

ARTICLE

Distribution of AAV8 particles in cell lysates and culture media changes with time and is dependent on the recombinant vector

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With clinical trials ongoing, efficient clinical production of adeno-associated virus (AAV) to treat large numbers of patients remains a challenge. We compared distribution of AAV8 packaged with Factor VIII (FVIII) in cell culture media and lysates on days 3, 5, 6, and 7 post-transfection and found increasing viral production through day 6, with the proportion of viral particles in the media increasing from 76% at day 3 to 94% by day 7. Compared to FVIII, AAV8 packaged with Factor IX and Protective Protein/Cathepsin A vectors demonstrated a greater shift from lysate towards media from day 3 to 6, implying that particle distribution is dependent on recombinant vector. Larger-scale productions showed that the ratio of full-to-empty AAV particles is similar in media and lysate, and that AAV harvested on day 6 post-transfection provides equivalent function in mice compared to AAV harvested on day 3. This demonstrates that AAV8 production can be optimized by prolonging the duration of culture post-transfection, and simplified by allowing harvest of media only, with disposal of cells that contain 10% or less of total vector yield. Additionally, the difference in particle distribution with different expression cassettes implies a recombinant vector-dependent processing mechanism which should be taken into account during process development.

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INTRODUCTION

Adeno-associated virus (AAV) is a powerful gene delivery vehicle capable of safely transducing a variety of tissues to provide long-term expression.^{1–4} As a result, AAV has long been considered a potential therapeutic vector, with the first clinical trial using AAV starting in 1995 (ref. 5), and the first AAV therapy receiving approval from a regulatory commission for clinical use in 2012 (ref. 6). With a number of clinical trials currently ongoing, the efficient clinical production of sufficient quantities of AAV to treat large numbers of patients remains a significant challenge.

Current cellular systems for producing AAV at scale include the use of adherent 293 cells in cell factories or roller bottles,^{7,8} suspension 293 cells,^{9–12} insect cells in suspension using the baculovirus system,¹³ HeLa cells stably expressing rep/cap genes,¹⁴ and coinfection with herpes simplex virus in baby hamster kidney cells.^{15,16} While each of these systems have unique advantages and disadvantages, none is dramatically more efficient than the rest at generating AAV on a per cell or per culture volume basis. Therefore, in place of a substantially more efficient system, it is necessary to optimize current AAV production methods to the greatest extent possible.

In addition to increasing production of AAV, streamlining the purification process is also essential to clinical production. Typically, cell lysates are harvested alone, though they can be harvested along

with cell culture media, with purification of AAV from both fractions. Producing AAV in adherent cell factories involves decanting media from the cell factories and adding ethylenediaminetetraacetic acid to detach the cells, followed by centrifugation and freeze-thawing and/or microfluidization.⁷ This process, when performed on up to hundreds of cell factories over the course of several weeks, is extremely cumbersome and time-intensive. A suspension system may make cell harvest more efficient, but would nevertheless likely involve either Triton-X100 or large-scale microfluidization.^{10,13} In addition, any methods which process cell lysates and culture media together have a greater burden in terms of DNA, RNA, and proteins, complicating downstream purification. An ideal method would restrict purification to one fraction (either lysate or, preferably, culture media) and thus minimize downstream processing.

Vandenberghe *et al.*¹⁷ and Lock *et al.*¹⁸ both described serotype-dependent release of AAV particles from cells into cell culture media during production. By using quantitative polymerase chain reaction (qPCR) to measure viral genomes, both papers show that the majority of viral genomes are located in the media at 72 hours with calcium phosphate-based transfection (Vandenberghe *et al.*¹⁷) or at 120 hours with polyethylenimine-based transfection (Lock *et al.*¹⁸). The notable exception was AAV2, which was closely associated with cells, apparently due to its heparin sulfate binding domain.¹⁷

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Furthermore, Okada *et al.*¹⁹ also showed a sevenfold increase in AAV8 particles in the cell culture media compared to the lysate.

These papers indicate that it should be possible to harvest cell culture media alone during AAV8 production, while losing only a small percentage of product and greatly streamlining the downstream process. To that end, we tested the distribution of AAV8 particles in cell lysates and culture media on days 3, 5, 6, and 7 post-transfection. Using an enzyme-linked immunosorbent assay (ELISA) kit to detect full and empty assembled AAV8 particles,^{20,21} we found, and show for the first time, a gradual shift in distribution from lysates to media with a daily increase in total AAV production through day 6, allowing for harvest of media only with an overall increase in yield compared to day 3. Furthermore and intriguingly, we found that this shift in distribution is dependent on the recombinant vector. Finally, we found that the full-to-empty particle ratio is equivalent in both lysate and media fractions, and that AAV harvested on day 6 is equivalent in function, after systemic injection in mice, to AAV harvested on day 3.

RESULTS

Distribution of AAV8 particles in cell lysates and culture media changes with time

Four groups ($n = 3/\text{group}$) of HEK 293T/17 cells on 15 cm plates were transfected to produce AAV8 packaged with a 5.1 kb

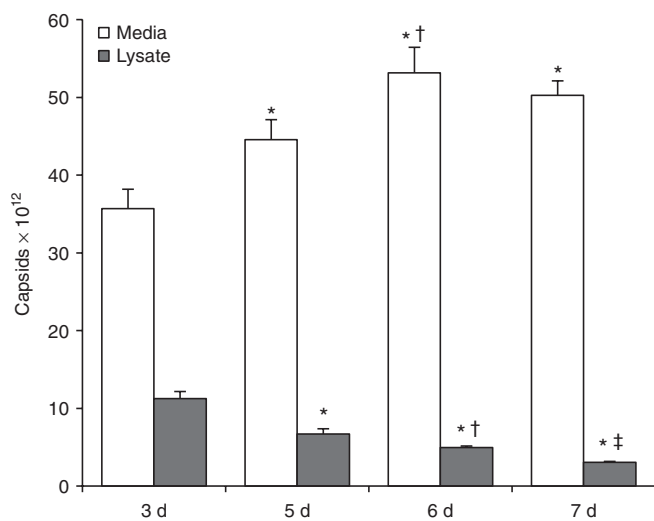


Figure 1 Distribution of adeno-associated virus (AAV)8 particles in cell lysates and culture media changes with time. Four groups ($n = 3/\text{group}$) of HEK 293T/17 cells on 15 cm plates were transfected to produce AAV-FVIII. Cells and culture media were harvested on days 3, 5, 6, and 7 and analyzed by AAV8 capsid enzyme-linked immunosorbent assay to determine the distribution of viral genomes. AAV-FVIII showed an increase in production when culture was extended from day 3 ($1.1 \times 10^{13} \pm 9.2 \times 10^{11}$ and $3.6 \times 10^{13} \pm 2.5 \times 10^{12}$ total capsids in the lysate and media, respectively) to day 5 ($6.7 \times 10^{12} \pm 6.7 \times 10^{11}$ and $4.5 \times 10^{13} \pm 2.6 \times 10^{12}$ total capsids in the lysate and media, respectively), day 6 ($5.0 \times 10^{12} \pm 1.9 \times 10^{11}$ and $5.3 \times 10^{13} \pm 3.3 \times 10^{12}$ total capsids in the lysate and media, respectively), and day 7 ($3.0 \times 10^{12} \pm 1.3 \times 10^{10}$ and $5.0 \times 10^{13} \pm 1.9 \times 10^{12}$ total capsids in the lysate and media, respectively) post-transfection, with day 5 showing a 9% increase over day 3 and day 6 showing a 24% increase over day 3 (* denotes significant difference versus day 3; † denotes significant difference versus day 5; and ‡ denotes significant difference versus day 6 as measured by analysis of variance, $P = 0.004$). Viral titer decreased from day 6 to 7, but was still 14% higher than on day 3. 76% of AAV-FVIII particles were in the culture media at day 3, and this proportion increased to 87% by day 5, 91% by day 6, and 94% by day 7.

single-stranded genome expressing the human Factor VIII (FVIII) gene.²² Cells and culture media (containing 10% fetal bovine serum in this and all subsequent experiments) were harvested on days 3, 5, 6, and 7 and analyzed by AAV8 capsid ELISA to determine the distribution of assembled viral particles in lysates and media. As shown in Figure 1, AAV-FVIII production increased when culture duration was extended from day 3 to day 5, day 6, and day 7 post-transfection, with day 5 showing a 9% increase over day 3 and day 6 showing a 24% increase over day 3 (all groups significantly different versus day 3 as measured by analysis of variance, $P = 0.004$). Viral titer decreased from day 6 to 7, but was still 14% higher than on day 3. Seventy-six percent of AAV-FVIII particles were in the culture media at day 3, and this proportion increased to 87% by day 5, 91% by day 6, and 94% by day 7.

Distribution of AAV8 particles in cell lysates and culture media is dependent on the recombinant vector

Six groups ($n = 3/\text{group}$) of 293T/17 cells on 10 cm plates were transfected to produce AAV-FVIII, or AAV packaged with human Factor IX (FIX),^{23,24} or AAV packaged with human Protective Protein/Cathepsin A (PPCA; which, along with FIX, is a self-complementary genome of approximately 2.4 kb).²⁵ Cells and culture media were harvested on day 3 or 6 to determine whether AAV distribution was influenced by recombinant vector. As shown in Figure 2, at days 3 and 6 post-transfection, the majority of AAV8 particles were located in the media for all three vectors that were packaged; however, this ratio changed more dramatically for FIX and PPCA than for FVIII. At day 3, 71% of the FVIII particles were in the media, while 65% of the FIX and 59% of the PPCA particles were in the media (the FVIII and PPCA groups were significantly different as measured by analysis of variance, $P = 0.008$). By day 6, 92% of FVIII, 90% of FIX and 88% of PPCA particles were in the media, indicating a greater shift in AAV particle distribution for FIX and, especially, PPCA. Interestingly, all three vectors showed a 14% increase in total AAV production by day 6 versus day 3.

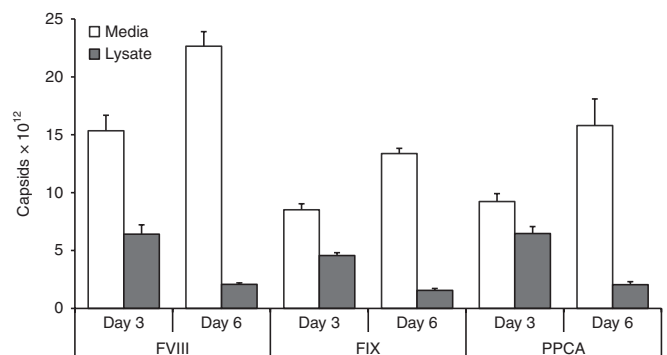


Figure 2 Distribution of adeno-associated virus (AAV)8 particles in cell lysates and culture media is dependent on the recombinant vector. Six groups ($n = 3/\text{group}$) of 293T/17 cells on 10 cm plates were transfected to produce AAV-FVIII, AAV-FIX, or AAV-PPCA. Cells and culture media were harvested on day 3 or 6 to determine whether AAV distribution was influenced by the recombinant vector. At days 3 and 6 post-transfection, the majority of AAV8 particles were located in the media for all three vector genomes, but this ratio changed more dramatically for FIX and PPCA than for FVIII. At day 3, 71% of the FVIII particles were in the media, while 65% of the FIX and 59% of the PPCA particles were in the media (the FVIII and PPCA groups were significantly different as measured by analysis of variance, $P = 0.008$). By day 6, 92% of FVIII, 90% of FIX and 88% of PPCA particles were in the media, indicating a greater shift in AAV particle distribution for FIX and, especially, PPCA.

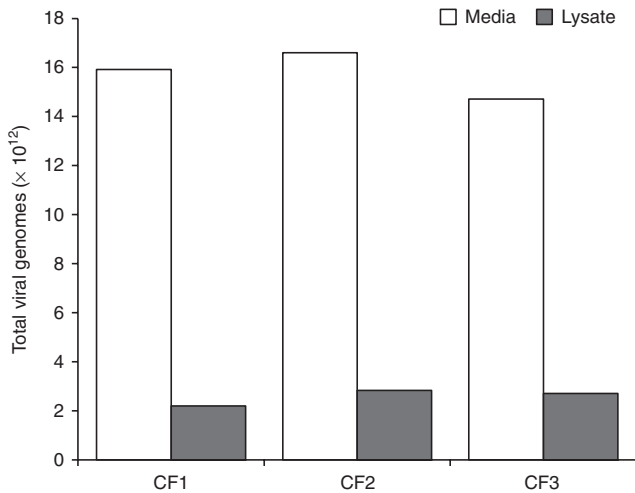


Figure 3 Viral genome distribution in cell lysates and culture media tracks with particle distribution. Three 10-stack cell factories (CF1, CF2, and CF3) containing 293T/17 cells were transfected to produce adeno-associated virus (AAV)-FVIII. Cell lysates and media were harvested 6 days after transfection, and were processed separately for each cell factory. Quantitative polymerase chain reaction revealed that an average of $1.6 \times 10^{13} \pm 9.6 \times 10^{11}$ and $2.6 \times 10^{12} \pm 3.4 \times 10^{11}$ total viral genomes were in the media and lysate, respectively, with an average of $85.9 \pm 1.8\%$ of FVIII genomes in the media across the three cell factories.

Viral genome distribution in cell lysates and culture media tracks with particle distribution

To determine whether AAV viral genome distribution followed the same pattern as AAV8 capsid distribution, three individual 10-stack cell factories containing 293T/17 cells were transfected to produce AAV-FVIII. This experiment was important to verify that empty AAV particles did not preferentially traffic to the culture media, which would result in a high ratio of empty to full particles. Cell lysates and media were harvested 6 days after transfection, and were processed separately for each cell factory through size-exclusion chromatography and anion exchange. As shown in Figure 3, qPCR revealed that an average of $1.6 \times 10^{13} \pm 9.6 \times 10^{11}$ and $2.6 \times 10^{12} \pm 3.4 \times 10^{11}$ total viral genomes were in the media and lysate, respectively. With an average of $85.9 \pm 1.8\%$ of FVIII genomes in the media across the three cell factories, the ratio of full-to-empty AAV particles is nearly equivalent in both media and the cell lysates.

AAV harvested at D6 provides comparable expression to AAV harvested at D3

To ensure that AAV harvested at later time points was as effective at providing therapeutic expression as AAV harvested at earlier time points, two preparations of three 10-stack cell factories of AAV-FVIII were purified after harvest of cell lysates and culture media at either day 3 or day 6 post-transfection. AAV-FVIII was injected into C57Bl/6 mice at a dose of 5×10^9 , 2×10^{10} , or 2×10^{11} viral genomes per mouse and plasma FVIII levels were analyzed 7 days postinjection ($n = 4-5$ /group). Figure 4 shows mean FVIII levels in the three dose ranges at both time points, with comparable expression between AAV preparations (21.0 ± 7.6 ng/ml for D3 versus 15.0 ± 6.0 ng/ml for D6 for the low dose; 189.2 ± 83.9 ng/ml for D3 versus 113.7 ± 68.5 ng/ml for D6 for the medium dose; and $1,181.5 \pm 371.5$ ng/ml for D3 versus $1,019.2 \pm 69.9$ ng/ml for D6 at the high dose). While AAV-FVIII harvested at day 3 post-transfection provides a slightly higher level of expression at all three time points, these differences were not

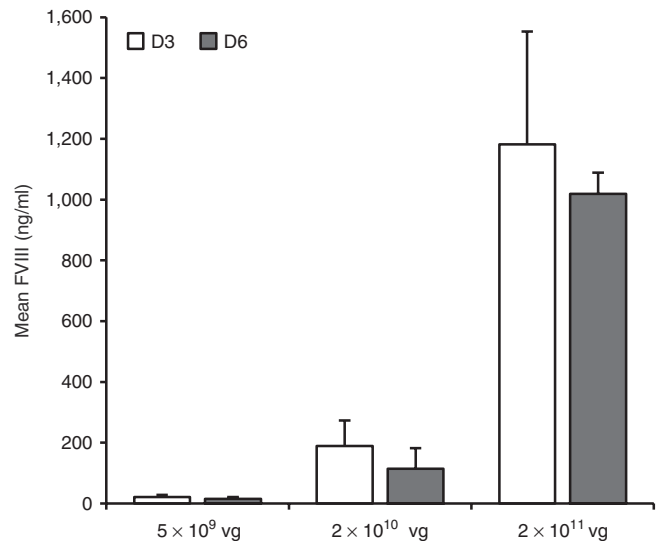


Figure 4 Adeno-associated virus (AAV) harvested at day 6 provides comparable expression to AAV harvested at day 3. Two preparations of three 10-stack cell factories of AAV-FVIII were purified after harvest of cell lysates and culture media at either day 3 or day 6 post-transfection. AAV-FVIII was injected into C57Bl/6 mice at a dose of 5×10^9 , 2×10^{10} , or 2×10^{11} viral genomes per mouse and plasma FVIII levels were analyzed 7 days postinjection ($n = 4-5$ /group). Expression was comparable between AAV preparations (21.0 ± 7.6 ng/ml for D3 versus 15.0 ± 6.0 ng/ml for D6 for the low dose; 189.2 ± 83.9 ng/ml for D3 versus 113.7 ± 68.5 ng/ml for D6 for the medium dose; and $1,181.5 \pm 371.5$ ng/ml for D3 versus $1,019.2 \pm 69.9$ ng/ml for D6 at the high dose). Though AAV-FVIII harvested at day 3 post-transfection provides a slightly higher level of expression at all three time points, these differences were not statistically significant ($P = 0.26$, $P = 0.19$, and $P = 0.37$ at the low, medium, and high doses, respectively).

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DISCUSSION

While previous studies have looked at the distribution of various AAV serotypes in the lysate and culture media, this is the first, to our knowledge, to demonstrate a gradual shift from lysate to media with time and a dependence on the recombinant vector. As reported previously,¹⁸ this shift takes place without any evident cytopathic effect: adherent 293 cells cultured in 10% fetal bovine serum appear under the microscope on day 6 post-transfection as attached, confluent, and similarly healthy to cells at day 3. Interestingly, however, our experience shows that changing media 24 hours after transfection to serum-free Dulbecco's Modified Eagle's Medium and maintaining culture until day 6 leads to a large amount of cell detachment and death, but similar AAV yields in the media (data not shown). Because cells remain healthy in extended culture in the presence of serum, it may be possible to provide further supplements to encourage increased AAV production at later time points.

Importantly, the shift of AAV8 particle distribution toward the media is not primarily an accumulation of empty capsids, as has been speculated.²⁶ Instead, qPCR shows that 86% of viral genomes are located in the media, compared to 90% of capsids. This indicates that the full-to-empty particle ratio may be slightly lower in the media than the lysate, but this difference is likely to be unimportant, especially with downstream procedures that separate full AAV particles from empty particles. Furthermore, in the case of working with adherent 293 cells, the added labor and complications of processing the lysate are not worthwhile for the small increase in yield.

The mechanism behind the dependence on recombinant vector is unknown. AAV-FVIII was more prevalent in the media by day 3 post-transfection, with between 71–76% of total particles in the media, compared to 65 and 59% for FIX and PPCA, respectively. This dependence on recombinant vector could include factors such as size, genome sequence, self-complementarity, vector production levels within the cell, or transgene toxicity, and would be an interesting topic for future studies to address. Nevertheless, this difference indicates that groups should optimize their processes for each recombinant vector that goes into production, rather than assuming a given time point will provide the same AAV particle distribution, even within a given serotype.

While most groups harvest AAV preps at some point between day 2 and day 5 post-transfection, we show here that extended culture to day 6 is optimal for maximizing yields of AAV8 packaged with FVIII, although this finding could well change with a different cellular production system or transfection method. Indeed, as Figure 1 shows, at day 6 there were more AAV-FVIII particles in the media alone than in the media and lysate combined at day 3. In some cases, extension to day 7 could prove worthwhile as well. While we saw a small decrease in production from day 6 to day 7, the difference in total number of capsids was not significant between those two time points. Importantly, there were no statistically significant differences in expression in mice between groups injected with AAV-FVIII harvested at day 3 or day 6 post-transfection, indicating that extended time in culture does not lead to degradation of the AAV8 particle or decreased transduction efficiency *in vivo*.

In conclusion, increasing the duration of culture post-transfection can significantly improve yields of AAV8 and shift the distribution of particles more greatly toward the culture media. This has important implications for large-scale production, during which the media may be harvested alone, eliminating the more time- and labor-intensive processing of cell lysate, which may contain 10% or less of the total particle yield. Additionally, the differences in particle distribution of different expression cassettes at day 3 post-transfection implies a recombinant vector-dependent processing mechanism which should be taken into consideration when developing upstream production processes.

MATERIALS AND METHODS

Cell culture and transfection

Adherent HEK 293T/17 cells were cultured in 10 cm plates, 15 cm plates, or 10-stack cell factories (Corning, Corning, NY) using Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum supplemented with 2 mmol/l GlutaMAX (Life Technologies, Grand Island, NY). AAV was produced by two-plasmid transfection using PEIpro (Polyplus-transfection SA, Illkirch, France) 1 day after seeding cells at a density of 7.26×10^4 cells/cm². Three different viral genomes flanked by AAV2 inverted terminal repeats were packaged into AAV8 capsids: human Factor VIII, human Factor IX, and human Protective Protein/Cathepsin A. While the FVIII genome was single-stranded, FIX and PPCA cassettes included a mutated inverted terminal repeat for packaging of self-complementary genomes. The backbones of genome-containing plasmids also contained the adenoviral helper genes required for AAV production, and the second plasmid in each transfection contained the AAV2 Rep and AAV8 capsid genes. Cell cultures were maintained from between 3 and 7 days post-transfection. For cultures lasting longer than 3 days, additional media (50% of the original transfection volume) was added at day 3 to provide cells with additional nutrients.

AAV harvest

At the time of harvest, culture media were pipetted off plates or decanted from individual cell factories, after which cells were detached using PBS with 5 mmol/l ethylenediaminetetraacetic acid. Cells were pelleted, resuspended in TD buffer, and subjected to five freeze/thaw cycles. Cell lysates

were treated with benzonase and centrifuged at high speed to pellet debris. The lysate supernatants containing AAV were removed and pellets were discarded.

Large-scale AAV-FVIII purification

After decanting from cell factories, culture media were filtered through 0.22 micron filters and concentrated by tangential-flow filtration using a 0.005 m² Pellicon XL Ultrafiltration Module with a 100 kDa cut-off (EMD Millipore, Darmstadt, Germany) for single-cell factories (1.5 l total) or a 0.1 m² Pellicon 2 membrane for triple-cell factory preparations (3–4.5 l). After concentration, media were treated with benzonase and filtered again prior to column chromatography.

Cell pellets from single-cell factory preparations were processed as described above and filtered through a 0.22 micron filter prior to column chromatography. Pellets from triple-cell factory preparations were lysed using an M-110L Microfluidizer Processor (Microfluidics, Newton, MA), centrifuged to remove debris, treated with benzonase, and filtered through a 0.22 micron filter.

After benzonase treatment and filtration, lysates and media were loaded onto a HiPrep Sephacryl S-300 HR size-exclusion chromatography column equilibrated with 10 mmol/l Tris-Bis Propane, 10 mmol/l Tris, and 200 mmol/l NaCl, at pH 9.0. The flow-through fraction containing AAV was diafiltered to a low-salt buffer and concentrated with a Pellicon XL module with a 100 kDa cut-off. AAV in concentrated diafiltrates was further purified by anion-exchange on a POROS HQ resin equilibrated with 10 mmol/l Tris-Bis Propane, and 10 mmol/l Tris, at pH 9.0. AAV was eluted from the column using a linear NaCl gradient. For the single-cell factory preparations, the lysate and media fractions were purified separately through column chromatography and never combined. For triple-cell factory preparations, lysate and media fractions were combined during post-size-exclusion chromatography concentration and diafiltration.

For triple-cell factory preparations, anion-exchange fractions containing AAV were pooled, concentrated, and underwent cesium-chloride gradient ultracentrifugation to separate viral genome-containing AAV particles from empty particles. CsCl fractions were then dialyzed against PBS and genome-containing fractions were pooled prior to mouse studies.

Supplementary Figure S1 shows a Coomassie Blue-stained SDS-PAGE gel demonstrating the purity of AAV-FVIII through anion exchange. Furthermore, an experiment to determine the activity of benzonase nuclease in concentrated culture media is described in the **Supplementary Materials and Methods** and demonstrated in **Supplementary Figure S1**.

AAV8 capsid ELISA

Assembled viral genome-containing and empty AAV8 particles were detected using the AAV8 Titration ELISA Kit (PRAAV8; Progen Biotechnik GmbH, Heidelberg, Germany). Briefly, raw culture media and benzonase-treated lysate samples were diluted in kit-provided sample buffer and incubated on the provided ELISA plate for one hour. Plates were then incubated with biotin-conjugated anti-AAV8, followed by a streptavidin peroxidase conjugate, and a substrate containing tetramethylbenzidine. Absorbance was measured on a Spectramax M2 spectrophotometer (Molecular Devices, LLC, Sunnyvale, CA) at 450 nm.

Quantitative PCR

qPCR was used to determine AAV-FVIII viral genome titers in anion exchange fractions from single-cell factories and in dialyzed CsCl fractions from triple-cell factories. Primers targeted the HLP promoter in the 5' region of the viral genome, or the middle of the FVIII gene: 5'-CAGGACGCTGTGGTTCTG-3' (HLP forward), 5'-TGCCTGAAGCTGAGGAGAC-3' (HLP reverse), 5'-GGAGATG AAGAAGGAGGACTTTG-3' (FVIII forward), and 5'-TCCACAGCAGCAATGAAG TAG-3' (FVIII reverse). Samples were run in duplicates of 25 µl in Bio-Rad iQ Sybr Green Supermix on the Bio-Rad iCycler with attached MyIQ real-time detection system (Bio-Rad Laboratories, Hercules, CA).

Mouse studies

All animal studies were performed according to protocols approved by the St. Jude Institutional Animal Care and Use Committee. To compare AAV-FVIII preparations purified from harvest on day 3 versus day 6 post-transfection, C57Bl/6 mice were injected via the tail vein with 5×10^9 , 2×10^{10} , or 2×10^{11} viral genomes of either day 3 AAV-FVIII prep or day 6 AAV-FVIII prep ($n = 4-5$ mice per group). At 7 days postinjection, blood was collected by retro-orbital bleed with ethylenediaminetetraacetic acid-coated capillary tubes

and plasma was isolated. The plasma concentration of human FVIII was determined by ELISA (ASSERACHROM VIII:Ag, DIAGNOSTICA STAGO, France), which does not cross-react with endogenous mouse FVIII.

Statistics

Data are expressed as the average plus or minus the standard deviation, where appropriate. Analysis was performed with Student's *t*-test in Excel (Microsoft, Redmond, WA) or one-way analysis of variance with a Tukey comparison in Minitab (Minitab, State College, PA). $P < 0.05$ was considered significant in all comparisons.

CONFLICT OF INTEREST

There are no institutional or corporate arrangements that would be considered a financial conflict of interest related to this manuscript.

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