Scalable production and purification of adeno-associated virus (AAV) vector using the ExpiSf Expression System

Introduction

The Gibco™ ExpiSf™ Expression System is a complete, chemically defined baculovirus–insect cell expression system that delivers superior protein yields and consistent performance run after run using a fast, streamlined workflow. The system includes the first chemically defined insect cell growth medium for superior run-to-run reproducibility. Here we demonstrate the versatility of the ExpiSf system beyond its use for expression of recombinant proteins. A dual-infection method was adopted to evaluate the production of two different recombinant adeno-associated virus (rAAV) serotypes, AAV2 and AAV6, in the ExpiSf system [1]. For this purpose, Gibco™ ExpiSf9™ cells were infected with two baculoviruses: RepCapX, where X denotes serotype 2 or serotype 6, and inverted terminal repeat (ITR)–green fluorescent protein (GFP). The RepCap baculovirus supplies the necessary viral genes for genome replication, genome packaging, and capsid assembly. The ITR–GFP baculovirus contains the gene of interest (encoding GFP) flanked by AAV ITR sequences. Production of rAAV particles was scalable from shake flask to bioreactor scale, yielding high viral genome titers (vg/mL) of infectious rAAV, and high rAAV yields were obtained following purification on a Thermo Scientific™ POROS™ GoPure™ column pre-packed with POROS™ CaptureSelect™ AAVX affinity resin.

Materials

- Gibco™ ExpiSf9™ Cells (Cat. No. A35243)
- Gibco™ ExpiSf™ CD Medium (Cat. No. A3767802)
- Gibco™ ExpiFectamine™ Sf Transfection Reagent (Cat. No. A38915)
- Gibco™ ExpiSf™ Protein Production Kit (Cat. No. A3767806)
- Invitrogen™ DNase I buffer (10X) (Cat. No. AM8170G)
- Invitrogen™ DNase I (Cat. No. 18047-019)
- Invitrogen™ Proteinase K (Cat. No. AM2546)
- Thermo Scientific™ HyPerforma™ Glass Bioreactor, 120 V, heat only (Cat. No. F100-2680-002)
- Thermo Scientific™ POROS™ GoPure™ AAVX Pre-packed Column, 0.5 x 5 cm, 1 mL (Cat. No. A36652)
- Invitrogen™ EVOS™ microscope

Methods

ExpiSf9 cell maintenance and P0 baculovirus production

ExpiSf9 cell growth and maintenance, and baculovirus production and titering were performed as described in the ExpiSf Expression System user guide (Pub. No. MAN0017532). Using a dual-infection method, two P0 (non-amplified) baculovirus stocks were produced by transfecting ExpiSf9 cells with a bacmid containing the gene encoding RepCap2 or RepCap6, and a bacmid containing the gene encoding GFP (the gene of interest) flanked by AAV ITR sequences (ITR–GFP). P0 baculoviruses were collected 3–4 days after transfection.

Small-scale rAAV production (125 mL and 250 mL shake flasks)

One day prior to infection (day –1), ExpiSf9 cells were seeded at a density of 3 x 10^6 viable cells/mL and immediately treated with Gibco™ ExpiSf™ Enhancer (included in the ExpiSf Protein Production Kit) (100 µL for 125 mL shake flask and 200 µL for 250 mL shake flask). The next day, 18–24 hours after the enhancer treatment, the viable cell density was determined using an automated cell counter, and the cultures were infected with two baculoviruses (one containing AAV RepCap2 or AAV RepCap6, and one containing ITR–GFP) using a multiplicity of infection (MOI) of 2 for each baculovirus stock. Samples were collected at 24-hour intervals post-infection, and crude lysates were prepared using a lysis buffer based on Triton™ X-100 detergent, following a standard procedure [1].

Large-scale rAAV production (3 L HyPerforma glass stirred-tank bioreactor)

To evaluate the scalability of rAAV production in the ExpiSf system, rAAV production was scaled up into a 3 L HyPerforma glass stirred-tank bioreactor. The bioreactor setpoints used were: dissolved oxygen (DO) at 40% controlled by air in the headspace and O_2 on demand through a macro sparge; temperature set to 27°C; dual impeller set to 180 rpm for a power input per volume of 9.5 W/m^3. Four days prior to infection, ExpiSf9 cells were seeded into the bioreactor at a density of 1 x 10^6 viable cells/mL at a ExpiSf™ CD Medium volume of 1 L. One day prior to infection, cells reached a density of 6–6.5 x 10^6 cells/mL and were diluted to 3 x 10^6 cells/mL with fresh ExpiSf CD Medium to a final culture volume of 2 L. At the same time, 8 mL of ExpiSf Enhancer was added to the 2 L culture. The next day, the culture was co-infected with two baculoviruses (one containing AAV RepCap2 or AAV RepCap6, and one containing ITR–GFP). An MOI of 2 was used for each baculovirus stock.

rAAV genome titering measurement

For determination of rAAV viral genome titers, quantitative PCR (qPCR) was adopted using GFP-specific primers and a GFP-specific probe, which target the gene of interest flanked by the ITR sequences of the packaged viral DNA. Prior to qPCR analysis, crude lysate samples were subjected to DNase I treatment to remove non-encapsulated AAV DNA and baculovirus DNA. In PCR tubes, 2 µL of crude lysates were incubated at 37°C for 60 minutes in 1X DNase I buffer containing 100 U DNase I (20 µL total reaction volume). Samples were then heated at 85°C for 20 minutes to inactivate DNase I and stop the reaction. Next, to release the packaged viral DNA, 20 µL of 2X Proteinase K buffer containing 20 µg of Proteinase K was added to the PCR tubes and incubated at 60°C for 1 hour, followed by a 10 minute incubation at 95°C to inactivate the Proteinase K. qPCR was performed on an Applied Biosystems™ QuantStudio™ 7 Flex Real-Time PCR System, and rAAV titers were determined based on a standard curve of linearized GFP-containing plasmid.

rAAV infectivity assay

HT1080 cells were seeded in 24-well plates at a density of 1 x 10^5 cells/well and infected with rAAV2–GFP or rAAV6–GFP crude lysate 24 hours after cell seeding. Note: Crude lysates were heated at 60°C for 30 minutes prior to infection to inactivate baculoviruses. Cells were observed 48 hours post-infection for infectivity. Light and fluorescence images were obtained on an EVOS microscope.

rAAV6–GFP clarification

Clarified rAAV6–GFP lysates were prepared as previously described [1].

rAAV6–GFP purification

Prior to purification, 400 mL of clarified rAAV6 lysate (from a 1.2 L culture volume) was adjusted to pH 7.2 using 1.5 M Tris, pH 11. In parallel, a POROS GoPure 1 mL column (0.5 x 5 cm), pre-packed with POROS CaptureSelect AAVX resin, was equilibrated with 10 column volumes (CV) of equilibration buffer. Next, the 400 mL of pH-adjusted clarified rAAV6 lysate was loaded onto the 1 mL AAVX column at a flow rate of 1 mL/min (corresponding to 1 minute of residence time). After the clarified lysate was loaded, the column was washed with 5 CV of equilibration buffer followed by three different intermediate washes, each 5 CV. rAAV6–GFP was eluted using a glycine buffer at pH 2.5.

Purification buffers used:

- Equilibration buffer: 100 mM Tris, 150 mM NaCl, 0.01% Pluronic™ F-68 surfactant, pH 7.2
- Wash buffer 1
- Wash buffer 2
- Wash buffer 3
- Elution buffer: 75 mM glycine, 50 mM NaCl, 0.5 M arginine, 0.01% Pluronic F-68 surfactant, pH 2.5

Results

A dual-infection method was adopted to evaluate the production of two different rAAV serotypes, AAV2 and AAV6, in the ExpiSf system. Addition of ExpiSf Enhancer increased AAV genome titers by 3.8-fold for rAAV2 and 3-fold for rAAV6 compared to control (no enhancer), with the highest titers obtained 3 days post-infection (Figure 1A). For comparison, rAAV2 and rAAV6 were produced in Sf9 cells grown in Gibco™ Sf-900™ II SFM following a previously reported protocol (Figure 1B) [1]. Equivalent AAV genome titers for both serotypes were obtained in the ExpiSf system and Sf9 cells in Sf-900 II SFM.

rAAV2 and rAAV6 were also produced in 250 mL shake flasks and 3 L bioreactors. Figures 2A and 2B show the growth kinetics of ExpiSf9 cells before and after infection for rAAV2 and rAAV6, respectively. Similar viable cell densities and viabilities were observed for shake flask and bioreactor scales. The scale comparison shows that ExpiSf production of rAAV2 and rAAV6 at bioreactor scale was 66% and 88% of the ExpiSf shake flask viral titers, respectively (Figure 2C). Importantly, rAAV2 and rAAV6 particles produced at both scales were infectious, as determined by an infectivity assay using crude lysates in HT1080 cells (Figure 2D).

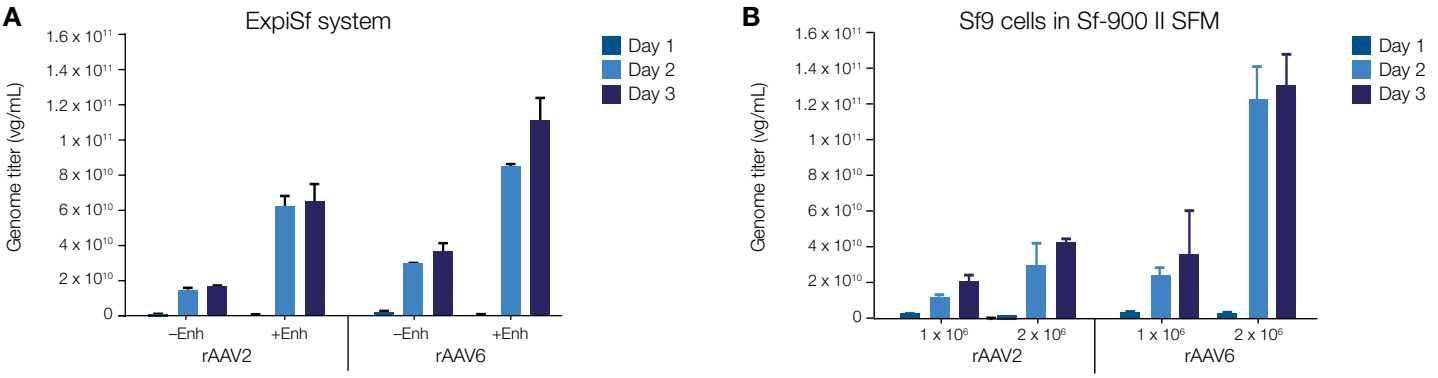

Figure 1. rAAV production in Sf9 cells. (A) AAV genome titers of crude lysates from ExpiSf9 cells cultured in ExpiSf CD medium. As controls, rAAV production runs were performed in the absence of enhancer. **(B)** rAAV genome titers of crude lysates from Sf9 cells seeded at two different densities (1 x 10^6 and 2 x 10^6 cells/mL) in Sf-900 II medium. Production runs in A and B were performed at 25 mL scale in 125 mL shake flasks. "–Enh" indicates no addition of ExpiSf Enhancer; "+Enh" indicates addition of ExpiSf Enhancer 18–24 hours prior to cell infection.

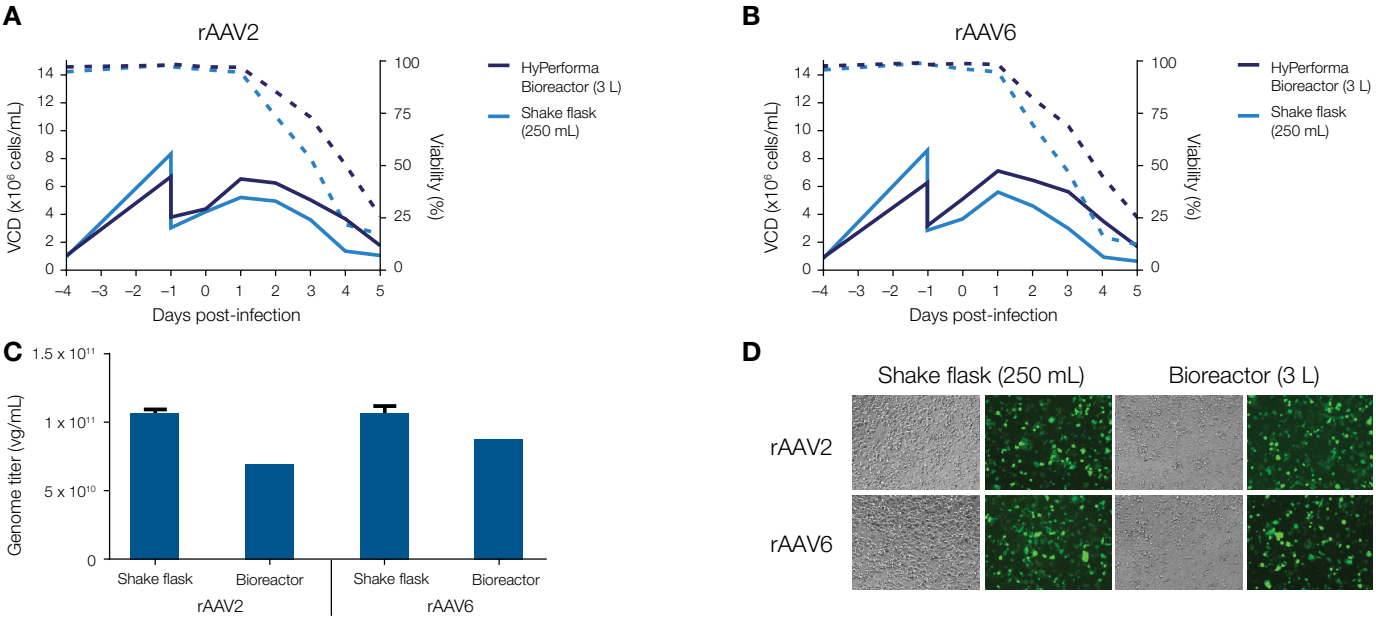

Figure 2. Scalable production of infectious rAAV in ExpiSf9 cells. Viable cell densities (VCD) and cell viabilities of ExpiSf rAAV production runs for **(A)** rAAV2 and **(B)** rAAV6 in 250 mL shake flasks and HyPerforma 3 L stirred-tank bioreactors. Solid lines represent viable cell density; dotted lines represent percent viability. **(C)** rAAV genome titers of ExpiSf cell lysates from 250 mL shake flasks and 3 L stirred-tank bioreactors. Samples were collected 3 days post-infection. **(D)** Light and fluorescence images of HT1080 cells infected with rAAV2 and rAAV6 crude lysates.

rAAV6 purification using POROS CaptureSelect AAVX affinity resin resulted in a sharp elution peak in the UV chromatogram, consisting of pure rAAV6 particles (Figures 3A and 3B). Transmission electron microscope imaging showed a mixture of full and empty particles (Figure 3C), and the rAAV6 elution fraction contained infectious rAAV as indicated by positive GFP fluorescence when inoculated onto HT1080 cells (Figure 3D). In addition, of the 3.5 x 10^{14} genome-containing rAAV6 particles loaded onto the AAVX column, 2.2 x 10^{14} genome-containing particles were recovered following elution (62% recovery), indicating that high yields of purified rAAV6 were obtained (Figure 3E). These data demonstrate that the ExpiSf system is readily adaptable to large-scale rAAV production, capable of producing high titers of infectious rAAV particles that can be purified with yields of 1.8 x 10^{14} vector genomes per liter of culture.

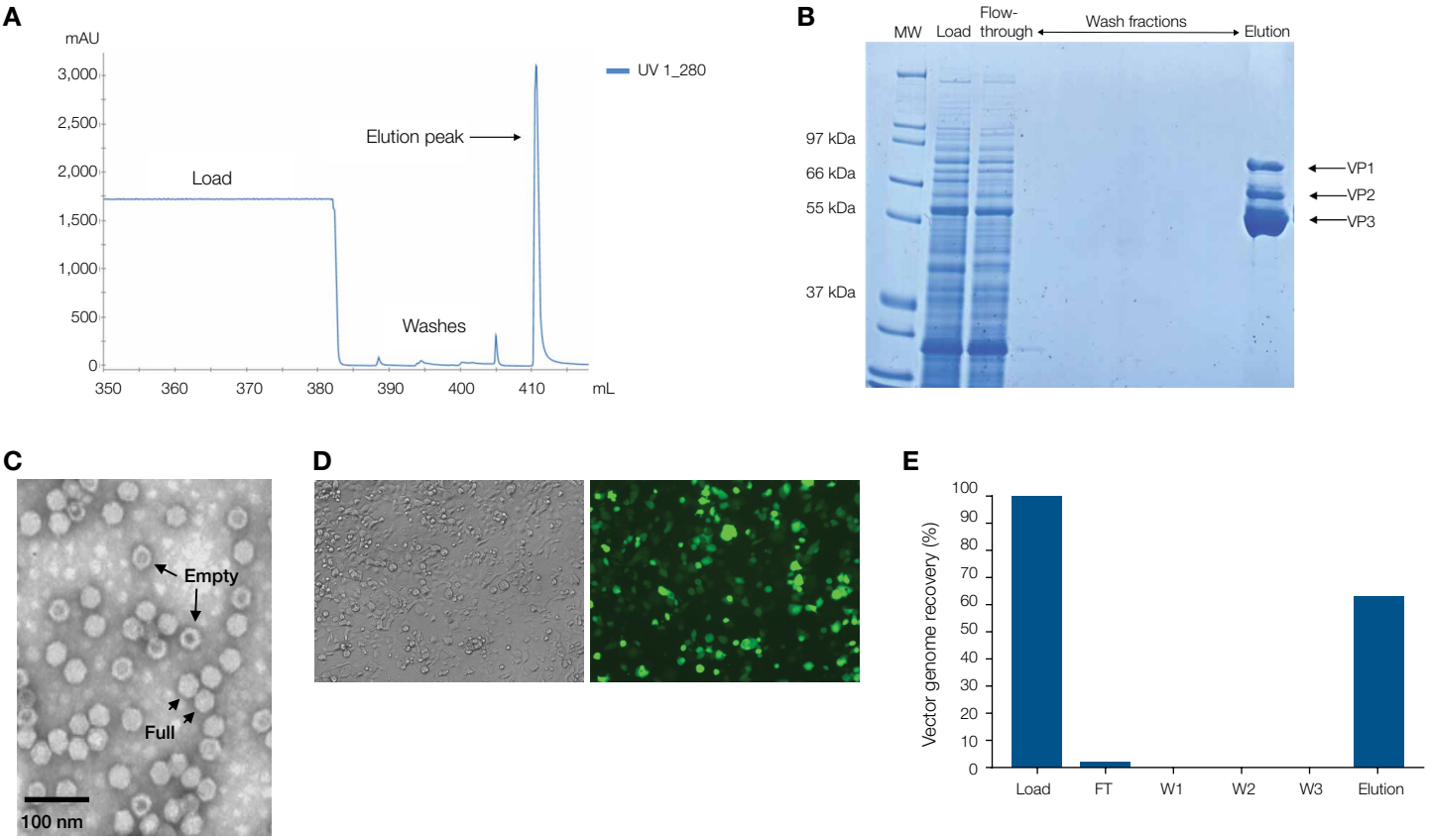

Figure 3. Purification of rAAV6 from ExpiSf production run. (A) ÄKTA chromatogram showing elution peak of rAAV6 purified on POROS CaptureSelect AAVX affinity resin. **(B)** Fractions from AAV6 purification run on a Coomassie stained gel. The capsid proteins VP1, VP2, and VP3 are indicated. **(C)** Transmission electron microscope image of purified rAAV6 particles. Empty and full particles are indicated in the image. **(D)** Light and fluorescence microscopy images of HT1080 cells infected with purified rAAV6 virus. **(E)** Vector genome recovery, relative to the load, in flow-through (FT), wash (W1–W3), and elution fractions of rAAV6 purification run.

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Conclusion

Here we show scalable high-titer production of recombinant AAV2 and AAV6 in the ExpiSf system. We show that infectious rAAV6 particles can be readily purified using the POROS CaptureSelect AAVX affinity resin. Taken together, we demonstrate the versatility of the ExpiSf system beyond protein expression, highlighting the use of this platform for the scalable production of recombinant AAV for gene therapy applications.

References

1. Smith RH, Levy JR, Kotin RM (2009) A simplified baculovirus-AAV expression vector system coupled with one-step affinity purification yields high-titer rAAV stocks from insect cells. *Mol Ther* 11:1888-1896.
2. User's manual for POROS CaptureSelect AAV8, AAV9 and AAVX affinity resins. Bedford MA, Thermo Fisher Scientific, August 2017.

Ordering Information

Product	Region	Quantity	Cat. No.
ExpiSf Expression System Starter Kit	NA, EMEA	1 kit	A38841
	Asia Pacific, Japan, Hong Kong, LATAM	1 kit	A39112
	China	1 kit	A39111
ExpiSf CD Medium	Global	1,000 mL	A3767802
ExpiSf9 Cells	Global	1 vial	A35243
ExpiFectamine Sf Transfection Reagent	Global	1 mL	A38915
ExpiSf Protein Production Kit	Global	1 L	A3767806

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