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APPLICATION NOTE No. 450 | **2022**

Adeno-associated Virus Production in Suspension Cell Culture Using the SciVario® twin Bioprocess Controller

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Abstract

Vaccines are essential tools for resisting pathogens and controlling global pandemics. Various types of virus vaccines, such as live attenuated, inactivated pathogen and subunit vaccines have been widely produced using mammalian cell culture. However, they often rely on attachment cell culture. For higher production capacity, increased yield, and most importantly, ease of production scale-up, it is more desirable to produce vaccines in suspension cell culture platforms. The widely available suspension bioreactors used in antibody production will allow a faster development process of versatile vaccine platforms. Novel technologies such as mRNA vaccines, Adeno virus vector and Adeno-associated virus (AAV) vector-based vaccines have all played a key role in controlling the recent COVID-19 pandemic. Cell culture produced AAV vectors have gained momentum as one of the most effective gene & protein delivery tools in vaccine production as well as gene therapy.
In this application note, we demonstrated the feasibility of an efficient and scalable AAV production platform based on suspension cell culture. We used a suspension-adapted HEK293 cell line (Expi293F) as the host and incorporated a Helper-Free AAV System to eliminate the requirement for wild-type adenovirus co-infection. To achieve high yield AAV capsid production, we performed the cellular transfection in a BioBLU 1c Single-Use Bioreactor controlled by the SciVario twin Bioreactor Control System. We monitored and analyzed metabolites, cell density and viability, reaching a robust AAV titer of around 10^{12} capsids/mL in line with the vaccine industry's typical yields [1].

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Introduction

Vaccines are one of the most effective tools used against viral pathogens and infectious diseases that prevents millions of deaths worldwide each year. There are various vaccine types on the market that vary on each person's health and preexisting conditions [2]. Each type of vaccine is developed specifically to train the immune system to fight certain infectious agents in the future.

Inactivated vaccines, one of the most commonly used types of vaccines, are made up of an inactivated version of a particular pathogen. They are not like a real infection and generally do not provide strong immunity like other type of vaccines, so people may need several booster shots to maintain immunity against viruses and bacteria. A few examples of inactivated vaccines are the Hepatitis A, Flu, and Polio vaccines.

Live-attenuated vaccines use weakened disease-causing organisms to trigger immune responses like natural infections, thus often creating a strong immune response. Chickenpox, yellow fever, rotavirus, or smallpox vaccines are examples of live-attenuated vaccines [3].

Subunit vaccines use only a specific part of the pathogen instead of the whole virus or bacteria. They give a very strong immune response that targets the germ's antigen [4]. Usually this type of vaccine has fewer side effects. Vaccines against COVID-19, hepatitis B, meningococcal or whopping cough, part of the DTaP (diphtheria, tetanus, and pertussis) combined vaccine are typically subunit vaccines. Most current COVID vaccines have targeted on one specific antigenic subunit, like the spike protein, however, not all subunit vaccines are designed to introduce protein antigens directly into the body. Recently, new types of vaccines, such as mRNA vaccines, have been designed to either deliver the genetic code of the pathogen subunit into patient cells, which would allow these cells to make antigens themselves [5], or deliver the subunit antigen via a virus vector (vector-based vaccines) [6]. In this case, the genetic code for an antigen is inserted into a low risk virus (the vector), which acts as a delivery system to introduce the code into the cell; instructing the cell's machinery to replicate the virus and produce antigens without causing the diseases. Lentivirus, adenovirus and AAV are among the most widely used vectors. Both adenovirus [7] and AAV [8] have been used in the production of COVID-19 vaccines.

Multiple factors must be considered to produce large amounts of antigens including the proper selection of media, cultivation conditions, cell platforms and vessel sizes. Vaccine production can be accomplished with either adherent or suspension cell lines, but each option requires a specific design of upstream and downstream processing. Previously, Eppendorf has released a number of applications on attachment cell culture, especially Vero cells, for vaccine applications [9-11].

However, when cultivating anchorage-dependent cells, the procedure is more labor-intensive, and attachment cultures lacks the large-scale bioreactor manufacturing solutions as compared to suspension cultures. Furthermore, a cell dissociation step is needed from its growth surface, which complicates the scale-up process and implies higher costs. We believe that in order to meet the growing demand of vaccines, adapting anchorage-dependent cells into suspension cell culture is a sound manufacturing strategy that could greatly simplify the upstream workflow, and increases titer yield while reducing costs.

Although numerous mammalian cell lines have been evaluated for viral vectors and vaccine production [12-16], the HEK293 cell line is one of the most widely used cell platforms for these demands. HEK293 cells have a wide variety of advantages including low-maintenance, rapid proliferation, and convenient application to both, transient and stable expression. Furthermore, they are easy to transfect and can produce large amounts of recombinant proteins and virus particles. Specifically, high yield adenoviral vectors (~4 x 10^{15} viral particles) have been obtained in stirred-tank bioreactor systems using microcarriers [17,18]. However, Expi293F cells, a new suspension adapted HEK293 cell line developed by ThermoFisher, is capable of achieving high cell productivity [19] and can accelerate the production process of different viral vectors.

AAV, a leading platform in gene delivery, has recently emerged as an important tool for the vaccine industry as well [20-24]. AAV particles contain a single-stranded DNA genome of about 4.7 kb, packed into a non-enveloped capsid of 60 proteins that are arranged in icosahedral symmetry. Twelve AAV serotypes have been found in humans, where AAV-2 is the most widely used vector in preclinical and clinical studies [25-27]. Each serotype has unique receptors, and tissue specificity is determined by the capsid serotype. Furthermore, the higher safety rating due to the fact that wild-type AAV is not currently known to cause disease in vivo, low immunogenicity due to AAV causes a very mild response in vivo and a wide range of infectivity (AAV infect quiescent and dividing cells) are the most important advantages of AAV vectors.

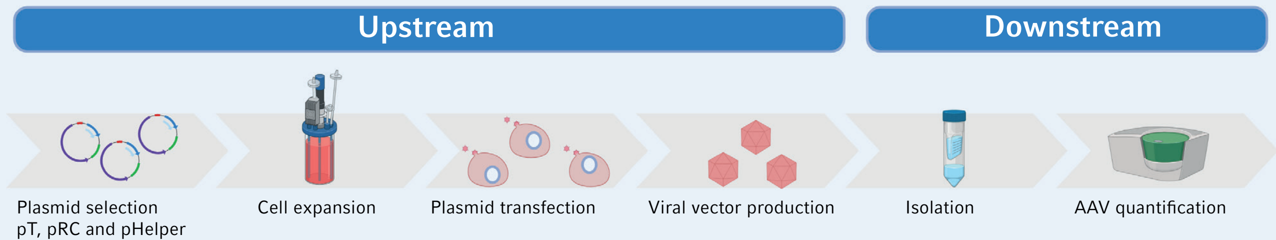

Fig. 1: Typical AAV-2 vector (capsids) production workflow. Created with BioRender.com

Under the current COVID-19 pandemic, the drive for more scalable vaccine production strategies are needed to meet the growing global demand with the increased risk to humans of exposure to new and deadly pathogens. To this end, we have developed an example of bioreactor-based AAV production application on a suspension cell culture platform through the AAV-2 capsid production process in a controlled environment using the SciVario twin bioreactor control system in conjunction with the BioBLU 1c Single-Use Bioreactor and using Expi293F cells. We analyzed cell growth, viability, and metabolic activity (glucose, ammonia, and lactate levels in the medium) throughout the run. We also determined the titer of AAV-2 capsids in the supernatant and in permeable cells of the cell pellet.

Material and Methods

Suspension Cell Line

The selection of a suitable cell line platform is one of the key factors to produce large scale of AAV capsids. In addition, another important feature of the HEK293 cell line is that they were transformed by the integration of adenoviral 5 (Ad5) genome fragment containing *E1A* and *E1B* genes, located on chromosome 19 [28,29]. *E1A* and *E1B* genes are essential helper factors for AAV replication.

We used the suspension adapted HEK293 cell line, Expi293F™ (ThermoFhisher Scientific, USA, A14528) because we previously demonstrated that it is a robust suspension cell line capable of achieving higher per cell productivity in high density culture [19].

AAV vector production workflow

We followed a typical strategy for producing high titer AAV to produce our AAV-2 vector, which includes several steps from plasmid development or selection, cell expansion, plasmid transfection, and viral vector production through isolation and titration (Figure 1).

Plasmids selection

We used a method that involves only two plasmids, the rep/cap plasmid containing the structural and packaging genes of AAV-2 (the replication (rep) and the capsid genes (cap)) and the helper plasmid (AAVpro® Packaging Plasmid (AAV2),Takara, USA, 6234). In this case, the helper plasmid contains adenovirus E2A, E4, and VA, genes required for efficient AAV production and not a wild-type adenovirus, making the system safer and convenient. We used the two plasmids to produce empty AAV-2 capsid vector without gene of interest to establish the feasibility.

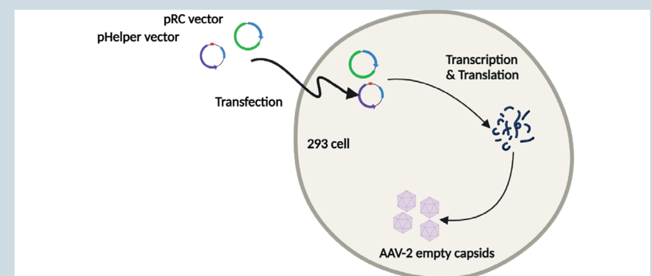

Fig. 2: Formation of empty AAV-2 capsids within cells without the gene of interest using AAV Helper Free Systems. Created with BioRender.com

Plasmid transfection & viral vector production

Once the cells are expanded and reach the selected transfection cell density, DNA complexes (with selected plasmids in a fixed ratio) are prepared and added to the system. We used FectoVIR®-AAV (PolyPlus+ transfection, France, 101000044) reagent for transfection. FectoVIR-AAV is a novel chemical-based and animal-free transfection reagent that has been engineered to address the current limits in AAV-2 viral vector yields and industrial scalability.

AAV-2 isolation, purification and capsid titer

Unlike other AAV serotypes, AAV-2 capsids are highly retained within cells and an isolation method is required. We used the AAVpro Purification Kit, (Takara, USA, 6232) to obtain the AAV-2 capsids.

We used an enzyme-linked immunosorbent assay (AAV-2 Xpress ELISA, Progen, USA, PRAAV2XP) for the quantitative determination of AAV-2 capsids titer from the supernatant and from the cell pellet.

AAV-2 capsids production in bioreactor

Inoculum preparation

We started the cell's expansion process in shaker as shown in Figure 3. We cultured the cells in a New Brunswick S41i CO_2 incubator Shaker (Eppendorf, Germany) (at 37 °C, 8 % CO_2 and at agitation speed of 125 rpm). We obtained more than 900 × 10^6 cells in the third passage. During the expansion process we kept the inoculation density, percentage fill of the shake flasks and other parameters constant. Finally, we prepared the inoculum containing 400 × 10^6 cells in 200 mL of Expi293F Expression Medium on each inoculation bottle.

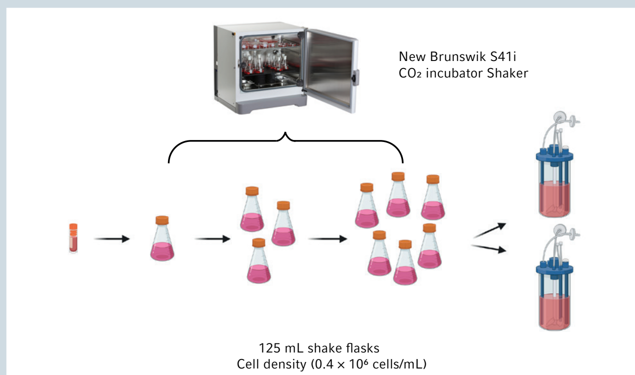

Fig. 3: Workflow of AAV suspension culture scale-up from shakers to bioreactors. Created with BioRender.com

Bioreactor control: SciVario twin

We used SciVario twin bioreactor control system to perform two batch cultures using BioBLU 1c Single-Use Bioreactors equipped with a single pitched-blade impeller. Each bioreactor unit is equipped with three universal port connectors for pH (port 1) and DO (port 2) sensors, a temperature control block that combines electrical heating and water cooling, agitation control and a gas module that includes one Thermal Mass Flow Controller (TMFC) with an ultra-high turndown ratio of 1:12,000, and 4 solenoid valves for automated 4-gas mixing (see Figure 4).

Sensor calibration

Prior to the preparation of the BioBLUs 1c Single-Use Bioreactors, we connected the ISM gel-filled pH sensors to the SciVario twin bioreactor control system, and they were automatically detected by the software. We performed the calibration process according to the operation's manual using buffer solutions of pH 7 and pH 4 as "zero" and "span" respectively. Then, we disconnected the pH sensors and sterilized them in an autoclavable pouch.

BioBLU 1c Single-Use Bioreactor preparation and process parameters

We outfitted each BioBLU 1c with a pH sensor (previously sterilized, see details above). We inserted the pH sensor in a PG 13.5 port under aseptic conditions inside the BioSafety Cabinet. In addition, we also connected the following into the headplate: A polarographic DO sensor (Mettler Toledo®), an exhaust condenser, a sampling dip tube, a 4-gas mixing line connected to the gas sparge port, and silicon tubing exten-

Fig. 4: AAV-2 capsids production in a batch culture using SciVario twin bioreactor control system and BioBLU 1c Single-Use Bioreactors

sions connected to liquid addition ports (one for inoculation/ medium addition, one for base addition and another for the addition of 0.1 % of antifoam (Pluronic®-F68 surfactant, Life Technologies®, USA)). Then, we placed the BioBLU 1c bioreactors in their respective temperature control block to keep the system at constant temperature. Finally, we added 800 mL of Expi293FTM Expression Medium into each bioreactor and conditioned for at least 24 hours under the parameters and setpoints listed in Table 1.

Parameters	Setpoints
Starting volume	800 mL
Ending volume	1 L
Initial agitation	155 rpm (0.4 tip speed)
Temperature	37 °C
Inoculation density	0.4 x 10^6 cell/mL
Cell culture medium	Expi293™ Expression Medium
DO Setpoint	40% (P=0.1; I=0.001)
pH Setpoint	7.0 (deadband = 0.2), cascade to CO_2 (acid) cascade to 0.45 M sodium bicarbonate (base)
Gassing range	Set O_2 % at 30 % controller output to 21 % and at 100 % controller output to 100 %. Set flow at 0 % controller output to 0.04 SLPH, and at 100 % controller output to 30 SLPH.

Cascade control of DO

We established the following DO cascade to control the culture with air only, without oxygen supplementation: "Set O_2 % at 30 % controller output to 21 % and at 100 % controller output to 100 %. Set flow at 0 % controller output to 0.04 SLPH and at 100 % controller output to 30 SLPH."

Expi293F™ cell culture in BioBLU 1c Single-Use Bioreactors

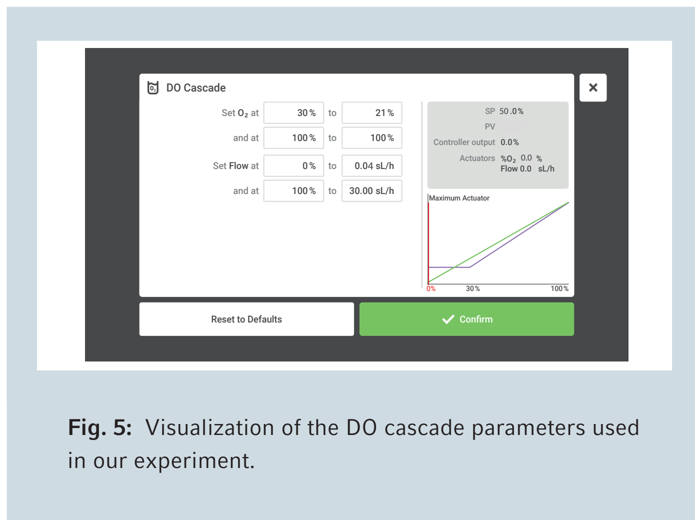

Fig. 5: Visualization of the DO cascade parameters used in our experiment.

We inoculated the BioBLU 1c Single-Use Bioreactors with 200 mL of the inoculum described above (see section "Inoculum preparation") at a cell density around 0.4 x 10^6 cell/mL, and cell viability greater than 95 %. We set the temperature at 37 °C, controlled the dissolved oxygen (DO) at 40 % and used Pluronic-F68 surfactant as needed. We employed a gel-filled pH sensor to control the pH during the cell culture run at 7.0 (deadband = 0.1), using a cascade to CO_2 (acid) and 0.45 M sodium bicarbonate (base). We took a sample from the bioreactor daily and measured the pH, the cell viability and density as well as the concentration of various metabolites offline.

Transfection procedure of Expi293F cells on BioBLU 1c Single-Use Bioreactor

We reached a transfection cell density of approximately 3 x 10^6 cells/mL three days after the bioreactor's inoculation. Then, plasmids at a 2:1molar ratio (1752 µg of pRC + 1245 µg of pHelper) were diluted in 50 mL of Expi293F Expression Medium (5% of the total volume of culture). Next, we vortexed the FectoVIR briefly and added 3 mL of the solution into the plasmids/medium solution all at once, we vortexed again and incubated the mixed solution for 30 minutes at room temperature. Finally, we added the transfection mix onto the cells in the bioreactor.

Cell viability and metabolic activity

We collected a sample daily from the BioBLU 1c Single-Use Bioreactors to determine the cell viability, cellular density, and the concentration of metabolites (glucose, ammonia (NH_3) and lactate), by connecting a sterile 5 mL syringe to the Luer Lock sample port. We collected and discarded 5 mL of dead volume and after switching to a new 5 mL sterile syringe, we collected another 3 mL as a viable sample for analysis. We used 1 mL to measure the metabolite levels employing a Cedex® Bio Analyzer (Roche, USA), 1 mL to measure the cell viability and density using a Vi-Cell® XR Viability Analyzer (Beckman Coulter®, USA) and 1 mL to check the pH offline using an Orion Star™ A211 pH meter (ThermoFisher Scientist, USA).

AAV-2 capsids isolation

72 hours after transfection we collected 60 mL of Expi293F cells and medium using a labtainer bag with line sets (ThermoFisher Scientific, USA, SH30658.11). We centrifuged the cells at 300 x g (Centrifuge 5430R, Eppendorf, 022620601) for 5 minutes and separated the cell pellet and the supernatant. We used the AAVpro Maxi purification kit to

isolate and purify AAV-2 capsids in the cell pellet or supernatant, following the manufacture's protocol instructions.

AAV-2 capsids titration

Following the viral particles isolation, we quantified the total amount of capsids in the cell pellet and the supernatant through AAV-2 Xpress ELISA kit. Immuno-titration using this kit offers a fast, sensitive and reproducible method for titration of assembled and intact empty AAV-2 capsids. Then, we finished the assay according to the manufacture's protocol instructions.

Results and Discussion

AAV-2 capsids production in bioreactor

The purpose of this application is to demonstrate the feasibility of bioreactor-based suspension cell culture method for AAV vector-based vaccine production. Previously, we have demonstrated the robust growth of Expi293F cells in suspension culture in bioreactors without AAV [23]. We established a plasmid transfection protocol using pCR:pHelper plasmid ratio of 2:1, and FectoVIR:total DNA ratio of 1:1. We carried out a transient transaction in the 1 L bioreactor using the transfection conditions previously mentioned and continued the bioreactor cell culture for a total of 7 days.

We inoculated both vessel for the AAV-2 capsids production with a cell density of approximately 0.4 x 10^6 cell/mL and carried out the transfections at day 3 with approximately 3 x 10^6 cells/mL each.

We observed a rapid increase of cell growth in both bioreactors between days 1 and 4 of culture, reaching a peak in viable cells density at 7 x 10^6 cells/mL (see Figure 6 A). We would like to point out that the bioreactors process in both vessels showed lower cell growth after transfection compared to a bioreactor process without transfection (data not shown, peak in viable cells density was approximately 13 x 10^6 cells/mL). Furthermore, we determined the consumption of glucose and production of lactate and NH_3 as well as maintained the concentration of lactate and NH_3 below 2 g/L and 2.3 mmol/L respectively during the whole run (See Figure 6 B).

As previously stated, we harvested 60 mL of cells and medium 72 hours after transfection and determined the AAV-2 capsids titer via ELISA, in the cell pellet and supernatant.

We observed lower AAV-2 capsids titer from the supernatant compared to the cell pellet in the AAV-2 production processes from both vessels (see Figure 7). We obtained very similar AAV titer from both bioreactors, demonstrating excellent reproducibility of AAV production using precision-controlled bioreactors.

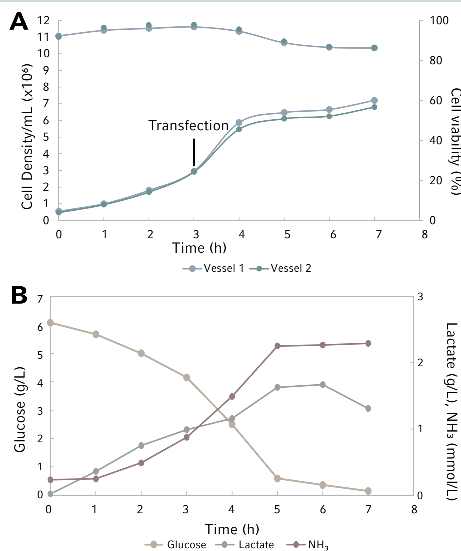

Fig. 6: Expi293F growth profile in BioBLU 1c Single-Use Bioreactor. **A:** Expi293F cell density and viability of two different vessels from inoculation (day 0) to transfection (day 3) and harvest (day 6). **B:** Metabolic profile of cell culture from vessel #1.

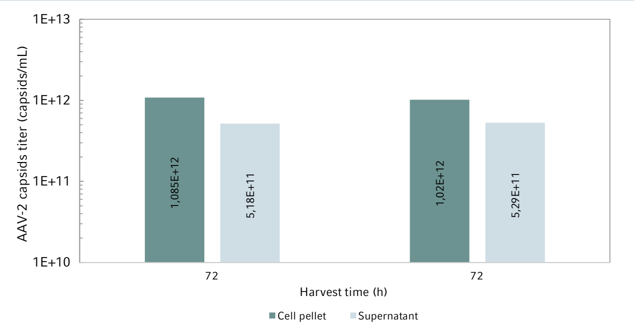

Fig. 7: AAV-2 capsids titer obtained in cell pellet and cell culture supernatant from transfected cells of both vessels.

Comparison of AAV-2 capsids production between shake flask and bioreactor

We successfully transfected Expi293F cells and quantified AAV capsid production titers in shake flasks as well as precision-controlled bioreactors. As shown in Figure 8A, the bioreactor process achieved higher cell density and viability than the shake flask process after transfection. Furthermore, we also observed that cells in the bioreactor maintained higher cell density after day 5 as compared to the decrease in cell density and viability observed in the shake flask.

We compared the AAV-2 capsid production from shake flasks and bioreactors (Figure 8B), and we observed much higher capsid titer from the precision-controlled bioreactor system.

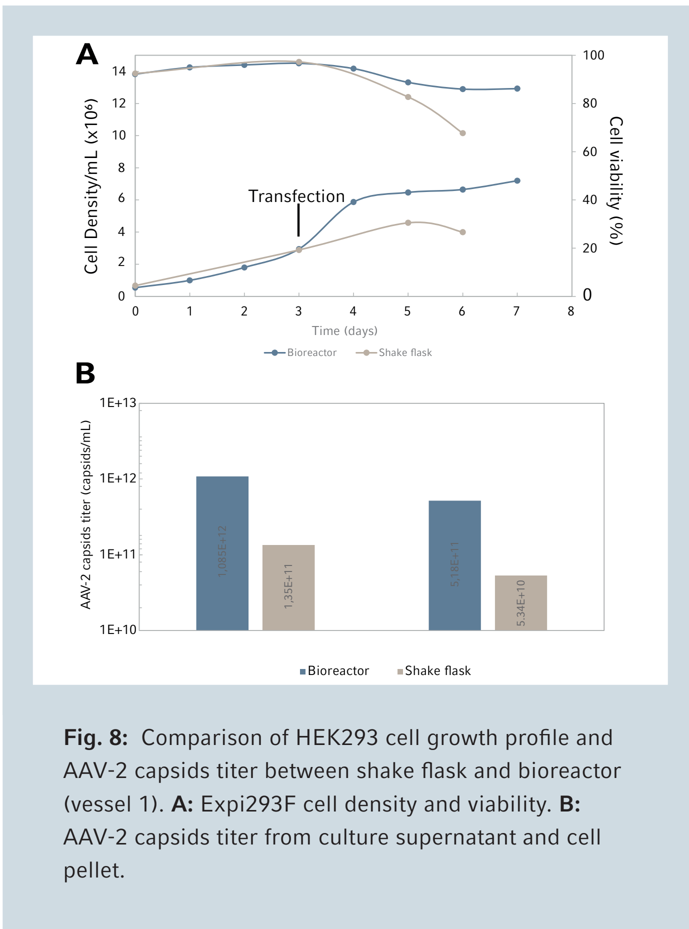

Fig. 8: Comparison of HEK293 cell growth profile and AAV-2 capsids titer between shake flask and bioreactor (vessel 1). **A:** Expi293F cell density and viability. **B:** AAV-2 capsids titer from culture supernatant and cell pellet.

Conclusions

This study underscores a suspension culture-based vaccine production platform as an easier alternative to attachment culture approaches. The suspension culture produced good yield on AAV-2 particles while using the standard stirred-tank suspension bioreactor process that's well established in the field of CHO cell antibody production. The suspension method will allow more straightforward scale-up into larger bioreactors similar to the well-established CHO culture process [26] and offers much desired simplicity, as well as access to a greater variety of production systems over attachment culture-based methods.

We began the suspension culture process at the shake flasks stage using a shaking incubator followed by bioreactor suspension culture using SciVario twin bioreactor control system and BioBLU 1c Single-Use Bioreactors. The efficient and simple setup of the SciVario twin allowed the precise control of the cell culture environment when compared to cell transfection in shake flask, leading to an AAV-2 capsid titer of approximately 10^{12} capsids/mL from the cell pellet compared to the approximately 10^{11} capsids/mL obtained under the same shake flask transfection conditions. Furthermore, the data obtained from both batch culture processes demonstrate the reproducibility of these experiments when using the SciVario twin as a controller. We believe that the Expi293F cell line together with advanced suspension stirred-tank bioreactors and controller from Eppendorf such as the SciVario twin [30], the BioFlo 320 [31], and the BioFlo 720 [29] offer ideal solutions towards the development of suspension-based vaccine platforms as well as viral vector production for gene therapy R&D.

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Ordering information

Description	Order no.
BioBLU® 1c Single-Use Bioreactor, cell culture, open pipe, 2 pitched-blade impellers, optical pH, sterile, 4 pieces	1386110500
SciVario® twin Fermenter/Bioreactor Control System, base unit, 100 – 240 V/50/60 Hz, for 2 vessels	7600100001
New Brunswick S41i, 170 L, CO2 incubator shaker with inner shelf and touch screen control, stackable up to 2-fold, 1 (2. optional) shelves, 120 V/60 Hz, orbit 2.5 cm (1 in)	S41I120010
Centrifuge MiniSpin® plus, non-refrigerated, with Rotor F-45-12-11, 120 V/50 – 60 Hz (US)	022620207
Centrifuge 5430 R, keypad, refrigerated, with Rotor FA-45-30-11 incl. rotor lid, 120 V/50 – 60 Hz (US)	022620601
Eppendorf Conical Tubes, sterile, pyrogen-, DNase-, RNase-, human and bacterial DNA-free, colorless, 50 mL	0030122178
Eppendorf Conical Tubes, sterile, pyrogen-, DNase-, RNase-, human and bacterial DNA-free, colorless, 15 mL	0030122151

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