV productivity. The efficiency of gene delivery relies on physicochemical properties of the transfection components such as total DNA amount or transfection reagent to DNA ratio, as well as on cell physiology[3].

Response surface methodology (RSM) statistical model, using a Design of Experiment (DoE) approach with JMP® statistical software[4], was selected to analyze the effect of two of the main factors impacting on rAAV productivity and packaging: viable cell density (VCD) and total DNA amount. Titration showed that transfection VCD has a strong impact on productivity with negligible impact due to very low or high total transfection DNA amounts. Conversely, a decrease in the amount of DNA during transfection correlates with an increase in the percentage of full capsids as measured by Analytical Ultracentrifugation (AUC) or Mass photometry (MP). DNA:PEI complex characterization indicated that increasing total DNA amounts led to greater complex size measured by Dynamic Light Scattering (DLS), which may suggest a decrease in transfection efficiency and therefore a lower packing capacity.

Introduction

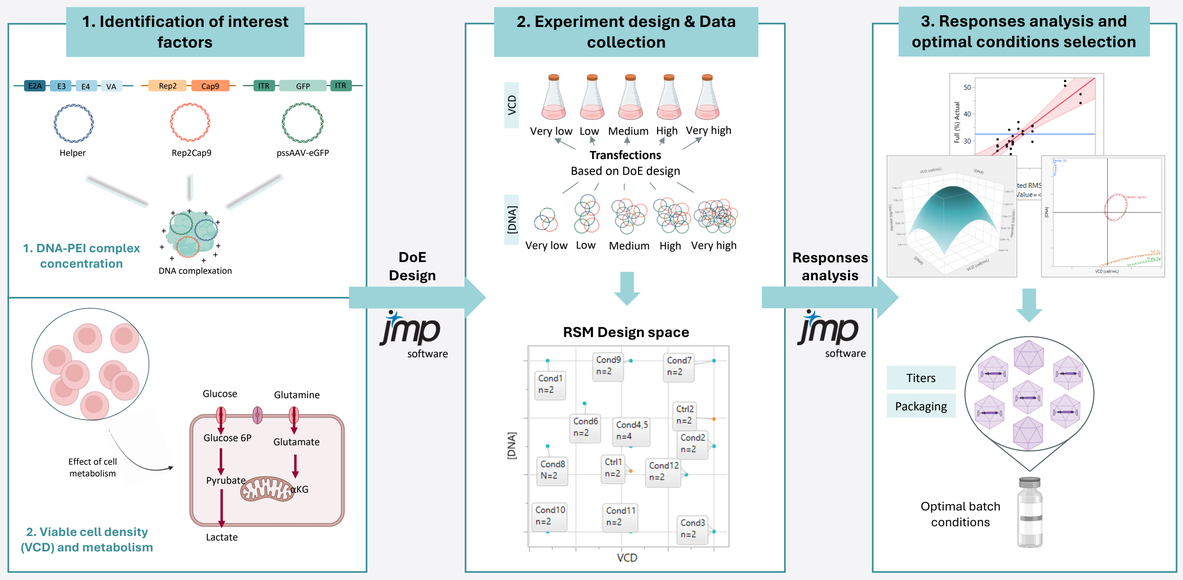
Viralgen has used the HEK293-derived Pro10™ suspension cell line in conjunction with its triple transfection platform process to produce both standard AAV serotypes and novel vectors applied in 29 approved clinical trials worldwide. There is a clear demand for viral vectors, which translates into a need to optimize the production process and increase productivity.

Design of Experiment (DoE) is a mathematical approach to design an experiment, interpret the results and make the choice of an optimal condition. DoE methodology is suitable to analyze various factors at a time and helps obtaining the greatest output by selecting the optimal combination of factors to maximize the desired response[5]. DoEs have started to be used in many fields as an alternative to the classic one-factor at a time approach, as it helps obtaining more responses in a unique experiment, decreasing costs and obtaining the desired outcomes faster[5,6].

Transfection DNA amount is usually calculated based on VCD. However, it is becoming increasingly clear that transfection is a multifactorial process where numerous variables simultaneously interfere with the final efficiency. In this study, a DoE was performed to address the question of how these multiple factors influence transfection efficiency in large-scale batches.

Experiment design

Figure 1. Aiming to optimize high-quality rAAV generation in a suspension-cell based production platform, the impact of transfection VCD and DNA concentration were evaluated by DoE approach. JMP statistical software was used for DoE design and evaluation, both DNA and VCD concentration considered continuous factors with a RSM design, and rAAV productivity and packaging efficiency were determined as outcome responses. A design of 12 conditions was computed by the software, which was performed in duplicates to give consistency to the experiment, and two additional conditions were added as controls (represented in orange in the design space scatterplot). Productivity was measured in the bulk harvest by polymerase chain reaction (PCR), and three orthogonal methods were used to evaluate the packaging efficiency: SEC-HPLC, AUC and MP. Figure created with BioRender.com.



Process parameters characterization

Cell metabolism

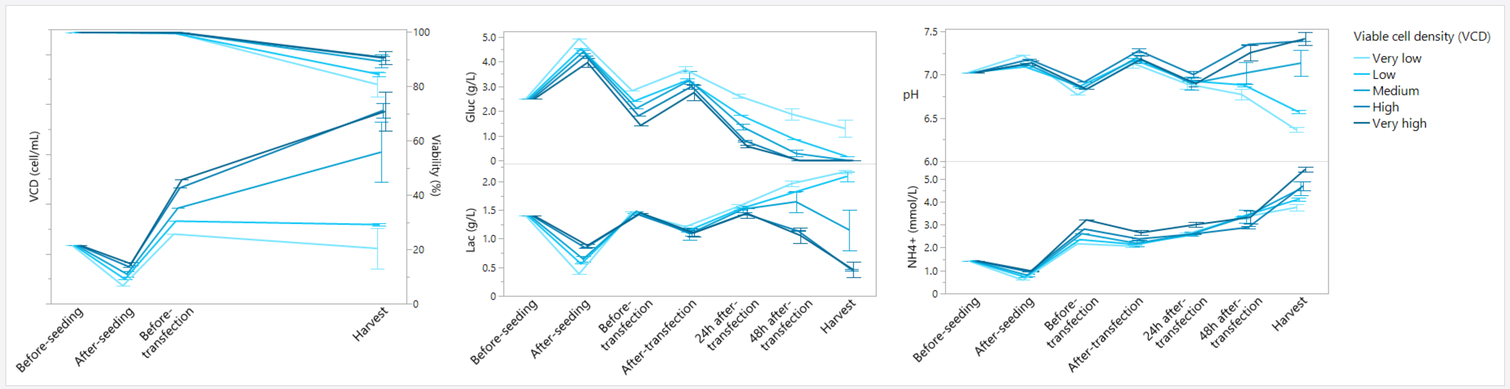


Figure 2. Distinct metabolic trends observed due to different VCD with negligible impact due to the DNA concentration used on transfection. Pro10™ cells were seeded at five different cell densities in shake flasks 48h before-transfection. Cells were split into different shake-flasks for transfection according to the DoE design (Fig. 1). VCD was determined by Vi-CELL™ XR counter before and after seeding, before transfection and on harvest. pH and cell metabolites were also measured before and after seeding and transfection, as well as daily after transfection using BioProfile® FLEX2 automated cell culture analyzer. pH, pO2, pCO2, glutamine, glutamate, glucose (Gluc), lactate (Lac), ammonia (NH4+), sodium, potassium and calcium were measured, but only the most significantly affected metabolites are presented above.

Cells transfected at lower cell densities presented lower VCD at harvest as expected, but lower viabilities were observed. No prominent impact on cell metabolism was observed due to very low or very high DNA concentration. In this sense, lactate re-consumption was observed in high and very high VCD 24h after transfection and 48h after transfection in medium VCD, along with glucose depletion. Accordingly, pH increment was observed in medium, high and very high cell densities.

PEI/DNA Complex characterization

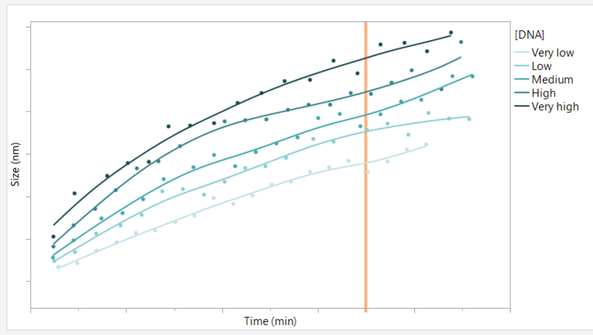


Figure 3. PEI/DNA complex sizes increased with higher PEI/DNA concentrations. PEI/DNA complexes were formed by adding first helper, repcap and transgene plasmids into culture media. Then, PEI transfection reagent was added to initiate complexation. Different cocktail concentrations were prepared according to the DoE design. After a specific complexation time they were added to the cells as showed in the scatterplot above (Fig 1). An aliquot of the transfection cocktail was measured by DLS with the Malvern Zetasizer Advance Ultra Red instrument from the beginning of the complexation to characterize the size of the complexes at the different DNA and PEI concentrations.

Increasing DNA and PEI concentration led to greater PEI/DNA complex sizes from the starting point of complexation. They continued increasing their size proportionally over time, entailing different complex sizes at the time of transfection (orange line).

Results & Discussion

Productivity

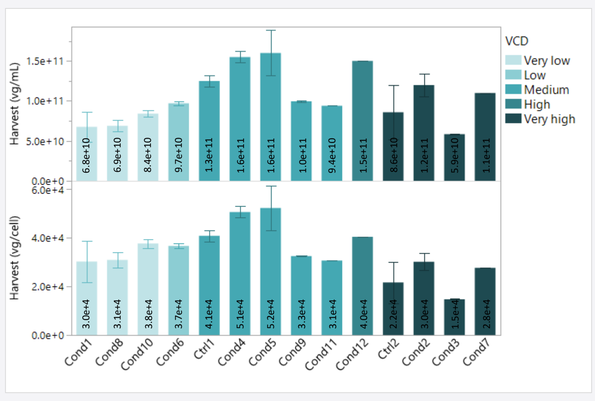
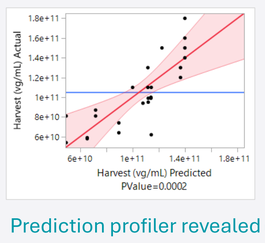


Figure 4a. Productivity is dependent on cell concentration. PCR rAAV titers from the crude harvest (after cell lysis and clarification) are shown in the graph, represented as rAAV concentration in viral genomes per mL (vg/mL) and viral genomes per cell (vg/cell).

Low titration in very high VCDs might be attributable to early nutrients depletion after transfection. Nevertheless, medium-to high VCDs presented greater productivity per cell than very low or low VCD conditions. This implies that having abundant nutrients in the media is not enough to boost rAAV generation. Transfection at too low cell density could cause toxicity, or autocrine factors might be insufficient to handle rAAV production.

DoE analysis provided a robust model for productivity. VCD resulted the most significant factor for productivity



Effect summary

Source	p value
VCD*VCD	0.00037
[DNA]*[DNA]	0.00499
VCD	0.06759
VCD*[DNA]	0.07597
[DNA]	0.62614

Figure 4b. Confidence curve for the line of fit on the effect leverage plot crossed the horizontal blue line representing the hypothesis, which means that the harvest productivity response was significant.

Quadratic effect of VCD resulted the most significant factor for productivity at harvest.

Prediction profiler revealed optimal titers would be obtained in medium to high cell density

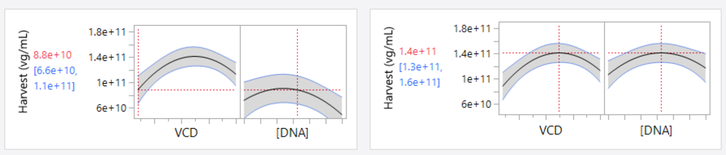


Figure 4c. Left profiler shows that independently of the DNA concentration used, low titers would be expected when selecting a too low cell density, and the profiler on the right shows the condition targeting the maximum desirability titers.

Packaging

Packaging efficiency decreased with higher DNA concentrations independently of the analysis method used

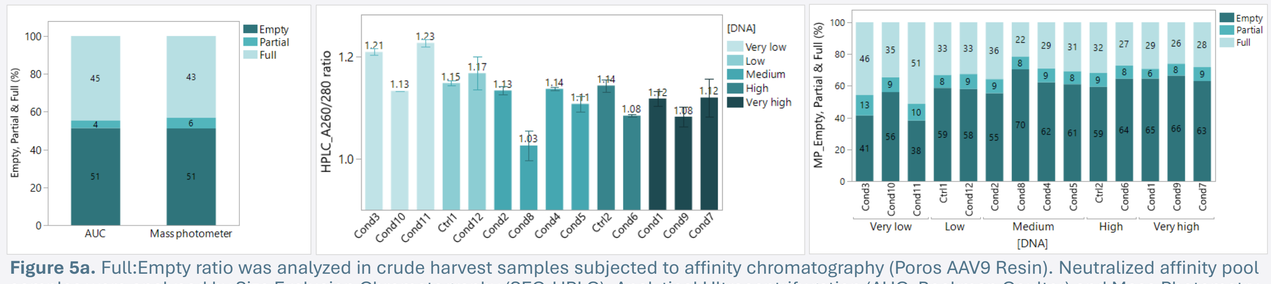
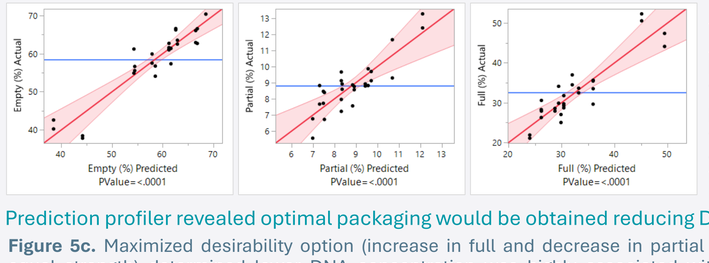


Figure 5a. Full:Empty ratio was analyzed in crude harvest samples subjected to affinity chromatography (Poros AAV9 Resin). Neutralized affinity pool samples were analysed by Size Exclusion Chromatography (SEC-HPLC), Analytical Ultracentrifugation (AUC, Beckman Coulter) and Mass Photometry (MP, SamuxMP). As previously described, our data also demonstrate no significant differences for F:E ratio between AUC and MP (left panel)[7]. Thus, MP was selected as a fast and simple orthogonal method. Both SEC-HPLC (Abs 260/280 nm) and MP show a certain correlation between the DNA concentration and percentage of filled capsids.

DoE analysis provided a robust model for empty, partial and full capsid percentages. DNA concentration showed greatest impact on packaging



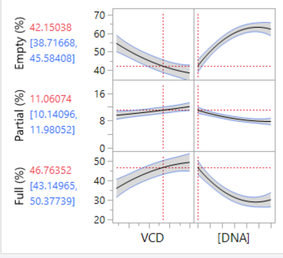
Effect summary

Source	p value
[DNA]	<0.00001
VCD	0.00001
[DNA]*[DNA]	0.00002
VCD*[DNA]	0.01895
VCD*VCD	0.13006

Figure 5b. Confidence curve for the line of fit on the effect leverage plot crossed the horizontal blue line in all cases, meaning that empty, partial and full particles percentage responses were significant.

Prediction profiler revealed optimal packaging would be obtained reducing DNA concentration

Figure 5c. Maximized desirability option (increase in full and decrease in partial and empty percentages with equal strength) determined lower DNA concentration was highly associated with increment on full capsids percentage. A possible explanation could be the size of the PEI/DNA complexes, which increase with larger DNA amounts. Larger complexes might obstruct cell entry as well as alter helper:repcap:transgene ratio, which could affect on transgene replication efficiency, and thus cause a decrease in encapsidation. A trend towards higher VCD incrementing the percentage of full capsids can also be observed. That might be a consequence of toxicity caused by PEI/DNA complexes in low VCDs, only being able to generate empty capsids and hindering transgene packaging, which comes at later timeframes. Despite the response of partial capsids fitted to the model, the percentage did not vary considerably, and therefore only empty and full percentage responses were used for optimal conditions selection.



Optimal conditions selection

Effect summary

Source	p value
[DNA]	<0.00001
VCD	0.00001
[DNA]*[DNA]	0.00002
VCD*VCD	0.00037
VCD*[DNA]	0.01895

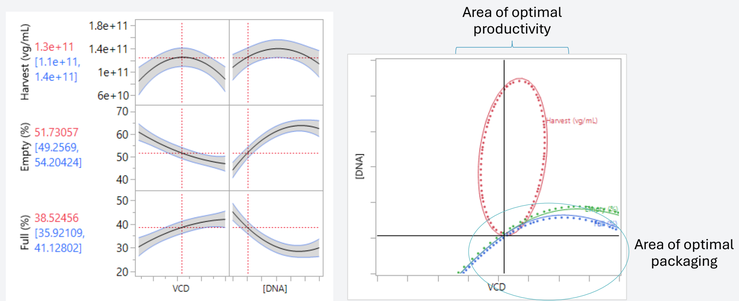


Figure 6. Joint analysis of productivity and packaging reveals both VCD and [DNA] strongly affect rAAV production efficiency. Transfection VCD plays a key role in rAAV productivity. However, the concentration of transfection DNA in a specific range does not have a large impact on rAAV production but does significantly affect packaging efficiency. This indicates that a combination of medium VCD together with a low concentration of DNA would result in obtaining a higher number of rAAVs at the same time as filled particles. However, it should be noted that very low concentrations of DNA are associated with lower rAAV production. Black lines in the contour profiler (right panel) visually show the intersection of optimal VCD and DNA concentrations to achieve maximum packaging efficiency without a decrease in harvest titers.

Conclusion

Transfection is a key step in rAAV manufacturing. Transfection parameters must be exhaustively controlled so that high quality rAAV productions could be achieved. Our study suggested that cell density and DNA concentration are key parameters that must be tightly controlled during the process. Despite the optimal transfection conditions obtained in this DoE experiment could be associated to the specific DNA combination used or to the production cell line employed, the utilization of this methodology could be applied to other transfection conditions. Application of this methodology to the manufacturing platform processes would aid upgrading rAAV production capabilities and would help meeting the highly increasing rAAV demand for the clinic.

Bibliography

1. Clément, N., & Grieger, J. C. (2016). Manufacturing of recombinant adeno-associated viral vectors for clinical trials. *Molecular therapy. Methods & clinical development*, *3*, 16002.

2. Grieger, J. C., Soltys, S. M., & Samulski, R. J. (2016). Production of Recombinant Adeno-associated Virus Vectors Using Suspension HEK293 Cells and Continuous Harvest of Vector From the Culture Media for GMP FIX and FLT1 Clinical Vector. *Molecular therapy: the journal of the American Society of Gene Therapy*, *24*(2), 287–297.

3. Sha, S., Maloney, A. J., Katsikis, G., Nguyen, T. N. T., Neufeld, C., Wolfrum, J., Barone, P. W., Springs, S. L., Manalis, S. R., Sinskey, A. J., & Braatz, R. D. (2021). Cellular pathways of recombinant adeno-associated virus production for gene therapy. *Biotechnology advances*, *49*, 107764.

4. JMP®, Version 17. SAS Institute Inc., Cary, NC, 1989-2024

5. Mancinelli, S., Turcato, A., Kisslinger, A., Bongiovanni, A., Zazzu, V., Lanati, A., & Liguori, G. L. (2021). Design of transfections: Implementation of design of experiments for cell transfection fine tuning. *Biotechnology and bioengineering*, *118*(11), 4488–4502.

6. Kasemiire, A., Avohou, H. T., De Bleye, C., Sacre, P. Y., Dumont, E., Hubert, P., & Ziemons, E. (2021). Design of experiments and design space approaches in the pharmaceutical bioprocess optimization. *European journal of pharmaceutics and biopharmaceutics: official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik e.V*, *166*, 144–154.

7. Wagner, C., Fuchsberger, F. F., Innthaler, B., Lemmerer, M., & Birner-Gruenberger, R. (2023). Quantification of Empty, Partially Filled and Full Adeno-Associated Virus Vectors Using Mass Photometry. *International journal of molecular sciences*, *24*(13), 11033