Case Study on an Insect Cell-Produced AAV6 Gene Therapy Vector for Neuromuscular Disease

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INTRODUCTION

Developing AAV gene therapies for systemic delivery presents unique manufacturing challenges, such as producing commercially viable titers, achieving acceptable full capsid ratios and complying with regulatory requirements. These conditions can be met with the use of the inherently safe and scalable insect cell system (Felberbaum). This presentation provides a case study of the production and activity of AVGN7, an AAV6-based gene therapeutic for treating rare neuromuscular diseases (Maricelli, et al.). The upstream manufacturing process employs a newly introduced expression platform comprised of a rhabdovirus-negative clonal Sf9 RV-Free cell line derived from a single cell printing method, as well as a co-developed chemically defined insect cell medium, ESF AdvanCD™. Additional process improvements include formulation of a lysis reagent specific to the baculovirus and insect cell system. Preliminary studies demonstrate the ability to produce high levels of AAV expression using a high cell density, high MOI (multiplicity of infection) methodology followed by increased recovery utilizing the optimized lysis reagent.

METHODS

Cell culture: Suspension cultures of S. *frugiperda* RV-Free Sf9 cells were maintained with constant orbital agitation at 27°C in polycarbonate Erlenmeyer flasks containing chemically defined ESF AdvanCD insect cell culture medium.

Plasmid and recombinant baculovirus construction: Two transfer vectors were designed using pBakPak8 with one harboring the hSmad7 gene sequence flanked by AAV2 ITR sequences (ITR construct) and a second plasmid containing the AAV2 rep genes and the AAV6 capsid (VP1, VP2 and VP3) (RepCap construct). Recombinant baculoviruses were generated by co-transfecting Sf9 RV-Free cells with either the ITR construct or the RepCap construct along with BestBac™, a linearized baculovirus backbone. Recombinant baculovirus stocks were titered using the gp64 staining method previously reported (Mulvania, et al.) to accurately determine the infectious titer and calculate the desired multiplicity of infection (MOI).

rAAV production in RV-Free Sf9 cells and purification by affinity chromatography: RV-Free Sf9 cells were seeded at a density of 3 million per mL in ESF AdvanCD. Culture volumes ranged from 500-2500 mL. Cells were grown overnight to ensure log phase cultures receptive to recombinant baculovirus infection. Cultures were infected at an MOI of 3 for the RepCap and the ITR constructs and incubated for approx. 72 hours. Whole cell cultures were treated with a commercially available lysis buffer (Mammalian Cell Lysis Buffer, Teknova) or a novel reagent developed by Expression Systems that simultaneously lysed the insect cells and baculovirus, treated with DNase to remove non-encapsidated DNA and clarified by depth filtration.

Clarified lysate was applied to an affinity chromatography column consisting of Poros GoPure AAVX resin. Following two washes with increasing concentrations of NaCl, capsids were eluted with 75 mM citric acid, 10 mM $MgCl_2$ directly into a 1 M tris neutralization buffer. Eluate was diluted 1:20 into an AEX equilibration buffer of 20 mM bis-tris propane, 10 mM $MgCl_2$, pH 8. Elution was performed with 20 mM bis-tris propane, 10 mM $MgCl_2$, 250 mM sodium acetate. Eluate was exchanged into PBS containing 2 mM $MgCl_2$ and sterile filtered.

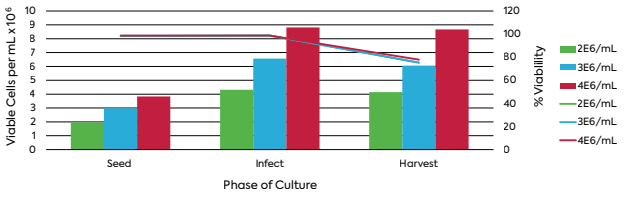
Quantification of rAAV: Nuclease-resistant rAAV genome titers were determined by digital PCR analysis using a Qiagen QIAcuity One Digital PCR System and the hSmad7-specific oligonucleotide pair 5'- CCT TGT CAT GTT CGC TCC TTA G -3', 5'- CTC TGC TTC TCC ACA GTC TTC -3' and probe 5'- CCA GTG CGC TTC TCT TTG TCC GA -3' with FAM fluorophore and Black Hole Quencher.

rAAV capsid titers were determined by Progen AAV6 Capsid ELISA kit specific to assembled capsids and analyzed using a four-parameter logarithmic regression.

RESULTS

To evaluate the most effective cell culture conditions to produce baculovirus-mediated rAAV, a seeding density titration was performed. 50 mL cultures were inoculated with 2, 3 and 4 million viable cells per mL and incubated overnight to ensure cells in optimal logarithmic growth phase and receptiveness to baculovirus infection. Cultures were infected at an MOI of 3 for both the RepCap and ITR recombinant baculoviruses and incubated for 72 hours. As expected, cultures seeded at a higher density contained more cells per L than those at lower density. The character of the cultures was equivalent across all cell densities as shown by equivalent drops in viability at harvest (Figure 1).

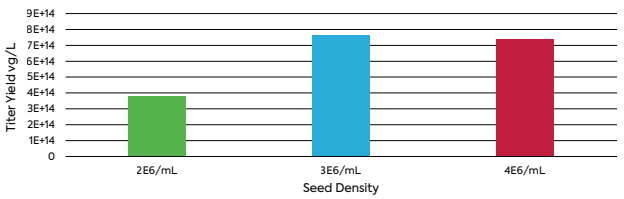
Figure 1: Seed Density Titration for Evaluation of rAAV Productivity



50 mL cultures were seeded at 2E6, 3E6 and 4E6/mL. Viable cell densities are represented by the solid bars across the phases of the expression cell culture with harvest occurring 72 hours post infection. The viability of the three culture conditions is represented by the lines.

Cultures were harvested, cells were separated by centrifugation and lysed to release rAAV particles. Non-encapsidated DNA was digested by treatment with DNase and rAAV capsids are denatured to solubilize genome template for digital PCR. rAAV yields from crude culture lysates increased two-fold from the increase in seed density from 2 to 3 million cells per mL. There was no appreciable increase in overall yield when increasing the seed density to 4 million cells per mL (Figure 2).

Figure 2: rAAV Yield as a Function of RV-Free Seed Density



5 mL samples were collected from each culture at 72 hours post infection, pelleted by centrifugation and lysed in a commercially available buffer. Non-encapsidated DNA was treated with DNase and $MgCl_2$. Viral genome copies were determined by digital PCR.

One process-oriented goal of the case study was to develop a scalable process for manufacturing which would eliminate time intensive centrifugation for recovery of rAAV containing cells. Therefore, it was decided to evaluate whole culture lysis followed by depth filtration as an alternative. Initial attempts with commercial lysis buffers led to turbid harvest material that fouled both filters and chromatography columns. Additionally, a key step in the lysis process, inactivation of infectious baculovirus, was not effectively performed (data not shown). A proprietary lysis reagent was developed consisting of two detergents and salts that simultaneously lysed the insect cells, releasing the rAAV and lysed the associated baculovirus. The lysate is subsequently treated with DNase, yielding a clear solution (Figure 3) that could be effectively depth filtered and applied to a column without causing fouling. Evaluation of rAAV yield was performed on whole culture lysates rather than pelleted cells for the continuation of the project.

Figure 3: Improved Clarity and Filterability of Whole Culture Lysate Using a BEVS-Compatible Formulation

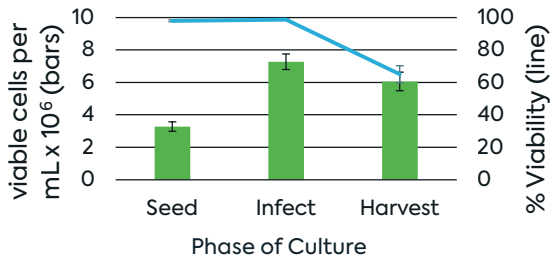
Two 500 mL cultures were used to express rAAV6 by coinfection with RepCap and ITR baculoviruses. 72 hours post infection the cultures were lysed by addition of 50 mL of 10x lysis reagent for 1 hour at standard culture incubation conditions (27°C at 135 rpm). After 1 hour, 20 unit/mL of DENARASE were added to cultures and incubated for an additional hour.

RESULTS

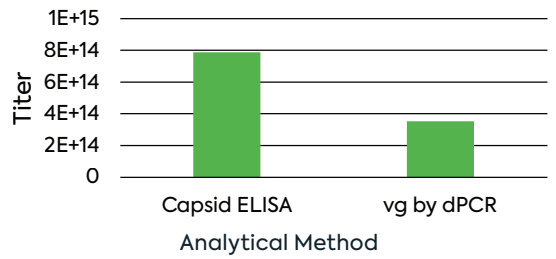
Consistent, predictable performance of cell culture expression is a key factor in a successful manufacturing platform. Growth of RV-Free Sf9 cells in ESF AdvanCD is reproducible and consistently achieves high density cultures in logarithmic growth (data not shown). Equally important is the use of a consistent and accurate method for determining the titer of the baculovirus stock used to infect the cells. Reproducible and scalable performance was evaluated at 200 mL and 5 L scale cultures seeding at a density of 3 million viable cells per mL and infecting the following day with the two recombinant baculoviruses at an MOI of 3 for each. Indeed, we found that the culture performance across nine expression runs ranging across 200-, 500-, 2500- and 5000-mL cultures was consistent (Figure 4A). Additionally, rAAV yield from the crude lysates of a subset of the samples (some cultures weren't analyzed) reliably produced titers greater than 10^{14} viral genomes per L measured by either capsid ELISA or dPCR (Figure 4B).

Figure 4: Reproducibility of Expression Across Varying Culture Volumes

A. AAV6 Expression Culture



B. AAV6 Yield by ELISA and dPCR

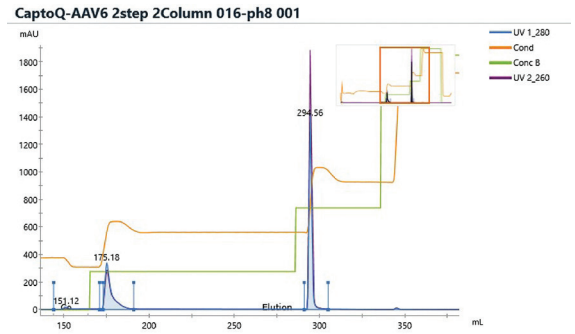


Cell counts (A) are averaged from 9 different expression cultures in volumes ranging from 200-, 500-, 2500- and 5000-mL. Cultures were seeded at $3x10^6$ viable cells per mL, incubated overnight and then infected at an MOI of 3 for both the RepCap and ITR containing baculoviruses. Harvest occurred at 72 hours post infection. Cultures were lysed with a proprietary lysis reagent and rAAV yield determined for a subset of cultures (B) by capsid ELISA and dPCR.

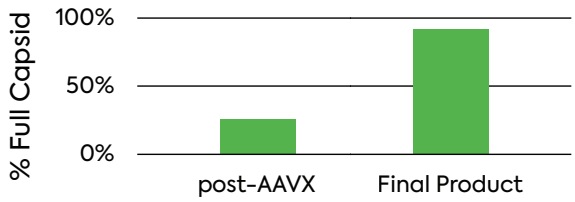
Downstream processes for purification include conventional affinity chromatography followed by enrichment of full capsids by anion exchange. Nuclease treated clarified lysates were loaded onto a chromatography column packed with Poros GoPure AAVX resin. Following two washes with Tris buffered saline with increasing NaCl, the bound material was eluted with citric acid at pH 3 into 1 M tris for immediate neutralization. The affinity column eluate was diluted 1:20 in 20 mM bis-tris propane (BTP) 10 mM $MgCl_2$ pH 8 to lower the conductivity to less than 5 µS/cm for ion exchange and enrichment of full capsids. Elution was performed with increasing sodium acetate concentrations, eluting empty capsids in the first peak and full capsid in the second peak (Figure 5A). The Capto Q AEX binding and elution conditions were modified from standard AAV enrichment conditions to reduce the binding affinity of AAV6. Efficacy of enrichment was determined by calculating the ratio of viral genomes to intact capsids in following AAVX purification and AEX enrichment followed by buffer exchange (Figure 5B). The final product contained greater than 90% full capsids, enriching from 26% full capsids following affinity purification.

Figure 5: Enrichment of Full Capsids Using AEX Chromatography

A.



B.



AEX enrichment using Capto Q column showing a fraction of mostly empty capsids in the first peak with a A260/A280 ratio <0.8. Enriched capsids are eluted in the second peak with a A260/A280 ratio >1.25. Binding and elution conditions were optimized to prevent tight binding to AEX resin and prevent damage to capsids. Full capsid proportion was calculated by dividing dPCR titer by the capsid ELISA titer. Percent full capsid is shown following affinity purification using AAVX resin and the final product enriched by anion exchange and formulated in final buffer.

DISCUSSION/CONCLUSION

Here we have presented the application of a novel insect expression platform for the generation of high titers of an AAV6 gene therapy vector for treatment of neuromuscular disease. The combination of an upstream process capitalizing on high cell densities with enrichment procedures specific to the AAV6 serotype results in a high yield of vector exceptionally enriched for full capsids. Utilization of the Sf9 RV-Free cell line allowed for the elimination of a known adventitious agent that is of concern in the production of biologics, particularly in the case of rAAV. RV-Free cells were co-developed in a novel chemically defined insect cell culture medium that allows growth and expression to occur at densities higher than are typically seen in insect cell culture, leading to a greater yield per liter. Isolating the AAV capsids requires lysing the insect cells using detergents that also inactivate the recombinant baculovirus used to produce the product. Commercially available lysis reagents are rarely optimized for the simultaneous performance of both activities, so a novel lysis reagent was developed that allowed for more efficient lysis and better recovery of the product by reducing fouling of filters and chromatography columns. Following the lysis with DNase treatment removed the non-encapsidated DNA, further clarifying the lysate. rAAV titers following this lysis treatment were reproducibly greater than 10^{14} particle titer as measured by AAV6 capsid ELISA. Affinity chromatography was performed using standard conditions for the Poros AAVX resin with a capsid recovery of approximately 60% of the starting material. Enrichment of capsids containing the genetic payload was performed using anion exchange with binding conditions optimized for enrichment of full capsids while keeping the binding affinity and residence time of the AAV6 relatively low to minimize loss of the encapsidated DNA. This lead to a highly purified population containing >90% full capsid, enriching from 26% full capsids following affinity purification.

References

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