Creation of a High-Yield AAV Vector Production Platform in Suspension Cells Using a Design-of-Experiment Approach

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INTRODUCTION
Recombinant adeno-associated virus (rAAV) vectors are a leading gene delivery platform, but vector manufacturing remains a challenge. New methods are needed to increase rAAV yields and reduce costs. Past efforts to improve rAAV production have focused on optimizing a single variable at a time, but this approach does not account for the interactions of multiple factors that contribute to vector generation. Here, we utilized a design-of-experiment (DOE) methodology to optimize rAAV production in a HEK293T suspension cell system. We simultaneously varied the transgene, packaging, and helper plasmid ratios, the total DNA concentration, and the cell density to systematically evaluate the impact of each variable across 52 conditions. The results revealed a unique set of parameters with a lower concentration of transgene plasmid, a higher concentration of packaging plasmid, and a higher cell density than previously described protocols. Using this DOE-optimized protocol, we achieved unpurified yields approaching 3 \times 10^{14} viral genomes (VGs)/L of cell culture. Additionally, we incorporated polyethylene glycol (PEG)-based virus precipitation, pH-mediated protein removal, and affinity chromatography to our downstream processing, enabling average purified yields of >1 \times 10^{14} VGs/L for rAAV-EGFPs across 13 serotypes and capsid variants.

Design-of-experiment (DOE) methodology has been successfully used to optimize biotechnological processes, such as antibiotics production, antibody generation, and embryonic stem cell expansion. Unlike OFAT-based optimization, a DOE-driven approach allows one to evaluate the impact of multiple interdependent factors on a given output. In this study, we utilized a DOE methodology to optimize rAAV vector production in a HEK293T suspension cell system. To the best of our knowledge, this represents the first application of DOE methodology to optimize rAAV vector production. We simultaneously varied the concentrations of transgene, packaging, and helper plasmids, the total DNA concentration, and the cell density to systematically evaluate the impact of each variable across 52 different conditions. Data analysis revealed a unique set of parameters that contribute to vector generation, antibody generation, and embryonic stem cell expansion.
with a lower concentration of pAAV, a higher concentration of pRC, and a higher cell density compared with previously described methods using OFAT-based approaches. This DOE-optimized protocol allowed us to achieve unpurified yields approaching \(3 \times 10^{14}/L\) cell culture. Additionally, we incorporated polyethylene glycol (PEG)-based virus precipitation, pH-mediated protein removal, and affinity chromatography to our downstream processing, enabling us to achieve average purified yields greater than \(1 \times 10^{14}VGs/L\) rAAV-EGFP vectors across 13 serotypes/capsid variants. These yields are significantly greater than the highest previously published rAAV yields of \(>1 \times 10^{13}VGs/L\) by triple transfection in suspension cells.

RESULTS
Creation of an OFAT-Optimized rAAV Suspension Cell Production Protocol
To create a suspension cell-based rAAV vector production system, we initially evaluated different production cell lines, cell densities, total plasmid DNA concentrations, and vector harvest times. For these studies, each parameter was assessed individually, and rAAV5-EGFP was used as a model serotype for optimizing production. We selected two cell lines for potential inclusion in our system: suspension HEK293T cells and suspension HEK293-6E cells. HEK293T cells express the SV40 large T antigen and they have previously been shown to produce more rAAVs than parental HEK293 cells.\(^9,17\) HEK293-6E cells are an improved variant of HEK293E cells and have increased transgene expression compared with HEK293E cells.\(^18\) We compared the transfection efficiency of HEK293-6E and HEK293T suspension cells using the plasmid pAAV-EF1α-EGFP (EF1α, elongation factor 1 alpha promoter) with Polyethyleneimine MAX (PEIMAX) as a transfection reagent. The fluorescence intensity of transfected cells and the percentage of EGFP-positive cells were determined at 24 and 48 h post-transfection (HPT) by flow cytometry. HEK293T cells had an 18.2-fold and 5.1-fold higher mean fluorescence intensity than HEK293-6E cells at 24 and 48 HPT, respectively (Figure 1A). Whereas HEK293T and HEK293-6E cells had a similar percentage of EGFP-positive cells at 48 HPT, HEK293T cells had a 2-fold higher percentage of EGFP-positive cells at 24 HPT compared with HEK293-6E cells (Figure 1B). Given the robust transfection efficiency and higher levels of transgene expression, suspension-adapted HEK293T cells were selected for further optimization of rAAV vector production.

We next evaluated the impact of the total amount of plasmid on rAAV vector production. rAAV was generated by triple transfection
using pHelper, pAAV, and pRC plasmids. The pRC plasmid was previously optimized to improve vector production based on a strategy similar to Li et al., Xia et al., and Koizumi et al. (see Materials and Methods and Figures S1 and S2). In these experiments, the total DNA was varied, but the plasmid ratio was fixed at 2:1:1 (pHelper/pRC/pAAV weight ratios, 1:1:1 molar ratio). This ratio has been used previously for rAAV production in suspension cells and adherent cells. A cell density of 0.5 x 10^6/mL was utilized based on previously published work in HEK293T, HEK293, and HEK293F cells. rAAV5-EGFP production was carried out in 20-mL cultures, and immunoblots for rAAV capsid protein in cell lysate were used as an indirect measurement of rAAV vector yield. As shown in Figure 1C, concentrations at 1.5 and 2 µg/mL generated the most rAAV capsid protein. Thus, a concentration of 1.5 µg/mL was selected to reduce the amount of total DNA needed for vector production. Utilizing the optimized plasmid concentration of 1.5 µg/mL, we then assessed the role of cell density on capsid protein expression. Cell densities ranging from 0.5 to 2 x 10^6/mL were evaluated. Although we initially predicted higher cell densities would result in higher levels of viral capsid protein, we observed that cell concentrations greater than 1 x 10^6 cells/mL produced less capsid protein than lower cell densities (Figure 1D). Finally, we examined the optimal virus harvest time. The largest amount of AAV capsid proteins was produced between 72 and 96 HPT (Figure 1E). We observed reductions in cell viability at 96 HPT, so 72 HPT was selected as the final harvest time.

After optimizing the cell line, total DNA plasmid concentration, cell density, and harvest time, we explored the ability of different media additives to improve rAAV5 production. Histone deacetylase inhibitors (HDACis) can induce transcriptional activation and increase gene expression. The HDACi sodium butyrate has been shown to improve antibody production, and we wondered whether this compound could potentially increase rAAV vector generation. Similar to Grünberg et al., we found a 5 mM concentration of sodium butyrate treatment improved rAAV5 capsid production (Figure 1F). Peptones have also been shown to increase recombinant protein production, and Hildinger et al. showed that soy peptone could increase rAAV vector production in HEK293 cells. Pham et al. previously screened 16 peptones, including soy peptone, for their ability to increase recombinant protein expression, and found Tryphtone N1 (TN1; 0.5%) was the best peptone for recombinant protein expression in transient HEK293 expression assays. Given these studies, we evaluated whether 5 mM sodium butyrate and 0.5% TN1 could improve rAAV vector capsid protein expression. We found either TN1 or sodium butyrate increased capsid protein expression, and the addition of both TN1 and sodium butyrate augmented capsid protein expression more than either compound alone (Figure 1F). Given these findings, sodium butyrate and TN1 were added to our rAAV vector production protocol.

**Table 1. Parameter Ranges Selected for Optimization and DOE-Optimized Values**

| Parameters          | DOE I Variation Range | DOE I Optimized | DOE II Variation Range | DOE II Optimized |
|---------------------|-----------------------|-----------------|------------------------|------------------|
| Cell density (10^6/mL) | 0.25–3                | 2.67            | 1–4                    | 2.45             |
| DNA amount (µg/mL)   | 0.5–4                 | 2.53            | 1.5–3.5                | 1.5              |
| pHelper/pAAV        | 1:5 to 5:1            | 1:5             | 1:5 to 5:1             | 1:5              |
| pHelper/pRC         | 1:5 to 5:1            | 1:0.23          | 1:1 to 6:1             | 1:0.31           |
| PEIMAX/DNA          | 1:1 to 4:1            | 4:1             | 3:1 (fixed)            | 3:1 (fixed)      |

**The OFAT-Optimized rAAV Production Protocol Works for rAAV5, But Not rAAV8**

Using our OFAT-optimized parameters, we produced a 1-L batch of rAAV5-EGFP in suspension HEK293T cells and compared the yield with virus generated in adherent HEK293T cells using a 10-layer cell stack containing 1 L of medium. Virus generated in adherent cells was produced via a standard calcium phosphate transfection using 4 mg total DNA per cell stack and a plasmid ratio of 2:1:1 (pHelper/pRC/pAAV weight ratio) (see Materials and Methods for additional details). In these experiments, the production yield of the suspension culture system was comparable (~80%) with rAAV5 produced in adherent cells (4.71 x 10^13 VGs/cell stack for adherent cells and 3.77 x 10^13 VGs/L for suspension cells). Given the successful generation of rAAV5 using the suspension cell protocol, we next evaluated whether this protocol could be used to effectively produce other rAAV serotypes. Unfortunately, a 1-L batch of rAAV8-EGFP produced using these parameters yielded only 18.5% of the total virus produced from one cell stack with 1 L of medium (2.41 x 10^13 VGs/L suspension cells versus 1.38 x 10^13 VGs/cell stack). These results suggested that the utility of OFAT-based optimization may be suboptimal and/or serotype specific. We therefore considered additional approaches for developing a suspension-cell-based rAAV production platform.
plasmid DNA and pAAV plasmid in combination with increased levels of pRC plasmid and high cell density were optimal for rAAV production (Table 1, DOE I optimized). These results differed significantly from the OFAT-optimized conditions for rAAV5 and the previously published ranges of these parameters discussed earlier. Therefore, we performed a second DOE study to confirm these results. The parameter ranges were set based on the results of the first study, and 20 different conditions (run in duplicate) were generated by statistical software (Table 1, DOE II variation ranges). To reduce the number of conditions tested, we fixed the PEIMAX/DNA ratio at 3:1.26 The results are shown in Table S2. Except for the total amount of plasmid DNA, the parameters identified in the DOE II study were strikingly similar to the DOE I experiments (Table 1, DOE II optimized). To ensure these findings would hold true in larger-scale rAAV vector production, we tested the conditions identified in the DOE I and DOE II experiments for their ability to generate rAAV8-EGFP at a 1-L scale. For these studies, rAAVs were purified with AVB-Sepharose, and the titers were determined by the CyQuant method. The yields of rAAV for the DOE I and DOE II conditions were 1.11 × 10^{14} and 1.12 × 10^{14} VGs/L after affinity purification, respectively. These yields were an average of 4.6-fold higher than the yield of rAAV8-EGFP generated using the OFAT-optimized method discussed earlier (2.41 × 10^{13} VGs/L after affinity purification). In parallel to these studies, we also generated rAAV8-EGFP in adherent HEK293T cells grown in 10-layer cell stacks. Virus generated in adherent cells was produced via a standard calcium phosphate transfection using 4 mg total DNA per cell stack and a plasmid ratio of 2:1:1 (pHelper/pRC/pAAV weight ratio) (see Materials and Methods for additional details). The average yield from the two 10-layer cell stacks was comparable (1.38 × 10^{14} VGs/L) to the yields in suspension cells generated using the DOE-optimized parameters. Although the volumetric yields in suspension cells were roughly equivalent to the yield of the cell stack, it should be noted that more cells were used in the 1-L suspension culture than in the cell stack (~2.5 × 10^{9} cells/L versus ~6.36 × 10^{8} cells/10-layer cell stack). Although the DOE-optimized method produces similar rAAV yields to traditional production in adherent cells, this suspension-based protocol is more user-friendly and less labor-intensive due to the nature of working with a
suspension cell culture. Because vector purification can result in the loss of vector, we also determined the genome-containing particles in crude lysate with the DOE II condition in suspension cells using a droplet digital polymerase chain reaction (ddPCR)-based titration method and achieved a yield of 2.93 × 10^14 VGs/L of cell culture for rAAV8-EGFP.

The DOE-Optimized Protocol Improves rAAV Production across Multiple AAV Serotypes

To determine whether the DOE-optimized method was specific to rAAV8, we compared virus production of rAAV1-, rAAV2-, rAAV5-, rAAV8-, and rAAV9-EGFP using the DOE- and OFAT-optimized protocols. For these studies we selected the conditions identified in the DOE II studies because this protocol required less total DNA. As shown in Figure 2A, the DOE-optimized method improved the average production yield by 6.1-fold compared with the rAAVs produced by the OFAT method. The yield of rAAV9 was not determined for this assay because the vector could not be purified by AVB-Sepharose. The cell lysates from these studies were also analyzed by immunoblot to quantify the amount of rAAV capsid produced. Based on band intensity, the DOE-optimized protocol generated 3.2-, 2.4-, 2.1-, 4.4-, and 2.9-fold more capsid proteins than the OFAT method in rAAV1, rAAV2, rAAV5, rAAV8, and rAAV9 vectors, respectively (Figure 2B). The DOE-optimized method was successfully used to produce large-scale rAAV-EGFPs for many serotypes, including rAAV1, rAAV2, rAAV5, rAAV6, rAAV8, and rAAV9, as well as the capsid engineered rAAVs DJ, DJ8, DJ9, DJ-mCherry, AAV2-mCherry, AAV2-BR1, and AAV9-PHP.eB (Figure 2C). The average yield for these affinity chromatography-purified viruses was 1.16 × 10^14 VGs/L, as determined by the CyQuant method (Figure 2C). We also examined whether DOE-derived plasmid ratios could be applied to adherent HEK293T cells using the calcium phosphate method for rAAV8 production. As shown in Figure 2D, we obtained comparable yields using the standard 2:1:1 ratio and the DOE-optimized ratio 1.5:0:31 (pHelper/pRC/pAAV) with less DNA (3 mg/L suspension cells for DOE-optimized method versus 4 mg/cell stack for adherent cells).

Improvement of Downstream Processing for rAAV Production

In addition to optimizing the parameters involved in rAAV vector production, we also developed several methods to improve the downstream processing of rAAV. When harvesting rAAV, cells and media are usually separated and processed separately. Although rAAV2 and rAAV5 viral particles are mostly cell associated, significant amounts of viral particles can be found extracellularly in other rAAV serotypes. To save sample processing time, we added a PEG-NaCl solution directly to the whole-cell culture at the time of harvest to precipitate both virus-containing cells and extracellular virus in one step. Following PEG-NaCl treatment, the solution can be centrifuged and the pellet resuspended in a smaller volume. This protocol modification quickly reduces the volume of the culture to facilitate further downstream processing. Additionally, these smaller working volumes reduce the amount of costly reagents, such as DNase or Benzonase, needed for sample processing, and make downstream steps, such as centrifugation, freeze-thaw, and purification, more manageable.

Following the PEG-NaCl precipitation and centrifugation, the resuspended pellets are subjected to three freeze-thaw cycles, Benzonase digestion, and centrifugation to remove cellular debris. The resulting supernatant contains rAAV, as well as large amounts of proteins and other impurities from the cells and medium. To remove these contaminants from the viral prep, we tested whether isoelectric-mediated protein precipitation could provide cleaner starting material for filtration, chromatography, or gradient purification. The isoelectric point (pI) of most cytoplasmic proteins is between pH 5 and 6, while the pI of rAAVs with a packaged genome (4.7 kb) is predicted to have a pI of 5.9. We evaluated whether reducing the pH could precipitate cellular proteins while a majority of the rAAV particles remain soluble. For these studies, we used rAAV1 encoding an empty vector. Following freeze-thaws, Benzonase digestion, and clarification of the lysate, we evaluated the amounts of protein precipitated and rAAV retained from supernatants at pH 5.5, 7.0, and 8.5. As shown in Table 2, adjusting the pH to 5.5 increased the subsequent precipitated pellet’s wet weight by 3.8-fold compared with pH 8.5. Importantly, an analysis of the corresponding viral supernatants showed that 90.5% of capsid proteins (based on band intensity) were recovered from pH 5.5 precipitation as compared with the control pH 8.5 (Figure 3A). We did not observe significant changes at pH 7.0. These results suggest a substantial amount of impurities can be removed prior to additional downstream processing, such as filtration, gradient centrifugation, or chromatography. We have successfully applied this pH-based precipitation method to multiple rAAV serotypes, except for rAAV2, which appears to precipitate at this lower pH (data not shown). These improvements combined with affinity purification (AVB-Sepharose, POROS CaptureSelect-AAV8, -AAV9, or -AAVX) enable us to purify rAAVs in less than 2 days. Since implementing this protocol in our lab, we have generated over 220 large-scale rAAVs (mostly 1-L scale) of different serotypes and genes of interest with an average production yield of 8.92 × 10^13 VGs/L, demonstrating the reproducibility of this protocol (Figure 3B). A flow scheme providing an overview of the optimized rAAV production system is shown in Figure 3C.

Our lab routinely titer rAAV by the CyQuant method and ddPCR using protocols adapted from Lock et al. with the primers and probe for BGHpA. Because titers can vary between researchers and labs, we compared our titers with the available rAAV8 reference standards generated via the AAV Reference Standard Working Group (AAVRSWG). As shown in Figure 3D, our ddPCR titers for the reference rAAV8 were well within the stated ranges and were equivalent to the titers defined by the AAVRSWG. In parallel, we also calculated

| pH | Wet Weight (g) |
|----|----------------|
| 8.5 | 0.27 |
| 7.0 | 0.32 |
| 5.5 | 1.03 |
the titers for two in-house AAV8-EGFP vectors. These titers were nearly equivalent (average 30.3% lower) to the titers calculated by the CyQuant method (Figure 3D). Because the CyQuant titration method is not PCR based, we were unable to titer the reference standards using the CyQuant method due to the low titer of the reference standard and the limited amounts of material available. These data validate our ability to achieve high rAAV yields using the DOE-optimized protocol in suspension cells.

**DISCUSSION**

Despite the promising advances of rAAV-mediated therapies in the clinic, vector manufacturing remains a challenge. Previous efforts to improve vector production have focused on modifying single variables at a time, but these approaches do not account for the multiple interdependent factors that impact rAAV vector production. To our knowledge, the DOE method has never been applied to rAAV vector production. In this study, we utilized DOE methodology to optimize rAAV production in a HEK293T suspension cell system by simultaneously varying the ratios of the transgene, packaging, and pHelper plasmids, the total DNA concentration, and the cell density. In total, we systematically evaluated over 52 different conditions and successfully identified a unique set of parameters for rAAV production that resulted in high yields of rAAV. Compared with previously published protocols that utilize the common 2:1:1 ratio (pHelper/pRC/pAAV weight ratio), we identified an optimal plasmid ratio of 1:5:0.31 (pHelper/pRC/pAAV weight ratio). Assuming the same amount of total input plasmid DNA, this results in a 5.1-fold decrease in the amount of pAAV DNA. Depending on the gene of interest, transgene expression from the pAAV plasmid can be toxic to production cells. Thus, a reduced amount of pAAV DNA may be beneficial for rAAV production with transgenes that impact cell viability and rAAV packaging. Another feature of the DOE-optimized protocol is the increased amount of pRC plasmid (1.19 mg/L), which comprises nearly 80% of the total plasmid DNA used in the transfection. Production of Cap proteins has been identified as a limiting factor for rAAV production, and increased expression of the cap gene by promoter modulation has previously been shown to increase rAAV production 10-fold. Thus, the increased amounts of pRC plasmid in the DOE protocol may contribute to the improved rAAV yields. Although the higher amounts of pRC may improve vector yields, the increased amounts of this plasmid may also result in higher numbers of empty particles. Additionally, although we obtained high-quality rAAV following affinity purification, this method does

*Figure 3. Overview of the rAAV Production System*

(A) Top: rAAV1-empty vector was produced in suspension HEK293T cells (1 L). Harvested cells and medium precipitation were resuspended, subjected to three freeze-thaw cycles and treated with Benzonase as described in the Materials and Methods. Parts of the total lysate were either not adjusted or adjusted to pH 7.0 or 5.5 and spun at 12,000 × g for 15 min. The pH of clarified supernatants was then adjusted to 8.0 and analyzed by immunoblot with anti-capsid protein antibody. Bottom: the bands of the immunoblot were quantified with Odyssey scanner. (B) The yields of 227 large-scale rAAV productions with different serotypes and different transgenes were produced using the DOE-optimized method. rAAVs were purified by affinity chromatography, and the titers were determined by the CyQuant (CQ) method. (C) Scheme of our rAAV production process (detailed in Materials and Methods). (D) Comparisons of in-house ddPCR titers with rAAV8 Reference Standard Stock (AAV8RSS) from ATCC. The ddPCR titers of internal rAAV8-EGFP preps (AAV8 #1 and AAV8 #2) were also compared with titers determined by the CQ method. Titers were performed in triplicate at three different dilutions. Error bars show standard deviations.
not specifically remove empty particles. We routinely administer these rAAVs to immune-competent mice and observe strong, sustained transgene expression in vivo, suggesting the levels of empty particles in these preparations do not have large, negative impacts on in vivo transduction. However, it is increasingly recognized that empty particles can impact rAAV-mediated gene delivery. Future studies will examine the changes in empty particle ratios between the OFAT and DOE protocols, and evaluate the possibility of implementing additional methods for empty particle removal. Lastly, in the DOE-optimized protocol, pHelper was reduced to one-third compared with plasmid ratios of 2:1:1 (pHelper/pRC/pAAV). Because the E2A and E4 genes may induce cytotoxicity, the reduction in the amount of the pHelper plasmid may be beneficial for rAAV production. In our initial studies using OFAT to optimize rAAV5 production, we observed viral capsid protein expression was decreased when the cell density was greater than 1 × 10^6 cells/mL (Figure 1D). Conversely, the data from the DOE-derived protocol suggested a cell density of 2.5 × 10^6/mL was optimal at the same total DNA concentration. This discrepancy could be due to the higher levels of the pRC plasmid in the DOE system, which may compensate for the higher cell density. These observations underscore the complex relationships between cell density and plasmid ratios, and highlight the advantages of the DOE methodology.

The DOE-optimized protocol allowed us to achieve yields approaching 3 × 10^14 VGs/L of crude lysate as determined by ddPCR. Importantly, the pre-purification yields obtained via this DOE-optimized method were comparable (by volume) to those seen in baculovirus vector/sf9 cell systems (2.5–3.5 10^14 VGs/L), which are currently viewed as one of the most promising platforms for rAAV vector production. Because these results were obtained utilizing an internally derived HEK293T suspension cell line and a modified pRC plasmid, this method may not be applicable to all HEK293T suspension cell lines or plasmids and will need to be evaluated on a case-by-case basis. In these studies, we also incorporated PEG-based virus precipitation, pH-mediated protein removal, and affinity purification to our downstream processing, enabling us to achieve average purified yields of 8.92 × 10^13 VGs/L across many serotypes and transgenes. Future studies will focus on optimizing purification techniques and exploring continuous harvesting and perfusion technologies to further increase the production yield, improve the viral particle-to-VG ratio, and reduce the cost of rAAV vector production. In conclusion, we have developed a rAAV production system in HEK293T suspension cells and utilized a DOE-based approach to identify unique production parameters enabling us to achieve high yields of rAAV for multiple serotypes.

MATERIALS AND METHODS

Cell Culture

HEK293T suspension cells were grown in FreeStyle 293 Expression Medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 2% fetal bovine serum (FBS) and 50 µg/mL G418. These cells were derived from parental HEK293T cells as determined with short tandem repeat (STR) profiling (IDEXX, Westbrook, ME, USA) and adapted to suspension culture in-house. HEK293-6E cells were cultured in FreeStyle F17 Expression Medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 6 mM glutamine, 0.1% F68, and 25 mg/L G418. For small scale (20 mL), suspension cells were cultured in a 125-mL Erlenmeyer flask (Corning Life Science, Tewksbury, MA, USA) agitated at 110 rpm. For rAAV vector production in large scale, cells were cultured in 1-L medium in a 3-L baffled Erlenmeyer flask (Corning) agitating at 65 rpm. Adherent HEK293T cells were cultured in DMEM supplemented with 10% FBS and penicillin (100 U/mL), streptomycin (100 µg/mL), and glucose (0.292 mg/mL) in 10-layer CellStack (Corning, Corning, NY, USA).

Plasmid Construction

pRC2/2 was generated by subcloning the AAV2 genome minus the inverted terminal repeats (ITRs) (bp 191–4,498) from pAV2 (ATCC 37216) into pBluescript II (Agilent Technologies). A modified AAV2 intron lacking the splice donor site was inserted upstream of the rep open reading frame (ORF) between the p5 promoter and the rep start codon to enhance AAV cap gene expression in a strategy similar to Li et al., Xiao et al., and Kozak. PACG2 and pXX2 were constructed according to Li et al. and Xiao et al. (see Figures S1 and S2). The pRC2/8 and other serotypes were constructed by replacing the cap ORF in pTrans2/2 with the AAV8 cap ORF or cap ORFs for other serotypes. To construct pPAV, we sub-cloned the ITRs of pAV2 into the pBluescript II backbone and then inserted the promoter/WPRE/BGHpA between the ITRs. The pHelper plasmids were constructed in-house using the similar strategy of Matsushita et al.

rAAV Vector Production in Suspension Cells

Cells were transfected using PEIMAX (Polyscience) with three plasmids (pHelper, pRC, and pAAV). Plasmids were mixed in OptiMEM (Life Technology) in 1/20 vol of the cells to be transfected and incubated for 5 min. PEIMAX was then added to DNA diluted in OptiMEM, and after incubation for an additional 10 min, the DNA-PEIMAX complex was added to the cells. Eighteen hours post-transfection, sodium butyrate (5 mM; Sigma) and protein hydrolysate TN1 (0.5%; Organotechnie) were added to the culture, and rAAV vectors from cell lysate were harvested about 65 HPT. Alternatively, TN1 was replaced by Yeastolate (GIBCO Difco TC Yeastolate UF) at the same concentration. For large-scale production of rAAV vectors, rAAV vectors in both the cells and the medium were harvested (see below).

rAAV Vector Production in Adherent Cells

For large-scale rAAV vector production, adherent cells in a 10-layer CellSTACK (6,360 cm²; Corning) were transfected with 4 mg (plasmid ratio 2:1:1, pHelper/pRC/pAAV weight ratio) or 3 mg (plasmid ratio 1:5:0.31) of total DNA using a CaPO₄ method in 2-L DMEM with 2% FBS, penicillin (100 U/mL), streptomycin (100 µg/mL), and glucose (0.292 mg/mL). After 24 h, the transfection...
medium was replaced with 1 L fresh medium supplemented with TN1 and sodium butyrate, and rAAV vectors in cells and medium were harvested at about 65 HPT.

**Purification of rAAV**
For large-scale 1-L production, both cells and rAAV vectors in the medium were precipitated with 1/4 cell culture volume of 40% PEG 8000 (Sigma), 2.5 M NaCl. After incubation on ice for 2 h, the cells and rAAV in the medium were precipitated by centrifugation at 4,000 rpm for 30 min (J6-MI; Beckman Coulter). The precipitation was resuspended in 80 mL NT buffer (150 mM NaCl, 50 mM Tris [pH 8.5]) and subjected to three cycles of freeze and thaw. After Benzonase treatment (50 U/mL for 1 h at 37°C), the cell debris were spun down at 4,000 rpm for 30 min. The supernatant was then slowly adjusted to pH 5.5 with acetic acid (except for rAAV2) and centrifuged at 12,000 × g for 15 min. The pH was then adjusted to 8.0 with NaOH, and the starting material was filtered through a 0.45-mm filter. The filtrate was then loaded onto an affinity column (AVB-Sepharose or POROS CaptureSelect AAV8, AAV9, or AAVX) and eluted with 50 mM glycine-HCl (pH 3.0) after extensive wash with 10 times of column volume of phosphate-buffered saline (PBS) until the 280 nm absorbance was close to baseline. The eluted rAAV vectors were immediately neutralized with 1/10 vol of 1 M Tris-HCl (pH 8.0), dialyzed overnight against PBS-MK buffer (PBS with 1 mM MgCl2 and 2.5 mM KCl [pH 8.0]), and centrifuged at 3,000 rpm for 10 min to remove any precipitates. Purified rAAV vectors were then concentrated to a desired concentration in dialysis cassettes with Slide-A-Lyzer Concentrating Solution (Thermo Fisher) or spin filter (Amicon Ultra Centrifugal Filter Units [Millipore-Sigma]; molecular cutoff, 100 kDa).

**rAAV Titration Using Branched DNA (bDNA) Assay**
For quantification of rAAV2/1, rAAV2, rAAV2/5, or rAAV2/8 produced in 20-mL cultures, rAAVs in cell lysates were first purified with AVB-Sepharose, and then the QuantiGene2.0 assay (Branched DNA Technology) was used to determine the VGs of rAAV using a rAAV prep with known viral VG as standard. The EF1α-specific probe sets for pAAV-EGFP were used. Serially diluted viral preps were incubated in lysis buffer with a specific probe set in bDNA capture plates at 55°C for overnight hybridization, and then chemiluminescent signals were read in a Perkin Elmer EnVision.

**DOE Design and Statistical Methods**
All experimental designs and statistical analyses were performed using JMP version 7.0.2 and version 9.0.0 under the Windows Vista System. For the purpose of characterizing the impact of cell density, total DNA amount, ratio of plasmid 1 (pHelper) to plasmid2 (pRC), and ratio of plasmid 1 to plasmid 3 (rAAV) on the production of rAAV8 vector in suspension HEK293T cells and maximizing such production with regard to these factors with TN1 and sodium butyrate fixed, two response surface design experiments were performed and statistically analyzed. Student’s t tests were performed where appropriate. p < 0.05 was considered statistically significant.

**SDS-PAGE and Immunoblot**
Proteins in cell lysates were separated on a 4%–20% reducing Tris-Glycine gel (Life Technologies). Following transfer, membranes were probed with anti-VP1, -VP2, and -VP3 monoclonal antibody (mAb) (Fitzgerald). The bands were visualized by either Pierce ECL Western Blotting Substrate (Pierce) or IRDye 800CW Secondary Antibodies and Odyssey Scanner. Protein bands were quantified on LI-COR Image Studio Software (Li-Cor). Protein concentration was determined by the BCA (Bicinchoninic Acid) method (Pierce).

**SUPPLEMENTAL INFORMATION**
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**AUTHOR CONTRIBUTIONS**
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**CONFLICTS OF INTEREST**
The authors are employees of Amgen Inc. (Thousand Oaks, CA, USA).

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Supplemental Information

Creation of a High-Yield AAV Vector Production Platform in Suspension Cells Using a Design-of-Experiment Approach

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Table S1: DOE I design and results

Table S2: DOE II design and results

Figure S1: Description of the modified pRC.
The modified package plasmid (pRC2/2) was generated by inserting the AAV2 intron (Blue) between the p5 promoter (Green) and rep start codon (Yellow), mutating the splicing donor (blue, underlined) and modifying upstream open reading frames (uORFs) in the intron. Purple: the mutated ATGs (to GTC); Red: remaining ATGs; Black and underlined: stop codon. There are three uORFs left: 1. the first ATG is in frame with the first stop codon (TGA); 2. the second ATG is in frame with the second and the third stop codons (both are TAA); 3. the third ATG is also in frame with the second and third stop codons.

Figure S2. Modified pRC (pRC2/2) improves capsid protein expression.
rAAV2-EGFP was produced in 293T cells (20ml at 2.5 x 10⁶/ml) using different package plasmids. pRC2/2 was modified in-house as described in the Materials and Methods and Supplemental Figure S1. Plasmids pACG2 and dpXX2 were derived from Li et al and Xiao et al.¹⁹, ²². The cells were transfected using a 2:1:1 plasmid ratio (pHelper:pRC/pACG2/pXX2:pAAV, weight ratio) and harvested at 65 hours post-transfection. Samples for total culture were taken immediately after mixing the culture. All samples (total culture, medium and cell lysate) were subjected to three cycles of freezing and thawing. Equal volumes of samples for pRC, pACG2 and pXX2 were loaded on SDS-PAGE and immunoblot were performed as in the Materials and Methods.
Figure S1: Sequence from p5 promoter to rep78 stop codon in the pRC plasmid.

GTCCTGTATTAGAGGTACGTGAGTGTTTTGCGACATTTTGCGACACCATGTGGTCACGCTGGGTATTTAAGCCCGAGTGAGCACGCAGGGTCTCCATTTTGAAACGCGGAGGTTTTGAC
GCTCTAGAGCGTCAGACGCAGAAAGCTTTCAGTCAACAGCAGTGCCATCCAAAACAAAGCTTTCTGTCACGTGGGCTCAATCTGTCCTGTCTTTCCCTGCAAGACAATGCGAGAGAATG
AATCAGAATTCAATATCTCGTCTCACTCACGACAGAAAGACTGTATGCTTTCCCTGTCAGAAATCTCAACCCGTTTCTGTCGTCACAAAAGGCATACAGAAAATGTCGTACATT
CATCATATCATGGAAAAGGTGCAGCAGCTTGACACTGCGCTGCGATCTGGATTTGTCAGTCTGCAATTTTGTCAGTCATCTTCTGAAATAAAGTACATAAATCAGGATG
Figure S2: Comparison of the novel pRC2/2 with published packaging plasmid for VP expression.