Highly Efficient Production of Adeno-associated Virus Using the Corning[®] Ascent[®] Fixed Bed Reactor System 5

Application Note

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Introduction

Cell and gene therapies (CGTs) are breakthrough medical innovations that are transforming how we treat and potentially cure certain diseases. With more than 2,600 CGT clinical trials underway¹, the manufacturing processes for both gene delivery vehicles (such as viral vectors) and therapeutic cells (such as stem cells) continue to be bottlenecks for the pharmaceutical industry. There is an urgent need for an automated, scalable, and costefficient manufacturing platform to meet the rapidly increasing clinical demands.

To address this increasing need, Corning has developed a novel fixed bed reactor (FBR) platform, the Corning Ascent FBR system, which combines the benefits of adherent bioproduction platforms with the scale and automation of suspension manufacturing systems. To meet various applications and production scale needs, the Ascent FBR system is designed to provide a broad range of cell growth surface areas from 1 m² to 1,000 m². The system is designed for use in CGT workflows such as adeno-associated virus (AAV), lentivirus, mesenchymal stem cells (MSCs), and pluripotent stem cells (PSCs) in addition to viral vaccine and other biologic production applications.

The Corning Ascent FBR System 5 (Figure 1) provides flexibility in protocol development and automated operation during use. The system is designed around a single-use bioreactor supported by a media conditioning vessel (MCV) with integrated disposable sensors for temperature, pH, and dissolved oxygen (DO) to monitor and control key process parameters. The system features a specially treated and packed polymer mesh (Figure 2) that enables uniform, low-shear fluid flow through the bioreactor bed. This promotes evenly distributed cell growth and enhances exposure of cells to nutrients and reagents. There are three sizes of bioreactor available for the Ascent FBR System 5: 1 m², 2.5 m², and 5 m². All fluid-contacting components are pre-assembled, single-use, and irradiated, allowing for immediate use.

The focus of this study was to demonstrate highly efficient production of AAV in the 2.5 m² Ascent FBR bioreactor and to compare its performance side-by-side with a comparable FBR system and standard 2D cell culture controls.

Figure 1. Corning Ascent FBR System 5 with bioreactor vessel (BRV) and media conditioning vessel (MCV) installed. *Actual instrument component appearance may differ from image*.



Figure 2. Corning Ascent FBR System 5 bioreactor vessel and magnified views of polymer mesh substrate.

Materials and Methods

Cell Culture

Before inoculation into the Ascent FBR bioreactor, HEK-293T cells (ATCC CRL-3216) were routinely cultured in DMEM (Corning 15-018-CM) supplemented with 6 mM L-glutamine (Corning 25-005-CI), Penicillin-Streptomycin Solution, 100X (Corning 30-002-CI), and 10% FBS (Corning 35-010-CV) in Corning CellBIND® surface-treated 2D flasks in a humidified environment at 37°C and 5% CO₂.

Seed Train

A 10-day seed train process was developed for cell amplification to obtain enough cells to seed into the Ascent FBR System 5, the comparable FBR system, and the two 2D controls (Figure 3). Three Corning HYPERStack® 12-chamber cell culture vessels (Corning 20012) were used for the N-1 step. HEK-293T cells were maintained between passage 3 and 6 post-thaw for this study.

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Figure 3. Overview of a seed train process from 175 cm² flask to 6,000 cm² Corning HYPERStack 12-chamber vessel to obtain enough cells to seed into a 2.5 m² bioreactor in the Corning Ascent FBR System 5.

System Setup and Batching

Prior to cell seeding, both the Corning[®] Ascent[®] FBR system and the comparable FBR system were set up and batched with complete cell culture medium.

For the Ascent FBR system, one set of the main consumable (Corning 6972) was installed on the Ascent FBR System 5. The consumable consists of two parts, one is the 2.5 m² BRV and the other is the 3.5L MCV with all tubing and in-line sensors already assembled and attached. Connection of MCV and BRV and all accessory reagent bottles, was done in an open lab environment, using AseptiQuik® sterile connectors (CPC AQG17004) supplied with the consumable set. Cell culture medium (1.1L) was then added to the MCV through a media addition bottle that was filled in a biological safety cabinet (BSC). The medium was circulated from the MCV through the bioreactor and returned to the MCV at a flow rate of 150 mL/min. for the remainder of the batching process. The controller was programmed to automatically control the conditions in the MCV to maintain the medium at 21% O₂, 5% CO₂, and 37°C. After 2 hours of batching, the in-line optical pH and DO sensors were calibrated, via an offline measurement for the former and an input of 100% for the latter. Once complete, the system was set to maintain the medium at a pH of 7.2 with 100% DO in the MCV and a minimum of 20% DO in the outlet of the bioreactor and allowed to equilibrate overnight.

For the comparable FBR system, the assembly and sterilization of the main consumables, sensors calibration, and system set up were all performed according to manufacturer's instructions and required sterile environment. Complete cell culture medium was added to the system, set to maintain the medium at a minimum of 50% DO and a pH of 7.2, and batched overnight.

Cell Seeding and Growth

On the day of cell seeding, HEK-293T cells cultured in the Corning HYPERStack[®] 12-chamber vessel were harvested using Accutase[®] (Corning 25-058-CI). The cells were pelleted by centrifugation and then resuspended in complete cell culture medium to a predefined concentration. For the Ascent FBR 2.5 m² bioreactor, 140 mL of medium containing 5.5 x 10⁹ cells was inoculated into the MCV using a Corning Ascent storage bottle with dip tube and aseptic connector (Corning 6980). The cells in the suspension were then circulated through the BRV at a flow rate of 100 mL/min. to allow cells to attach to the substrate inside the BRV. The seeding of cells onto the comparable FBR was performed according to the manufacturer's protocol. The seeding density for both FBRs was 22,000 cells/cm². A Corning CellBIND surface-treated T-75 flask and a HYPERStack 12-chamber vessel were used as 2D controls. The seeding density for the T-75 flask and HYPERStack 12-chamber vessel were at 10,000 and 12,000 cells/cm², respectively, to achieve similar cell density as the FBR systems at the time of transfection.

The cells in both FBR systems were cultured for 3 days under controlled conditions. The consumption of glucose and L-glutamine and release of lactate and ammonia from the cells were monitored daily via media sampling and measurement on the BioProfile® FLEX2 automated cell culture analyzer (Nova Biochemical). A one-time addition of 2L of fresh medium to the Ascent MCV occurred between 24 to 48 hours to support cell growth. No media refeed was needed for either 2D controls or comparable FBR system. The cells in each vessel typically multiplied by approx. 10-fold in 3 days and reached a density of approx. 200,000 cells/cm² prior to transfection.

Transfection

An AAV2-GFP helper-free packaging system (Cell BioLabs VK-402) was used to transfect HEK-293T cells. Transfection complexes were made with PElpro® (PolyPlus 115-010) and 3 plasmid DNAs at a ratio of 2:1 (PElpro:DNA) and a mass ratio of the three plasmid DNA was 1:1:1 (pAAV-GFP: pHelper: pAAV-RC2). The total amount of plasmid DNA for each vessel was calculated based on total surface area with a pre-defined DNA density of 0.18 µg/cm².

Two hours prior to transfection, spent medium from the 3-day culture was removed from each system and exchanged with the same volume of fresh medium to remove cellular waste. For the two FBR systems, the pH was adjusted from 7.2 to 7.0. Once the system stabilized, the transfection complex was added aseptically to each system. Twenty-four hours post-transfection, the transfection medium was replaced with the same volume of fresh medium for the Ascent FBR system and 2D controls. For the comparable FBR system, media exchange was performed at 4 hours post-transfection according to manufacturer's recommendations. Transfected cells were cultured for an additional 2 days before harvest. During this period, metabolites were monitored daily and fresh medium and nutrients, such as glucose, were added to support cell growth. A small amount of cell culture medium was collected from each system at 48 and 72 hours post-transfection and stored at -80°C for later viral titer analysis.

Adeno-associated Virus Harvest

At 72 hours post-transfection, AAV vector was harvested from the Ascent FBR and comparable FBR systems using an in situ lysis method. Briefly, cell culture medium was first drained completely from the system, and several pieces of substrate samples were removed from the bioreactor beds for later analysis. Pre-warmed 37°C lysis buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM MgCl₂, 0.1% Tween[®] 20, and 25 units/mL Benzonase) was then added—1L to the Ascent FBR and 700 mL to the comparable FBR system. pH of the controllers was set to pH 8.0 and the lysis buffer was circulated through each bioreactor continuously for 3 hours. The lysis buffer was completely drained from the system and collected, and the bioreactor was washed sequentially with DPBS for 30 minutes and with high salt solution (500 mM NaCl) for another 30 minutes. Each wash was collected for analysis. For the comparable FBR system, in addition to the DPBS and high salt solution wash, an extra two-hour high salt wash was performed after the bioreactor was stored at -80°C overnight, per the manufacturer's recommendation. All volumes collected were measured and aliquots of lysis buffer, DPBS wash, and high salt wash were collected and stored at -80°C for later viral titer analysis. For 2D controls, all transfected cells were first harvested from the vessel using Accutase[®]. Then an aliquot of cell harvest solution containing 1×10^7 cells was pelleted by centrifugation, resuspended in 1 mL lysis buffer, and incubated at 37° C for 1 hour. The resulting cell lysate was stored at -80°C for later viral titer analysis.

Crystal Violet Staining

Crystal Violet staining was used to visualize cells on the bioreactor substrates. Substrate samples removed from the bioreactors were submerged into 1:20 dilution of Gram Crystal Violet staining solution (BD 212525) to DPBS in a 100 mm Petri dish and incubated for 5 minutes. The stained substrate samples were then rinsed with distilled water to remove excess dye, air-dried, and imaged using a HP ScanJet Scanner.

Cell Harvest from FBR Bioreactor Substrates

FBR bioreactor substrate samples were placed into a 100 mm Petri dish containing 20 mL of Accutase and incubated at room temperature for 40 minutes under agitation using an orbital shaker. At the end of incubation, cell harvest solution was pipetted over the substrate several times to ensure complete removal of all cells from the substrate surface. The cell harvest solutions were then collected for cell enumeration using a Beckman Coulter Vi-CELL[™] XR cell viability analyzer.

Flow Cytometry

Cells harvested from bioreactor substrate samples and 2D controls were used for flow cytometry analysis. Flow samples were prepared by passing the cell solutions through a 40 µm cell strainer (Corning 431750) to remove any large cell clumps. Cells were then pelleted by centrifugation, and the pellet was resuspended in DPBS to a final concentration of 0.5 x 10⁶ cells/ mL. Samples were run on a BD FACSCalibur™ Flow Cytometer using pre-determined settings to count 30,000 events for each sample. Mock-transfected cells were used as a negative control to determine percentage of GFP expressing cells (GFP+ cells).

Adeno-associated Virus (AAV) Titer Quantitation

Cell culture media collected at 48 and 72 hours post-transfection, cell lysate, PBS wash, and high salt wash samples were sent to Welgen, Inc. for AAV titer analysis. Welgen, Inc. used real-time quantitative polymerase chain reaction RT-qPCR to measure copy numbers of GFP gene in AAV. The samples were briefly treated with DNase I prior to being isolated using a DNA extraction kit (Applied Biosystems 4403319). RT-qPCR assays were then performed on the StepOne[™] PCR system (Applied Biosystems 4376357) by adding the diluted extracted samples to a master mix solution consisting of water, PrimeTime® Gene Expression master mix (IDT 1055770), TaqMan[®] probe, and forward and reverse primers against the GFP insert (F: GAACCGCATCGAGCTGAA, R: TGCTTGTCGGCCATGATATAG, Probe: ATCGACTTCAAGGAGGACGGCAAC). A standard curve was constructed using the purified pAAV-CMV GFP plasmid DNA and the results given as genome copies (GC)/mL.

Results and Discussion

Cell Distribution

To evaluate how cells were distributed throughout the Corning® Ascent® FBR bioreactor, mesh disk samples were removed from top, middle, and bottom sections of the bioreactor. The mesh disks were then stained with crystal violet solution to visualize the cells. The images in Figure 4A show uniform cell distribution across each disk as well as throughout the top, middle, and bottom of the bioreactor.



Figure 4. Crystal Violet staining images of mesh disk samples removed from the Corning Ascent FBR (A) before cell harvest; (B) after cell harvest.

Total Cell Yield

Since the Ascent FBR bioreactor substrate is a woven polymer mesh, cells growing on the surface of the mesh disks can be easily harvested by incubating in a cell disassociation reagent, such as Accutase. Cells harvested from the mesh disks can then be used to estimate total cell yields of the Ascent FBR system. The average total cell yields on day 3 post-transfection from three replicate experiments was determined to be approx. $4.3 \pm 0.6 \times 10^5$ /cm². After cell harvest, the mesh disks were stained with crystal violet solution to evaluate harvest efficiency. Figure 4B showed that cells were almost completely removed from mesh disk (note the lack of staining on the disks after harvest); the average harvest yield was >95%.

Since intact cell removal is not practical for the comparable FBR, a lysate-based nucleus count method was used to determine total cell numbers. The average total cell yields on day 3 post-transfection from three replicate experiments was determined to be approx. $3.2 \pm 1.3 \times 10^5$ /cm², which is 26% less than what was obtained from the Ascent FBR system.

The very high viable cell harvest yield from the Ascent FBR bioreactor substrate allows flexibility in the method used in the recovery of virus and supports the use of the Ascent FBR system in cell seed train scale-up prior to virus production. We have demonstrated highly efficient aseptic cell harvest from the Ascent FBR System 5 performed in a fully automated mode, resulting in both harvest yield and cell viability of greater than 95%.

GFP Expression

Since an AAV2-GFP helper-free packaging system was used for this study, the transfected cells express GFP and could be visualized using a fluorescent microscope for the different substrates used. The fluorescent images of both FBR substrates as well as a T-75 flask are shown in Figure 5. Images were only taken on one focal plane since FBR substrates from both the Ascent FBR and comparable FBR represent 3D surfaces.



Figure 5. Representative fluorescent images of GFP+ cells on different substrates. (A) T-75 flask; (B) Corning Ascent FBR system; (C) comparable FBR system. Images were obtained using Thermo Fisher EVOS® FL microscope. Magnification (4X).



Figure 6. Representative flow cytometry profiles of cells harvested from 2.5 m² Corning Ascent FBR bioreactor substrates and 2D controls. Purple line = negative control of non-transfected cells. Black line with green fill = net GFP expression.

Transfection Efficiency

%GFP+ cells, in conjunction with mean fluorescence intensity (MFI), can be used as an indicator of transfection efficiency since there is a good correlation between %GFP+ cells and transfection efficiency. We anticipate high viral titer from those FBR systems achieving high levels of %GFP+ cells and MFI.

For the Ascent FBR system, %GFP+ cells and MFI from cells harvested from the top, middle, and bottom of the bioreactor were very similar (Figure 6). This indicated an even distribution and uptake of transfection complex within the bioreactor, resulting in a uniform transfection across the 2.5 m² packed mesh bed which was consistent with uniform cell distribution data. The average %GFP+ cells in the Ascent FBR bioreactor was >90%, which was comparable with what were obtained from standard 2D controls. The %GFP+ cells for the comparable FBR substrates were not assessed due to poor cell harvest efficiency from the substrate.

Adeno-associated Virus (AAV) Titer Yield

RT-qPCR was used to determine titers of AAV harvested from different cell culture substrates. The total AAV titer yield consisted of titers of samples collected from different processes used to generate and harvest the AAV: cell culture media (48 and 72 hours post-transfection), cell lysates, PBS wash and high salt washes. The results are summarized in Table 1. The average total AAV titer yield produced by the Ascent FBR 2.5 m² bioreactor was $1.1 \pm 0.2 \times 10^{15}$ GC/vessel, which was 7.9 times higher than that obtained from the comparable FBR bioreactor with similar surface area.

Table 1. Comparison of AAV titer yields produced from differentsubstrates.

Vessel	Surface Area	No. of Runs	GC/Vessel	GC/cm ²
Corning® Ascent® FBR System 5	2.5 m ²	3	$1.1 \pm 0.2 \times 10^{15}$	$4.3 \pm 0.9 \times 10^{10}$
Comparable FBR system	approx. 2.5 m ²	3	$1.4 \pm 0.5 \times 10^{14}$	5.2 ± 1.9 x 10 ⁹
Corning HYPERStack® 12-chamber	0.6 m ²	2	$2.5 \pm 0.6 \times 10^{14}$	3.7 ± 0.3 x 10 ¹⁰
Corning T-75 flask	0.0075 m ²	3	4.5 ± 3.4 x 10 ¹²	$6.0 \pm 4.4 \times 10^{10}$

The productivity of the Ascent FBR system and the comparable FBR system, measured by GC/cm^2 , were also compared. The average GC/cm^2 for the Ascent FRB system was $4.3 \pm 0.9 \times 10^{10}$, which was 8.3-fold higher than that for the comparable FBR system.

The productivity of the Ascent FBR system as compared to the 2D controls showed that GC/cm² of the Ascent FBR system was comparable to that of the HYPERStack 12-chamber vessel and the T-75 flask, showing no statistically significant difference between the Ascent FBR system and 2D controls (Figure 7). However, the difference between the Ascent FBR system and comparable FBR system was statistically significant as seen with a p <0.05.

The GC/cm² of the T-75 flask showed a relatively large variation when compared with other substrates. This was most likely due to overgrowth of cells over a 6-day culture period on a relatively small surface area. Cells dislodged from the surface were seen during media exchange and DPBS wash processes. In contrast, the HYPERStack 12-chamber vessel, with much larger surface area, had more reproducible results, which makes it a better 2D control than the T-75 flask.

The average total AAV titer yield obtained from the HYPERStack 12-chamber vessel and the T-75 flask were 2.5×10^{14} and 4.5×10^{12} , respectively. When plotted together with the total AAV titer yield from the Ascent FBR 2.5 m² bioreactor, there was a strong linear correlation (r² = 0.9999) between total AAV titer yield and surface area (Figure 8). This result demonstrated that the Ascent FBR system is a linear-scalable platform for AAV production.



Figure 7. Comparison of productivities of AAV produced from different systems. *Paired t-test, p < 0.05, N=3.



Figure 8. Linear correlation between total AAV yield and vessel surface area.

Conclusions

- The side-by-side experiments demonstrated that AAV titer yield (total GC and GC/cm²) in the Corning[®] Ascent[®] FBR 2.5 m² bioreactor were 8-fold higher than those generated in a comparable FBR system with similar surface area.
- The productivity of AAV (GC/cm²) in the Ascent FBR system was comparable to that of traditional 2D tissue culture vessels, which implies minimal optimization efforts to take existing 2D protocols into the Ascent FBR system. Further optimization specific to the Ascent FBR system could increase productivity further.
- The Ascent FBR bioreactor's novel design enables evenly distributed cell growth and subsequent uniform transfection efficiency and AAV production.
- The ability to harvest viable cells aseptically from the Ascent FBR bioreactor substrate allows the Ascent FBR System 5 to be used as seed train for manufacturing scale, as production platform for cell therapy workflows and for other potential applications.

References

1. The Alliance for Regenerative Medicine: Regenerative Medicine in 2021: A Year of Firsts and Records.

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