Response Surface Methods Point the Way to Higher rAAV Yields at Lower Levels of Transfection Reagent

Adverum Biotechnologies describes a multifactor design of experiments methodology that can optimize rAAV vector production while maintaining a specified N/P ratio

By Kara Bautista, Pratik Jaluria, PhD, and George Todorov

Researchers at Adverum Biotechnologies have demonstrated that a novel multifactor design of experiments (DOE) methodology can optimize production of a recombinant adeno-associated virus (rAAV) vector. The methodology, an advanced form of DOE that incorporates response surface methods (RSMs), focused on the relative amounts of transfection agent polyethylenimine (PEI) and DNA.

This methodology is unique because of the way it keeps the ratio of PEI nitrogen to DNA phosphate—the N/P ratio—within a specified range. The N/P ratio is well known to influence the rAAV yields obtained via transient transfection. Vector yield is reported via qPCR quantitation of the vector genome (VG) copies packaged by the virus particles.

Transient transfection is a means to introduce nucleic acids into cells and can be performed in a variety of ways. A common way to transfect mammalian cells involves complexing negatively charged plasmid DNA with a positively charged, polymer-based transfection reagent, such as PEI. Once this complex is formed, cells take up the complex through endocytosis and express genes encoded in the DNA.

In the case of rAAV, multiple plasmids which carry the key components needed to make the gene therapy vector are delivered to cells. These components include a replicase gene, a capsid gene, adenovirus helper genes, and the vector genome, which includes the therapeutic transgene.[1] This allows the cells to produce an rAAV that encapsidates the intended transgene. Following a series of purification steps, the packaged viral vector becomes the gene therapy drug.

Optimizing the rAAV yield from cells is important in terms of process efficiency, process robustness, and manufacturing-related costs. A number of factors are known to impact rAAV yields in transient transfection, including the cell type, media, DNA quality and amount, the ratio of the different plasmids, transfection reagent, and transfection method.

For PEI-based transfections, the N/P ratio is particularly important and impacts transfection efficiency. This parameter denotes the molar ratio of nitrogen in the PEI molecule to the phosphorous amount found in the DNA backbone.[2] Adjusting the N/P ratio is known to change both the size and morphology of the PEI-DNA complex.[3]

For adherent mammalian cultures, the optimal N/P ratio for maximum rAAV yield is recognized to be higher than that for suspension cells, with Huang *et al.*[2] reporting an optimal N/P of 40 for HEK293T using 1.05 µg/mL plasmid DNA. For suspension cultures, the optimal N/P ratio is reported to be lower, in the 10–13 range, but it is also dependent on DNA amount and transfection methodology.[4] Other groups report using an N/P ratio as low as 6.[5]

Recently, members of Adverum's Early Stage Process Development Team expanded their knowledge of multifactor testing techniques via a customized DOE workshop organized by Stat-Ease, Inc. (Minneapolis, MN). Then, using Design-Expert® software (DX) published by Stat-Ease, and with the help one of their statisticians, the group team laid out an RSM experiment to optimize transfection conditions.

The experiment aimed to maximize VG at lower N/P ratios than previously studied and, ideally, to use less of a costly transfection reagent. The study was performed using 80 mL HEK293 suspension cultures in shake flasks. A low N/P ratio range between 3.8 and 15.3 was targeted. The N/P ratio was calculated as described by Huang *et al.*[2]:

$$\text{PEI (µL)} = 3 \times \text{DNA (µg)} \times \text{(N/P ratio)} / \text{(PEI concentration (mM))}$$

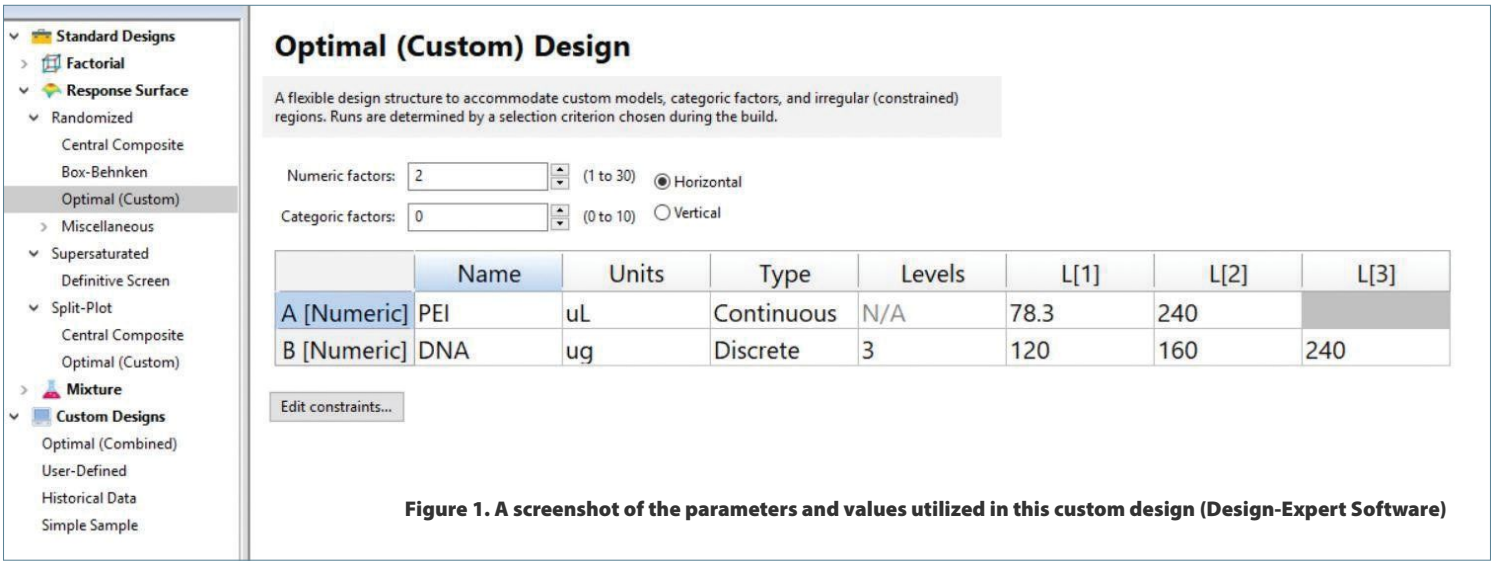

Figure 1. A screenshot of the parameters and values utilized in this custom design (Design-Expert Software)

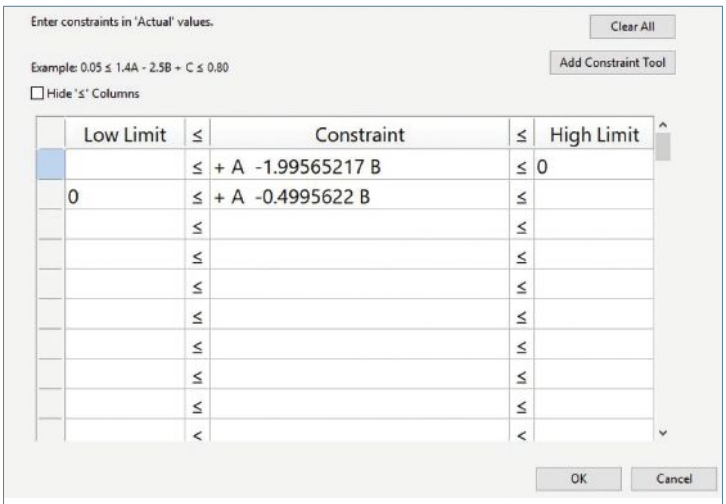

Figure 2. Display of the constraints applied to the model. The constraints were calculated using the N/P ratio's minimum and maximum acceptable values (Design-Expert Software).

A significant consideration in the design of the experiment was deciding how to define each parameter (PEI amount, DNA amount, and N/P ratio). It should be noted that the N/P ratio is more of an artifact of inputs than an absolute value. Many different PEI and DNA combinations can result in the same N/P ratio. Therefore, it was important to define PEI and DNA amounts within a range that agreed with historical data. Ultimately, the DNA amount was held as a discrete variable and PEI amount was allowed to fall within a specified range, as shown in *Figure 1*.

The N/P ratio was used as a constraint in this experiment. To define the constraint, the N/P ratio equation was rearranged and simplified into a direct relationship between the two variables constituting the N/P ratio, where A is PEI and B is DNA. The lower and upper limits of the N/P ratio were defined in advance and plugged into the simplified equation to obtain constraint parameters, as displayed below:

$$N/P = 23A/3B$$

$$N/P \times 3/23 = A/B$$

$$3.83 \times 3/23 = 0.49956522 \text{ (lower limit of N/P)}$$

$$15.3 \times 3/23 = 1.99565217 \text{ (upper limit of N/P)}$$

Figure 2 depicts how the constraints were displayed once they were entered into the software.

The input and output variables are presented as follows:

- Factor levels set at –1 for low and +1 for high.
- Response data normalized to a mean of 100 and then directly scaled to the actual results.

A full three-level factorial design of 9 runs (3 × 3) serves well for an initial RSM experiment focusing on only two factors. It provides enough data to fit the standard quadratic model needed by an RSM experiment to fit nonlinear surfaces such as peaks and some curvature. However, after the Adverum team entered the N/P ratio constraints (translating to A/B ratio ranging from 0.5 to 2) into Design-Expert, combinations of this full factorial became biochemically infeasible. Recognizing this, the program steered them to a custom design. They then accepted the recommended defaults for building the experiment per optimal statistical criteria.

This design included 6 points ideally spaced to fit the 6 coefficients in the two-factor quadratic model. The team also opted for 5

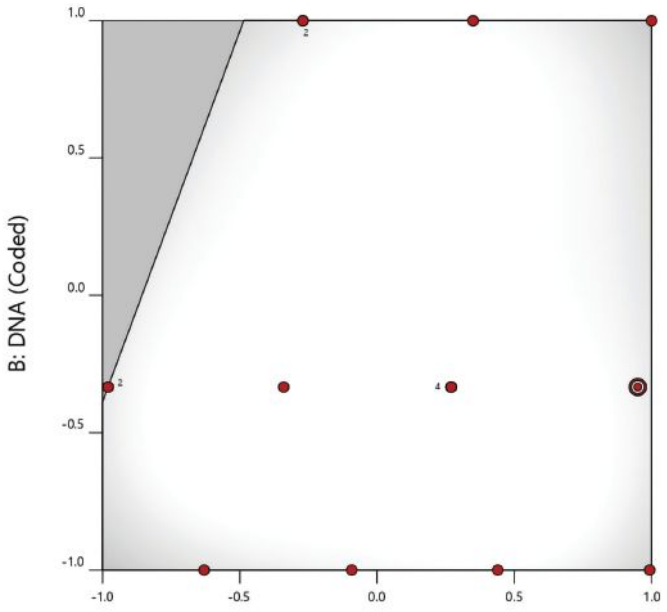

Figure 3. Experiment design constrained by the N/P ratio

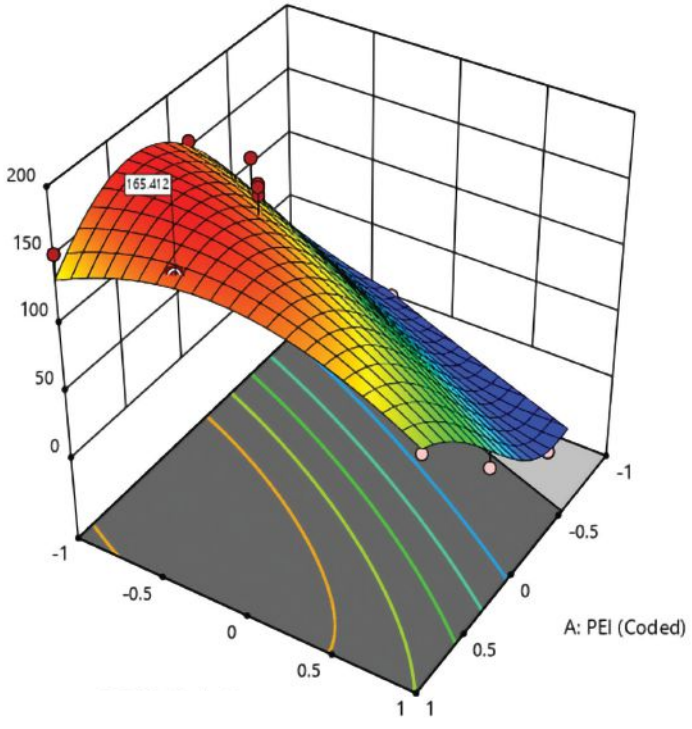

Figure 4. Response surface

more "check" runs to fill gaps between model points. Furthermore, they accepted the software's recommendation to replicate 5 of the points (now 11 = 6 model plus 5 check runs) chosen for maximum leverage on the fitting. Replication provides great value by enabling the measurement of pure error, which then makes it possible to assess statistical significance, if any, of the model's lack of fit.

Figure 3 shows the points selected by the program. Note the following details:

- A portion of the space at the upper left got cut off by the N/P ratio constraint, necessitating a computer-generated design rather than a standard 3 × 3 RSM experimental template.
- Numbers appear by some of the points, indicating replication of these points.
- A run is circled at the lower right—this being run 1, which turned out to be near where the numerical optimization recommended the factors be set for maximum production.

Table 1. Optimal RSM experiment with results

	A: PEI	B: DNA	Total VG
Run	Coded	Coded	Scaled
1	0.95	-0.33	165.5
2	-0.63	-1.00	107.9
3	-0.34	-0.33	57.8
4	-0.27	1.00	2.2
5	0.44	-1.00	116.7
6	1.00	1.00	115.2
7	0.27	-0.33	146.9
8	0.27	-0.33	177.4
9	0.35	1.00	45.9
10	-0.98	-0.33	1.0
11	0.27	-0.33	171.6
12	-0.27	1.00	2.5
13	-0.98	-0.33	1.2
14	0.27	-0.33	179.4
15	-0.09	-1.00	156.6
16	0.99	-1.00	152.3

The Table lists the 16 runs of the design, done in randomized order, alongside the total VG results (scaled).

These data produced a highly significant ($p < .0001$, 0.9 R^2 adjusted) quadratic model (VG = f(A, B, AB, A^2, B^2) which produces the 3D response surface shown in *Figure 4*.

The response surface map revealed a broad ridge of PEI-DNA combinations producing maximal VG, including the conditions of Run 1 (flagged). The results of the study indicated that there are various N/P ratios between 8 and 15.3 which can yield the same total amount of vector genomes. When the DNA was held constant and a lower amount of PEI (and, consequently, a lower N/P ratio) was used, the same VG yield was achieved, allowing more efficient use of a costly PEI reagent.

These results have directed Adverum's transfection optimization efforts, narrowing the effective DNA and PEI parameters. The use of a multifactor method, that is, an RSM experiment, greatly accelerated the experiment. This efficiency provides time for more experimentation beyond current boundaries on N/P ratios.

This experiment has pointed Adverum toward future DOE methods that have a narrower DNA range and wider PEI range. A wider PEI range may allow a continuation of curvature to be seen in the 3D contour plot and further characterize the response. Further optimization will involve optimizing the plasmid ratio within the optimal DNA amount. DOE will enable a more efficient screening and birds-eye view as conditions narrow through rounds of experimentation. GEN

Kara Bautista was a process development associate, Pratik Jaluria, PhD, was executive director of process development and manufacturing, and George Todorov is associate director of the Early Stage Process Development Team at Adverum Biotechnologies. Website: https://adverum.com.

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