

Rapid, Simple, and Versatile Manufacturing of Recombinant Adeno-Associated Viral Vectors at Scale

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Abstract

Adeno-associated viral (AAV) manufacturing at scale continues to hinder the application of AAV technology to gene therapy studies. Although scalable systems based on AAV–adenovirus, AAV–herpesvirus, and AAV–baculovirus hybrids hold promise for clinical applications, they require time-consuming generation of reagents and are not highly suited to intermediate-scale preclinical studies in large animals, in which several combinations of serotype and genome may need to be tested. We observed that during production of many AAV serotypes, large amounts of vector are found in the culture supernatant, a relatively pure source of vector in comparison with cell-derived material. Here we describe a high-yielding, recombinant AAV production process based on polyethylenimine (PEI)-mediated transfection of HEK293 cells and iodixanol gradient centrifugation of concentrated culture supernatant. The entire process can be completed in 1 week and the steps involved are universal for a number of different AAV serotypes. Process conditions have been optimized such that final purified yields are routinely greater than 1×10^{14} genome copies per run, with capsid protein purity exceeding 90%. Initial experiments with vectors produced by the new process demonstrate equivalent or better transduction both *in vitro* and *in vivo* when compared with small-scale, CsCl gradient-purified vectors. In addition, the iodixanol gradient purification process described effectively separates infectious particles from empty capsids, a desirable property for reducing toxicity and unwanted immune responses during preclinical studies.

Introduction

THE USE OF RECOMBINANT adeno-associated viral (rAAV) vectors for clinical gene therapy applications has become widespread and is largely due to the demonstration of long-term transgene expression from rAAV vectors in animal models with little associated toxicity and good overall safety profiles in both preclinical and clinical trials (Snyder and Flotte, 2002; Moss *et al.*, 2004; Warrington and Herzog, 2006; Maguire *et al.*, 2008; Mueller and Flotte, 2008; Brantly *et al.*, 2009). Most early AAV gene therapy studies were performed with serotype 2 vectors, but vector systems based on other AAV serotypes with more efficient gene delivery and different tissue specificity are currently in human trials and their use will likely increase (Brantly *et al.*, 2009; Neinhuis, 2009).

A major requirement for the development and eventual marketing of a gene therapy drug is the ability to produce the gene delivery vector at a sufficient scale. In the past this requirement has been a barrier to the successful application

of rAAV vectors, but more recently several innovative production systems have been developed that are compatible with large-scale production for clinical application. These new systems use adenovirus, herpesvirus, and baculovirus hybrids to deliver the rAAV genome and *trans*-acting helper functions to producer cells and have been reviewed (Clement *et al.*, 2009; Virag *et al.*, 2009; Zhang *et al.*, 2009). The ease of introduction of the required genetic elements to the producer cell line through rAAV hybrid virus infection permits efficient rAAV vector production and, importantly, up-scaling of the process to bioreactors. These systems are particularly suited to final clinical candidate vectors but because of the need to make the hybrid viruses for each vector, they are less suited to early development and preclinical studies in which several combinations of transgene and vector serotype may need to be evaluated.

Although much preclinical rAAV-based gene therapy work has been performed in mice, results obtained in larger animals are often considered more predictive of actual

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clinical outcomes. Large animal studies require higher rAAV vector doses and, to satisfy these demands, a versatile production system that can rapidly produce a variety of test vectors at scale without the need for time-consuming production of intermediates is required. Transient transfection by calcium phosphate coprecipitation of plasmid DNAs containing the AAV vector genome, the AAV capsid gene, and the *trans*-acting helper genes into HEK293 cells (a process known as “triple transfection”) has long been the standard method to produce rAAV in the research laboratory (Grimm *et al.*, 1998; Matsushita *et al.*, 1998; Salvetti *et al.*, 1998; Xiao *et al.*, 1998). Transfection-based methods remain the most versatile of all rAAV production techniques and permit simultaneous manufacture of different rAAV vectors. However, calcium phosphate triple transfection has generally not been considered ideal for large-scale rAAV production because of a lack of compatibility with suspension culture systems. Polyethylenimine (PEI) has been used as a transfection reagent to produce AAV vectors for some time (Grieger *et al.*, 2006; Reed *et al.*, 2006), but some promising results using this reagent have demonstrated the production of rAAV2 vectors in mammalian cell suspension culture with unpurified yields of $1\text{--}3 \times 10^{13}$ vector particles per liter, which are comparable to yields from attached-mode transfection systems (Durocher *et al.*, 2007; Hildinger *et al.*, 2007). The advantages of PEI-based transfection are that it can also be performed in serum-free medium without the need for the medium exchanges that are typically required with conventional calcium phosphate-mediated transfection (Durocher *et al.*, 2007). These features translate into lower cost and the elimination of concerns surrounding animal-derived serum such as the presence of prions and other adventitious agents.

A further impediment to the scale-up of rAAV vector production occurs during downstream processing of the vector. At small scale, the most prevalent method used for rAAV vector purification involves multiple rounds of overnight cesium chloride gradient centrifugation (Zolotukhin *et al.*, 1999). This purification method can be performed easily with standard laboratory equipment, is generally high-yielding, and when performed carefully gives vector of reasonable purity. The drawbacks of this technique, however, are first that prolonged exposure to CsCl has been reported to compromise the potency of rAAV vectors (Zolotukhin *et al.*, 1999; Auricchio *et al.*, 2001; Brument *et al.*, 2002) and second that the gradients have a limited loading capacity for cell lysate, which can in turn limit rAAV purification scale-up. An alternative gradient medium, iodixanol, has also been used to purify rAAV vectors (Hermens *et al.*, 1999; Zolotukhin *et al.*, 1999). This isotonic medium was developed originally as a contrast agent for use during coronary angiography and the low associated toxicity and relative inertness are advantages over cesium chloride from both safety and vector potency points of view (Zolotukhin *et al.*, 1999). However, iodixanol shares the same drawback as cesium chloride in that the loading capacity for rAAV production culture cell lysate and thus the scalability of rAAV purification are limited. To overcome these gradient-specific constraints, researchers have gravitated toward ion-exchange chromatography and more recently affinity purification using single-domain heavy chain antibody fragments to purify AAV at scale (Auricchio *et al.*, 2001; Brument *et al.*, 2002; Kaludov *et al.*, 2002; Zolotukhin *et al.*, 2002; Davidoff *et al.*,

2004; Smith *et al.*, 2009). Using these techniques, yields, scalability, and purity are all enhanced. However, there remain vector-related impurities such as empty capsids, which are not generally separated from fully functional vector particles by chromatography-based techniques. Although some progress has been made with AAV2 vectors to develop ion-exchange-based resolution of empty and full vector particles (Qu *et al.*, 2007; Okada *et al.*, 2009), CsCl gradient centrifugation remains the best characterized method for removing empty particles from rAAV vector preparations.

We observed that in contrast to AAV2, most other AAV serotypes were released primarily into the medium of calcium phosphate-transfected production cultures and not retained in the cell lysate (Vandenberghe *et al.*, 2010). This observation confirms a similar finding reported for AAV8 (Okada *et al.*, 2009). Because this distribution occurs in the absence of cell lysis, we reasoned that the production culture medium would represent a relatively pure source of rAAV vector and that the lower level of cellular contaminants may improve the loading capacity and resolution of purification gradients. Here we describe a scaled rAAV production method suitable for large animal studies, which is based on PEI transfection and supernatant harvest. The method is high-yielding, versatile for the production of vectors with different serotypes and transgenes, and simple enough that it may be performed in most laboratories with a minimum of specialized techniques and equipment. In addition, we demonstrate the use of iodixanol gradients for the separation of genome-containing vectors from empty particles.

Materials and Methods

Cell culture

Late-passage HEK293 cell cultures were maintained on 15-cm plates in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Manassas, VA) with the addition 10% fetal bovine serum (FBS; HyClone Laboratories, South Logan, UT). The cells were passaged twice weekly to maintain them in exponential growth phase. For small-scale transfections 1×10^6 HEK293 cells were seeded per well of 6-well plates and 1.5×10^7 cells were seeded into 15-cm dishes. For large-scale production HEK293 cells from 16 confluent 15-cm plates were split into two 10-layer cell stacks (Corning, Corning, NY) containing 1 liter of DMEM–10% FBS 4 days before transfection. The day before transfection, the two cell stacks were trypsinized and the cells were resuspended in 200 ml of medium. Cell clumps were allowed to settle before plating 6.3×10^8 cells into each of six cell stacks. The cells were allowed to attach for 24 hr before transfection. Confluency of the cell stacks was monitored with a Diaphot inverted microscope (Nikon, Melville, NY) from which the phase-contrast hardware had been removed in order to accommodate the cell stack on the microscope stage.

Plasmids

The plasmids used for all transfections were as follows: (1) *cis* plasmid pENNAAVCMVeGFP.RBG containing an enhanced green fluorescent protein (eGFP) expression cassette flanked by AAV2 inverted terminal repeats (ITRs); (2) *trans* plasmids pAAV2/1, pAAV2/6, pAAV2/7, pAAV2/8, and pAAV2/9 containing the AAV2 *rep* gene and capsid protein

genes from AAV1, 6, 7, 8, and 9, respectively; and (3) adenovirus helper plasmid pAdΔF6. Fifteen- to 50-mg lots of >90% supercoiled plasmid were obtained (Puresyn, Malvern, PA) and used for all transfections.

Calcium phosphate transfection

Small-scale calcium phosphate transfections were performed by triple transfection of AAV *cis*, AAV *trans*, and adenovirus helper plasmids as previously described (Gao *et al.*, 2002). Briefly, the medium on 85–90% confluent HEK293 monolayers in 6-well plates was changed to DMEM–10% FBS 2 hr before transfection. Plasmids at a ratio of 2:1:1 (1.73 μg of adenovirus helper plasmid/0.86 μg of *cis* plasmid/0.86 μg of *trans* plasmid per well) were calcium phosphate precipitated and added dropwise to plates. Transfections were incubated at 37°C for 24 hr, at which point the medium was changed again to DMEM–10% FBS. The cultures were further incubated to 72 hr postinfection before harvesting the cells and medium separately. For large-scale transfection of cell stacks the plasmid ratio was kept constant but all reagent amounts were increased by a factor of 630. The transfection mix was added directly to 1 liter of DMEM–10% FBS and this mixture was used to replace the medium in the cell stack. The medium was changed at 24 hr posttransfection. Cells and medium were harvested 72 or 120 hr posttransfection either directly or after further incubation for 2 hr in the presence of 500 mM NaCl. When vector present in the cells was to be quantified, the cells were released by trypsinization and lysates were formed by three freeze–thaw cycles.

Small-scale vector preparation

Forty 15-cm plates were transfected by the calcium phosphate method and cell lysates were prepared 72 hr posttransfection with three successive freeze–thaw cycles (–80°C/37°C). Cell lysates were purified by two rounds of cesium chloride centrifugation and pure gradient fractions were concentrated and desalted, using Amicon Ultra-15 centrifugal concentrator devices (Millipore, Bedford, MA).

Small-scale polyethylenimine transfection

For polyethylenimine (PEI)-based triple transfections of HEK293 cells in six-well plates the same plasmid amounts were used as described for calcium phosphate transfections. PEI “Max” (Polysciences, Warrington, PA) was dissolved at 1 mg/ml in water and the pH was adjusted to 7.1. Two micrograms of PEI was used per microgram of DNA transfected. PEI and DNA were each added to 100 μl of serum-free DMEM and the two solutions were combined and mixed by vortexing. After 15 min of incubation at room temperature the mixture was added to 1.2 ml of serum-free medium and used to replace the medium in the well. No further medium change was carried out. For 15-cm plate the plasmid ratio was kept constant but the amounts of plasmid and other reagents used were increased by factors of 15.

Large-scale polyethylenimine transfection

Large-scale PEI-based transfections were performed in 10-layer cell stacks containing 75% confluent monolayers of HEK293 cells. Plasmids at a ratio of 2:1:1 (1092 μg of adenovirus helper plasmid/546 μg of *cis* plasmid/546 μg of *trans*

plasmid per cell stack) were used. The PEI “Max”/DNA ratio was maintained at 2:1 (w/w). For each cell stack, the plasmid mix and PEI were each added to a separate tube containing serum-free DMEM (total volume, 54 ml). The tubes were mixed by vortexing and incubated for 15 min at room temperature, after which the mixture was added to 1 liter of serum-free DMEM containing antibiotics. The culture medium in the stack was decanted and replaced with the DMEM–PEI–DNA mix, and the stack was incubated in a standard 5% CO₂, 37°C incubator. At 72 hr posttransfection 500 ml of fresh serum-free DMEM was added and the incubation was continued to 120 hr posttransfection. At this point, Benzonase (EMD Chemicals, Gibbstown, NJ) was added to the culture supernatant to a final concentration of 25 units/ml and the stack was reincubated for 2 hr. NaCl was added to 500 mM and the incubation was resumed for an additional 2 hr before harvest of the culture medium (at this point the culture medium was called the downstream feedstock). When cell-associated vector was to be quantified, the cells were released by trypsinization and lysates were formed by three sequential freeze–thaw cycles (–80°C/37°C).

Downstream processing

Ten liters of feedstock culture medium from six cell stacks was clarified through a 0.5-μm Profile II depth filter (Pall, Fort Washington, NY) into a 10-liter Allegro medium bag (Pall). The clarified feedstock was then concentrated by tangential flow filtration (TFF), using a NovaSet-LS LHV holder with customized 0.25-inch (inner diameter) tubing and ports (TangenX Technology, Shrewsbury, MA) and a 0.1-m² Sius-LS single-use TFF screen channel cassette with a 100-kDa molecular weight cutoff (MWCO) HyStream membrane (TangenX Technology). A 125-fold concentration to 85 ml was performed according to the manufacturer’s recommendations with a transmembrane pressure of 10–12 psi maintained throughout the procedure. The TFF filter was discarded after each run and the system was sanitized with 0.2 N NaOH between runs. The concentrated feedstock was reclarified by centrifugation at 10,500×g and 15°C for 20 min and the supernatant was carefully removed to a new tube. Six iodixanol step gradients were formed according to the method of Zolotukhin and colleagues (1999) with some modifications as follows: Increasingly dense iodixanol (OptiPrep; Sigma-Aldrich, St Louis, MO) solutions in phosphate-buffered saline (PBS) containing 10 mM magnesium chloride and 25 mM potassium were successively underlaid in 40 ml of Quick-Seal centrifuge tubes (Beckman Instruments, Palo Alto, CA). The steps of the gradient were 4 ml of 15%, 9 ml of 25%, 9 ml of 40%, and 5 ml of 54% iodixanol. Fourteen milliliters of the clarified feedstock was then overlaid onto the gradient and the tube was sealed. The tubes were centrifuged for 70 min at 350,000×g in a 70Ti rotor (Beckman Instruments) at 18°C and the gradients were fractionated through an 18-gauge needle inserted horizontally approximately 1 cm from the bottom of the tube. Fractions were diluted 20-fold with water into an ultraviolet (UV)-transparent 96-well plate (Corning) and the absorbance was measured at 340 nm. A spike in OD₃₄₀ (optical density at 340 nm) readings indicated the presence of the major contaminating protein band and all fractions below this spike were collected and pooled. Pooled fractions from all six gradients were combined, diafiltered against 10 volumes of

the final formulation buffer (PBS–35 mM NaCl), and concentrated 4-fold to ~10 ml by tangential flow filtration according to the manufacturer's instructions, using a 0.01-m² single-use Sius TFF cassette with a 100-kDa MWCO HyStream screen channel membrane (TangenX Technology) and an LV Centramate cassette holder (Pall). A transmembrane pressure of 10 psi was maintained throughout the process. The holdup volume of the apparatus was kept low, using minimal lengths of platinum-cured silicone tubing (inner diameter, 1.66 mm) (Masterflex; Cole Parmer Instrument, Vernon Hills, IL). In addition, all wettable parts were pretreated for 2 hr with 0.1% Pluronic F-68 (Invitrogen, Carlsbad, CA) in order to minimize binding of the vector to surfaces. The TFF filter was discarded after each run and the system was sanitized with 0.2 N NaOH between runs. Glycerol was added to the diafiltered, concentrated product to a final concentration of 5% and the preparation was aliquoted and stored at –80°C.

Vector characterization

DNase I-resistant vector genomes were titered by TaqMan PCR amplification (Applied Biosystems, Foster City, CA), using primers and probes directed against the polyadenylation signal encoded in the transgene cassette. The purity of gradient fractions and final vector lots were evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and the proteins were visualized by SYPRO ruby staining (Invitrogen) and UV excitation. Purity relative to nonvector impurities visible on stained gels was determined with GeneTools software (Syngene, Frederick, MD). The empty particle content of vector preparations was assessed by negative staining and electron microscopy. Copper grids (400-mesh, coated with a Formvar/thin carbon film; Electron Microscopy Sciences, Hatfield, PA) were pretreated with 1% alcian blue (Electron Microscopy Sciences) and loaded with 5 μ l of vector preparation. The grids were then washed, stained with 1% uranyl acetate (Electron Microscopy Sciences), and viewed with a Philips CM100 transmission electron microscope. Ratios of empty to full particles were determined by direct counting of the electron micrographs.

Relative vector potency assessment

Early-passage HEK293 cells were plated to 80% confluency in 96-well plates and infected with AAV vector at a multiplicity of infection (MOI) of 10,000 in the presence of wild-type adenovirus type 5 (MOI, 400). Forty-eight hours postinfection GFP fluorescence images were captured digitally and the fluorescence intensity was quantified as described previously (Wang *et al.*, 2010), using ImageJ software (Ferreira and Rasband, 2010). For *in vivo* analysis of transduction, C57BL/6 mice were injected intravenously with 1×10^{11} genome copies of AAV vector. The animals were necropsied 9 days postinjection, the livers were sectioned and imaged for GFP fluorescence as described previously (Wang *et al.*, 2010), and fluorescence intensity was quantified with ImageJ software.

Results

Comparison of transfection reagents for rAAV production

The standard upstream method for producing rAAV vectors at small scale in our laboratory (total yield, $\sim 1\text{--}2 \times 10^{13}$

genome copies [GC]) is based on calcium phosphate-mediated triple transfection of HEK293 cells in forty 15-cm tissue culture plates. Although this method reproducibly yields vectors of various AAV serotypes with good titers in both the cell pellet and the culture medium (Vandenberghe *et al.*, 2010), it is technically cumbersome, requires the presence of animal serum, and involves two medium changes. For scaled rAAV production we reasoned that a less complicated, more robust transfection agent such as polyethylenimine (PEI) would be required. The production of rAAV7 vector carrying an eGFP expression cassette (rAAV7-eGFP) after either calcium phosphate- or PEI-mediated triple transfection, was quantified by quantitative PCR (qPCR) of DNase-resistant vector genomes in both cells and media of six-well plate HEK293 production cultures (Fig. 1a). With either transfection method, rAAV7-eGFP production was found to partition equally between the cells and culture medium at similar levels, despite stronger expression of the eGFP transgene in the calcium phosphate-transfected cells. The results indicate that, interestingly, transgene expression levels in the production culture are not predictive of rAAV production yields but more importantly that rAAV7-eGFP is released to the culture medium at similar levels irrespective of the transfection technique.

Effect of serotype and salt addition on rAAV release to the culture medium

Having established the release of rAAV7-eGFP to the culture medium after PEI triple transfection, an immediate

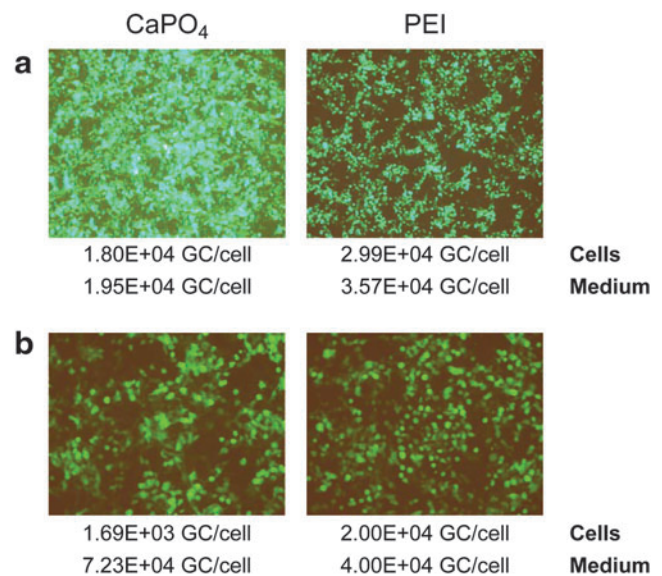


FIG. 1. Comparison of transfection agents for rAAV7 productivity and release to the culture medium. **(a)** Six-well plates were seeded with HEK293 cells and transfected with three plasmids carrying the vector genome, AAV2 *rep/* AAV7 *cap* genes, and adenovirus helper functions, respectively, using polyethylenimine (PEI) or calcium phosphate as the transfection reagent. DNase-resistant vector genome copies (GC) present in cell lysates and the production culture medium at 72 hr posttransfection were quantified by qPCR. **(b)** Ten-layer Corning cell stacks containing HEK293 cells were triple transfected by both PEI and calcium phosphate methods and vector genome copies in the culture supernatant and cells were determined 120 hr later.

goal was to demonstrate similar release with other AAV serotypes. In addition, we were interested to determine whether we could manipulate the 45% of detectable vector that remained associated with the cells (Fig. 1a) into the culture medium. It was found that by simply postponing the harvest until 120 hr after PEI transfection, as opposed to the standard 72 hr, the total vector in the culture medium could be doubled (data not shown). Adopting this strategy, 15-cm plates of HEK293 cells were triple transfected with PEI. *Trans* plasmids encoding five different AAV serotype capsid genes were included in the various transfection mixes and after a 120-hr incubation the culture medium and cells were harvested either immediately, or 2 hr after addition of 500 mM NaCl. The encapsidated AAV genomes in the cell lysates and culture medium were then quantified by qPCR (Fig. 2). Each of the five AAV serotypes tested could be released to the supernatant after 5 days of incubation without salt addition at levels between 61.5 and 86.3% of the total GC yield. This result confirmed our observation during early development runs that increased incubation time post-transfection led to higher titers of AAV vector in the culture medium. Incubation of production cultures with salt has been demonstrated to cause release of AAV2 to the supernatant presumably through a mechanism mediated by cellular stress (Atkinson *et al.*, 2005). The high-salt incubation performed here led to a further ~20% GC release of AAV6 and AAV9 vectors to the culture medium, but elicited little change with the other serotypes.

Effect of scale-up on rAAV7 vector yields

The major goal at the outset of this work was to develop a scaled AAV production system that could be performed in most laboratories using standard equipment to support large animal preclinical studies. Hence we chose Corning 10-layer cell stacks to scale up the PEI-based transfection because this type of tissue culture vessel can be accommodated by standard laboratory incubators. Initially a single 10-layer cell stack was seeded with 6.3×10^8 HEK293 cells such that the

monolayers would be 75% confluent the next day. To assess the confluency of the bottom HEK293 monolayer before transfection, a standard laboratory microscope was adapted by removing the phase-contrast hardware such that the cell stacks could be accommodated. One cell stack was triple transfected with the relevant plasmids to produce AAV7-eGFP vector, using either calcium phosphate or PEI (see Materials and Methods), and then incubated to 120 hr post-infection before quantification of DNase-resistant vector genomes in both cells and medium (Fig. 1b). Per-cell yields from the PEI-transfected cell stack were similar to those obtained previously in 6-well and 15-cm plates (Fig. 1a and Fig. 2). The overall yield from the culture medium in this experiment was 2.2×10^{13} GC per cell stack. The calcium phosphate-transfected stack produced significantly lower vector yields per cell than was observed previously in plates and this effect may result from a lack of diffusion of CO₂ into the central areas of the cell stack. On the basis of the 10-layer cell stack transfection results, PEI was chosen as the transfection reagent for further development of the scaled procedure.

Downstream processing of rAAV7-eGFP production culture medium

A primary goal in developing the scaled production process was to maintain flexibility such that any AAV vector could be purified by a generic method. Separation of vector from contaminants on the basis of density and size can be applied to multiple vector serotypes. Hence we chose to concentrate the rAAV7 vector in the culture medium by tangential flow filtration (TFF) to volumes small enough to permit purification over iodixanol density gradients. Preclarification of the production culture medium through a 0.5- μ m depth filter was necessary to remove cellular debris and detached cells and to prevent clogging of the TFF membrane. A 130-fold concentration was then achieved with a disposable, 100-kDa cutoff, screen channel TFF membrane while maintaining a transmembrane pressure of 10–12 psi throughout the

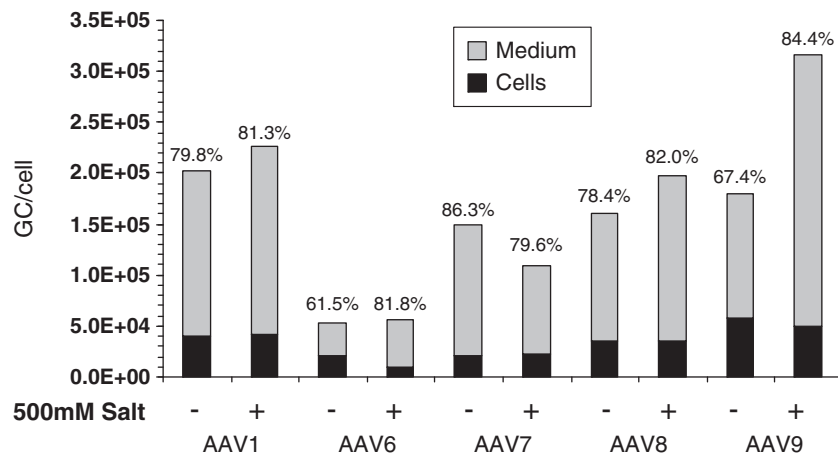


FIG. 2. Productivity and release of various serotypes after PEI transfection in the presence or absence of 500 mM salt. Fifteen-centimeter plates of HEK293 cells were triple transfected with PEI and DNA mixes containing one of the five different AAV capsid genes indicated. Five days posttransfection culture media and cells were harvested either with or without exposure to 0.5 M salt and DNase-resistant vector genome copies (GC) were quantified. Genome copies produced per cell are represented with the percentage of vector found in the supernatant indicated above each column.

TABLE 1. IN-PROCESS AND FINAL YIELDS (GENOME COPIES) OF AAV VECTOR PILOT PRODUCTION RUNS

Pilot runs (no.)	Serotype	Transgene	TFF1/iodixanol gradient				TFF2/buffer exchange		
			Feedstock	TFF1 retentate	Iodixanol fraction pool	Percentage of feedstock	Final product	Percentage of iodixanol pool	Total process yield (%)
1	AA V9	eGFP	6.41×10^{14}	3.58×10^{14}	2.24×10^{14}	42.15	1.82×10^{14}	81.34	28.46
8	AA V9	eGFP	1.03×10^{15}	2.26×10^{14a}	1.05×10^{14}	10.20	6.66×10^{13}	63.57	6.48
9	AA V9	eGFP	3.12×10^{14}	3.76×10^{14}	1.63×10^{14}	52.33	8.38×10^{13}	51.37	26.88
10	AA V8	eGFP	9.83×10^{14}	1.22×10^{15}	4.32×10^{14}	43.97	2.66×10^{14}	61.50	27.04
11	AA V8	eGFP	9.24×10^{14}	1.01×10^{15}	3.26×10^{14}	35.29	2.00×10^{14}	61.27	21.62
12	AA V8	eGFP	1.51×10^{15}	1.57×10^{15}	6.06×10^{14}	40.19	3.67×10^{14}	60.58	24.35
5	AA V6	eGFP	3.35×10^{13}	5.69×10^{13}	6.37×10^{12}	22.92	2.48×10^{12}	38.84	7.39
7	AA V6	eGFP	1.01×10^{14}	1.32×10^{14}	1.57×10^{13}	15.58	4.58×10^{12}	29.12	4.54

Abbreviations: AAV, adeno-associated virus; EGFP, enhanced green fluorescent protein; TFF, tangential flow filtration.

^aLoss due to mechanical failure.

process. The disposability of the membrane avoided the need to defoul and sanitize between runs and therefore added to the reproducibility of the process. The production culture medium was treated with nuclease (Benzonase) to degrade contaminating plasmid and cellular DNA, and 500 mM salt was added before concentration to minimize aggregation of the vector to both itself (Wright *et al.*, 2005) and to contaminating proteins during processing. These two treatments were subsequently determined to increase recoveries from the iodixanol gradient (data not shown). During development of the downstream process and performance of full-scale pilot runs (Table 1), no significant loss of vector was observed at any point due to the concentration process.

Iodixanol gradient purification of AAV vectors has been fully described (Zolotukhin *et al.*, 1999) and the step gradient used here is adapted from this work. Minor modifications to the volumes of the gradient layers were made in order to achieve better resolution of vector from contaminants

(Materials and Methods). Fourteen milliliters of TFF retentate containing concentrated AAV7-eGFP vector from the production culture medium of one cell stack were loaded onto a 27-ml iodixanol step gradient and centrifuged for 1 hr at $350,000 \times g$. The gradient was then fractionated from the bottom of the tube and the fractions ($275 \mu\text{l}$) were analyzed for vector content, iodixanol concentration, and vector purity by qPCR, optical density at 340 nm (Schroder *et al.*, 1997), and SDS-PAGE, respectively. Representative profiles of one such gradient are shown in Fig. 3. A linear gradient of iodixanol concentration indicated by the decreasing OD₃₄₀ readings was observed up until fraction 22. After this point the readings increased (Fig. 3a) and corresponded to a spike in contaminating protein visualized by SDS-PAGE (Fig. 3b) and by the naked eye in the form of a thin band present in the gradient. The OD₃₄₀ spike was likely due to overlapping absorbance of protein and iodixanol at this wavelength and this phenomenon provided us with an accurate and

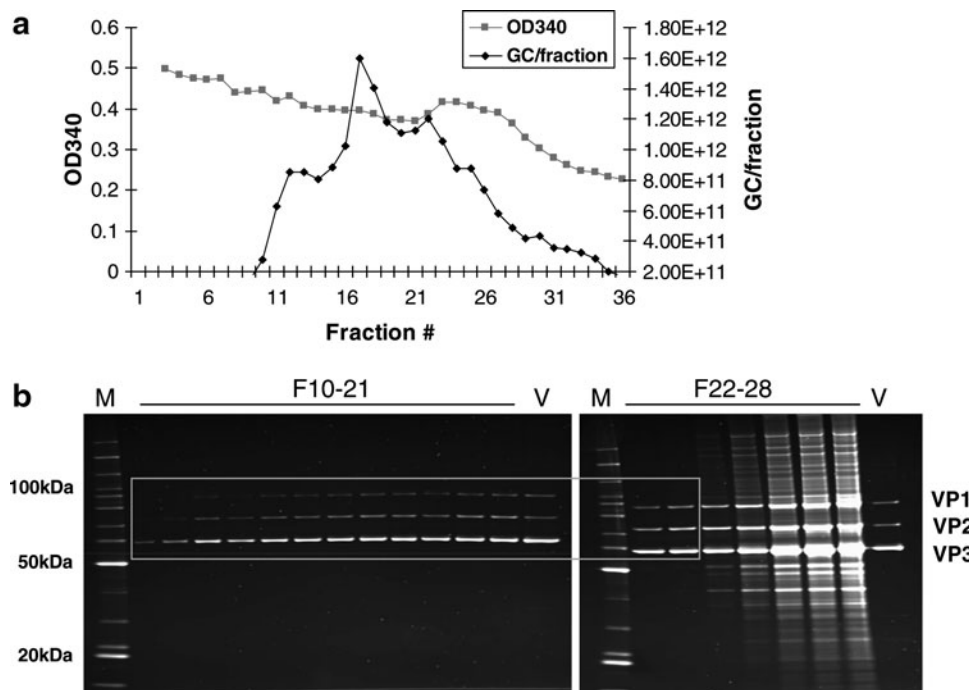


FIG. 3. Large-scale iodixanol gradient-based purification of rAAV7 vector from concentrated production culture supernatants. **(a)** rAAV7 vector from cell stack culture medium was concentrated and separated on iodixanol gradients and fractions were harvested from the bottom of the tube (fraction 1). Iodixanol density was monitored at 340 nm and genome copy numbers for each fraction were obtained by qPCR. **(b)** Equal genome copies (1×10^{10}) for each fraction were loaded onto SDS-polyacrylamide gels and proteins were visualized with SYPRO ruby stain. V, validation lot; M, molecular weight marker. AAV capsid proteins VP1, VP2, and VP3 are indicated. The pure AAV vector peak is indicated by the boxed area on the SDS-polyacrylamide gels.

reproducible method of detecting the emergence of the contaminating protein band.

The peak of vector genomes was observed toward the bottom third of the gradient, between fractions 12 and 22, at an OD₃₄₀-extrapolated iodixanol concentration range of 1.31 to 1.23 g/ml (Fig. 3a), just below the start of the contaminating cellular protein band (fractions 23 to 28). This peak coincided with those fractions containing pure vector particles as judged by the presence of AAV capsid proteins without contaminating cellular protein (Fig. 3b). Approximately 50% of the vector genomes consistently comigrated with the contaminating protein and could not be resolved despite attempts to do so by using different iodixanol concentrations, spin times, salt concentrations, and detergents (data not shown). Interestingly, despite loading equal genome copies of each fraction (10¹⁰ GC) on the SDS-polyacrylamide gels, fractions 26, 27, and 28 contained elevated levels of the capsid proteins VP1, VP2, and VP3 (Fig. 3b). This result suggested the presence in these fractions of either empty capsids or capsid assembly intermediates with no associated or packaged genome. Separation of empty capsids from fully infectious capsids by iodixanol gradients has been demonstrated previously (Potter *et al.*, 2002).

Large-scale pilot production run recoveries and yields

The development work described previously demonstrated that pure rAAV7 vector could be produced at high titer from a single cell stack, using a combination of PEI transfection and iodixanol gradient purification. To properly characterize the production process and demonstrate reproducibility and applicability to other AAV serotypes, full-scale pilot production runs were initiated, each using six cell stacks. The goal for each run was to produce in excess of 10¹⁴ GC of final purified vector; the final process employed is summarized in Fig. 4 and fully detailed in Materials and Methods. Three runs each of rAAV8-eGFP and rAAV9-eGFP were performed along with two runs of rAAV6-eGFP. In-process samples were taken at various stages to assess vector loss throughout as follows: (1) feedstock samples were taken after treatment of the culture medium with Benzonase/0.5 M salt and clarification; (2) retentate samples were taken after TFF concentration; (3) iodixanol gradient fraction samples were taken after gradient harvest and fraction pooling; and (4) final product samples were taken after buffer exchange and final concentration by a second TFF procedure. The recoveries of encapsidated vector genome copies at each of these stages for the various runs are listed in Table 1.

The mean recovery of rAAV8 and rAAV9 vector in the feedstock was 9.0 × 10¹⁴ GC whereas for rAAV6 vectors the mean recovery was 6.7 × 10¹³ GC. Similar low yields of rAAV6 vectors were seen in transfections during development (Fig. 2) and are also consistently observed in our standard small-scale AAV production process.

A 125-fold concentration of the feedstock from 10 liters to 85 ml (Table 1: TFF1 retentate) resulted in no loss of vector except for one instance, in which the loss was due to a mechanical failure. In several cases there was an apparent increase in yield on concentration but this was attributed to error introduced by extra dilution of the retentate, which was necessary to overcome inhibition of the qPCR. As was the case during development runs, there was loss of the vector

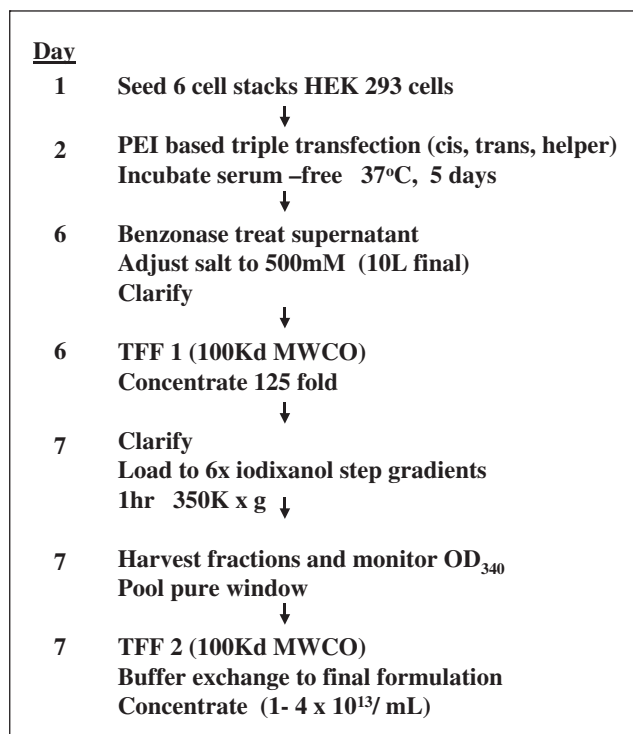


FIG. 4. Summary of the large-scale vector production process. The major process steps and corresponding timeline are shown.

during pilot iodixanol purification runs with recoveries between 35 and 50% of feedstock for AAV8 and AAV9 vectors. More loss was seen with AAV6 vectors during purification (80–85% of feedstock) and the reason for this remains unclear. Final concentration and buffer exchange led to further losses although this was most pronounced with AAV6 vectors, possibly because of the lower titer of the starting material and therefore a larger fraction of vector was absorbed to the surfaces of the TFF apparatus. Excluding the run in which mechanical loss occurred, the average overall process yield for AAV8 and AAV9 vectors was 2.2 × 10¹⁴ GC (~26% of feedstock).

Characterization of large-scale production lots

The vector lots produced in the pilot runs were characterized for capsid protein purity by SDS-PAGE analysis and for empty particle content by electron microscopy. Only a few minor bands in addition to the AAV capsid proteins VP1, VP2, and VP3 could be visualized by SDS-PAGE analysis in each of the rAAV8 and rAAV9 large-scale production lots, and the estimated purity exceeded 90% in all but a single case (Fig. 5). These results compared favorably with our standard small-scale process, in which vector purities exceeding 85% are routinely achieved. Estimates of empty particle content of the large-scale production lots were determined by direct observation of negatively stained vector particles on electron micrographs (Fig. 6). Empty particles are distinguished on these images by an electron-dense central region of the capsid in comparison with full particles, which exclude the negative stain. In comparison with unpurified preparations, in which the empty-to-full ratio can be

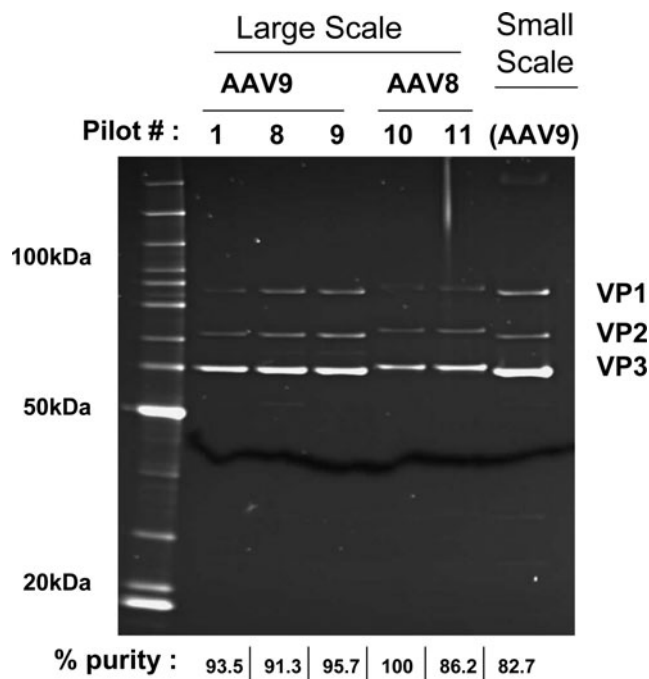


FIG. 5. Purity of large-scale rAAV production lots. Equal genome copies (1×10^{10}) of large-scale AAV8 and AAV9 vector preparations were loaded onto SDS-polyacrylamide gels and proteins were visualized by SYPRO ruby staining. All protein bands were quantified and the percent purity of the capsid (VP1, VP2, and VP3 proteins indicated over total protein) was calculated and indicated below the gel. The purity of the large-scale lots was compared with a small-scale CsCl gradient-purified AAV9 vector.

as high as 30:1 (Sommer *et al.*, 2003), the empty particle content of the pilot production lots ranged from 0.4 to 5%.

An essential quality of any rAAV production lot is the ability of the vector to deliver and express the gene of interest in cells. The potency of the rAAV8 and rAAV9 large-scale production lots relative to vectors produced by our small-scale process was assessed *in vitro* by eGFP expression and in C57BL/6 mouse livers after intravenous injection (Figs. 7 and 8, respectively). By both *in vitro* and *in vivo* analysis, all rAAV8 and rAAV9 vectors manufactured by the new production method exhibited equal or higher potency (up to 3.5-fold) when compared with identical vectors produced by the standard small-scale approach. Although rAAV6 vector yields were consistently low, the large-scale production lots still showed 2-fold transduction improvement compared with rAAV6-eGFP produced at small scale.

Discussion

The demand for rAAV vectors for clinical gene therapy continues to grow and as the current progress in the field accelerates, unprecedented quantities of vector for use in late-stage clinical trials may soon be needed. In parallel, a growing demand for vector to satisfy the complex requirements of preclinical studies is likely to rise as researchers rely increasingly on large animal data for improved prediction of clinical outcomes in humans. Several new processes for the production of rAAV vectors with yields sufficient to fuel

late-stage clinical trials are currently migrating from development laboratories to production suites both in industry and academic institutions (Clement *et al.*, 2009; Virag *et al.*, 2009; Zhang *et al.*, 2009). However, these processes often involve time-consuming construction of intermediates such as hybrid viruses and packaging cell lines and are therefore ill-suited to the preclinical environment, where several combinations of transgenes and AAV serotypes must often be tested under strict timelines. Furthermore, the majority of preclinical work is performed in academic institutions, where access to the high-technology equipment used in many large-scale production processes can be limited. To support the vector requirements of our preclinical group, we set out to develop a scaled production process that would yield sufficient vector for large animal studies while retaining the flexibility and simplicity to rapidly generate any desired rAAV product in standard AAV laboratories.

The production process we describe is based on PEI triple transfection, which allows us to maintain some unique properties of transfection-based production techniques, such as quick and easy substitutions of various AAV serotype/transgene combinations. A novel feature of the new process is that the majority of the vector can be harvested from the culture medium rather than from the production cells and thus the bulk of cellular contaminants present in the cell lysate is avoided. The upstream process is extremely efficient and yields up to 2×10^5 GC per cell or 1×10^{15} GC per lot of six cell stacks (Fig. 2 and Table 1). The choice of iodixanol gradient centrifugation for the downstream process was influenced by our desire to maintain a generic purification process for all serotypes. The isotonic, relatively inert nature of iodixanol has proven advantages with regard to maintaining vector potency (Zolotukhin *et al.*, 1999) and to overall product safety. By applying concentrated production culture medium to iodixanol step gradients we were able to obtain highly pure and potent rAAV vector with acceptable yield in a single 1-hr centrifugation step. The whole process is rapid (7 days total; Fig. 4) and cost-effective. The average overall yields for AAV8 and AAV9 vectors were 2.2×10^{14} GC with an overall process recovery of 26%.

In the current format the production method is partially serum-free because the cells are grown in 10% fetal bovine serum before transfection. However, with animal product-free medium commercially available for 293 cells, the process should be easily adaptable to be completely serum-free and meet safety regulations. Similarly, the process is cGMP compatible because all containers are sealed and manipulations are performed within the confines of a biosafety cabinet. Therefore in addition to preclinical studies, the process is also adaptable for early-stage clinical trials, where vector demand is low, and for certain applications such as the treatment of inherited retinal diseases, where low vector doses are anticipated.

An observation that we made during development of the upstream process is that rAAV of various serotypes is released to the supernatant in both calcium phosphate- and PEI-transfected cultures (Fig. 1). The nature of this release mechanism is unclear but appears to occur in the absence of obvious cytopathology. Although the release may be influenced directly by the transfection reagents used, an active export pathway for AAV virions is a possibility. The type of transfection technique did not greatly influence the amount

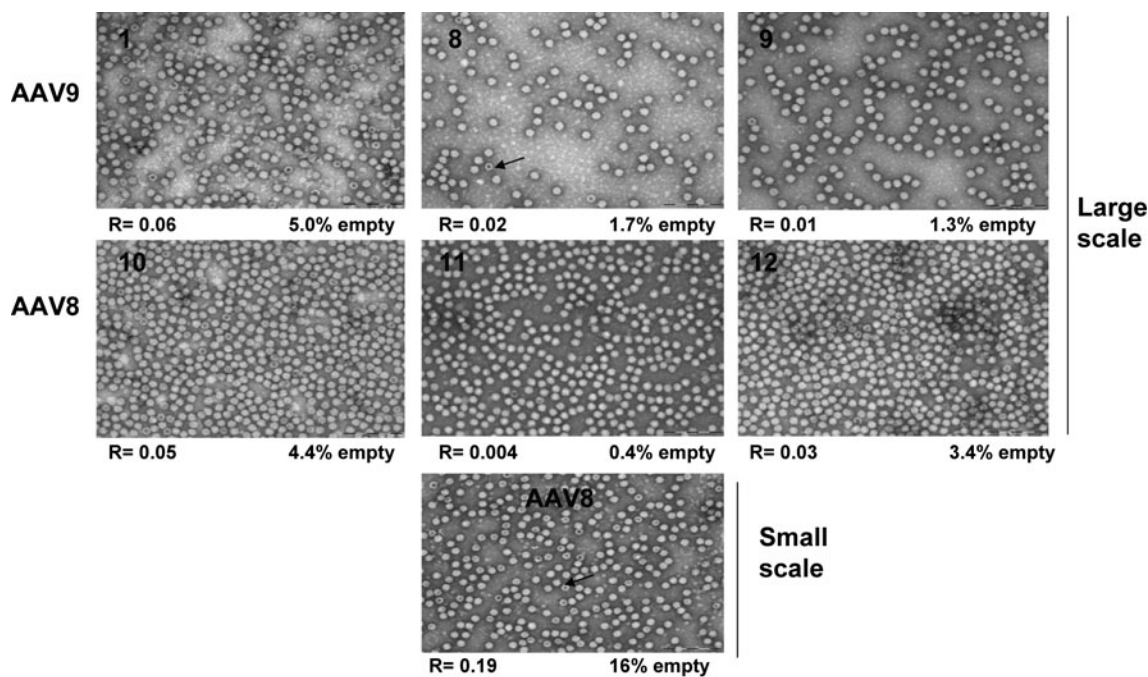


FIG. 6. Determination of empty-to-full particle ratios in large-scale rAAV8 and rAAV9 production lots. Large-scale rAAV8 and rAAV9 vector preparations were negatively stained with uranyl acetate and examined by transmission electron microscopy. The pilot run number is indicated on each image. Empty particles can be distinguished on the basis of the electron-dense center and are indicated by arrows. The ratio of empty to full particles and the percentage of empty particles are shown below the images. A small-scale AAV8 vector preparation was included in the analysis for comparison.

of vector released to the culture medium, but extending the incubation period posttransfection led to substantial increases in release. These results contrast with the data of Okada and colleagues, who found an average of 7-fold more AAV8 vector in the culture medium versus the cell pellet at 72 hr posttransfection (Okada *et al.*, 2009). These researchers used calcium phosphate transfection along with an active gassing system and it is possible that the resulting different culture conditions contributed to the increase in release at the earlier time point. Alternatively, in our experience quantification of AAV genomes in a complex cell lysate by qPCR can be prone to nonspecific inhibition of the PCR and it is possible that the cell lysate amounts were underestimated. Interestingly, in experiments not reported here, when the medium was harvested and replaced on successive days posttransfection, the recovery of rAAV7 vector in the culture medium remained constant. This observation suggests that the incorporation of perfusion culture techniques to the process may increase upstream yields further still. We chose to restrict our efforts to adherent HEK293 cultures for reasons of simplicity and convenience, but given the use of PEI transfection in the production of rAAV in suspension cultures (Durocher *et al.*, 2007; Hildinger *et al.*, 2007), the adaptation of our upstream process to bioreactors appears feasible. A major advantage of such an approach would be the ability to use the same upstream process for both pre-clinical and clinical vector manufacture, which is desirable from a regulatory standpoint.

Our demonstration that most AAV serotypes can be efficiently harvested from the production culture medium (Fig. 2;

and Vandenberghe *et al.*, 2010) indicates that the new process will likely be widely applicable to most AAV vectors. However, for some AAV serotypes modifications will be required. For example, rAAV2 is mostly retained in the cell (Vandenberghe *et al.*, 2010) and release of this serotype to the culture medium would need to be manipulated, perhaps by a combination of pH, temperature, and salt treatment (Atkinson *et al.*, 2005). rAAV6 vectors were not efficiently produced in either the cells or the culture medium after PEI triple transfection (Fig. 2 and Table 1) and in our hands cannot be reproducibly manufactured at high titer by standard calcium phosphate transfection and cesium chloride gradient purification. These findings therefore appear to reflect fundamentally different efficiencies of AAV6 capsid assembly and genome packaging, or perhaps a lower stability of the AAV6 virion.

Ion-exchange, hydrophobic interaction, and affinity column chromatography are the methods of choice for capture of AAV vector from large volumes of culture medium. These methods must often be developed specifically for each AAV serotype and therefore for preclinical vector production, a generic purification method to accommodate multiple serotypes is a better solution. The TFF concentration/iodixanol gradient method we have developed is a generic downstream approach to rAAV purification and in the studies presented here produced a vector peak that was pure and relatively free of empty particles (Figs. 5 and 6). Possible alternatives to the TFF concentration step we have employed include precipitation of the vector from the supernatant, using ammonium sulfate or polyethylene glycol (PEG). However, at the scale described these procedures would

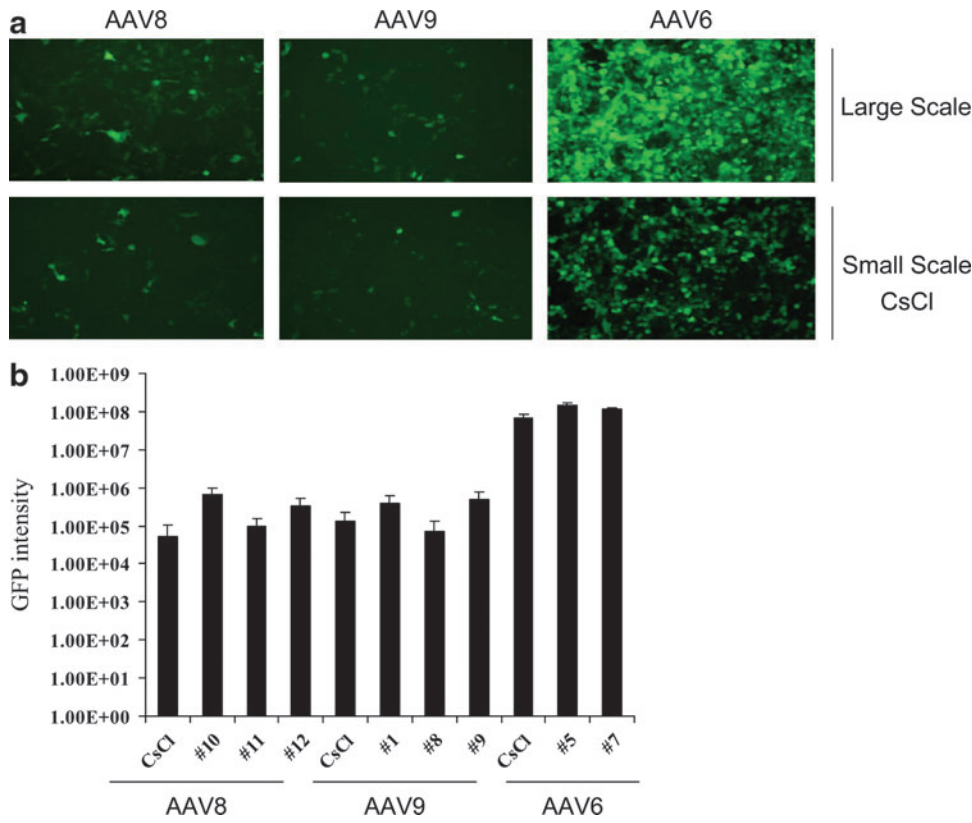


FIG. 7. Relative transduction of rAAV8, rAAV9, and rAAV6 vectors *in vitro*. **(a)** HEK293 cells were infected in triplicate with rAAV-eGFP vector lots produced by both large- and small-scale processes at an MOI of 1×10^4 GC/cell in the presence of adenovirus. GFP transgene expression was photographed 48 hr postinfection. **(b)** eGFP fluorescence intensity was quantified directly from the digital images by determining the product of brightness levels and pixels over background levels.

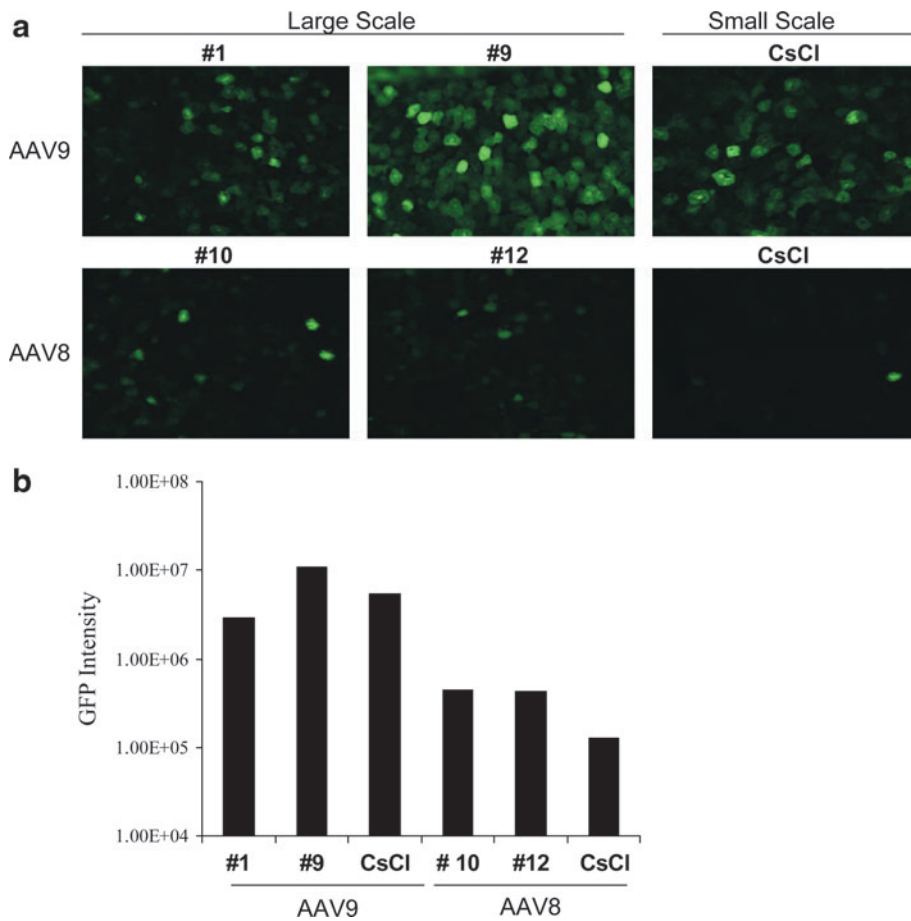


FIG. 8. Liver transduction with rAAV8 and rAAV9 large-scale production lots. **(a)** C57BL/6 mice were injected intravenously with 1×10^{11} GC of rAAV8-eGFP and rAAV9-eGFP vectors produced by both small- and large-scale processes. eGFP fluorescence was compared in liver sections 9 days postinjection. **(b)** eGFP fluorescence intensity was quantified directly from the digital images by determining the product of brightness levels and pixels over background levels. Each column represents the average intensity value of liver samples from two animals.

involve centrifugation of large volumes of supernatant on a routine basis and this is especially a problem with ammonium sulfate, for which up to 50% saturation is required to precipitate AAV. Furthermore, it has been our experience that PEG precipitation can negatively impact the potency of AAV vectors. TFF, on the other hand, eliminates the need for centrifugation and the concentration process we describe can be accomplished in less than 2 hr. A further advantage of the TFF process is that small proteins will pass through the filter membrane, which leads to additional purification.

Flexibility in terms of purification of different serotypes could not be achieved without cost, because the average loss of vector on the iodixanol gradient was 57% and mainly resulted from an inability to completely resolve the vector from contaminating protein and empty particles (Table 1). Removal of empty vector particles is a preferred feature of any rAAV purification protocol because, depending on the production process, this vector-specific contaminant can be present in vast excess in unpurified feedstock. A lack of removal of empty particles from the final product exposes the recipient of the vector to a large source of AAV antigen that can lead to unwanted immune responses and toxicity (Wright, 2009). Common column chromatography-based purification techniques do not discriminate against empty particles, although ion-exchange methods that are capable of this function have been published for AAV1, AAV2, and AAV8 (Qu *et al.*, 2007; Okada *et al.*, 2009). Like most bulk capture ion-exchange-based column chromatography processes, these methods for removal of AAV empty capsids must be tailored to a particular AAV serotype and the shallow elution gradients employed are likely to present technical challenges during scale-up. A more robust procedure might involve direct capture of AAV particles from the production culture medium on ion-exchange or affinity chromatography columns and subsequent density gradient purification of the concentrated column eluate for separation of empty particles. The integration of such a strategy in place of the TFF concentration step described for our process would likely have the added advantage of decreasing contaminant cellular protein in the gradient load, increasing resolution of the iodixanol gradient, and thus ultimately increasing yield of the vector.

The potency of the rAAV8 and rAAV9 vectors produced by the new process described was demonstrated in both *in vitro* and *in vivo* transduction assays to be at least equivalent to if not slightly better than that of identical vectors produced by our routine small-scale production method (Figs. 7 and 8). This result might be expected because iodixanol is less harsh than cesium chloride in terms of impacting AAV transducing activity (Zolotukhin *et al.*, 1999) and, in addition, we have been unable to demonstrate a difference in potency linked to the source of the rAAV vector (cells vs. culture medium; Vandenberghe *et al.*, 2010). The delayed harvest featured in the new process at 120 hr posttransfection was originally a concern regarding vector potency but appears to have a negligible, if any, effect.

In conclusion, the large-scale rAAV vector production process presented here has been tailored toward the needs of AAV gene therapy laboratories involved in preclinical trials

and is anticipated to satisfy most requirements of these studies including the preclinical requirement for flexible vector manufacture. With some of the modifications suggested, the potential exists to scale up the new method to supply rAAV vectors for clinical applications while retaining inherent advantages of the process such as reagent simplicity, process speed, and clearance of vector-specific impurities.

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Author Disclosure Statement

M.L., and L.H.V. are inventors on patents licensed to various biopharmaceutical companies, including ReGenX. J.M.W. is a consultant to ReGenX Holdings, and is a founder of, holds equity in, and receives a grant from affiliates of ReGenX Holdings; in addition, he is an inventor on patents licensed to various biopharmaceutical companies, including affiliates of ReGenX Holdings.

References

- Atkinson, M.A., Fung, V.P., Wilkins, P.C., Takeya, R., K, Reynolds, T.C., and Aranha, I.L. (2005). Methods for generating high titer helper free preparations of release recombinant AAV vectors. U.S. Patent No. 0266567.
- Auricchio, A., Hildinger, M., O'Connor, E., Gao, G.P., and Wilson, J.M. (2001). Isolation of highly infectious and pure adeno-associated virus type 2 vectors with a single-step gravity-flow column. *Hum. Gene Ther.* 12, 71–76.
- Brantly, M.L., Chulay, J.D., Wang, L., Mueller, C., Humphries, M., Spencer, L.T., Rouhani, F., Conlon, T.J., Calcedo, R., Betts, M.R., Spencer, C., Byrne, B.J., Wilson, J.M., and Flotte, T.R. (2009). Sustained transgene expression despite T lymphocyte responses in a clinical trial of rAAV1-AAT gene therapy. *Proc. Natl. Acad. Sci. U.S.A.* 106, 16363–16368.
- Brument, N., Morenweiser, R., Blouin, V., Toubanc, E., Raimbaud, I., Cherel, Y., Folliot, S., Gaden, F., Boulanger, P., Kroner-Lux, G., Moullier, P., Rolling, F., and Salvetti, A. (2002). A versatile and scalable two-step ion-exchange chromatography process for the purification of recombinant adeno-associated virus serotypes-2 and -5. *Mol. Ther.* 6, 678–686.
- Clement, N., Knop, D.R., and Byrne, B.J. (2009). Large-scale adeno-associated viral vector production using a herpesvirus-based system enables manufacturing for clinical studies. *Hum. Gene Ther.* 20, 796–806.
- Davidoff, A.M., Ng, C.Y., Sleep, S., Gray, J., Azam, S., Zhao, Y., McIntosh, J.H., Karimipour, M., and Nathwani, A.C. (2004). Purification of recombinant adeno-associated virus type 8 vectors by ion exchange chromatography generates clinical grade vector stock. *J. Virol. Methods* 121, 209–215.
- Durocher, Y., Pham, P.L., St-Laurent, G., Jacob, D., Cass, B., Chahal, P., Lau, C.J., Nalbantoglu, J., and Kamen, A. (2007). Scalable serum-free production of recombinant adeno-associated

- virus type 2 by transfection of 293 suspension cells. *J. Virol. Methods* 144, 32–40.
- Ferreira, T., and Rasband, W. (2010). *The ImageJ User Guide*, version 1.43. Available at <http://rsb.info.nih.gov/ij/docs/user-guide.pdf> (accessed August 2010).
- Gao, G.P., Alvira, M.R., Wang, L., Calcedo, R., Johnston, J., and Wilson, J.M. (2002). Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. *Proc. Natl. Acad. Sci. U.S.A.* 99, 11854–11859.
- Grieger, J.C., Choi, V.W., and Samulski, R.J. (2006). Production and characterization of adeno-associated viral vectors. *Nat. Protoc.* 1, 1412–1428.
- Grimm, D., Kern, A., Rittner, K., and Kleinschmidt, J.A. (1998). Novel tools for production and purification of recombinant adeno-associated virus vectors. *Hum. Gene Ther.* 9, 2745–2760.
- Hermens, W.T., ter Brake, O., Dijkhuizen, P.A., Sonnemans, M.A., Grimm, D., Kleinschmidt, J.A., and Verhaagen, J. (1999). Purification of recombinant adeno-associated virus by iodixanol gradient ultracentrifugation allows rapid and reproducible preparation of vector stocks for gene transfer in the nervous system. *Hum. Gene Ther.* 10, 1885–1891.
- Hilding, M., Baldi, L., Stettler, M., and Wurm, F.M. (2007). High-titer, serum-free production of adeno-associated virus vectors by polyethyleneimine-mediated plasmid transfection in mammalian suspension cells. *Biotechnol. Lett.* 29, 1713–1721.
- Kaludov, N., Handelman, B., and Chiorini, J.A. (2002). Scalable purification of adeno-associated virus type 2, 4, or 5 using ion-exchange chromatography. *Hum. Gene Ther.* 13, 1235–1243.
- Maguire, A.M., Simonelli, F., Pierce, E.A., Pugh, E.N., Jr., Mingozzi, F., Bannicelli, J., Banfi, S., Marshall, K.A., Testa, F., Surace, E.M., Rossi, S., Lyubarsky, A., Arruda, V.R., Konkle, B., Stone, E., Sun, J., Jacobs, J., Dell'Osso, L., Hertle, R., Ma, J.X., Redmond, T.M., Zhu, X., Hauck, B., Zelenia, O., Shindler, K.S., Maguire, M.G., Wright, J.F., Volpe, N.J., McDonnell, J.W., Auricchio, A., High, K.A., and Bennett, J. (2008). Safety and efficacy of gene transfer for Leber's congenital amaurosis. *N. Engl. J. Med.* 358, 2240–2248.
- Matsushita, T., Elliger, S., Elliger, C., Podsakoff, G., Villarreal, L., Kurtzman, G.J., Iwaki, Y., and Colosi, P. (1998). Adeno-associated virus vectors can be efficiently produced without helper virus. *Gene Ther.* 5, 938–945.
- Moss, R.B., Rodman, D., Spencer, L.T., Aitken, M.L., Zeitlin, P.L., Waltz, D., Milla, C., Brody, A.S., Clancy, J.P., Ramsey, B., Hamblett, N., and Heald, A.E. (2004). Repeated adeno-associated virus serotype 2 aerosol-mediated cystic fibrosis transmembrane regulator gene transfer to the lungs of patients with cystic fibrosis: A multicenter, double-blind, placebo-controlled trial. *Chest* 125, 509–521.
- Mueller, C., and Flotte, T.R. (2008). Clinical gene therapy using recombinant adeno-associated virus vectors. *Gene Ther.* 15, 858–863.
- Neinhuis, A. (2009). Dose-escalation study of a self complementary adeno-associated viral vector for gene transfer in hemophilia B. Available at <http://clinicaltrials.gov/ct2/show/NCT00979238> (accessed August 2010).
- Okada, T., Nonaka-Sarukawa, M., Uchibori, R., Kinoshita, K., Hayashita-Kinoh, H., Nitahara-Kasahara, Y., Takeda, S., and Ozawa, K. (2009). Scalable purification of adeno-associated virus serotype 1 (AAV1) and AAV8 vectors, using dual ion-exchange adsorptive membranes. *Hum. Gene Ther.* 20, 1013–1021.
- Potter, M., Chesnut, K., Muzyczka, N., Flotte, T., and Zolotukhin, S. (2002). Streamlined large-scale production of recombinant adeno-associated virus (rAAV) vectors. *Methods Enzymol.* 346, 413–430.
- Qu, G., Bahr-Davidson, J., Prado, J., Tai, A., Cataniag, F., McDonnell, J., Zhou, J., Hauck, B., Luna, J., Sommer, J.M., Smith, P., Zhou, S., Colosi, P., High, K.A., Pierce, G.F., and Wright, J.F. (2007). Separation of adeno-associated virus type 2 empty particles from genome containing vectors by anion-exchange column chromatography. *J. Virol. Methods* 140, 183–192.
- Reed, S.E., Staley, E.M., Mayginnes, J.P., Pintel, D.J., and Tullis, G.E. (2006). Transfection of mammalian cells using linear polyethylenimine is a simple and effective means of producing recombinant adeno-associated virus vectors. *J. Virol. Methods* 138, 85–98.
- Salveti, A., Oreve, S., Chadeuf, G., Favre, D., Cherel, Y., Champion-Arnaud, P., David-Ameline, J., and Moullier, P. (1998). Factors influencing recombinant adeno-associated virus production. *Hum. Gene Ther.* 9, 695–706.
- Schroder, M., Schafer, R., and Friedl, P. (1997). Spectrophotometric determination of iodixanol in subcellular fractions of mammalian cells. *Anal. Biochem.* 244, 174–176.
- Smith, R.H., Levy, J.R., and Kotin, R.M. (2009). A simplified baculovirus-AAV expression vector system coupled with one-step affinity purification yields high-titer rAAV stocks from insect cells. *Mol. Ther.* 17, 1888–1896.
- Snyder, R.O., and Flotte, T.R. (2002). Production of clinical-grade recombinant adeno-associated virus vectors. *Curr. Opin. Biotechnol.* 13, 418–423.
- Sommer, J.M., Smith, P.H., Parthasarathy, S., Isaacs, J., Vijay, S., Kieran, J., Powell, S.K., McClelland, A., and Wright, J.F. (2003). Quantification of adeno-associated virus particles and empty capsids by optical density measurement. *Mol. Ther.* 7, 122–128.
- Vandenberghe, L.H., Xiao, R., Lock, M., Lin, J., Korn, M., and Wilson, J.M. (2010). Efficient serotype-dependent release of functional vector into the culture medium during adeno-associated virus manufacturing. *Hum. Gene Ther.* 21, 1251–1257.
- Virag, T., Cecchini, S., and Kotin, R.M. (2009). Producing recombinant adeno-associated virus in foster cells: Overcoming production limitations using a baculovirus-insect cell expression strategy. *Hum. Gene Ther.* 20, 807–817.
- Wang, L., Wang, H., Bell, P., McCarter, R.J., He, J., Calcedo, R., Vandenberghe, L.H., Morizono, H., Batshaw, M.L., and Wilson, J.M. (2010). Systematic evaluation of AAV vectors for liver directed gene transfer in murine models. *Mol. Ther.* 18, 118–125.
- Warrington, K.H., Jr., and Herzog, R.W. (2006). Treatment of human disease by adeno-associated viral gene transfer. *Hum. Genet.* 119, 571–603.
- Wright, J.F. (2009). Transient transfection methods for clinical adeno-associated viral vector production. *Hum. Gene Ther.* 20, 698–706.
- Wright, J.F., Le, T., Prado, J., Bahr-Davidson, J., Smith, P.H., Zhen, Z., Sommer, J.M., Pierce, G.F., and Qu, G. (2005). Identification of factors that contribute to recombinant AAV2 particle aggregation and methods to prevent its occurrence during vector purification and formulation. *Mol. Ther.* 12, 171–178.
- Xiao, X., Li, J., and Samulski, R.J. (1998). Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. *J. Virol.* 72, 2224–2232.
- Zhang, H., Xie, J., Xie, Q., Wilson, J.M., and Gao, G. (2009). Adenovirus-adeno-associated virus hybrid for large-scale

recombinant adeno-associated virus production. *Hum. Gene Ther.* 20, 922–929.

Zolotukhin, S., Byrne, B.J., Mason, E., Zolotukhin, I., Potter, M., Chesnut, K., Summerford, C., Samulski, R.J., and Muzyczka, N. (1999). Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. *Gene Ther.* 6, 973–985.

Zolotukhin, S., Potter, M., Zolotukhin, I., Sakai, Y., Loiler, S., Fraitas, T.J., Jr., Chiodo, V.A., Phillipsberg, T., Muzyczka, N., Hauswirth, W.W., Flotte, T.R., Byrne, B.J., and Snyder, R.O. (2002). Production and purification of serotype 1, 2, and 5 recombinant adeno-associated viral vectors. *Methods* 28, 158–167.

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