Design of experiments in rAAV manufacturing: Optimization of plasmid ratio for better upstream yield

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❶ Introduction

- There are over 200 clinical trials utilizing recombinant adeno-associated virus (rAAV) for gene therapy. A major concern is the cost of manufacturing viral vectors.
- A widely employed method for large-scale production of rAAV vectors involves co-transfecting HEK293-derived producer cell lines with three plasmids. These materials not only influence rAAV Critical Quality Attributes (CQAs) for purity and potency, but also positively affect manufacturability parameters like productivity.
- One of the most expensive raw materials for making rAAVs by transient transfection are the plasmids, that code for the different parts of the virus. Identifying better amounts of plasmids needed to produce the most, functional rAAVs – albeit from a seemingly endless number of possible combinations – is an important factor to driving down the cost of these therapies.
- Design of experiments (DoE) is a systematic approach to selecting conditions for tests. Optimal designs maximize a criterion based on user input, through computationally-assisted optimization algorithms. It allows thousands of possible combinations to be reduced to a few dozen, while keeping enough statistical power to determine the most relevant conditions.
- Here, we report the application of a design of experiments approach to simultaneously increase productivity and decrease production costs through reduction in starting material (Figure 1).

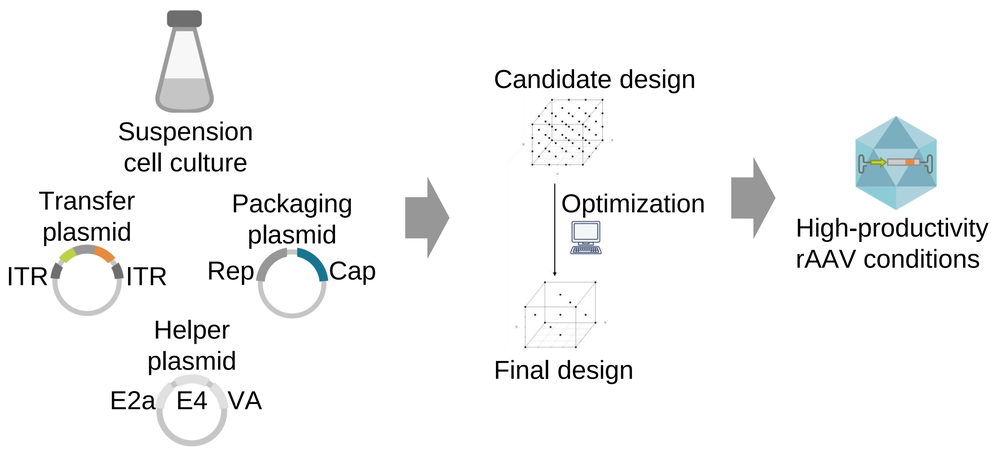

Figure 1. *Graphical representation of the method. The production of rAAV employs the use of the mammalian cell culture, which is transfected by a transfection reagent to introduce the 3 plasmids necessary for rAAV production. The conditions for the upstream process can be varied across a wide range, leading to over 4851 different combinations. A DoE optimization process is used to decrease the number of combinations needed to be tested, uncovering the conditions that lead to successful rAAV production.*

❷ Methods

- The rAAV samples were produced with the Viral Production Cells 2.0 (Gibco™) in Viral Production Media (Gibco™). Cells were harvested 3 days post-transfection with the AAV lysis buffer (Gibco™).
- The cell lysate was clarified by centrifugation. The clarified lysate was measured by ITR-qPCR, where the result was expressed in VG/mL of cell culture.
- A design was developed through an R-based DoE pipeline developed internally, using optimal design of experiments. A D-optimal mixture design augmented with an initial I-optimal mixture design was chosen for this test. The factors tested were the amount of plasmid DNA, and plasmid ratio.
- For the DoE run, we produced rAAV using the plasmids pAAV-Rep2Cap3 and pAAV-CMV-eGFP-WPRE in 28 independent 3-plasmid transient transfections in 10 mL cultures in 50 mL mini-bioreactors (Figure 2). The follow-up confirmation of DoE results through ridge analysis was performed in triplicates, in similar conditions. Testing of different serotypes and a different transfer plasmid, pAAV-CMV-eGFP-SV40, were performed in duplicates, in similar conditions.

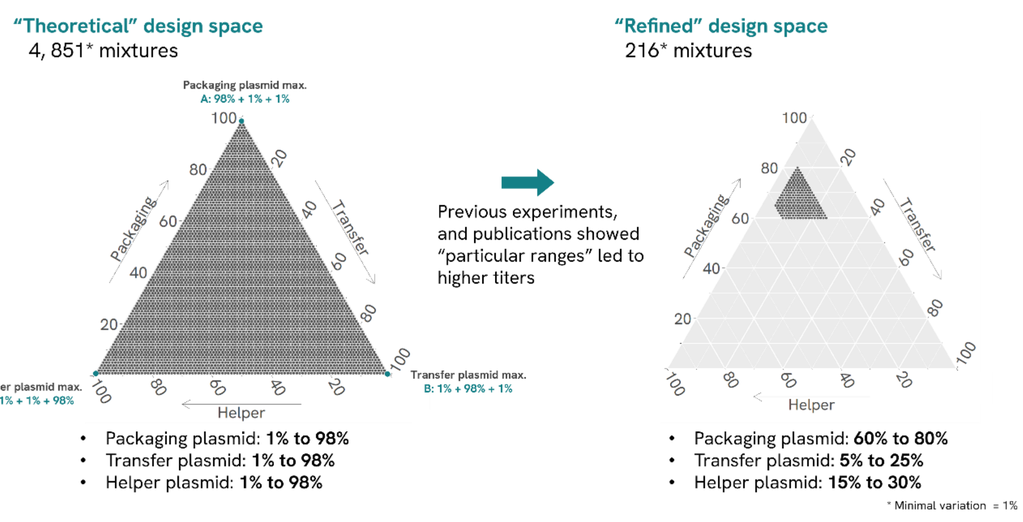

Figure 2. *The design space for the DoE. On the left, all the possible different combinations of the three plasmids are displayed as a black dot. On the right, the constrained design space that was used for design generation.*

❸ Response surface model identified all plasmid concentrations as significant for productivity

- The design chosen had a statistical power of >0.82 for all conditions tested, with an effect size of 3 and alpha of 0.05.
- The transfected cell cultures were harvested after 3 days, clarified, and analyzed by ITR-qPCR. The titers obtained varied from 1.40E11 to 5.66E11 VG/mL of cell culture.
- ANOVA testing showed that the ratio of pHelper was significant (p=<0.0001). DNA concentration, ratio of pTransfer and pRepCap were not significant.
- The results were fitted to a response surface model (RSM). Since this is a mixture design, we used a slack-variable approach. The model had an adjusted R-squared of 0.7664, and a p-value of <0.0001. All the factors tested were significant for the model (p=<0.05).
- The contour plots (Figure 3) represent each row as a different variable chosen as the slack-variable. How each variable influences productivity is shown as a color gradient.

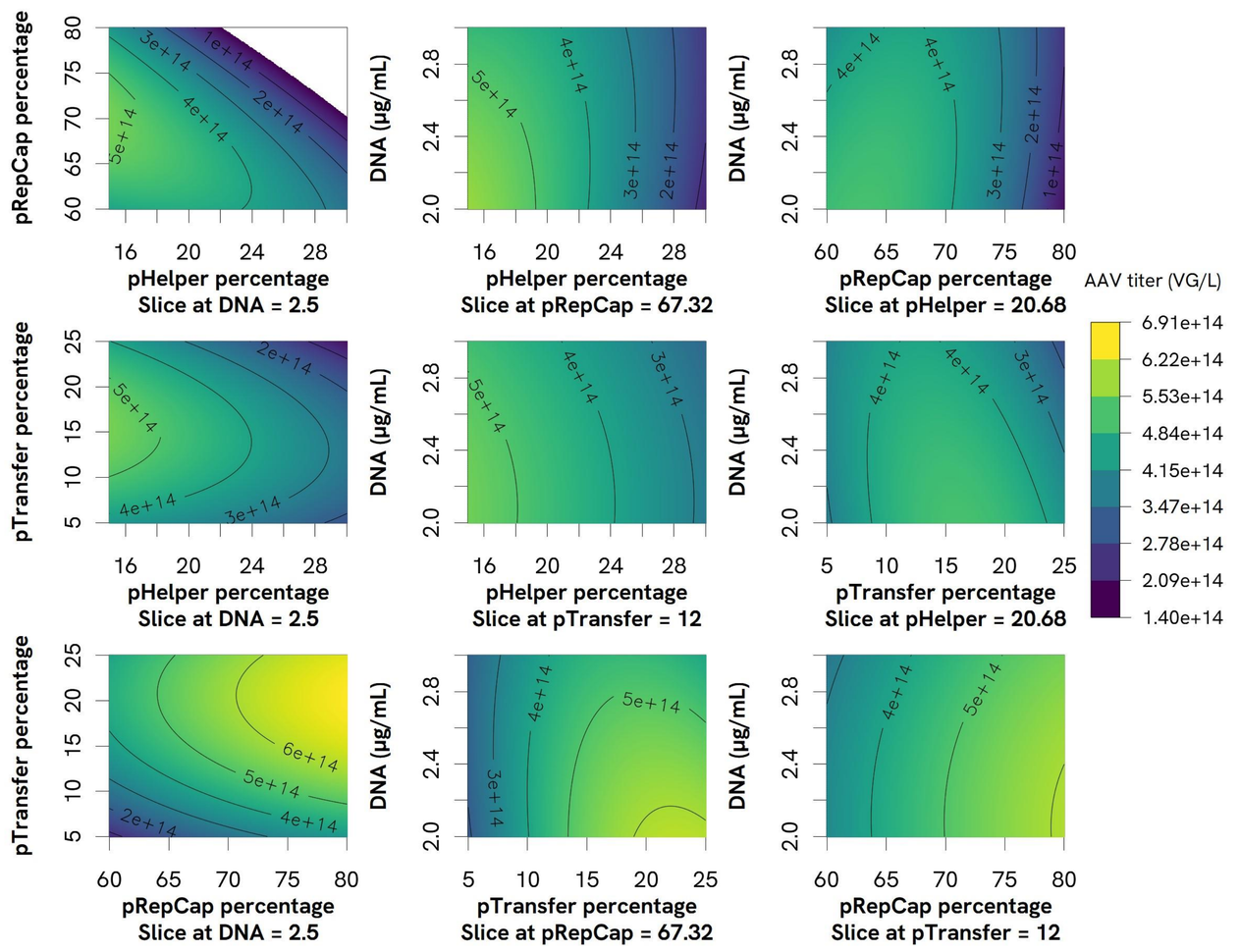

Figure 3. *The response surface model built from data generated from the DoE. Slices are made at the center of the design space.*

❹ Better conditions for plasmid ratio and DNA concentration were confirmed

- To confirm the results of the RSM, and to better determine the improved ratios of plasmids and concentration of DNA, we conceived a follow-up experiment using ridge analysis. This method aims to find the optimal conditions considering all the different factors that make up the model.
- The ridge analysis was used as a basis for the conditions shown below (Figure 4). Titers were improved up until condition c, when at which point the productivity decreased again as the pHelper concentration decreased.

	Packaging plasmid (%)	Transfer plasmid (%)	Helper plasmid (%)	Plasmid concentration (µg/mL)
a	60	20	20	1.5
b	**70**	**15**	**15**	**2**
c	**75**	**15**	**10**	**2**
d	77.5	19.5	3	2

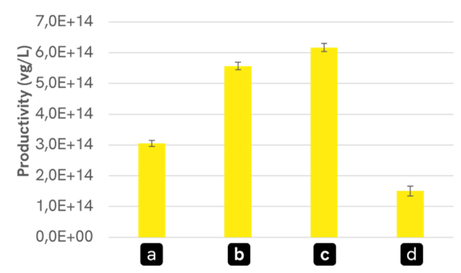

Figure 4. *Follow-up tests based on the ridge analysis of the RSM. Values close to those suggested by the ridge analysis were tested. Error bars represent standard deviation (n=3).*

- Conditions from group c were tested across multiple serotypes (AAV2, 3, 5 and 8), and 2 different transfer plasmids (Figure 5, n=2).
- Most serotypes achieved a gain in productivity when tested with the conditions found with the RSM. Serotype 5 had a slight drop in titers, while AAV2 with pAAV-CMV-eGFP-SV40 had the highest relative increase in productivity, at almost 300% more than previously. This highlights that even very similar constructs behave differently, requiring specific optimization for each production.

Figure 5. *Comparison of standard plasmid ratio with new plasmid ratio. Serotypes tested were AAV2, AAV3, AAV5 and AAV8, across 2 different transfer plasmids. Error bars represent standard deviation (n=2).*

❻ Conclusion

- The method of employing DoE, followed by ridge analysis, increases productivity and brings down costs, as evidenced by the 3x higher titer in certain productions tested with the conditions found.
- An R-based DoE generation allows a decrease in costs by process optimization, greater flexibility compared to other methods available, and a broader range of statistical testing.
- Taken together, these results demonstrate the possibilities of utilizing a DoE approach for process development before proceeding with non-GMP productions for preclinical studies. Thus, a DoE approach should not only be considered for developing a cost-effective GMP manufacturing process, but also as a cost-effective non-GMP manufacturing process for preclinical batches.

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