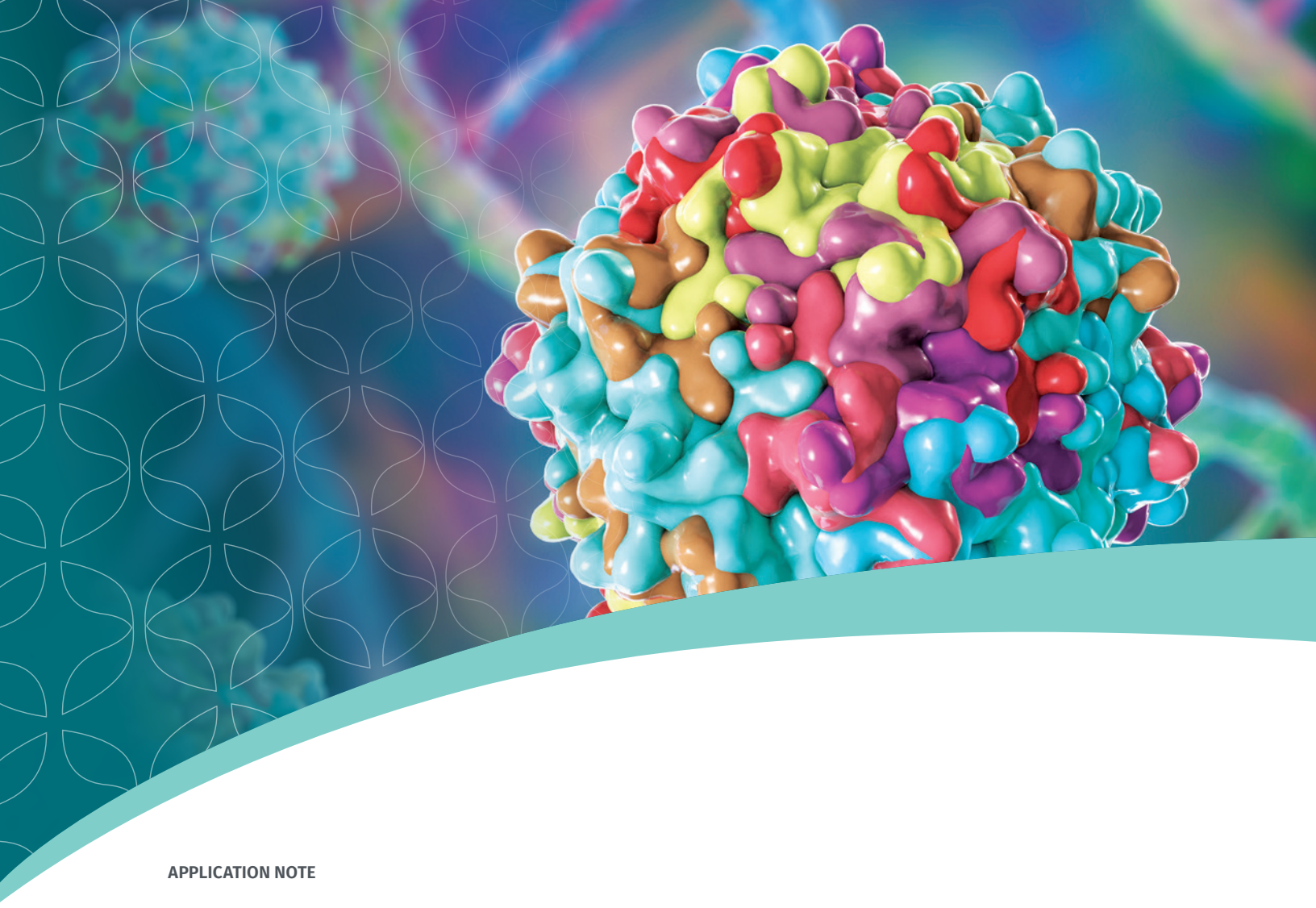

APPLICATION NOTE

Cultivation of Sf9 insect cells in Thomson Optimum Growth® flasks using ESF AdvanCD™ cell culture medium for production of SEAP and AAV

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Summary

This application note describes the cultivation of Sf9 suspension cells using ESF AdvanCD™ chemically defined cell culture media together with Thomson Optimum Growth® flasks. Thomson Optimum Growth flasks sized at 125 mL, 250 mL, 500 mL and 5 L were used to demonstrate equivalence between scales. Using an animal-origin-free, chemically defined medium that supports robust cell growth, a high-density infection strategy was employed. Expression of secreted alkaline phosphatase (SEAP) protein or AAV2 was induced by the baculovirus expression vector system (BEVS).

Introduction

Expression Systems provides high-performance insect cell culture media, namely ESF 921 and ESF AF, to the global bioprocessing market. Here we highlight the next generation ESF AdvanCD, a chemically defined insect cell culture medium free of any animal-origin components.

The present study focuses on the cultivation of Sf9 insect cells and utilizes BEVS to produce SEAP and AAV2. Expression Systems utilized 125 mL, 250 mL, 500 mL and 5 L Thomson Optimum Growth flasks as the single-use disposable bioreactors to demonstrate optimized cell cultivation and scalability.

The baffled Thomson Optimum Growth flasks are designed for high aeration and low shear to maintain cell viability. Increased aeration allows for a range of working volumes from 20-60% of the vessel volume. The 0.2 µm vented cap simultaneously maintains sterility and allows for ample gas exchange. Single-use sterile shake flasks are a cost-effective culture vessel compared to small-scale glass bioreactors and reduce the risk of contamination and detergent carryover from reusable glass flasks.

Culture vessels were inoculated at a density of $2.5x10^6$/mL Sf9 cells in log phase of growth in

The combination of Thomson Optimum Growth flasks (pictured above) and Expression Systems ESF AdvanCD cell culture medium offer an effective solution for scaling up protein production utilizing baculovirus and insect cell expression platforms.

ESF AdvanCD chemically defined medium. After expanding the cells for 24 hours using the ESF AdvanCD medium, the cells were infected by the baculovirus at a multiplicity of infection (MOI) of 3. Shake flask cultures were incubated for up to 4 days, with peak SEAP expression occurring 72 hours post infection and peak AAV2 production occurring 48 hours post infection.

Materials and Methods

Overview of procedure setup:

- **Day minus 4:** Inoculum production with Sf9 suspension cells in shake flasks.
- **Day minus 1**: Seed culture vessels with Sf9 cells at a density of 2.5x10^6 cells/mL in ESF AdvanCD.
- **Day 0:** Sample cultures to determine viable cell density and infect at an MOI of 3.
- **Day 1:** Sampling.
- **Day 2:** Sampling, analytics, and harvesting.
- **Day 3:** Sampling, analytics, and harvesting.

Media

ESF AdvanCD, a chemically defined medium without components of animal origin, was used as supplied. Designed in parallel with clonally derived Sf9 cells, ESF AdvanCD supports growth to high cell densities and high recombinant protein yields and protein expression when used with BEVS.

Sf9 Cell Inoculum

Expression cultures were seeded from a stock culture that was grown using conditions to maximize healthy Sf9 cells in log phase of growth. The Sf9 cell inoculum (stock culture) was produced in a single use shake flask with a volume of 500 mL. The stock cells were inoculated at a cell density of about 0.5E6 cells/ml and cultured until they had reached a cell density of about 7E6 cells/mL. The flasks were shaken at a rate of 135 rpm and amplitude of 25 mm. Before seeding, the cell density was determined to estimate the required volume of the inoculum and expression cultures were seeded in ESF AdvanCD at a cell density of 2.5x10^6 cells/mL at the respective volumes.

SEAP Expression Scale Up Experiment

SEAP expression cultures were performed in 125 mL and 500 mL Optimum Growth flasks with working volumes of 50 mL and 200 mL, respectively. Flasks were seeded as described above and allowed to grow overnight before infection at an MOI of 3 with the recombinant baculovirus (rBV) containing the SEAP gene.

SEAP Culture Conditions

Culture volume	50 mL and 200 mL
Flask size	125 mL and 500 mL
Agitation speed	135 rpm
Temperature	27° C
Seeding density	2.5x10^6 cells/mL
Infection density	~6x10^6 cells/mL
Cultivation time	4 days

Samples were taken once a day by pipette. In-process testing was performed using a Vi-CELL BLU cell viability analyzer (Beckman) to determine total and viable cell density, viability, and the cell diameter.

To determine levels of SEAP expression, 5 mL samples of the suspension cell cultures were transferred to conicals, and cells were removed

from the supernatant by centrifugation at 5,000 x g for 2 min. The clarified supernatant was transferred to new tubes and approximately 2 mL were stored at 4°C until the photometric analysis.

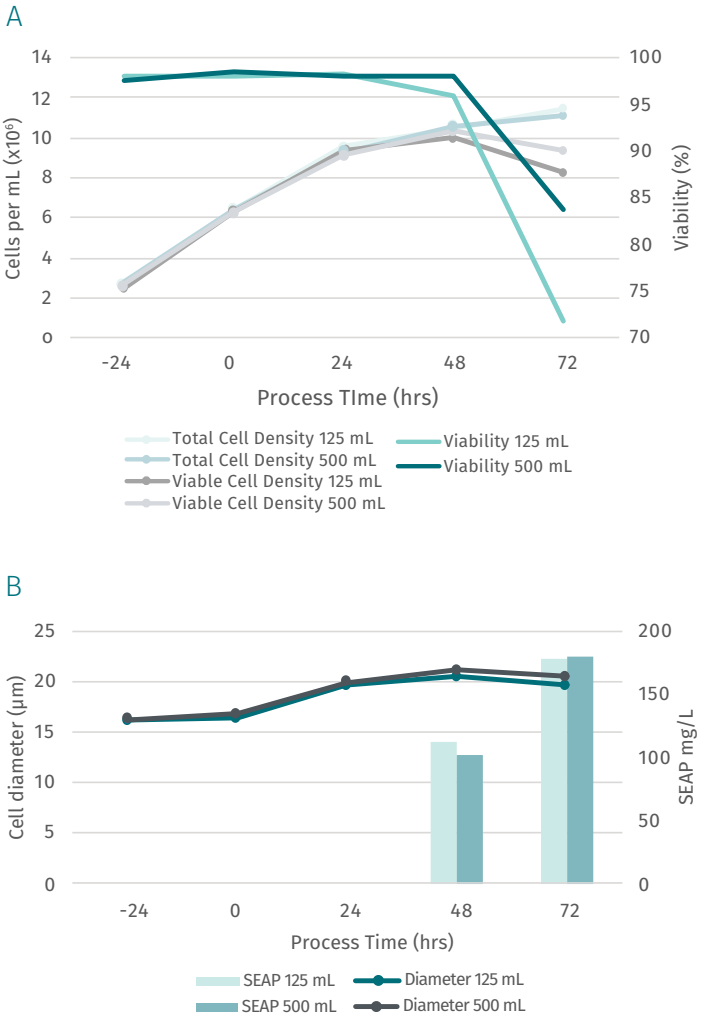
SEAP Quantitation

SEAP expression supernatants were serially diluted 1:400 in a SEAP assay buffer. A purified SEAP protein standard was serially diluted 1:2 a total of 7 times in the SEAP assay buffer along SEAP assay buffer blank to generate a standard curve. 10 µL of diluted standards and samples were added to a round bottom assay plate in duplicate and diluted with 10 µL of RODI water before being incubated at 65°C for 30 min. to deactivate endogenous alkaline phosphate proteins. Samples were briefly centrifuged to cool and then 100 µL of 1-Step PNPP substrate was added to each well. Samples were incubated at 25°C for 30 minutes before being read for absorbance and 405 nm wavelength. Standards were used to generate a 3rd degree polynomial standard curve which was used to quantitate the samples.

SEAP Results

Figure 1 depicts the profiles of total cell density, culture viability, and SEAP activity over a cultivation time of 4 days. Starting from the initial cell density of $2.5x10^6$ cells/mL, the cells grew to an average density of $5.95x10^6$ cells/mL in the SEAP experiment, corresponding to a doubling time of 19.2 hours. Growth rate was not calculated following infection as the baculovirus prevents cells from undergoing mitosis. Cultures were infected using a multiplicity of infection (MOI) of 3.

Figure 1. Data from the Sf9 SEAP expression culture in 50 mL and 200 mL working volumes. (A) Total and viable cell density and viability. (B) SEAP expression and average cell diameter.



SEAP expression levels were assessed at 48 and 72 hours post infection. As expected, SEAP levels were higher at 72 hours post infection and the cells started to die as a result of the lytic nature of the baculovirus infection. The viability in the 50 mL culture was 71.8% and the 200 mL culture was 83.7% when study was terminated.

SEAP expression was assessed following robust infection of the culture as determined by increased average cell diameter (Fig. 1B), which is an indication of the successful infection, as well as staining of the cell surface for the baculovirus envelope protein, gp64 (data not shown). Due to the virus infection, the cells were enlarged about one day after the virus infection, so that the average cell diameter increased from about 16.6 µm to 19.8 µm.

SEAP expression levels were determined to be equivalent between the two different culture volumes. At 48 hours post infection the yield of the 50 mL culture was 112.5 mg/L, and the 200 mL culture was 101.3 mg/L, at 72 hours post infection the yields were 178.3 and 179.8 mg/L respectively. These approximately equivalent results between culture volumes reiterated prior comparative experiments (data not shown).

AAV2 Expression Scale Up Experiment

AAV2 expression cultures were performed in duplicate in 125 mL, 250 mL, 500 mL and 5 L Optimum Growth flasks with working volumes of 50 mL, 100 mL, 200 mL and 2 L, respectively. Flasks were seeded as described above and allowed to grow overnight before infection at an MOI of 3 each with a RepCap rBV and an ITR-GFP rBV.

AAV2 Culture Conditions

Culture volume	50 mL, 100 mL, 200 mL and 2 L
Flask size	125 mL, 250 mL, 500 mL and 5 L
Agitation speed	135 rpm
Temperature	27° C
Seeding density	2.5x10^6 cells/mL
Infection density	~3.2x10^6 cells/mL
Cultivation time	3 days

Samples were taken once a day by pipette. In-process testing was performed using a Vi-CELL BLU cell viability analyzer to determine total and viable cell density, viability, and the cell diameter.

To determine AAV2 yields, 5 mL samples were taken from each culture and the cells pelleted by centrifugation as above, and the pellet was frozen at -80°C until analyzed.

AAV Quantitation

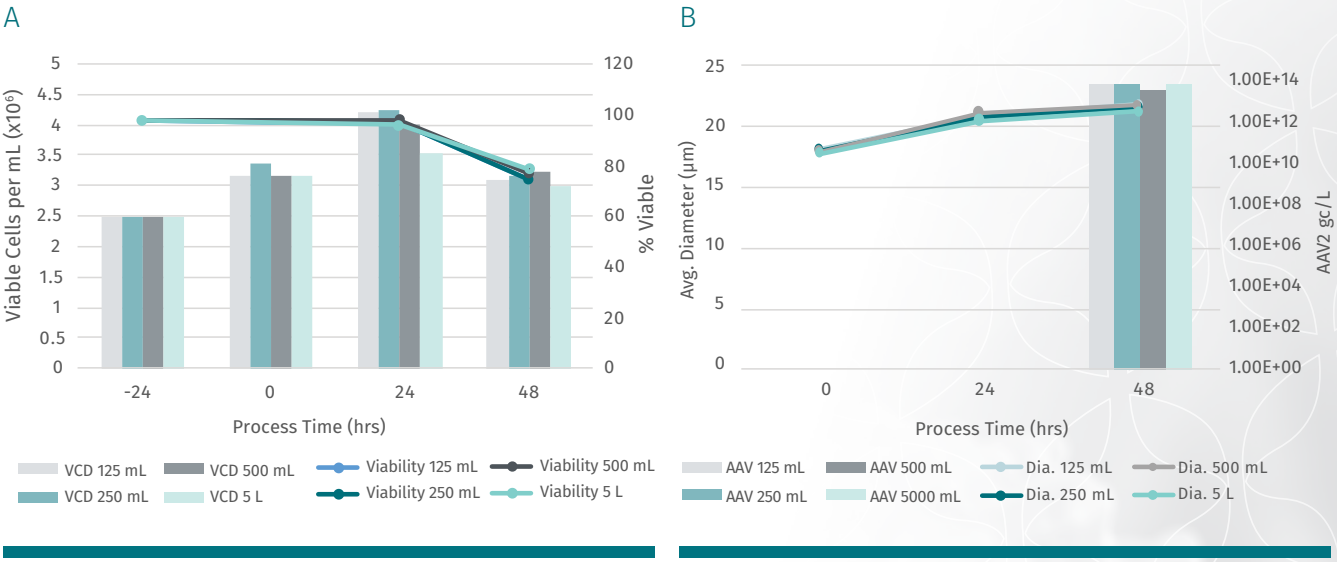
AAV expression pellets were thawed on ice and resuspended in 1 mL PBS. The cells were lysed by three freeze-thaw cycles in an isopropanol dry ice bath and a 37ºC water bath. The lysate was transferred to 1.5 mL microcentrifuge tubes and the cell debris was pelleted by centrifugation at 15000 rpm for 10 min. at 4ºC. Clarified lysate transferred to a new microcentrifuge tube. The lysates were treated with Benzonase* and incubated at 37ºC for 30 min. followed by addition of EDTA to a final concentration of 100 mM to stop the reaction. Benzonase-treated samples were then incubated at 95ºC for 10 min. A fraction of the lysate was then treated with Proteinase K and incubated at 55ºC for 30 min. followed by incubation at 95ºC for 15 min. to inactivate the Proteinase K. After cooling on ice, the lysate was used as template in a SYBR Green qPCR reaction. Two qPCR reactions were prepared for each sample. Reaction #1 included primers that bind within the EGFP gene. Reaction

As was done for SEAP, cultures were seeded at 2.5x10^6 viable cells per mL and allowed to grow overnight.

#2 (intended to quantitate ITR flanked EGFP expression cassette that has not been excised from the baculovirus genome) included a primer that binds to the ITR and a primer that binds to a sequence within the recombinant baculovirus genome. The qPCR reactions were cycled following standard SYBR Green qPCR methods and copy number calculated. The copy number determined from reaction #2 was subtracted from that of reaction #1 to arrive at the copy number of encapsidated viral genomes per mL of lysate and that value was used to calculate the viral genomes per liter of culture.

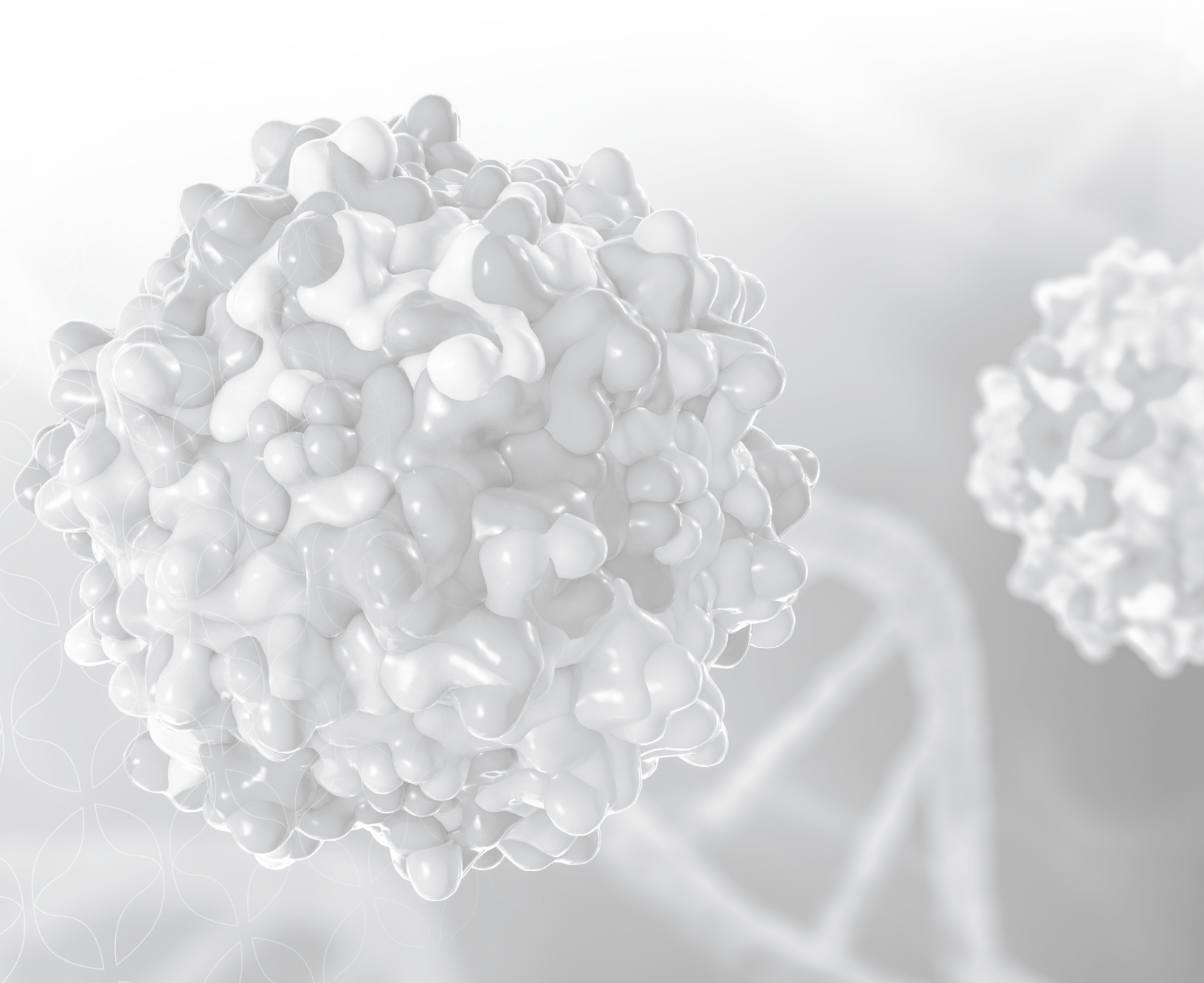
Cultures examining AAV2 expression were performed in duplicate at 50 mL, 100 mL, 200 mL and 2 L working volumes. As was done for SEAP, cultures were seeded at $2.5x10^6$ viable cells per mL and allowed to grow overnight. Cultures were infected with two recombinant baculoviruses, one expressing RepCap and the other ITR-GFP, both at an MOI of 3. AAV2 expression levels were assessed at 48 hours post infection. **As shown in Figure 2,** robust virus infection was demonstrated by increased cell diameter at 24 hours post infection. The increased diameter corresponded to AAV2 production levels of approximately $1x10^{14}$ genome copies (gc) per L at 48 hours post infection for culture volumes of 50 mL, 100 mL and 2 L. The 200 mL condition demonstrated lower yields averaging $5x10^{13}$ gc/L for reasons unknown. Importantly, culture parameter data and yields were approximately equivalent across the four culture volumes.

Figure 2. Data from the Sf9 AAV expression culture in 50 mL, 100 mL, 200 mL and 2 L working volumes (flask size 125 mL, 250 mL, 500 mL, and 5 L). (A) Viable cell density (VCD) and viability. (B) AAV2 expression and average cell diameter.



Conclusion

The combination of Expression Systems ESF AdvanCD cell culture medium and Thomson Optimum Growth flasks offer an effective solution for scaling up protein production utilizing baculovirus and insect cell expression platform. Consistent cell growth and expression levels were observed between various flask sizes and culture volumes, providing confidence in a robust and scalable platform.



Find out more at expressionsystems.com and htslabs.com

Expression Systems		Thomson	
ESF AdvanCD™	54-018	Optimum Growth® 125 mL Flask	PN# 931110
Virus Stabilization Additive	95-010	Optimum Growth® 250 mL Flask	PN# 931111
Transfection Medium	95-020	Optimum Growth® 500 mL Flask	PN# 931112
Sf9 Cells in ESF AdvanCD	94-030	Optimum Growth® 1.6 L Flask	PN# 931113
TniPRO™ Cells in ESF AdvanCD	94-031	Optimum Growth® 2.8 L Flask	PN# 931114
BestBac 2.0	91-002	Optimum Growth® 5 L Flask	PN# 931116

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