# Continuous Collection of Adeno-Associated Virus from Producer Cell Medium Significantly Increases Total Viral Yield

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The ability to efficiently produce large amounts of high-titer recombinant adeno-associated virus (AAV) is a prerequisite to the continued success of AAV as a gene therapy tool targeted toward large-animal preclinical studies or human clinical therapeutics. Current manufacturing procedures necessitate laborious and time-consuming purification procedures to obtain AAV particles of sufficient titer and purity for these demanding biomedical applications. The finding that AAV can be harvested and purified from producer cell medium may represent an efficient alternative to purifying AAV from cellular lysates. Here we sought to determine the maximum duration of time, and frequency within which AAV can be harvested from producer cell medium, in order to maximize the yield obtained from a single transfection preparation. Human embryonic kidney 293T cells were transfected with polyethylenimine to produce AAV2/5 expressing green fluorescent protein (GFP), and cellular medium was harvested every 2 days until a maximum duration of 19 days posttransfection. AAV2/5-GFP was released into producer cell medium at a steady state until 7 days posttransfection, at which time titers dropped dramatically. Harvesting medium every two days resulted in the maximum yield of AAV from a single preparation, and the cumulative yield of AAV harvested from the producer cell medium was 4-fold higher than the yield obtained from a traditional purification of AAV from cellular lysates. The AAV2/5 harvested from medium within the 7-day collection time-course mediated high levels of transduction *in vivo,* comparable to AAV2/5 harvested from cellular lysates. AAV purified from cell lysates showed increasing amounts of empty particles at 5 and 7 days posttransfection, whereas AAV purified from cell medium did not show an increase in the amount of empty particles throughout the 7-day time course. Finally, we extended these findings to AAV2/9, demonstrating that a comparable ratio of AAV2/9 particles are also released for up to 7 days posttransfection.

## INTRODUCTION

Recombinant adeno-associated virus (AAV) is one of the most frequently used viral vector systems for gene therapy research and clinical applications.[1–3] One major advantage of AAV is the ability to rapidly initiate transgene expression in both dividing and nondividing cells, which can be maintained in nondividing cells over the lifetime of an individual.[4,5] Further, AAV has a high biosafety rating because of its naturally replication-incompetent and nonpathogenic nature. However, despite these major advantages and the success already achieved with AAV, concerns regarding the viability of AAV as a gene therapy tool have arisen as a result of the relatively cumbersome and time-intensive process necessary to purify high-quality virus from cell lysates.[5] This limitation is particularly relevant to large-animal preclinical studies and human clinical applications, which necessitate the ability to produce large amounts of high-titer AAV in a simple, efficient, and cost-effective manner.

AAV production laboratories are addressing this problem by implementing new and innovative ways to produce AAV in large quantities, such as the use of insect cells or cell suspensions within bioreactors.[6,7] However, because of the use of hybrid helper viruses or expensive technical equipment, these production techniques may not be

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sufficiently flexible or cost effective to fit the needs of researchers performing important gene therapy research. Further, these techniques still rely on the laborious task of purifying viral particles from cellular lysates. The discovery that AAV can be harvested from cellular medium may provide a solution to these problems. Specifically, recent work has demonstrated that after the standard co-transfection technique for AAV production in human embryonic kidney 293T (HEK 293T) cells, large amounts of AAV are released into the culture medium.[8–10] The released AAV vectors contain intact genomes, are infectious, and are easily purified with standard iodixanol gradient centrifugation.[8–11] The release of AAV to the cellular medium is serotype dependent, and in most instances the majority of total viral particles produced are released into the medium.[8,9] Owing to the simplicity, flexibility, and scalability of this novel production technique, it is possible that purification of AAV from cellular medium may represent a solution to the increasing demands of gene therapy research. However, the duration of time, and frequency within which AAV can be harvested from producer cell medium after a single transfection has not been fully characterized.

Lock et al.[8] demonstrated that AAV can be harvested from the medium at 72 hours posttransfection, and that by increasing incubation time, the proportion of virus in the medium increases. This finding prompted us to investigate the maximum time and frequency within which functional AAV particles can be harvested from a single transfection preparation of AAV. HEK 293T cells were transfected to produce AAV2/5 expressing green fluorescent protein (GFP), and cellular medium was harvested every 2 days until a maximum duration of 19 days posttransfection. After that, AAV was purified from the medium, and the number of viral genomes at each individual time point was quantified to determine the optimal length and frequency that AAV can be harvested from a single preparation. Finally, the ratio of empty versus full particles of medium-purified AAV was assessed, and the biological activity was evaluated *in vivo* after stereotaxic injection to the central nervous system. From this work, we report the novel observation that the total yield of intact and biologically active AAV can be increased four-fold by harvesting particles from producer-cell medium for seven days posttransfection.

## METHODS

### Virus preparation

HEK 293T (ATCC) cultures were maintained in a triple flask (Sigma-Aldrich) in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals) and 1% penicillin/streptomycin (Life Technologies). Cells were passaged bi-weekly in order to maintain the cells in the log phase of growth. A fully confluent triple flask was used to seed six triple flasks for each individual vector production group.

AAV5 vectors encoding humanized GFP, or AAV9 vectors encoding a blue fluorescent protein (BFP) under control of the hybrid chicken $\beta$-actin/cytomegalovirus (CBA/CMV) promoter, were produced as described previously.[12] Additionally, AAV5 expressing GFP under control of the synapsin promoter was used for experiments testing whether saturation of producer cell medium diminishes AAV release. Viruses were created by co-transfection of HEK 293T cells with the AAV transgene plasmid and a helper plasmid encoding capsid proteins for AAV serotype 5 or 9, as well as adenovirus helper functions. Transfected HEK 293T cells were maintained at 37°C for 24 hr in DMEM containing 10% FBS, after which the culture medium was replaced with serum-free DMEM for the reminder of incubation. After the change to serum-free DMEM, culture medium was collected every 24 or 48 hr and replaced with fresh serum-free DMEM, until the termination of the respective time course (Fig. 1). Harvested medium was stored at 4°C until further processing. At the termination of the time course, cells were harvested and lysed, and virus was purified using an iodixanol gradient as described previously.[12] To purify AAV from the medium, the harvested medium was centrifuged at $4000\times g$ for 5 min to pellet cellular debris, and clarified through a 0.5 $\mu$m Mini-profile II capsule filter (Pall). The clarified medium was then concentrated to 12 ml by tangential flow filtration (TFF), using two parallel Minimate TFF capsules (Pall) with a 100 kDa molecular weight cutoff. Clarified and concentrated medium or cellular lysates were then loaded onto an iodixanol concentration gradient and purified by ultracentrifugation as previously described.[12] DNAse 1 (Benzonase; Sigma-Aldrich)-resistant vector genomes were titered with a dot blot assay,[12] using a biotinylated probe against the promoter.[13] A near-infrared-conjugated antibody (IRDye 800; Li-Cor Biosciences) was used to detect the probe and this complex was quantified using a Li-Cor Odyssey scanner (Li-Cor Biosciences).

### Animals and viral injection

Experiments were conducted on young adult (220 g) male Sprague Dawley rats in accordance with guidelines of the Michigan State University

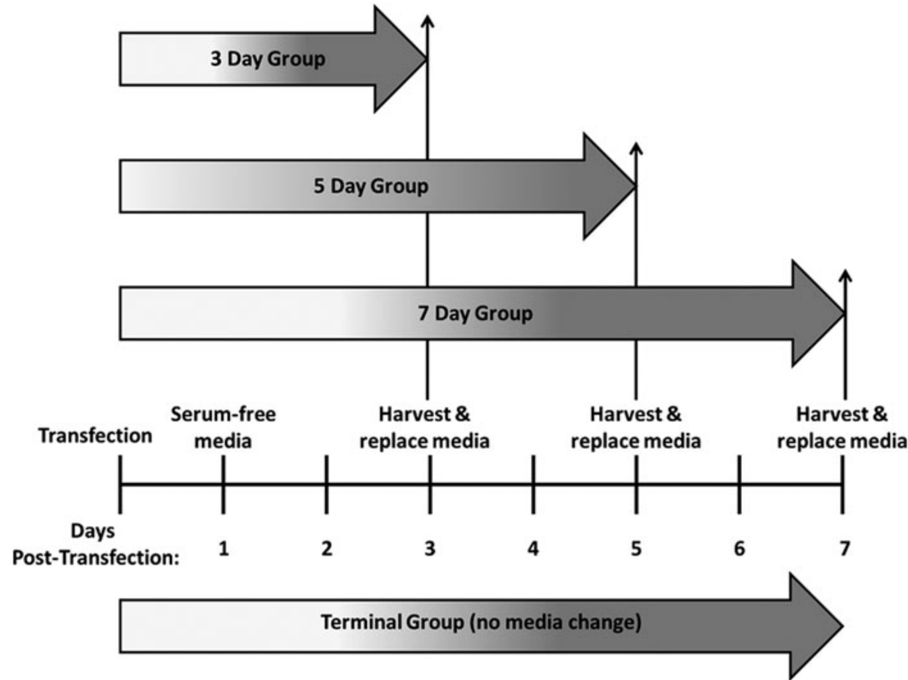



**Figure 1.** Seven-day AAV purification time course. Polyethylenimine was used to co-transfect six confluent triple flasks of HEK 293T cells with the AAV genome along with a plasmid encoding capsid proteins and all necessary helper functions. One-day posttransfection, cellular medium was replaced with fresh serum-free DMEM. Then, medium from each group was collected every 2 days and replaced with fresh serum-free medium (3-, 5-, and 7-day groups). To compare the amount of AAV within the medium to that purified from cellular lysates, producer cells from individual groups were harvested at 3 (3-day group), 5 (5-day group), or 7 days (7-day group), respectively. To determine if allowing the cells to incubate with no medium change would result in an additive accumulation of AAV in the supernatant, an additional group was transfected in an identical manner, and the same serum-free medium was kept on the producer cells until 7 days posttransfection, at which point both cells and medium was harvested (terminal group). AAV, adeno-associated virus; DMEM, Dulbecco's modified Eagle's medium; HEK, human embryonic kidney.

Institutional Animal Care & Use Committee (AUF 10/12-196-00). Rats were housed two per cage and maintained in a light-controlled (12 hr light/dark cycle; lights on 0600 hr) and temperature-controlled (22 ± 1°C) room, and provided with food and water *ad libitum*. All surgery was performed under 2% isoflurane anesthesia. After induction of anesthesia, rats were placed in a stereotaxic frame and the surgical site was scrubbed with Betadine before incision. A single incision was made along the rostrocaudal axis of the skull, and tissue overlying the skull was retracted to expose the skull surface. A Hamilton syringe (Hamilton) with a 30-gauge blunt-tip needle was fitted with a siliconized pulled glass micropipette with an opening of 60–80 $\mu$m to use for injections. Single-site striatal injections were made at the following coordinates relative to Bregma: anterior/posterior: 0.0 mm; medial/lateral: ±2.7 mm; dorsal/ventral: –4.0 mm. As there is no bilateral connectivity between the rodent striata, individual striatum from separate hemispheres were used as an $n = 1$. Thus, each subject received two separate injections (one per hemisphere) of randomized vectors from each production group. Purified virus from all production groups was injected at a titer of $1.0 \times 10^{13}$ viral genomes (vg)/ml. A total of 2 $\mu$l was injected at a rate of 0.5 $\mu$l/min using an automated micropump (World Precision Instruments). To prevent reflux the needle was left in place for one minute after the injection, after which the needle was retracted 1 mm, and thereafter left in place for 4 additional minutes before completely retracting the needle. The hole in the skull was filled with sterile bone wax, and the skin was closed using surgical staples. Rats were checked daily for signs of infection/distress.

### Tissue collection and processing

One month after AAV injections, when transgene expression reaches peak levels,[14] animals received a lethal injection of sodium pentobarbital and were transcardially perfused with Tyrode's solution followed by 4% paraformaldehyde. Brains were removed and postfixed in 4% paraformaldehyde in tris-buffered saline (TBS) overnight, followed by cryoprotection in 30% sucrose. Six separate series of coronal sections (40 $\mu$m) encompassing the entire rostrocaudal axis of the brain were prepared with a microtome.

### Immunohistochemistry

Immunohistochemistry (IHC) was performed on free-floating sections. Sections were washed in TBS containing 0.25% Triton-X 100 and blocked in 10% normal goat serum. Blocked sections were then incubated in a primary rabbit anti-GFP antibody (AB290; Abcam), followed by a biotin-conjugated, goat anti-rabbit secondary antibody (ap132b; Millipore), or a goat anti-rabbit 680LT near-infrared secondary antibody (Li-Cor). Bound peroxidase was visualized with 0.05% 3-3′-diaminobenzidine tetrahydrochloride (Sigma) with 0.01% hydrogen peroxide using an ABC Elite kit (Vector Laboratories).

### Unbiased stereological cell counting

Unbiased stereological counting of striatal GFP-positive (GFP+) cells was performed as previously described.[14] In brief, using Stereo-Investigator software (Version 4.03; Microbrightfield, Inc., 2000), sections were viewed on a screen at low magnification (4×) and the transduction area containing GFP+ cells was delineated through the rostrocaudal extent of the striatal nuclei. For estimates of GFP+ cells, every sixth section was sampled. GFP+ cells were counted using the optical fractionator method.[14] Approximately 6–9 sections per animal were needed to count the entire transduced striatum. Counting of GFP+ cells was performed using a 60× oil objective on an Olympus BX53 microscope equipped with a motorized stage. The coefficient of error for each estimate was calculated and was less than 0.1 (Gundersen, $m=1$).[15]

### Protein quantification

Quantification of GFP protein levels in the striatum was made indirectly by measuring near-infrared signal with an Odyssey near-infrared scanner after IHC using the LiCor 680LT secondary antibody. After IHC for GFP, mounted and coverslipped sections containing the entirety of the striatum were scanned with the Odyssey infrared analyzer. The striatum was then outlined using Odyssey software, and the total signal intensity at the 680 wavelength within the delineated area was quantified.[16]

### Electron microscopy

The ratio of empty to full particles within vector preparations was quantified with electron microscopy after negative staining. Formvar-coated copper mesh grids (Electron Microscopy Sciences) were loaded with 10 $\mu$l of virus from each production group. Grids were then washed with $dH_2O$ and stained with 1% filtered uranyl acetate (Electron Microscopy Sciences) before being viewed on a TEM 1400 plus electron microscope (JEOL). Ratios of empty versus full particles were determined by directly counting negatively stained and total particles from micrographs. Power analysis was conducted to determine the necessary sampling size based on an $\alpha$ of 0.05 for the examined comparisons and the expected standard error of measurement for viral particle counting. A sample size of five per group yields a power of 0.95. Five images were randomly acquired from preparations corresponding to individual samples within each production group. A minimum of 1500 total particles were counted for each production group.

### qPCR analysis of AAV genomes

Quantification of AAV genomes within the striatum was performed as previously described.[17] Total DNA was extracted from the AAV-injected striatum by incubating tissue in extraction buffer (200 m*M* tris, 250 m*M* NaCl, 25 m*M* EDTA, 0.5% SDS) with proteinase K and RNase A at 55°C, followed by phenol/chloroform extraction and ethanol precipitation of the DNA. DNA concentration was determined using a Nanodrop. qPCR primers for the AAV genome (Integrated DNA Technology) and GAPDH (ABI Research) were conjugated to a FAM or VIC reporter, respectively. All primers were ensured to produce a single product as assessed by melt curve analysis. GAPDH was used as the reference gene and detection was similar across treatment groups. Samples for PCRs were run in triplicate and averaged to give a single mean value per sample. Changes between treatment groups were analyzed by the differences in $\Delta C_t$, which compares the $C_t$ value of the AAV genome to that of the *GAPDH* control gene. Data are presented as fold change over corresponding control tissue (identical region of the striatum from noninjected rats) as determined by the $\Delta\Delta C_t$, which represents the $\Delta C_t$ normalized to a calibrator, in this case noninjected control tissue.

### Statistical analysis

The experimenter was blind to all experimental conditions during data collection and analysis. One-way analysis of variance (ANOVA) tests were used to test for statistical significance between two or more groups with a single independent variable. A $p$-value of less than or equal to 0.05 was considered statistically significant. If the ANOVA revealed an interaction of statistical significance, *post-hoc* analysis was followed by between-group comparisons using Tukey's test.

## RESULTS

### Time course of AAV5 release into cellular medium

Previous studies have shown that functional AAV can be harvested from the medium of HEK 293T producer cells in a simple and effective method.[8,9] The majority of protocols describing AAV purification from medium (or cellular lysates) have harvested medium 3 days posttransfection.[8,9,18] Here we wanted to advance these findings and determine the maximum duration of time within which functional AAV particles could be harvested from the medium of HEK 293T producer cells. To do so we began by analyzing the amount of AAV in the cellular medium from 3 to 7 days posttransfection. We chose to initially study the time course of release of AAV5 because of its highly efficient neuronal tropism that is commonly utilized in our laboratory. Polyethylenimine (PEI) was used to transfect six confluent triple flasks of HEK 293T cells with the AAV genome (containing a GFP transgene under control of the CBA/CMV hybrid promoter enhancer, flanked by AAV2 terminal repeats) along with a helper plasmid encoding all necessary helper functions and AAV serotype 5 capsid proteins. One day posttransfection, cellular medium was replaced with fresh serum-free DMEM. Then, medium from each group was collected every 2 days and replaced with fresh serum-free medium. To compare the amount of AAV within the medium to that purified from cellular lysates, producer cells from individual group were harvested at 3, 5, and 7 days posttransfection (Fig. 1). Medium and cells were processed and AAV was purified as previously described,[9,12,19] after which titers of DNAse-resistant viral genomes were quantified. Three days posttransfection there were high numbers of viral genomes from both the medium and the cell lysate (Fig. 2A). The amount of AAV detected in the medium fraction remained constant out to the 7-day time point, whereas there was a small, nonsignificant decrease in AAV purified from the cell lysates at these later time points (Fig. 2A). We observed no significant degree of cell death within any groups during the 7-day time course (data not shown).

The fact that the amount of AAV in the medium remained constant out to 7 days posttransfection in the absence of overt cellular lysis, while the medium was harvested and replaced every 2 days, suggests that there is a steady-state release of AAV into the cellular medium. To determine if allowing the cells to incubate with no medium change would result in an additive accumulation of AAV in the supernatant, an additional group was transfected in an identical manner, and the same serum-free medium was kept on the producer cells until 7 days posttransfection, at which point both cells and medium were harvested (Fig. 1, terminal group). Surprisingly, the amount of AAV in this "terminal" group did not show the anticipated additive accumulation of virus in the medium. Instead, the detected viral genomes were comparable to that of the 3-, 5-, and 7-day time points (Fig. 2A), suggesting that the steady-state release of AAV into the medium dissipates with prolonged incubation in the same medium.

We next wanted to determine whether the lack of accumulation of viral particles in the medium of the terminal group was because of impaired release of AAV, as the result of nutrient starvation or saturation of the medium with viral particles. In this experiment HEK 293T producer cells were transfected as described above to produce AAV2/5-CBA-GFP. However, one day posttransfection, groups of two triple flasks of HEK 293T producer cells received (1) fresh serum-free medium (control group), (2) used 3-day-old serum-free medium transferred from mock-transfected cells (used medium group), (3) conditioned 3-day-old serum-free medium transferred from cells that were transfected and actively releasing AAV2/5 containing a GFP transgene under control of the synapsin promoter (transfected-used medium group), or (4) fresh serum-free medium injected with $8.45\times10^{11}$ viral particles per flask (which is the average amount of virus released into the medium of producer cells at 3 days posttransfection) of purified AAV containing a GFP transgene under control of the synapsin (AAV-fresh medium group). Medium was harvested at 3 and 5 days posttransfection, at which point the medium was replaced with fresh medium, used medium, transfected-used medium, or AAV-fresh medium, identical to those described above. If the cessation of virion accumulation within the medium of HEK 293T producer cells was because of a lack of nutrients, we would expect to see a decreased total number of viral particles produced in groups receiving used medium. If the cessation of virion accumulation in the medium of HEK 293T producer cells was because of a saturation of the producer cell medium with viral particles, we would expect to see a decrease in the total number of viral particles produced in groups receiving medium transferred from cells actively releasing AAV, and in cells receiving fresh medium injected with purified AAV. Medium was processed as described above, and the total number of AAV2/5-CBA-GFP viral particles purified

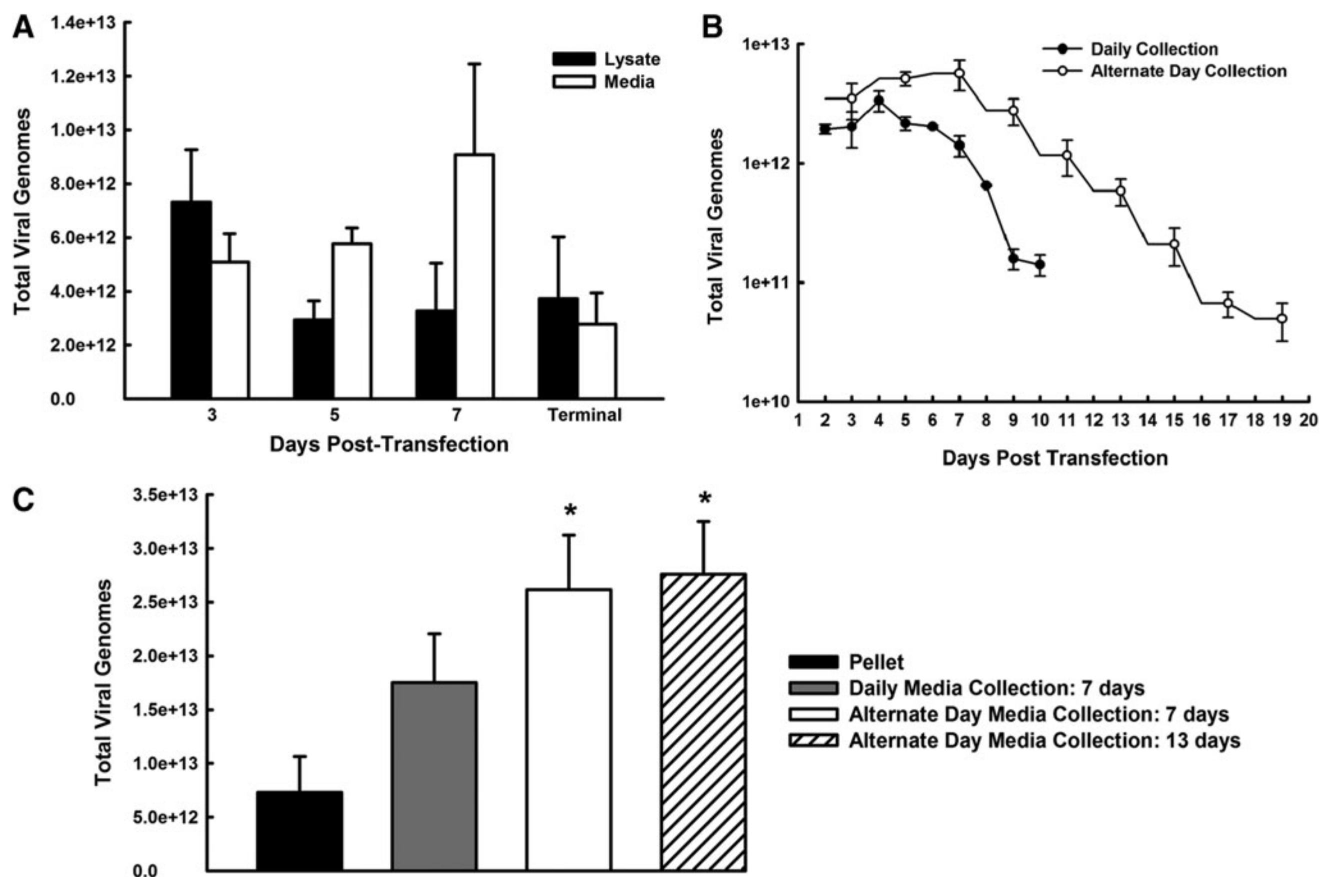



**Figure 2.** Characterization of the time course of AAV5-GFP release into cellular medium. AAV5-GFP was produced in HEK 293T cells by polyethylenimine transfection of the AAV-GFP genome and a plasmid encoding capsid proteins for AAV serotype 5, and all necessary helper functions. Each individual production group consisted of six triple flasks of HEK 293T cells. **(A)** Medium from HEK 293T producer cells was harvested at 3, 5, and 7 days posttransfection. Individual AAV production groups were terminated and cells harvested at 3, 5, and 7 days posttransfection, respectively. An additional group of producer cells was incubated for the entirety of time course with no medium change, and cells were harvested 7 days posttransfection (terminal group). AAV was purified and total DNase1-resistant viral genomes were quantified. Columns represent total viral genomes collected at each respective time point +1 SEM ($n=3$/group) after purification from cellular lysates (black column) or cellular medium (white columns). **(B)** To determine the duration in which viable AAV particles are released into the cellular medium, medium from HEK 293T producer cells was harvested every 2 days, after the switch to serum-free medium (alternate days group, open circle). Medium was harvested until the cells were no longer adherent. To determine the frequency at which viable AAV particles can be harvested, cellular medium was collected every day from a separate group of HEK 293T producer cells, after the switch to serum-free medium (daily group, closed circle). AAV was purified from medium at all time points and total DNase1-resistant viral genomes were quantified. Individual data points represent the total number of viral particles collected at each time point, averaged over experimental replicates ±1 SEM ($n=2$–3/group) **(C)** The cumulative yield of AAV5-GFP was determined by quantifying the total amount of virus harvested from a 3-day lysate preparation (black column), a 7-day medium collection paradigm in which medium was harvested daily (gray column), a 7-day medium collection paradigm in which medium was harvested on alternate days (white column), or a 13-day medium collection paradigm in which medium was harvested on alternate days (white striped column). Columns represent total viral genomes collected at each respective time point +1 SEM ($n=2$–3/group). Collecting and purifying AAV5-GFP from medium that was harvested on alternate days for 7 days posttransfection resulted in significant 4-fold increase in the number of AAV genomes detected over that of AAV purified from 3-day cellular lysates. There was no significant increase in the number of viral genomes detected by collecting the cellular medium for 13 days as compared with 7 days. *Significantly different from the 3-day lysate group ($p<0.05$). GFP, green fluorescent protein.

was quantified using a probe specific to the CBA promoter (i.e., will not detect the AAV/2/5-synapisn-GFP virus used to treat the cells). Supplementary Fig. S1 (Supplementary Data are available online at wwwliebertpub.com/hgtb) shows that there was no change in the total number of viral particles purified from any group at any time point. To confirm the presence of viral particles in the medium used to treat cells in the transfected-used medium group, we also quantified the number of AAV2/5-synapsin GFP genomes, using a probe specific to the synapsin promoter. The average number of AAV2/5-synapsin-GFP viral genomes within the transfected-used medium was $7.33\times10^{11}$, $9.72\times10^{11}$, and $7.1\times10^{11}$ at the 3-, 5-, and 7-day time point, respectively. This indicates that neither nutrient deficiency nor saturation of medium with viral particles can account for the lack of an additive accumulation of virions observed in the terminal group (Fig. 2A).

Because virion accumulation dissipates when medium remains on producer cells for longer periods of time, we next wanted to investigate the maximum duration that AAV could be collected from producer cell medium when medium was harvested and replaced at regular intervals. Six triple flasks of HEK 293T cells were transfected as describe above, and medium was collected and replaced every 2 days until the point when the cells were no longer adherent. Peak levels of AAV purified from the medium were observed at 7 days posttransfection, after which point there was a drop in the number of viral genomes detected (Fig. 2B, open circles). Despite this drop after the 7-day time point, there was still an appreciable amount of virus purified from the medium from 9 to 13 days posttransfection, after which titers dropped precipitously. These results demonstrate that a significant amount of intact viral particles can be successfully harvested and purified from the medium of producer HEK 293T cells for almost two weeks posttransfection.

Because there is a steady-state release of AAV when medium is replaced every 2 days, and that this steady-state accumulation of AAV into the medium ceases when the same medium was left on producer cells for 7 days, we next wanted to determine the effects of harvesting and replacing medium every day. When medium from transfected HEK 293T producer cells was harvested every day, there was a similar steady-state release of AAV into the medium; however, after the 4-day time point the number of genomes detected when the medium was collected every day was less than half of the genomes detected in AAV purified from the medium that was collected on alternate days (Fig. 2B). Further, during daily collections, AAV release into the medium dropped precipitously after 7 days posttransfection, mirroring the drop observed in the alternate-day collection paradigm (Fig. 2B).

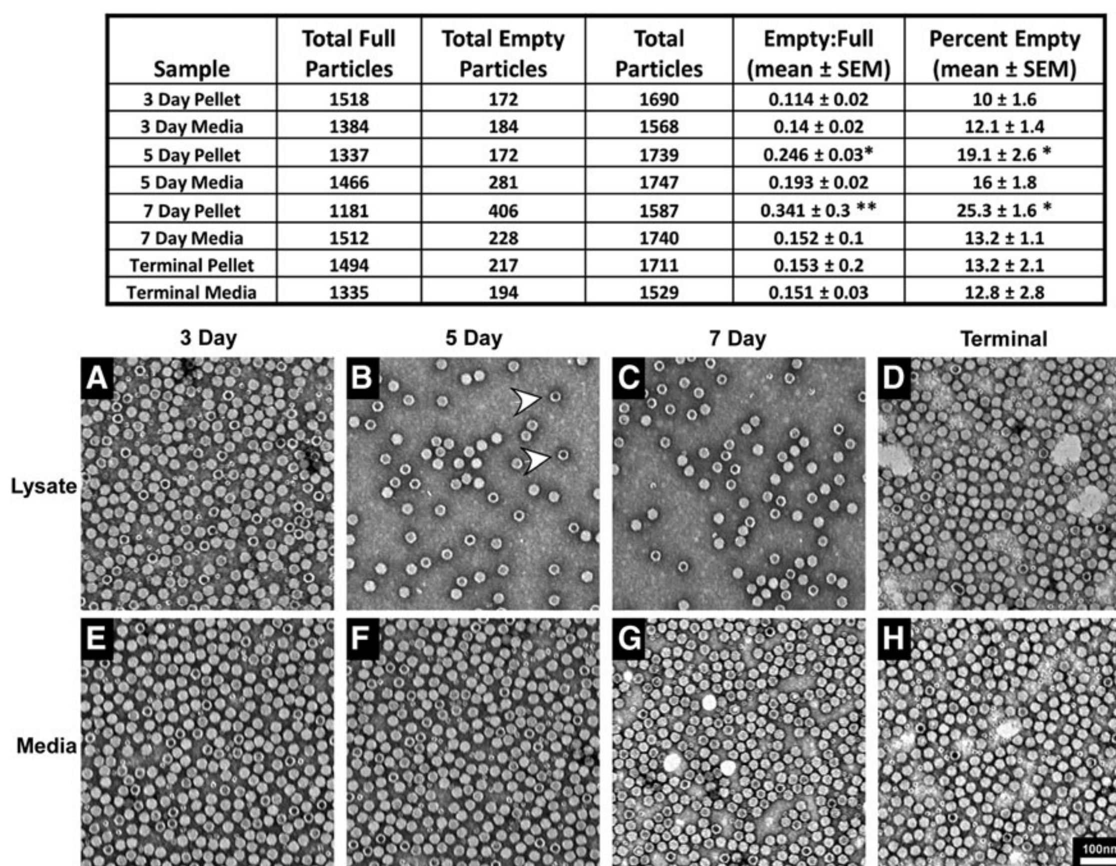
Together, these data demonstrate that intact AAV5 particles are released into the medium at a steady state where they can be regularly harvested on alternate days until approximately 7 days posttransfection, after which there is only a minuscule increase in total viral yield. This ability to harvest virus from the medium of producer cells on a regular basis drastically increases the yield obtained from a single preparation of AAV. For example, a comparison of the total yield of AAV5 from a traditional cell lysate preparation harvested at 3 days posttransfection (the prototypical AAV production paradigm[12]) versus the total yield obtained from an identical preparation in which medium was harvested every 2 days until 7 days posttransfection results in an approximately 4-fold increase in total viral particles (Fig. 2C). There is no significant accumulation of total viral particles beyond the 7-day posttransfection time point, and daily collections result in a slightly decreased yield compared with the alternate-day collection paradigm (Fig. 2C).

### Structural integrity of AAV5 released into the medium

Previous reports have demonstrated that physically intact AAV can be harvested from producer cell medium; however, the effects of prolonged harvest on the ratio of empty versus full particles have not been investigated. As such, we next sought to confirm that the ratio of empty versus full particles of the AAV5 released into the medium was both consistent throughout the 7-day collection period, and was on a comparable level to that of AAV5 produced through conventional means, that is, purified from cellular lysates harvested at 3 days posttransfection. Empty vector particles can be identified after negative staining with uranyl acetate using electron microscopy.[8,11] Empty vectors appear darker because of the presence of an electron-dense core resulting from the pooling of uranyl acetate on top of the empty capsid (arrowhead in Fig. 3B). Negatively stained empty particles and total particles were counted directly from electron micrographs obtained from vector preparations purified from cell lysates and medium harvested at 3, 5, and 7 days posttransfection. The table in Fig. 3 shows the mean ratio of empty to full particles and the corresponding percentage of empty particles per production group. There was no increase in the ratio of empty to full particles in AAV purified from producer cell medium throughout the 7-day time course (Fig. 3 table and E–H). Surprisingly, there was a progressive increase in the ratio of empty versus full particles observed from 3 to 7 days posttransfection in AAV purified from cellular lysates (Fig. 3 table and A–D). Curiously, this increase was not observed in the terminal pellet group (which was also harvested at 7 days posttransfection with no medium changes; Fig. 3 table and D).

### Biological activity of AAV5 released into the medium

Finally, we wanted to analyze the biological activity of AAV5 purified from cellular medium. Again, although AAV harvested from medium has demonstrated infectivity,[8–11] the effects of continual harvest on the biological activity of AAV are unknown. We chose to target stereotaxic injections of AAV to the rat striatum, as this allows for simple quantification of transduced cells within a contained area. We performed striatal injections of AAV5 purified from cellular lysates or medium harvested at 3, 5,



| Sample | Total Full Particles | Total Empty Particles | Total Particles | Empty:Full (mean ± SEM) | Percent Empty (mean ± SEM) |
|---|---|---|---|---|---|
| 3 Day Pellet | 1518 | 172 | 1690 | 0.114 ± 0.02 | 10 ± 1.6 |
| 3 Day Media | 1384 | 184 | 1568 | 0.14 ± 0.02 | 12.1 ± 1.4 |
| 5 Day Pellet | 1337 | 172 | 1739 | 0.246 ± 0.03* | 19.1 ± 2.6 * |
| 5 Day Media | 1466 | 281 | 1747 | 0.193 ± 0.02 | 16 ± 1.8 |
| 7 Day Pellet | 1181 | 406 | 1587 | 0.341 ± 0.3 ** | 25.3 ± 1.6 * |
| 7 Day Media | 1512 | 228 | 1740 | 0.152 ± 0.1 | 13.2 ± 1.1 |
| Terminal Pellet | 1494 | 217 | 1711 | 0.153 ± 0.2 | 13.2 ± 2.1 |
| Terminal Media | 1335 | 194 | 1529 | 0.151 ± 0.03 | 12.8 ± 2.8 |



**Figure 3.** The ratio of empty to full particles of AAV harvested from cellular lysates and medium. AAV purified from the cellular lysate or cellular medium collected at 3, 5, and 7 days posttransfection was negatively stained with 1% uranyl acetate and viewed under a transmission electron microscope. Empty particles are easily identified by an electron-dense circle at the center of the capsid (arrow head in **B**). The number of empty and full particles was counted directly from electron micrographs. Five images were acquired at random from each sample preparation, and the ratio of empty particles versus full particles was quantified. The table lists the number of full particles, empty particles, and total particles counted, as well as the average empty-to-full particle ratio and average percentage of empty particle per preparation (listed as mean ±1 SEM; $n=5$/group). Representative images of electron micrographs from each production group are shown in **(A–H)**, respectively. Scale bar in **(H)** is 100 nm and applies to **(A–G)**. *Significantly greater than all medium groups and the 3-day and terminal lysate groups ($p<0.05$). **Significantly greater than all groups ($p<0.05$).

and 7 days posttransfection. Adult male rats received 2 $\mu$l of AAV5-GFP (normalized to $1\times10^{13}$ vg/ml) stereotactically injected into the striatum, and were sacrificed 1 month postsurgery. The number of cells expressing the GFP transgene was quantified using unbiased stereological cell counting. All AAV5 vectors purified from the medium or lysate displayed equal levels of transduction, with no significant differences in the number of striatal GFP+ cells detected between any groups analyzed (Fig. 4). Representative images of striatal GFP+ cells after transduction with AAV5-GFP purified from cell lysates or medium harvested at 3, 5, or 7 days posttransfection are shown in Fig. 4B–I. Counting the number of GFP+ cells does not provide any information as to how much transgene product is made by each cell (i.e., the multiplicity of infection [MOI] or efficiency of intracellular processing within each cell). Consequently, in addition to quantifying the number of GFP+ cells, we also analyzed GFP protein expression in the striatum by quantifying the total signal at the 680 wavelength after immunohistochemical detection of GFP using a LiCor 680 secondary antibody. There was no significant difference in the overall GFP protein levels detected in the striatum after delivery of AAV5-GFP purified from cell lysates or medium harvested at 3, 5, or 7 days posttransfection (Fig. 5A–I). That being said there was a nonsignificant trend toward increased GFP protein expression in animals that had received AAV5-GFP purified from the cell lysate as compared with AAV5-GFP purified from the cellular medium (Fig. 5A–I). To confirm that there was no difference in the infectivity of AAV derived from medium versus cellular lysate, we also quantified the number of viral genomes present in the striatum of the same animals using qPCR (Fig. 5J). There was no difference in the number of intact viral ge-

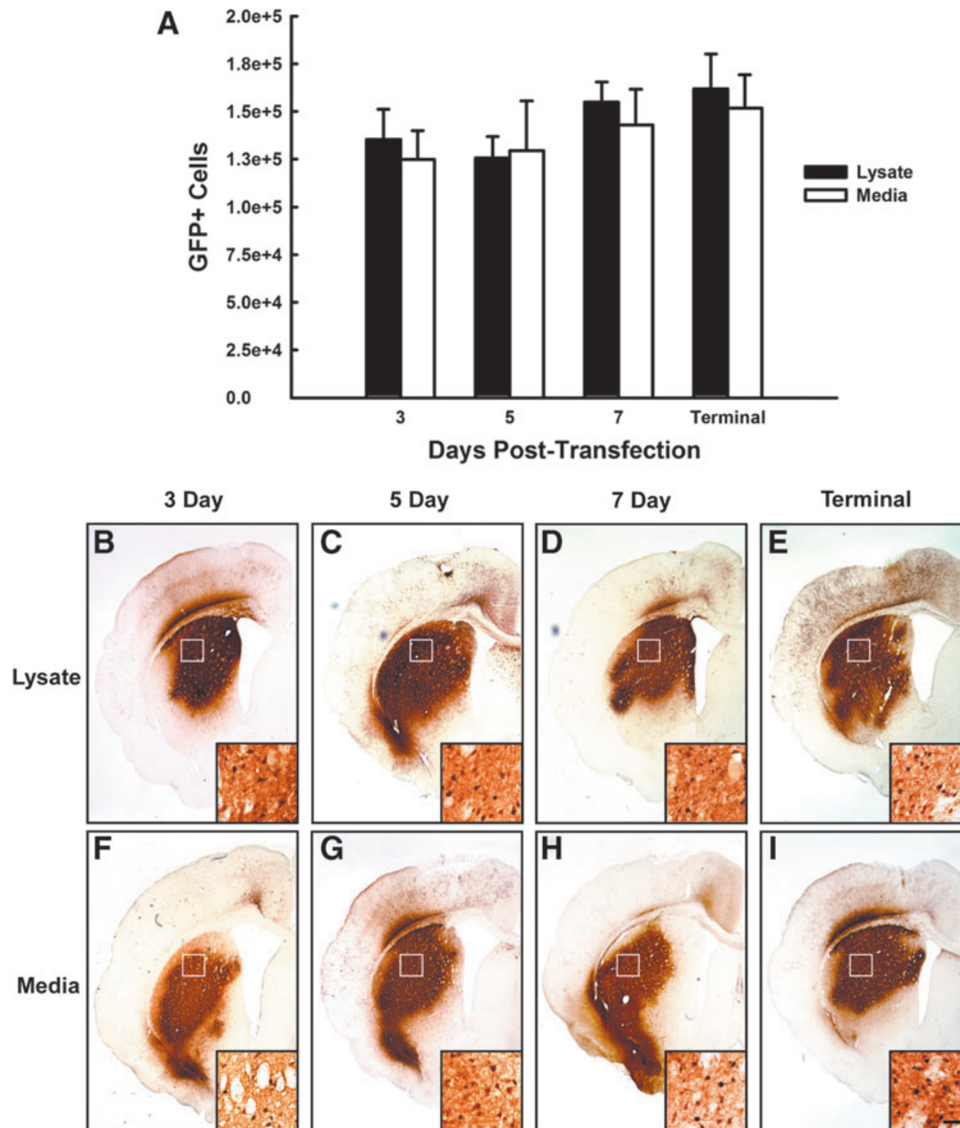



**Figure 4.** Quantification of GFP-positive cells in the striatum after transduction with AAV5-GFP purified from cellular lysates or cellular medium. Adult male rats received 2 $\mu$l unilateral injections of AAV5-GFP ($1\times10^{13}$ vg/ml) into the striatum. One month postsurgery, animals were sacrificed, and brains were removed, sectioned, and stained for GFP. Numbers of GFP-positive (GFP+) cells were estimated using unbiased stereology. Columns in **(A)** represent mean numbers of GFP+ cells, +1 SEM ($n$=6/group), in animals receiving AAV5-GFP purified from cellular lysates (black columns) or cellular medium (white columns) collected at the 3-, 5-, and 7-day posttransfection or terminal groups, respectively. Representative images of striatal transduction for each vector production group are shown in **(B–I)**. Insets in **(B–I)** are high-magnification images of the area within the white box of the transduced striatum. Scale bar in the inset in panel I represents 50 $\mu$m and applies to all other insets. Color image available online at www.liebertpub.com/hgtb

nomes detected in the striatum of animals injected with AAV-GFP derived from either medium or cellular lysates (Fig. 5J). Taken together, these data indicate that the biological activity of AAV derived from medium and traditional cellular lysate purification is equal.

### Time course of AAV9 release into cellular medium

Here we have detailed the maximum duration and optimal harvesting frequency to maximize AAV5 yield from cellular medium after a single PEI transfection preparation in HEK 293T cells. However, although other AAV serotypes are released into producer cell medium,[8–11] they may not display the same prolonged release of intact viral particles into cellular medium. To confirm that the ability to harvest virus from cellular medium for up to one week posttransfection was not limited to AAV5-GFP, we performed a similar experiment by packaging an AAV genome expressing BFP into the AAV9 capsid. Groups of two triple flasks were transfected as described above and medium was collected every 2 days after the change to serum-

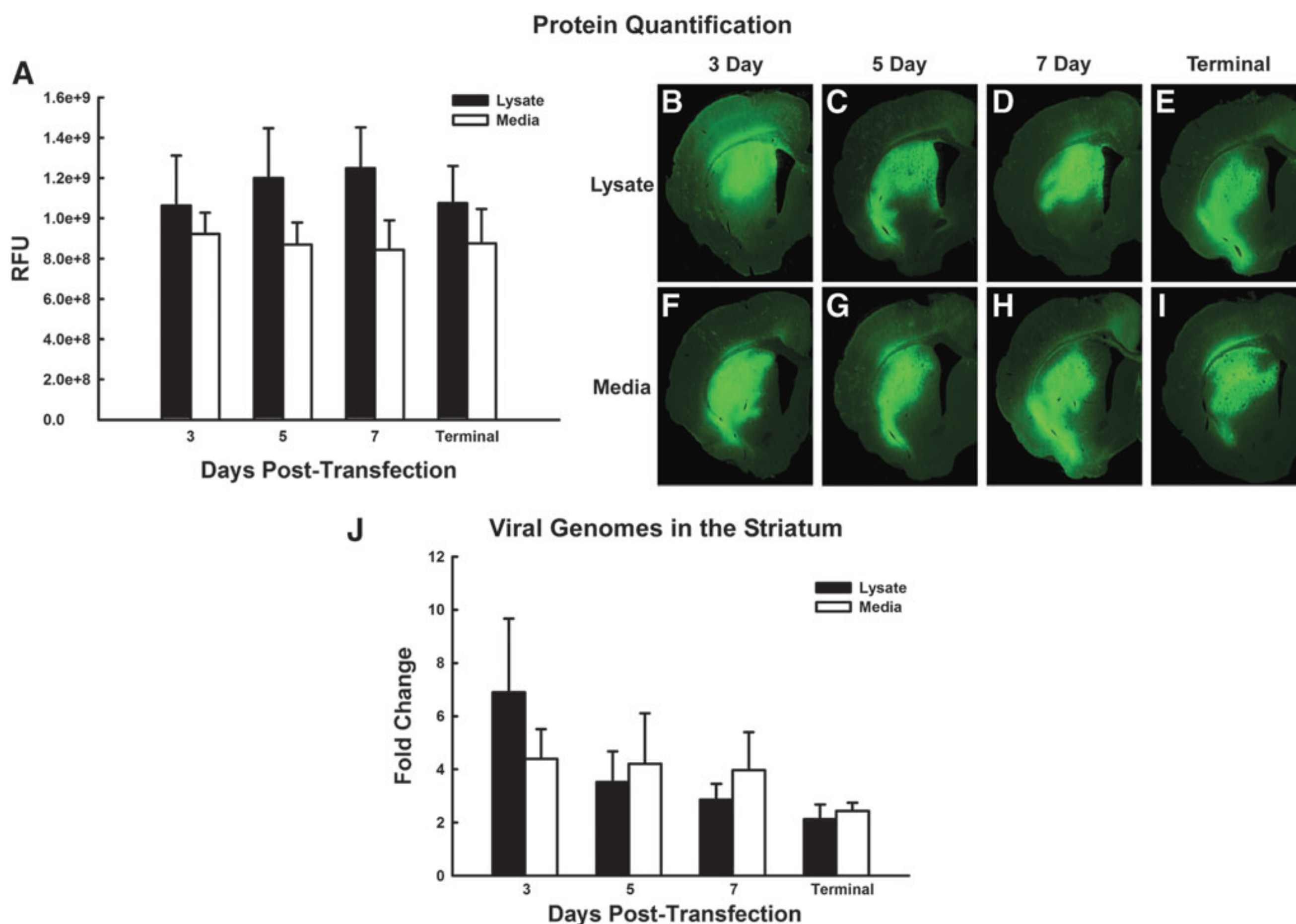



**Figure 5.** Quantification of GFP protein and AAV genomes in the striatum after transduction with AAV5-GFP purified from cellular lysates or cellular medium. Adult male rats received unilateral injections of AAV5-GFP ($1 \times 10^{13}$ vg/ml) into the striatum. **(A–I)** One month postsurgery, animals were sacrificed, and brains were removed, sectioned, and stained for GFP using a 680LT secondary antibody. Stained sections were scanned on an Odyssey infrared analyzer and the area of transduction was delineated and signal at the 680 wavelength quantified. Columns represent the mean total signal intensity +1 SEM ($n = 6$/group) in the striatum of animals receiving AAV5-GFP purified from cellular lysates (**A,** black columns) or cellular medium (**A,** white columns) collected at the 3-, 5-, and 7-day posttransfection or terminal groups, respectively. **(J)** To confirm that the infectivity of AAV purified from cellular medium is equal to that purified from cellular lysates, the number of viral genomes within the striatum was quantified by qPCR. One month postsurgery, genomic DNA was extracted from tissue sections containing the striatum adjacent to the injection site. Quantification of AAV genomes was performed by qPCR analysis using probes directed against the promoter of the AAV genome and GAPDH. Levels of AAV genomes were normalized to GAPDH. Columns represent fold change (as determined by the $\Delta\Delta$Ct method) over striatal tissue from noninjected control animals, +1 SEM ($n = 6$/group). Color image available online at www.liebertpub.com/hgtb

free medium, out to 7 days posttransfection. We also analyzed a "terminal group," in which the same serum-free medium was kept on the producer cells until 7 days posttransfection, at which point both cells and medium were harvested. Similar to AAV5, large quantities of AAV9 were detected in the medium of producer cells out to 7 days posttransfection (Fig. 6). Further, there was no significant difference between the amount of AAV purified from cellular medium versus lysate at any time point. Finally, again similar to AAV5, there was no additive accumulation of AAV9 in producer cell medium in the terminal group. These results indicate that the optimized method for continuous collection of AAV described herein with AAV5 is also applicable to other AAV serotypes.

## DISCUSSION

The discovery that a substantial portion of AAV is released into the cellular medium of production HEK 293T cells during packaging presents an opportunity to increase the efficiency and yield of vector production.[8–10] However, to date there has been no investigation into the maximum duration of time within which functional AAV particles can be harvested from a single AAV vector preparation. The novel work presented here details the time course of AAV5 release into the cellular medium, including an analysis of the structural and biological integrity of released viral particles. Specifically, we have determined that structurally intact and biologically active AAV5 particles are released

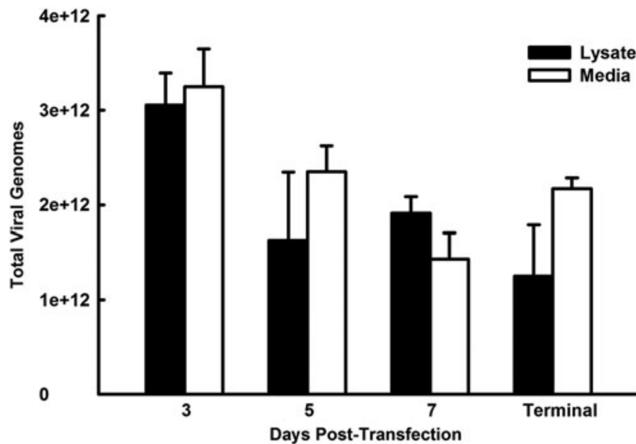



**Figure 6.** Quantification of AAV9 particles purified from producer cell medium for 7 days posttransfection. AAV9-BFP was produced in HEK 293T cells by polyethylenimine transfection of the AAV-BFP genome and a plasmid encoding capsid proteins for AAV serotype 9, and all necessary helper functions. Each individual production group consisted of two triple flasks of HEK 293T cells. Medium from HEK 293T producer cells was harvested at 3, 5, and 7 days posttransfection. Individual AAV production groups were terminated and cells harvested at 3, 5, and 7 days posttransfection, respectively. An additional group of producer cells was incubated for the entirety of time course with no medium change, and cells were harvested 7 days posttransfection (terminal group). AAV was purified and total DNase1-resistant viral genomes were quantified. Columns represent total viral genomes collected at each respective time point +1 SEM ($n$=2–3/group) after purification from cellular lysates (black column) or cellular medium (white columns).

into the medium at a steady state until approximately 7 days posttransfection. The released AAV5 can be harvested on alternate days, combined, and purified in a simple and efficient manner in order to increase total vector yield by approximately 4-fold over a conventional preparation of AAV purified from cellular lysates. Although we have detailed the time course of release of AAV5 and shown that AAV9 is also released for up to 7 days posttransfection, alternative serotypes have displayed differential release profiles,[9] and as such the optimal duration and frequency of collection for other serotypes may differ and should be determined empirically.

There were no differences in the ratio of empty versus full viral particles observed between cellular lysate AAV purified at 3 days posttransfection and AAV purified from medium at any time point examined. However, with the exception of the terminal lysate group, there was a progressive increase in the ratio of empty versus full particles in AAV purified from cellular lysates after the 3-day posttransfection time point. The reason why the number of empty particles would increase in the lysate-derived AAV but not in the medium-purified AAV is not clear. Presumably, the presence of empty particles at longer time points represents the decrease in the finite number of AAV genomes transfected into the producer cells, after packaging into capsids. With this in mind, it is possible that the release of AAV into the cellular medium is a nonrandom event, favoring genome-containing capsids over empty capsids. However, it must be noted that EM has been criticized to lack an absolute quantitative nature, and as such these observations may be viewed as qualitative or semiquantitative at best.

Despite the increased number of empty particles in the 5- and 7-day lysate AAV preparation, AAV purified from cell lysates and medium (at all time points examined) mediated efficient transduction *in vivo*, and there was no difference in the number of transduced cells between any of the groups examined. This result may seem surprising in the face of the increased ratio of empty versus full particles in the 5- and 7-day lysate-purified AAV; however, the amount of virus injected was normalized based on the number of genome copies. Thus, although there may have been an increased number of empty particles within these groups, all animals received the same number of full, genome-containing virions, accounting for the equal number of cells transduced. Although it is possible to achieve equal levels of transduction by normalizing the number of genome copies injected, because of the increased number of empty particles within the 5- and 7-day lysate-derived AAV, there must have been a greater overall number of capsids injected, increasing the possibility of receptor saturation or eliciting an immune response. As such, though it is possible to harvest viable AAV from cellular lysates at the 5- and 7-day time points, this AAV should be used with caution.

Although there were no significant changes between the medium- or cell lysate-purified AAV in terms of the numbers of cells transduced or the GFP protein expression after transduction, there was a nonsignificant trend toward an increase in GFP protein levels in the striatum of animals receiving AAV purified from cell lysates. As all vectors used the same promoter, the slight increase in the GFP protein, in the presence of an equal number of transduced cells, suggests that either the MOI or the efficiency of intracellular processing of the cell lysate-derived virus may be increased. This result was surprising as this has not been reported in other publications,[8,9] and there was actually an increase in the number of empty particles in the AAV purified from cellular lysates at 5 and 7 days posttransfection. To address this surprising finding we quantified the number of viral genomes within the striatum of the same animals

using qPCR. Quantification of viral genomes present in striatal tissue one month postinjection likely reflects the relative number of infectious particles that have delivered their genome to the nucleus, as the majority of AAV particles remaining in the cytosol or outside of the cell will be degraded or transported away from the injection site.[20,21] We did not detect any differences between the numbers of intact viral genomes within the striatum of animals injected with AAV purified from cellular medium versus cellular lysate, confirming that the infectivity of AAV derived from these two purification procedures is commensurate.

The steady-state accumulation of AAV in the medium was optimal when the medium was collected on alternate days. The total yield of AAV5-GFP was reduced when collections occurred daily, and allowing AAV to accumulate in the same medium for a prolonged period of time (7 days) resulted in an apparent cessation of release. That the total viral yield was reduced when medium was collected daily could simply be because of physical agitation of the cells during medium changes, as we observed the cells to detach at a much earlier time when medium was changed daily. This is reflected by the drop in titers observed around the 5–7-day time point during daily collections, as compared with a commensurate drop in titer at the 11–13-day time point during alternate-day collections. Currently, the mechanism by which AAV is released into the medium is unknown. AAV is not associated with any cytopathogenic effects (as evidenced by the lack of cell death during the production time course), and currently there is no known active egress pathway for AAV to escape the cell. Recent studies have identified AAV within exosomes; however, the amount of AAV harvested within these exosomes is a miniscule fraction of total AAV produced (approximately 0.01–0.2%),[22] and thus cannot account for the large amount of free AAV purified from the medium. It is possible that AAV is released in exosomes, which subsequently rupture, resulting in free AAV in the culture medium. However, this possibility remains unfounded and further research into the mechanism(s) by which functional AAV is released into the culture medium is needed. Accordingly, because the mechanisms regulating AAV release into the medium are poorly understood, we currently do not understand why the additive accumulation of AAV in the medium breaks down upon prolonged incubation. We reasoned that this could be the result of viral particles saturating the medium and preventing further release, or it could result from a lack of nutrients and accumulation of metabolic byproducts when the medium is not refreshed, slowing vector production and release. Here we tested the possibility that the cessation of virus accumulation within the medium was caused by a saturation of the medium or a lack of essential nutrients. Through this experimentation we found that neither lack of nutrients nor saturation of the medium with AAV slowed the release of AAV5 into producer cell medium over a 7-day time course. Accordingly, it is likely that the cessation of virion accumulation upon prolonged incubation in the same medium reflects active breakdown of virion caused by proteolytic activity in the medium. Based on these data, we have determined that the optimal frequency for continual harvest of AAV is an alternate-day collection paradigm.

The ability to continually harvest and purify viable AAV particles from the medium of production cells will most certainly act as a boon to the field of AAV gene therapy. Although AAV is a commonly used viral vector system that has witnessed great success in both preclinical and clinical settings, current manufacturing procedures necessitate laborious and time-consuming purification procedures in order to obtain AAV particles of sufficient titer and purity for biomedical applications. Specifically, evolving gene therapy studies and therapeutics targeted toward large animals or humans require large amounts of high-titer AAV. Accordingly, efficient manufacturing of high-quality AAV vectors has been foreseen as a potential rate-limiting step to the future success of AAV in the field of gene therapy.[5] We believe that the ability to continually harvest viral particles from the medium of producer cells may represent a novel solution to this potential roadblock. As this production technique increases the yield of intact viral particles by several fold, with minimal effort and virtually no added cost, the continuous harvest of AAV particles will also be of tremendous benefit to small laboratory-scale production efforts. Further, the biological activity of the released AAV is at least commensurate to that of conventional cell lysate-purified AAV, and in other reports released AAV has actually shown increased biological activity.[8,9] The ability to harvest AAV from the medium offers several unique safety advantages over AAV purified from cell lysates. Because of the large amount of virus released into the medium, it is not completely necessary to harvest intracellular virus. This would obviate the need to separate virus from cellular contaminants such as lipids, proteins, or carbohydrates that increase the potential to elicit an immune response if not purified correctly. Further, harvesting virus from serum-free medium eliminates the potential contamination of viral preparations

from zoonotic proteinacious infections particles derived from serum used in medium preparations.

Beyond safety considerations, AAV purified from medium also has practical production benefits. Previous methods for purifying AAV have almost exclusively relied on cellular lysis because of the canonical belief that AAV is not normally released from producer cells in any significant amount. However, the freeze–thaw cycles that are normally used to lyse producer cells decrease the yield of viral preparations.[23] As such, the ability to avoid the freeze–thaw cycles (or other methods of cellular lysis) is an added benefit to the purification of AAV from cellular medium. The purification of virus from medium could be improved by altering the culture conditions (osmolarity, pH, salt content) in order to maximize virus release.[8,24] Alternatively, the nature of the AAV capsid itself can be manipulated in order to maximize release. For example, abolishing the heparin-binding capacity of AAV vectors increases the proportion of vector collected from the medium.[9] Finally, the purification of AAV vectors from medium is scalable, and yield and efficiency could potentially be maximized using a system to continually perfuse and collect virus-laden medium from cells, or alternatively the use of a bioreactor for very large batches of AAV. Indeed a recent report has described the use of a nonadherent HEK 293 cell suspension system in which medium is collected continuously to purify GMP-grade AAV.[25]

In conclusion, here we have extended prior work showing that AAV is released in culture medium. Previous publications have demonstrated that structurally intact and infectious AAV particles can be harvested from HEK 293 producer cell medium.[8–11] However, the frequency and duration within which viable, infectious particles can be efficiently harvested has not previously been determined. Here we have detailed the time course of release of functional AAV5, and described the novel observation that viable, biologically active particles can be harvested at regular intervals from cellular medium for up to 7 days posttransfection. AAV9 was also collected from the medium for up to 7 days posttransfection, although the maximum duration and optimal interval for harvesting AAV9 from cellular medium must be empirically determined. In line with the rigor previously used to ensure the intact physical nature and high biological activity of purified AAV,[8–11] we have provided a thorough and previously unreported characterization of the relative infectivity and empty-to-full particle ratios for both cell lysate- and cell medium-derived AAV for the 7-day collection time course. The data presented herein represent an advance in AAV production as the ability to dramatically increase the yield of viral production through a simple and efficient method will save both time and resources, and can expand the potential of AAV in the field of gene therapy.

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## AUTHOR DISCLOSURE

The authors have no competing financial interests.

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