

REVIEW

Toward exascale production of recombinant adeno-associated virus for gene transfer applications

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To gain acceptance as a medical treatment, adeno-associated virus (AAV) vectors require a scalable and economical production method. Recent developments indicate that recombinant AAV (rAAV) production in insect cells is compatible with current good manufacturing practice production on an industrial scale. This platform can fully support development of rAAV therapeutics from tissue culture to small animal models, to large animal models, to toxicology studies, to Phase I clinical trials and beyond. Efforts to characterize, optimize and develop

insect cell-based rAAV production have culminated in successful bioreactor-scale production of rAAV, with total yields potentially capable of approaching the 'exa-(10^{18}) scale.' These advances in large-scale AAV production will allow us to address specific catastrophic, intractable human diseases such as Duchenne muscular dystrophy, for which large amounts of recombinant vector are essential for successful outcome.

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Introduction

Currently, adeno-associated virus (AAV) serotypes 1, 2, 4, 5, 6 and 8 vectors are produced using baculovirus expression vectors (BEVs) in insect cells. Newly developed improvements to production processes utilizing on-line permittivity measurements, multiplicity of infection (MOI) analysis, cell density at time of infection (TOI) and baculovirus stability studies have increased both our understanding of the production process as well as the yields of recombinant AAV (rAAV).^{1–3} Obtaining $\geq 3 \times 10^{14}$ particles of rAAV per liter of cell culture is now possible, thus a 3501 production run may yield $\approx 10^{17}$ rAAV particles, sufficient for toxicological studies and preclinical, dose escalation experiments in large animal models.

Adeno-associated virus

The AAV vectors or rAAV are derived from human, non-human primate and other mammalian species AAV isolates. AAVs belong to the dependovirus genus of the parvovirus family. The icosahedrally symmetric and non-enveloped capsids contain a single-stranded, linear DNA genome unique to the *Parvoviridae*. For efficient replication, the dependovirus genus requires co-infection with a helper virus, typically adenovirus or herpes simplex virus. In the absence of helper virus co-infection, AAV DNA integrates into the cellular genome forming a

stable provirus that may be activated and 'rescued' upon subsequent helper virus infection. Interestingly, in tissue culture experiments, the provirus frequently localizes to a defined region on human chromosome 19 referred to as AAVS1.^{4–7} However, since vectors produced from AAV are devoid of all virus genes, locus-specific integration is not observed, and random integration occurs at a low frequency.

The AAV genome is 4680 nt and contains two large open-reading frames (ORFs) encoding the non-structural and structural proteins (Figure 1) (reviewed by Smith and Kotin⁸). The left ORF codes for the Rep proteins, necessary for virus DNA replication. Two promoters, p5 and p19, regulate expression of the *rep* gene, producing two transcripts that undergo alternative splicing to yield four Rep proteins. Rep 78 and Rep 68 are derived from the p5 promoter transcripts, whereas Rep 52 and Rep 40 are products of p19 promoter transcripts. In effect, the p19-derived proteins are N-terminal truncations of the p5-derived proteins. The p5 Rep proteins contain a sequence-specific DNA-binding domain,⁹ as well as sequence- and strand-specific endonuclease¹⁰ and ligase activities.¹¹ Within the common C-terminal moiety of Rep are sequences associated with nucleoside triphosphate binding and hydrolysis (reviewed by Smith and Kotin⁸).

The *cap* gene, regulated by the promoter at map position 40 (p40), codes for the three AAV structural proteins, VP1, VP2 and VP3, with calculated molecular masses of 87, 73 and 62 kDa, respectively. In the virion, VP1, VP2 and VP3 occur in approximately 1:1:10.¹² This ratio reflects the stoichiometry of intracellular *cap* gene products and is regulated by splicing and utilization of an atypical initiation codon. Translational initiation from the first in-frame AUG codon produces VP1. Differential splicing removes the VP1 initiation codon allowing scanning ribosomes to initiate translation of VP2 from

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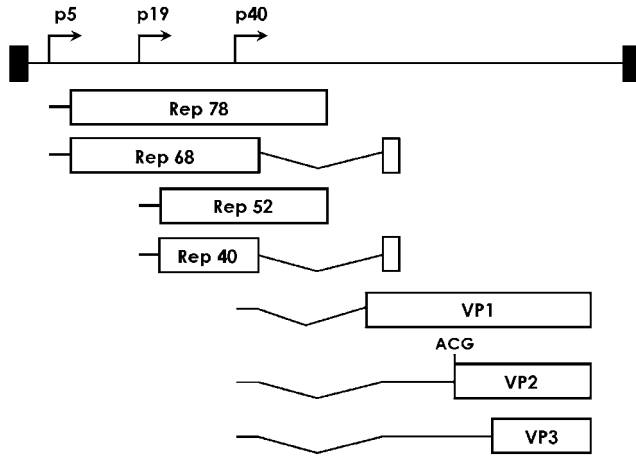


Figure 1 Transcriptional map of AAV genome. The wild-type AAV genome is represented as a line with three right-angle arrows indicating the positions of the three promoters at map positions 5 (p5), 19 (p19) and 40 (p40). The solid black boxes flanking the coding sequences identify the locations of the terminal palindromes or inverted terminal repeats. The four non-structural Rep proteins are translated from the unspliced and spliced transcripts regulated by the p5 promoters, Rep 78 and Rep 68, and the p19 promoters, Rep 52 and Rep 40, respectively. The structural virion proteins, VP1, VP2 and VP3, are translated from the p40 transcript that is encoded by the *cap* gene. One spliced form of the p40 transcript results in predominantly VP1. Utilization of an alternate splice acceptor removes the VP1 initiation codon and translation inefficiently initiating at a non-methionine codon (ACG) produces VP2. The first AUG codon encountered in the VP2/VP3 transcript is the initiation codon for VP3. Thus, through alternative splicing and non-AUG initiation codon use, the stoichiometry of the capsid proteins is regulated at a 1:1:10 ratio for VP1:VP2:VP3. AAV, adeno-associated virus.

an ACG triplet producing VP2 in an inefficient process (Figure 1). However, the majority of the ribosomes bypass the ACG VP2 initiation triplet and scan through to the first AUG codon initiating VP3 translation (reviewed by Smith and Kotin⁸).

AAV as a vector for gene transfer

Separating and reducing the overlapping sequences of the *cis*- and *trans*-acting factors¹³ enabled rAAV production and reduced the large amounts of recombinant wild-type or wild-type-like particles generated from the original method.¹⁴ However, these methods also utilized infectious adenovirus to provide helper functions, requiring the production, characterization and removal of the infectious adenovirus reducing the overall vector yield while increasing production cost. Three independent reports described that co-transfecting adenovirus early genes in a non-infectious plasmid reconstituted the adenovirus helper functions and rendered the cell permissive for rAAV production.^{15–17} In addition, differentially modulating levels of the AAV rep gene products either by substituting heterologous virus promoters for the native AAV promoters^{16,18} or attenuating translational initiation with a non-AUG codon improved rAAV yield.¹⁷

Other production methods that were developed employed recombinant herpes simplex viruses, recombinant

Table 1 Comparison of surface area and volume for rAAV production

Vg	Cells	Volume (L)	Surface (cm ²)
1 × 10 ¹⁰	1 × 10 ⁶	0.0005	10
1 × 10 ¹¹	1 × 10 ⁷	0.005	100
1 × 10 ¹²	1 × 10 ⁸	0.05	1000
1 × 10 ¹³	1 × 10 ⁹	0.5	10 000
1 × 10 ¹⁴	1 × 10 ¹⁰	5	100 000
1 × 10 ¹⁵	1 × 10 ¹¹	50	1 000 000
1 × 10 ¹⁶	1 × 10 ¹²	500	10 000 000
1 × 10 ¹⁷	1 × 10 ¹³	5000	100 000 000
1 × 10 ¹⁸	1 × 10 ¹⁴	50 000	1 000 000 000
1 × 10 ¹⁹	1 × 10 ¹⁵	500 000	10 000 000 000

Abbreviations: vg, vector genome; rAAV, recombinant adeno-associated virus.

The first column, vg, presents the number of virus particle required. The second column lists the number of cells needed to produce the vectors in column one assuming that 10⁴ vg are produced per cell. Columns three and four list the relative volume (in liters) and surface (in cm²) required to grow the number of cells. Thus, to produce 10¹⁵ particles requires either 50 l or 1 000 000 cm², which corresponds to either one small bioreactor or about 5700 × 15 cm diameter tissue culture plates. Increasing the production 10-fold requires either a 500 l bioreactor or 57 000 plates, and so on.

adenoviruses and recombinant baculoviruses. The reported yields of rAAV produced with these systems ranged the gamut,³ but no publications describing large-scale production processes had appeared. One mammalian cell-based system that has been developed and is in production for clinical grade vector involves either packaging or producer cell lines, and infectious adenovirus^{19–22} has reportedly been developed into a scalable process by Targeted Genetics Corps. (Seattle, WA), but the system is proprietary and no published descriptions are available.

The most commonly used processes rely on efficient transient transfection of adherent mammalian cells, often human embryonic kidney 293 cells.^{17,23,24} Alternative processes utilize stable cell lines bearing the vector genome (vg) and AAV rep and cap genes that are subsequently infected with adenovirus 5.^{19,20,25} Production of rAAV using recombinant herpes simplex virus containing the vg and the rep and cap genes has been described.^{26,27} Using mammalian cells, rAAV production processes have evolved into efficient and reproducible processes. However, the major deficiency with each mammalian cell-based production system is the difficulty to scale-up the process. The two processes utilizing adherent cells are limited by surface area requirements. For example, if human embryonic kidney 293 cells are transfected at a density of 10⁵ cells per cm² and if each cell yields 10⁴ rAAV particles then it follows that a surface area of 10 cm² will yield approximately 1 × 10¹⁰ particles and 100 cm² 1 × 10¹¹, and so on. The relationship between adherent and suspension cells in terms of surface area to volume is approximately 10 cm² ≈ 0.5 cm³. As illustrated by the values in Table 1, as the vg requirements increase, providing the solid support for adherent cells becomes unrealistically large, whereas the volumetric requirements for all except at the largest production scale is manageable with available systems.

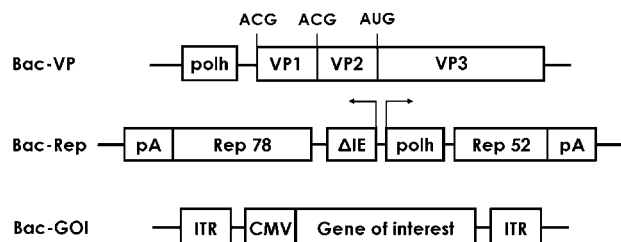


Figure 2 Diagrams representing the baculovirus constructs used to produce rAAV. Three BEVs were engineered that provide the *trans*-acting elements necessary for rAAV production. VP1, VP2 and VP3 are derived from one ORF (Bac-VP). The stoichiometry of the VPs are regulated at the translational level by substituting an ACG codon for the VP1 methionine initiation codon, in addition to other modifications as described by Urabe *et al.*³⁷ The Rep proteins are provided by Bac-Rep and the high Rep 52:Rep 40 level is regulated transcriptionally; the much stronger polyhedrin promoter (polh) overexpresses Rep 52 relative to the weaker promoter modified from the *OpIE* promoter as described by Urabe *et al.*³⁷ A third baculovirus carrying the vector genome, Bac-GOI, has the AAV inverted terminal repeats flanking the gene of interest (GOI). In the presence of the Rep proteins, the inverted terminal repeats function as AAV origins of replication. In co-infected cells, the vector genome is found replicated to relatively high copy numbers independently of Bac-GOI replication. BEV, baculovirus expression vector; ORF, open-reading frame; rAAV, recombinant adeno-associated virus.

Overview of rAAV production in Sf9 cells

To extend the results of therapeutic gene transfer beyond *in vitro* studies and small animal models, the impediment of using adherent cells for rAAV production had to be overcome. The production of biologically active, recombinant dependovirus in invertebrate cells followed a critical and unexpected experimental result demonstrating that AAV Rep 78 supported AAV DNA replication in Sf9 cells. Although it was known that mammalian virus structural proteins assemble into virus-like particles in insect cells, for example *Papillomaviridae*,^{28–31} *Reoviridae*^{32–34} and *Caliciviridae*,^{35,36} virus or vector DNA replication and packaging in invertebrate cells was never examined.

Similar to rAAV production in mammalian cells, insect cells require both *cis* and *trans* factors from AAV for replication, packaging and particle formation. These factors are provided by three baculovirus constructs including Bac-VP, Bac-Rep and Bac-GOI encoding for capsid proteins, replication proteins and the gene of interest, respectively (Figure 2). The AAV terminal palindromes, referred to as the inverted terminal repeats, function as primers for DNA synthesis and are considered important for encapsidation. Replication and packaging require two of the four AAV non-structural proteins: one of the p5 proteins, either Rep 78 or Rep 68, and one of the p19 proteins, either Rep 52 or Rep 40.

Bioprocess developments

To improve rAAV production and processing particularly related to the Sf9-BEV system, different analyses have been used including end point and kinetic studies, which described conditions for time of harvest and MOI. Insect cells in suspension grow best within a narrow window of pH, temperature, dissolved oxygen, cell density and agitation. These parameters define a mini-

mum set of conditions controlled through feedback loops to maintain set values. The pH of an insect culture is usually monitored passively without regulation. Additionally, the production or consumption of various metabolites have also been measured, such as O₂ consumption, CO₂ generation, ammonia, glucose and lactic acid, providing a means of profiling the culture conditions and perhaps identifying optimal conditions and times for BEV infection and rAAV harvesting.³⁸ In addition, cell diameter, which changes during the course of BEV infection, is often monitored.^{39,40}

Permittivity

Continuously monitoring the cell culture allows the modifications to be made nearly instantaneously. For example, the level of dissolved oxygen is controlled with a feedback loop to maintain specified set points. The pH and temperature are the other parameters often controlled in real time through feedback loops. The effects of continuously controlling various parameters have been described in several publications leading to improved recombinant protein production in insect cell cultures using BEVs.^{41–45} Few on-line options are available to measure biomass and cell viability. Breaching the sterile barrier allows cell counting and viability measurements non-continuously with off-line, laboratory instruments. Online optically based probes that measure absorption or optical density do not discriminate between viable and non-viable cells. An alternative system measures capacitance, also referred to as the dielectric permittivity (De_{fc}), to estimate cell density and biomass in cell cultures. As baculovirus infection progresses, the host cell cycle is arrested and the cell diameter increases thereby changing the biomass, which can be monitored by a permittivity probe.⁴¹

Zeiser *et al.*⁴¹ described the dielectric spectroscopy profile of Sf9 cell culture, and recently those findings were extended to characterize the BEV infection phases during the large-scale production of rAAV in stirred-tank bioreactors.² The different crucial process stages that can be identified by spectroscopic analysis for the production of rAAV include synchronous baculovirus infection. This is observed around 18 hpi.³⁸ After synchronized infection, the De_{fc} signal decreased reaching the minimum value approximately 24–27 hpi. Later, De_{fc} increased attaining a second maximum about 48 hpi, then near-linearly decreased until terminating the process. This second maximum value of De_{fc} has been correlated with the optimal harvesting time of rAAV. This analysis provides a detailed characterization of the conditions and kinetics of rAAV production essential for detecting differences between production runs, for estimating the optimal time for infection and for temporally adjusting the infected culture harvest time, thereby providing lot-to-lot reproducibility facilitating the large-scale production optimization and bioprocessing scale-up.

Time course of expression of component proteins of rAAV

Correlating vector production with the permittivity profile provides a real-time, on-line method for assessing

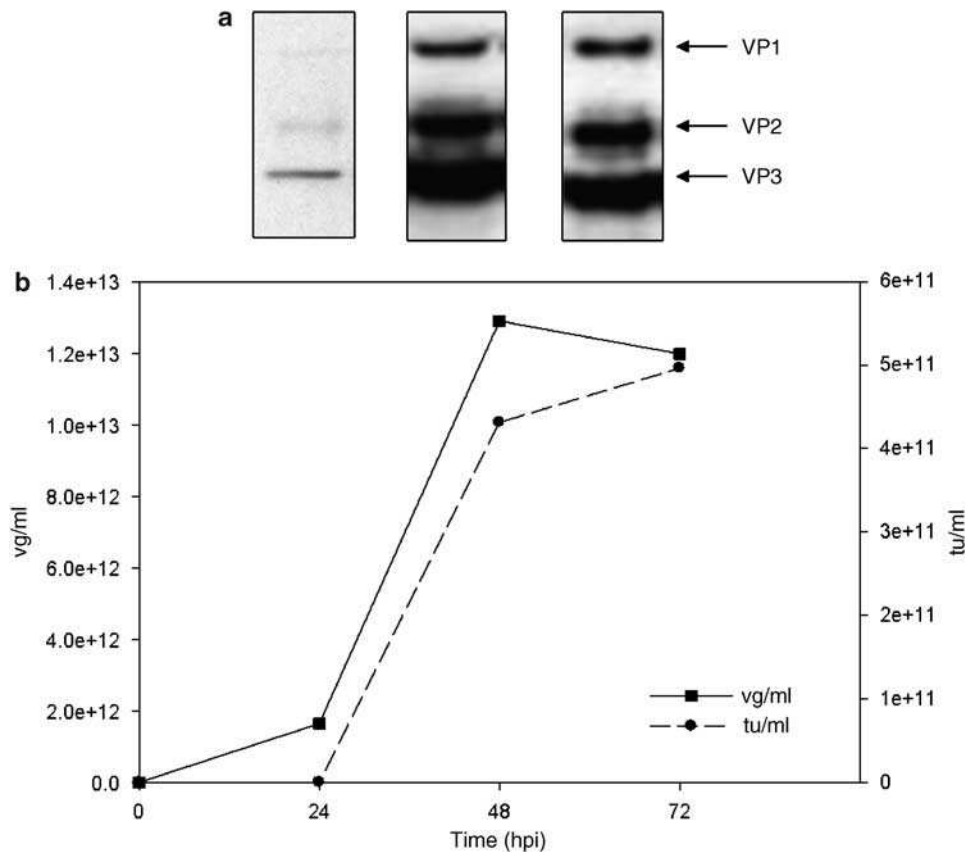


Figure 3 Time course production of rAAV. The kinetics of rAAV production in *Sf9* cells infected with three BEVs, Bac-VP, Bac-Rep and Bac-GFP. At 24 h intervals, the infected cell culture was sampled and (a) analyzed for capsid proteins by polyacrylamide gel electrophoresis and western blot with anti-VP primary antibody. (b) The production of DNase resistant particles (vg per ml (■)) was determined by quantitative PCR and transducing units (tu per ml (●)) was analyzed by flow cytometry analysis of human embryonic kidney 293 cells treated with rAAV-GFP processed from the *Sf9* cell culture. BEV, baculovirus expression vector; rAAV, recombinant adeno-associated virus.

the progress of the cell growth and infection, as well as determining the optimal harvesting time. Figure 3a shows the expression of capsid antigens in the triple-infected insect cell culture.² At 24 hpi, the lack of temporally regulated expression of the *rep* and *cap* genes is likely to cause an early accumulation of empty capsid particles before the vector DNA has replicated to the levels for efficient encapsidation (data not shown). At 24, 48 and 72 hpi, the three capsid proteins VP1, VP2 and VP3 are detected in isopycnic CsCl gradient fractions corresponding to filled capsids (1.41 g cm^{-3}) (Figure 3a).

Using quantitative PCR to detect the distribution of vgs across the CsCl gradient, the amount of vg determined at 24 hpi was low (Figure 3b). However, at 48 hpi, the production of vg substantially increased. The total yield of vg per liter of cell culture at 48 hpi was $\sim 1 \times 10^{13}$. The distribution profiles of vg across 48 and 72 hpi were similar. The vg distribution agreed with the positions of the capsid proteins as determined by western blots and corresponded to densities where full particles were expected.

The distribution of transducing units (tu) across the gradient was restricted to fractions of densities corresponding to full capsids (1.41 g cm^{-3}), whereas empty capsids were found in fractions of approximately 1.35 g cm^{-3} . The yield obtained of tu per liter of cell culture was $\sim 2.0 \times 10^{12}$. These results demonstrated that permittivity analysis can be used to determine accurately

both the optimal times for baculovirus infection and rAAV harvest.

Baculovirus stability

Ideally, commercializing rAAV production requires a robust, reliable, economical and current good manufacturing practice (cGMP) compliant process. The poor stability of BEVs characterized by the loss of recombinant gene products after multiple passages of recombinant baculovirus^{46–48} compromises the efforts for large-scale rAAV production. As a means to partially overcome this deficiency, strategy of using a separate BEV for each of the AAV Rep genes was described as a potential solution to the instability problem.⁴⁷ Indeed, the Rep BEVs were stable over multiple passages, yet, now each rAAV producer cell required simultaneous infection with four different baculoviruses; this is not practical and therefore other strategies were sought.

Using an internal promoter, wild-type AAV expresses Rep 52 from the Rep 78 open reading. Hermens *et al.*⁴⁹ developed a strategy to express Rep 78 and Rep 52 from a single ORF. Rather than using two promoters, this new approach substitutes a non-methionine codon for the initiation codon of rep 78. Translation inefficiently initiates at the rep 78 non-AUG triplet, and presumably ribosome scanning then encounters the rep 52 AUG

initiation codon initiating translation of the smaller Rep protein. The single ORF eliminates the gene duplication that resulted from separate rep ORFs.³⁷

Determining the maximum amplification capability of each BEV may be used to extrapolate the maximum volume of insect cell culture, and consequently the yield of rAAV. Therefore, the stabilities of the three BEVs were determined over the course of six expansion passages.¹ After each passage, BEVs were titered with soft agar overlay and 24 plaques were randomly selected and analyzed for expression of recombinant proteins. Analyses of the passage-amplified BEVs indicated that the VP expression was the least affected by serial passage. In passage 4, 23 out of 24 plaque-isolated BEVs retained GFP expression decreasing to 20 out of 24 in passage 5. However, in passage 6, no further decline was observed.

The Bac-Rep was the least stable of the three BEVs, declining precipitously after passage 4. Expression of Rep 78 and Rep 52 has cytotoxic effects when expressed in mammalian cells and this is most likely true for invertebrate cells as well⁵⁰ or heterologous viruses^{51–53} including baculovirus. However, since the titers of the low passage Bac-Rep are not substantially lower than other BEVs, it is likely that the instability is due to the organization of the Rep transcription cassettes in the Bac-Rep, which is essentially an extended inverted repeat.

Minimum MOIs for rAAV production

Using the minimum MOI required to obtain high yields of rAAV conserves baculovirus stocks and reduces the generation of defective interfering particles. Originally, the rAAV production system analyzed MOI range of 1–9 and determined that an MOI of 3 was optimal, that is higher MOIs might produce more rAAV, but the increased yield was not linearly proportional to the increased baculovirus.^{37,54,55} Subsequently, MOIs ranging from 0.01 to 3 were analyzed, and again, the MOI=3 resulted in the highest rAAV yield; however, at the lower MOIs, for example 0.03, the rAAV yield was only 3 × less than the highest yield. The biological activity of the vectors produced at each MOI was similar. Thus, using an MOI of 0.03 (100 × less baculovirus than MOI=3) suffered only a 3 × reduction in rAAV production.¹

Cell density at TOI

Infecting cells in early log phase Sf9 cells, approximately 2×10^6 cells per ml, provides sufficient time for cell growth^{42,44} prior to synchronous infection arrests cells at the S phase-to-G2/M phase transition.⁵⁶ Late log phase cell cultures accumulate noxious metabolites and deplete nutrient pools thereby limiting the recombinant protein production. According to the Poisson distribution, at MOI of ≥ 3 , the cells are >95% infected and growth is arrested. However, at the lower MOIs analyzed, for example ≤ 1 PFU per cell, only a small percentage of the cells are infected with the input baculovirus and the majority of the cells to divide until the virus released from the primary infected cells overtakes the culture.

Using the previously determined optimum MOI=0.3, a range of initial cell densities was evaluated for rAAV production. The best results were obtained with an initial

cell density of 1×10^6 cells per ml and obtaining the maximum yield of rAAV of $\sim 2 \times 10^{13}$ tu l⁻¹ after 72 h post-infection.¹

Production of rAAV in a stirred-tank bioreactor

Since the initial conditions of MOI and TOI were established in small volume shake-flask cultures, the optimal conditions were tested in a 10 l stirred-tank bioreactor using an MOI=0.03 and TOI of 1×10^6 cells per ml. Cells were harvested and processed as described previously⁵⁷ and following isopycnic density centrifugation, fractions were analyzed for vg and tu. Vector particles were found in gradient fractions with densities corresponding to filled capsids, 1.41 g cm⁻³. Extrapolating from the gradient fractions, the production of $>2 \times 10^{14}$ vg l⁻¹ and $>2 \times 10^{15}$ vg for the entire 10 l culture was produced.

Using the optimized MOI (0.03) for rAAV production, it is informative to calculate the maximum amount of cells and therefore the theoretical number of rAAV that may be produced from 1 ml of passage 1 BEVs (10^8 PFU). The baculovirus stability analyses demonstrated that each of the three BEVs used to produce rAAV are stable through passage 4. Large-scale production of rAAV may require larger baculovirus stocks than those obtained by four amplification passages. Each of the three passage rounds result in 200-fold baculovirus amplification, therefore, the baculovirus yields 4×10^{12} and 8×10^{14} PFU are passages 3 and 4, respectively. Calculating the rAAV yield based on 5×10^4 particles per cell obtained at the TOI as determined, the theoretical yields of rAAV using different MOIs including MOI=0.03, 1 and 3 are presented (Table 1). These results and calculations suggest that large quantities of rAAV may be produced using BEVs in Sf9 cells using the conditions described above.

At low cell density, the addition of nutrients and removal of noxious metabolites during the process are not necessary. The yield of rAAV obtained per liter of cell culture increased 10 times ($\sim 2 \times 10^{14}$ particles per l) relative to the original protocol. Thus, the combination of low MOI and low initial cell density (0.03 PFU per cell and 1×10^6 cells per ml, respectively) yields a 200-fold conservation of baculovirus stocks without loss of either product yield or quality. Implementing these conditions may lead to the development of rAAV production virtually at any scale.

Bioprocessing

Efficient recovery of rAAV is as important as production-inefficient, low yield recovery negates the large production values obtained in stirred-tank bioreactors. Suitable rAAV recovery and purification processes initially developed for small scale production have to be linearly scalable to minimize the time, expense and effort involved with orders-of-magnitude increases in volume and vector. Figure 4 shows the steps of upstream and downstream processing considered for process optimization. Each step in the process should be cGMP compliant. The techniques that are both scalable and

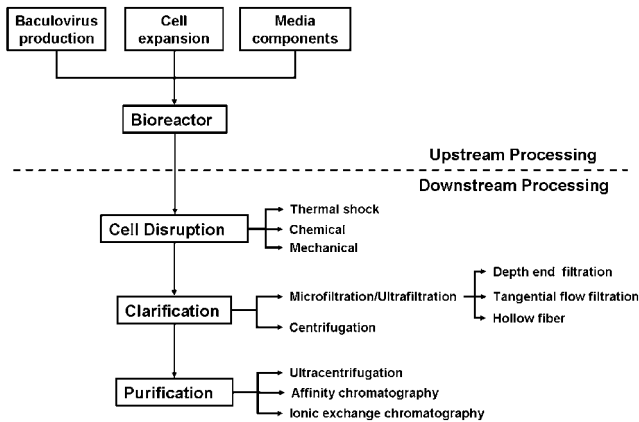


Figure 4 Bioprocessing diagram for the production of rAAV. The sequence of upstream processes involves growing and titrating the BEV stocks and expanding the Sf9 cells with the media components. The bioreactor is then inoculated and the cell culture volume increased by adding media components until the desired volume and cell density are obtained. Synchronously infected cell culture results in arrested cell growth and viability and permissivity are used to determine optimum harvest time. The downstream processing steps involve cell disruption to recover both intra- and extracellular rAAV particles. The cell lysate is then clarified and the rAAV particles then recovered from the clarified cell lysate. BEV, baculovirus expression vector; rAAV, recombinant adeno-associated virus.

cGMP compliant include column chromatography and filtration processes, for example tangential flow filtration. Conventional rAAV processing involves multiple rounds of CsCl isopycnic gradients. Although effective, repeated banding in CsCl gradients is time consuming, recalcitrant to scale-up and relatively inefficient in terms of recovery and importantly, not a good choice for cGMP production. Much recent work has focused on the development of liquid chromatography-based strategies for the purification of rAAV.^{58–66} Cells are disrupted mechanically using either microfluidizers or homogenizers, thermally by repeated freezing and thawing cycles, or chemically using surfactants (Figure 4). Freezing and thawing is not convenient for processing large volumes and variable efficiency of cell disruption may result from thermal transfer rates, heat capacities of the heating and cooling sources, the vessel material, and cell density. Microfluidizers or homogenizers provide a uniform and reproducible process for liberating rAAV from the cell and also reduces the amount of rAAV particles sequestered in cell membranes and other aggregates derived from cell debris. However, commercial-scale microfluidizers are very expensive and constitute another piece of large equipment requiring cGMP validation before, during and after each use. Chemical cell lysis involves the simplest protocol and is the least expensive process. Although originally developed using deoxycholate to disrupt the Sf9 cytoplasmic membrane,³⁷ other surfactants are also available.

Recently, an immunoaffinity-based rAAV purification protocol was developed by Amsterdam Molecular Therapeutics BV (Amsterdam, The Netherlands) and BAC BV (Naarden, The Netherlands) is commercially available (AVB High Performance Sepharose, GE Healthcare, and Bioscience Corp. Piscataway, NJ, USA). The medium consists of a recombinant single-chain antibody conjugated to the agarose-based chromatography resin.

The AVB medium is highly specific, has high binding capacity and is linearly scalable. These characteristics provide a one-column process for producing near homogenous preparations of rAAV from filtered cell lysates. The immunoaffinity capture protocol is likely to supplant other liquid column chromatography methods.

Gene therapy applications

The overwhelming majority of *in vivo* gene therapy publications using rAAV are based on murine models. Although inexpensive and convenient, the mouse studies have had limited predictive value for larger animal models, including primates. For example, the human equivalent of a genetic defect in mice may be readily abrogated with an rAAV infusion, yet with few exceptions, experiments with larger animal models have not been performed. Limited vector production remains a major obstacle for conducting large animal studies. For some diseases, for example Duchenne muscular dystrophy, the relevant mouse model has been studied extensively, for example the dystrophin null mdx mouse;^{67–74} however, the clinically relevant canine golden retriever (Duchenne) muscular dystrophy model has had only several, limited trials with antisense oligonucleotides and none with rAAV. If rAAV is not produced in sufficient quantities for a canine trial, then prospects of producing doses for a human trial are not encouraging. The recently developed scalable production and processing methods allow feasibility and efficacy studies using large animal models of human disease.

Conclusions

In conclusion, the successful exascale manufacture of rAAV is a result of using individual optimized steps during the process. These steps include well-characterized genetic constructs for highly regulated gene expression, production of stable baculovirus stocks, control of production parameters (temperature, dissolved oxygen, agitation speed) monitor of production parameters (pH, permissivity), precise determination of MOI, TOI and harvesting time, and minimizing the number of efficient recovery and purification unit operations.

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