*Rev*IT™ AAV Enhancer: Rev up AAV genome production in upstream manufacturing

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INTRODUCTION

In recent years, there has been an exponential increase in the number of registered clinical trials examining the use of recombinant adeno-associated virus (AAV)-based gene therapies, in part due to its ability to effectively deliver genes to target cells with minimal side effects. FDA approvals of AAV therapies are steadily increasing with two announced already in 2023, bringing the total number to five at the time of writing this paper [1-3]. These therapies represent some of the costliest drugs on the market with the most expensive, Hemgenix®, priced at roughly $3.5 million per dose [4]. The high price point is driven partly by the lack of efficient methods to produce sufficient AAV particles. A given therapy may need anywhere from 10^{11} to 10^{16} viral genomes per dose [5]. Using historical processes, numerous cell stacks or large stirred tank bioreactors may only be sufficient to produce a handful of doses per run, which presents a severe bottleneck in the manufacturing of these therapies [6]. Therefore, there is an urgent need to improve the overall process of AAV production to reduce the cost burden of these treatments. One area for improvement is the triple transfection step in the upstream manufacturing of AAV gene therapies.

Mirus has a legacy in nucleic acid delivery and was founded as a gene therapy and research tools provider in 1996. Conventional single component transfection reagents, i.e. polyethylenimine (PEI), are not sufficient to drive the steps necessary for maximum recombinant AAV production. The Mirus approach to transfection platform design is a combinatorial and multi-functional formulation that incorporates proprietary polymer and lipid technology. Mirus *Trans*IT® reagents are comprised of both polyamine-containing polymers and lipids that assist with overcoming various barriers during the transfection process. The polymer facilitates nucleic acid condensation, binding, and uptake by the cells, whereas the lipid promotes endosomal escape. This creates greater flexibility during product development and allows for the optimization of the reagent formulation to enable both high efficiency transfection and low cellular toxicity. Mirus supports both research and commercial grade manufacture of AAV and lentivirus through the *Trans*IT-VirusGEN® GMP Transfection Reagent and associated GMP grade kits which promote high titer recombinant virus production and percent full AAV capsids.

To further address the challenge of increasing the production of recombinant AAV in upstream manufacturing, Mirus Bio has developed an enhancer of AAV production, *Rev*IT™ AAV Enhancer, which can be used in conjunction with *Trans*IT-VirusGEN® Transfection Reagent or conventional polymeric transfection reagents. *Rev*IT™ AAV Enhancer produces 2-3x higher genome titers in suspension HEK 293 cells. This enhancer is simple to use, easily integrates into existing workflows, and improves AAV genome titers when added to many different types of transfection reagents. *Rev*IT™ AAV Enhancer produces high quality titers across a range of AAV serotypes and cell growth media, and when combined with the *Trans*IT-VirusGEN® Transfection Reagent this enhancer allows for the use of lower amounts of plasmid DNA which represents a key cost-saving opportunity. Through increasing recombinant AAV titers, *Rev*IT™ AAV Enhancer paves the way for driving down upstream manufacturing costs, resulting in a lower cost per therapeutic dose.

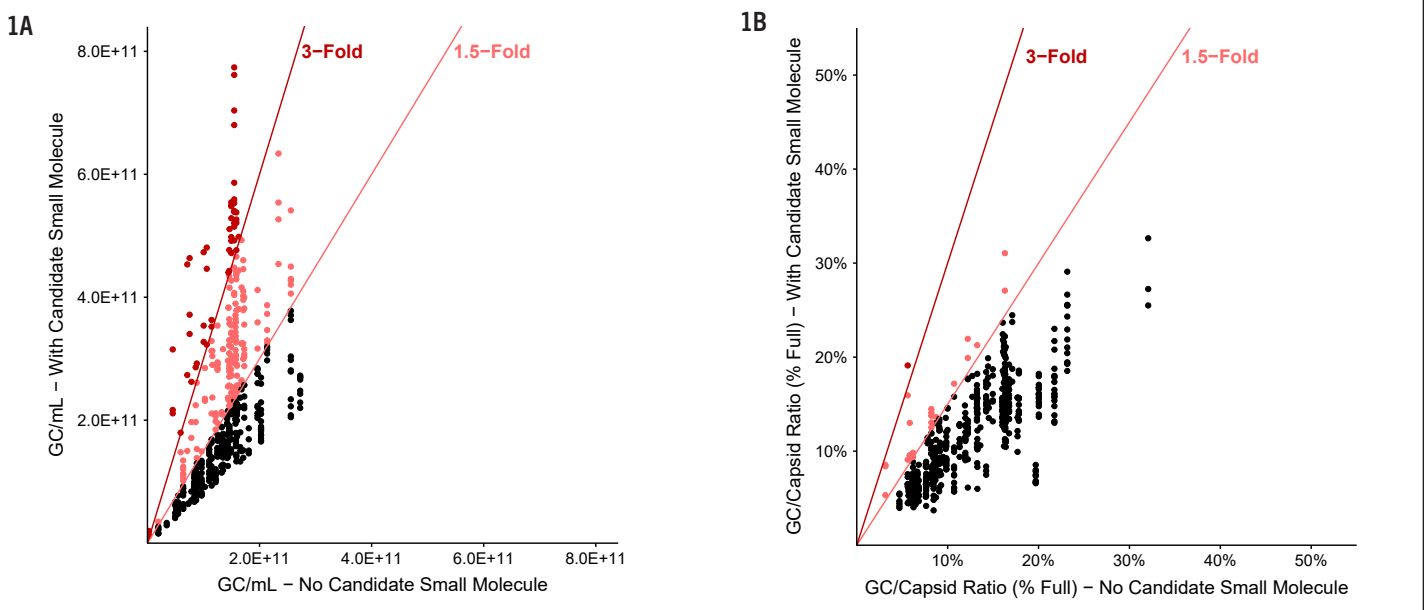
Discovery of small molecules enhancers for recombinant AAV production via triple transfection

To identify enhancers of AAV production, candidate small molecules which spanned many classes of cellular pathway modulators were selected via literature-based searches and evidence-based hypotheses. The key performance indicators (KPIs) for these molecules were set at a ≥1.5-fold increase in genome titers OR a ≥1.5-fold increase in the percentage of full AAV capsids as measured by the GC/capsid ratio, assuming no substantial decrease in genome titers. The initial range of test concentrations of each small molecule were determined, whenever possible, by previous uses of the molecules in HEK 293 cells in primary literature. Test concentrations were refined through empirical testing and optimization of experimental conditions. Figure 1 represents hundreds of iterative testing conditions across a range of operators, suspension HEK293 subtypes, complex formation times, transfection durations, and pDNA doses, and are specific to the effect of the candidate small molecule enhancers on AAV8 production. The data displayed in these graphs were filtered to only display enhancers which achieved genome titers that were within 20% of the "No Candidate Small Molecule" control. Several conditions for the small molecules tested achieved at least a 1.5-fold increase in genome titers (pink dots, Figure 1A), and a subset achieved greater than a 3-fold increase (red dots, Figure 1A). Interestingly, most of the small molecules tested had little effect on the percentage of full capsids (black dots, Figure 1B). This screening resulted in the identification of *Rev*IT™ AAV Enhancer, which increases genome titers 2-4x in multiple suspension HEK 293 subtypes, serotypes, and even allows for the reduction of overall pDNA usage while maintaining high genome titers.

*Rev*IT™ AAV Enhancer has a wide time of addition window, allowing for flexible workflows

To establish a window of time in which *Rev*IT™ AAV Enhancer is effective for increasing AAV genome production, time course experiments were performed. *Rev*IT™ AAV Enhancer was added 30-60 minutes pre-transfection (Figure 2A), immediately post-transfection, directly into the *Trans*IT-VirusGEN® transfection complex, and 30-60 minutes post-transfection (Figure 2B). At all times of addition, *Rev*IT™ AAV Enhancer increased AAV8 genome titers 2-fold or

FIGURE 1. Screening a collection of small molecules in the production of AAV8 leads to the identification of *Rev*IT™ AAV Enhancer, which boosts AAV genomes titers without negatively impacting percent full capsids.



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more over control conditions without a decrease in the percentage of full capsids, indicating that the timing of usage for this product is quite flexible. The ability to add *Rev*IT™ AAV Enhancer directly into the complex also eliminates the need for an additional line on a bioreactor when scaling up cultures, allowing for a simpler workflow.

Improved genome titers across multiple serotypes using *Rev*IT™ AAV Enhancer

The efficacy of *Rev*IT™ AAV Enhancer was assessed across multiple serotypes (AAV2, AAV5, AAV8, and AAV9) in 293-VP 2.0 cells (Thermo Fisher) using *Trans*IT-VirusGEN® and the single-component polymeric transfection reagents FectoVIR®-AAV, and PEIpro® (Figure 3). In all serotypes, using *Rev*IT™ AAV Enhancer in con-

FIGURE 2. *Rev*IT™ AAV Enhancer has a flexible window of time in which it can be added to cultures.

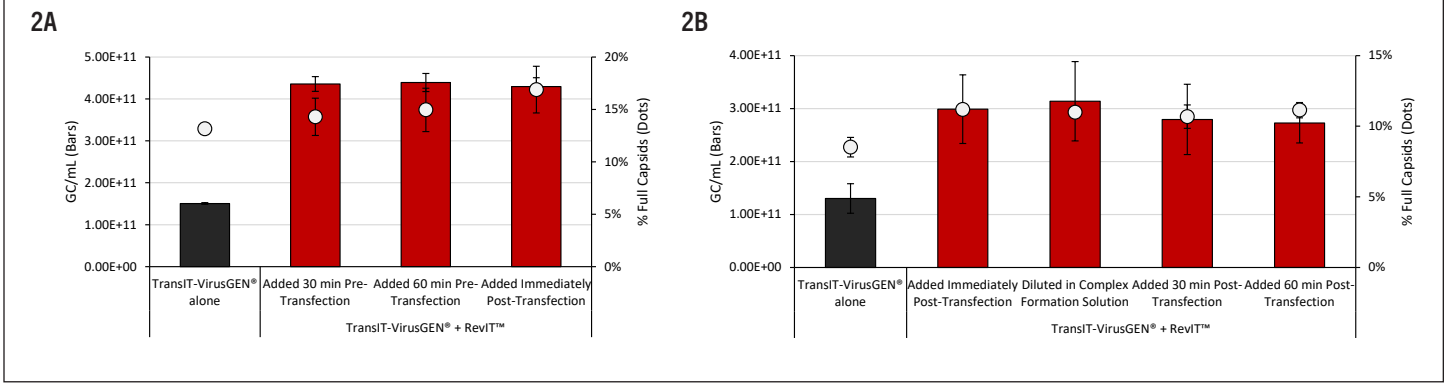
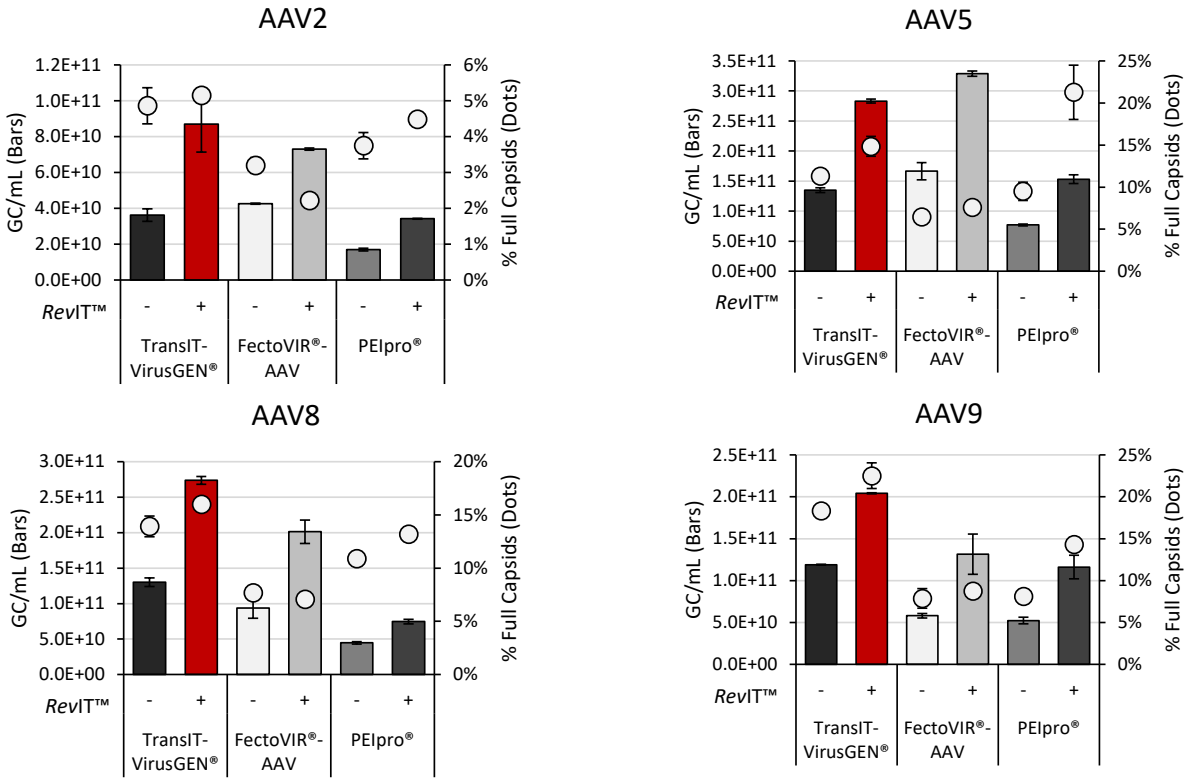


FIGURE 3. *Rev*IT™ AAV Enhancer increases genome titers across multiple serotypes and transfection platforms.



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junction with *Trans*IT-VirusGEN® increased genome titers 1.7 to 2.4-fold compared to the *Trans*IT-VirusGEN® control. The *Rev*IT™ AAV Enhancer + *Trans*IT-VirusGEN® condition also outperformed FectoVIR®-AAV alone and PEIpro® alone with up to 6-fold higher genome titers and 2.9-fold higher percent full capsids. *Rev*IT™ AAV Enhancer increased genome titers 1.7 to 2.2-fold with FectoVIR®-AAV, and PEIpro® compared to their respective controls, demonstrating the broad-spectrum applicability of *Rev*IT™ to other transfection reagents. In addition, *Rev*IT™ AAV Enhancer was tested in serotypes AAV2, AAV5, AAV8, and AAV9 using 293-VP 2.0 cells grown in either Viral Production Medium (VPM, Thermo Fisher) or BalanCD HEK 293 Medium (Irvine Scientific) and there was little to no difference in performance across these growth media formulations (Figure 4). Together, these data demonstrate broad serotype and growth medium compatibility when using *Rev*IT™ AAV Enhancer in conjunction with *Trans*IT-VirusGEN® and single-component polymeric transfection reagents.

FIGURE 4. *Rev*IT™ AAV Enhancer is effective in multiple cellular growth media formulations.

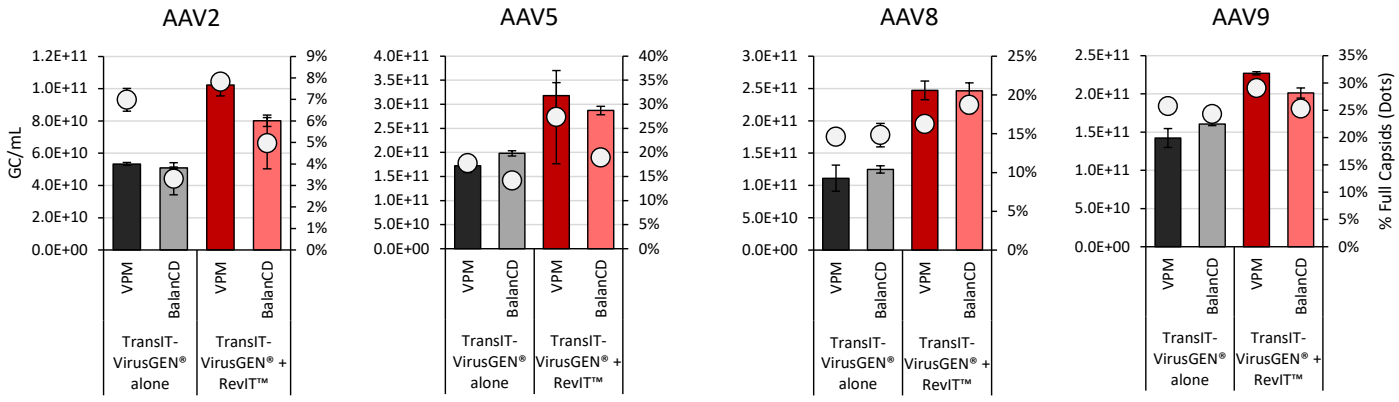
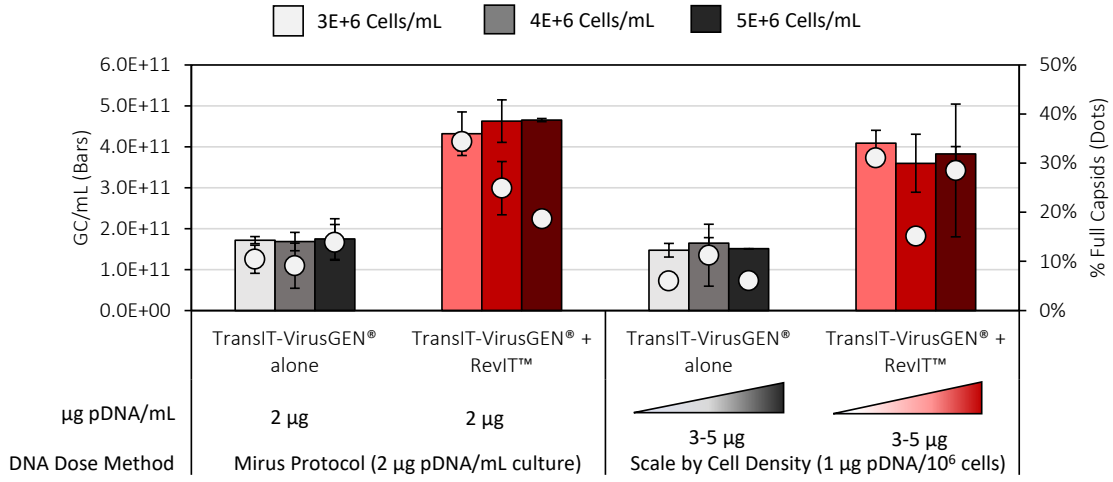


FIGURE 5. pDNA dose of 2 µg/mL cell culture is sufficient to generate high genome titers with the *Trans*IT-VirusGEN® Transfection Reagent and *Rev*IT™ AAV Enhancer.



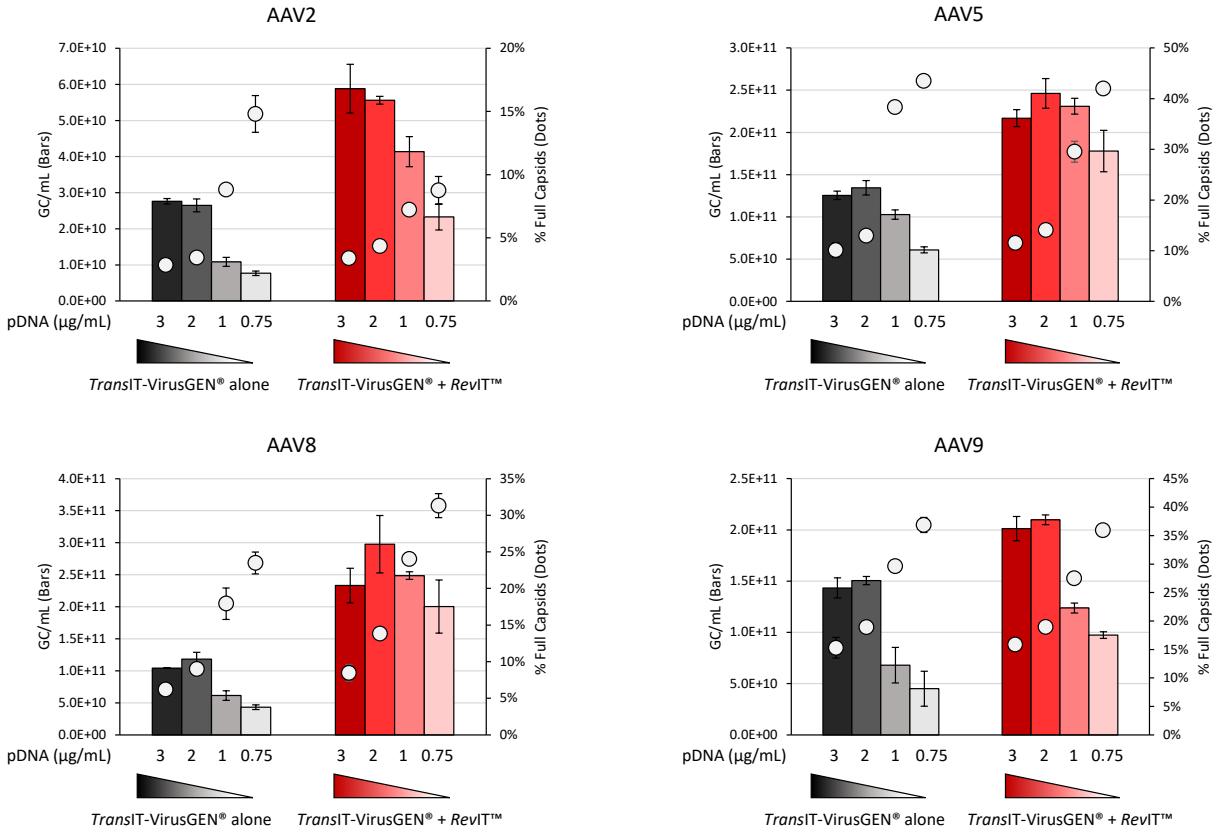
Cost savings is achieved from using *Rev*IT™ AAV Enhancer and lower pDNA doses

Protocols for commonly used single-component polymeric transfection reagents recommend plasmid DNA (pDNA) doses of 1 µg per 10^6 cells (e.g. 3 µg/mL at a density of 3×10^6 cells/mL) for triple transfection mediated AAV production in suspension cells. However, the Mirus protocol recommends dosing 2 µg pDNA per 1 mL of cell culture when using *Trans*IT-VirusGEN® regardless of cell density, which results in a 33% decrease in pDNA usage. Figure 5 demonstrates that scaling pDNA dosage by cell density does not improve viral titers, even when *Rev*IT™ AAV Enhancer is employed, indicating that maintaining a pDNA dose of 2 µg/mL cell culture with *Trans*IT-VirusGEN® can reduce the usage of valuable pDNA and save on manufacturing costs. We further tested even lower amounts of pDNA with *Rev*IT™ AAV Enhancer (Figure 6) and found that *Rev*IT™ AAV Enhancer allowed for a decrease in pDNA doses to as low as 0.75 µg/mL in some serotypes while still maintaining high genome titers. This allows for up to a 75% decrease in pDNA usage compared to a traditional DNA dose method of 3 µg/mL pDNA for a density of 3×10^6 cells/mL. Additionally, we found decreasing pDNA doses also leads to a higher percentage of full capsids, demonstrating that drastic cost savings and higher quality AAV can be achieved using lower pDNA doses in conjunction with *Rev*IT™ AAV Enhancer.

Conclusion

The overall goal of gene therapies is to target common genetic diseases, which will require both localized and systemic application of AAV. In order to make this a reality, all components of the manufacturing process need to be evaluated and optimized to reduce cost-of-goods and increase the number of patient doses obtained per run. *Rev*IT™ AAV Enhancer substantially increases AAV genome titers across multiple serotypes and transfection platforms, including the *Trans*IT-VirusGEN® Transfection Reagent and polymeric transfection reagents. Simple optimization will allow for fast and easy integration of *Rev*IT™ AAV Enhancer into existing AAV manufacturing workflows. These attributes, along with the ability to decrease the amount of pDNA, can lead to considerable savings in AAV-based gene therapy manufacturing costs.

FIGURE 6. Lower pDNA doses can be used to achieve high genome titers and percent full capsids with *Rev*IT™ AAV Enhancer.



Methods

Cell Culture

Viral Production Cells 2.0 (293-VP 2.0; Thermo Fisher Scientific, Cat. No. A49784) were maintained in 8% CO2 at 37°C on a 2.5 cm orbital shaker at 125 rpm in either Viral Production Medium (VPM; Thermo Fisher Scientific, Cat. No. A4817901) supplemented with 4 mM GlutaMAX™ (Thermo Fisher Scientific, Cat. No. 35050061) or BalanCD HEK293 Medium (Irvine Scientific, Cat. No. 91165) supplemented with 4 mM GlutaMAX™.

Transfection

The day before transfection, cells were passaged to ensure the cells were actively dividing and would reach a density of 4.0-5.0x10^6 cells/mL the day of transfection. The day of transfection, cells were seeded in 6-well non-tissue culture treated plates (Corning, 351146) at a density of 3.0x10^6 cells/mL. A 1 mg/mL DNA solution composed of a 1:1:1 plasmid concentration ratio of a transfer vector (Agilent, 240074-51), an AAV2 RepCap Vector (Aldevron, pALD-AAV2) OR an AAV5 RepCap Vector (Aldevron, pALD-AAV5) OR an AAV8 RepCap vector (Genemedi, P-RC09) OR an AAV9 RepCap vector (Genemedi, p-RC13), and a helper vector (Agilent 240071-54) was formed in water. For each milliliter of culture, 3 µL of *Trans*IT-VirusGEN® (Mirus Bio LLC, MIR 6700) and 2 µg of DNA were added to a final volume of 100 µL Phosphate-buffered saline (PBS; Corning, 21-040-CV) for a final transfection reagent to DNA ratio of 1.5:1. For any experiments where the concentration of plasmid DNA was an experimental variable, the amount of *Trans*IT-VirusGEN® Transfection Reagent was maintained at 3 µL/mL of cell culture across all pDNA doses. This mixture was incubated at room temperature for 30 minutes to allow transfection complexes to form, after which 100 µL was added per mL of culture for a final complexation volume of 10%. For FectoVIR®-AAV (5% complexation volume) and PEIpro® (10% complexation volume) transfections, 1 µL per million cells and 1 µg DNA per million cells for a final transfection reagent to DNA ratio of 1:1 was used. *Rev*IT™ AAV Enhancer was added either directly into the complex or immediately post-transfection. All experiments were harvested 72 hours post-transfection.

Harvest

To harvest AAV, cells were incubated with 0.1X volume of a 10X Cell Lysis Buffer, composed of 500 mM Tris pH 8, 10% Tween®20, 20 mM MgCl2, and 1000 U/mL Recombinant Dr. Nuclease (Syd Labs, BP4200) at 37°C for 1.5 hours while shaking. Cells were then incubated with 0.1X volume of 5 M NaCl at 37°C for 30 minutes while shaking. The lysate was centrifuged at 4,100 x g for 10 minutes to remove cell debris, and the supernatant transferred to a clean tube and stored at -80°C.

Genome and Capsid Quantitation

Genome quantitation was performed using digital PCR (dPCR) on a QIAcuity Digital PCR System (Qiagen, 911001). AAV lysates were diluted in a water-based buffer consisting of 1X GeneAmp PCR Buffer (Thermo Fisher Scientific, 4379878) and 0.05% Poloxamer 188 (Mirus Bio LLC, MIR 6230). Each reaction contained 1X QIAcuity Probe PCR Mastermix (Qiagen, 250102), 0.8 µM CMV Forward Primer (IDT; 5'- TTCCTACTTGGCAGTACATCTACG -3') and CMV Reverse Primer (IDT; 5'- GTCAATGGGGTGGAGACTTGG -3'), 0.4 µM CMV Probe (IDT; 5'- /56-FAM/TGAGTCAAA/ZEN/CCGCTATCCACGCCCA/3IABkFQ/ -3'), and 4.5 µL diluted AAV lysate in a final volume of 15 µL. Samples were loaded into a QIAcuity Nanoplate (Qiagen, 250021) and cycled using the following parameters: 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. Capsids were quantified using an ELISA kit for the appropriate serotypes (Progen, PRATV, PRAAV5, PRAAV8, PRAAV9).

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