Direct Seeding and Harvest of Cryopreserved HEK293 Cells in the Corning® Ascent® Fixed Bed Bioreactor System

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Application Note

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Introduction

Adherent human embryonic kidney (HEK293) cells are widely used in the pharmaceutical industry for transfection, vaccine production, and manufacturing of recombinant proteins due primarily to the cell's ability to reduce immunogenicity and offer superior glycosylation profiles as compared to nonmammalian cells. Often, due to the large numbers of cells required for production, cells are subject to a lengthy scaled-up process in progressively larger volumes. During this process, cells can undergo changes that potentially increase lot-to-lot variability in the quality and quantity of the product being generated. One approach to develop consistency and reduce scale-up time between experiments or production processes is direct inoculation from a single large-volume cell bank, thereby ensuring that the cells are uniform for each batch.

The Corning Ascent Fixed Bed Bioreactor (FBR) System is an automated bioreactor platform that enables control of pH, dissolved oxygen (DO), and temperature of media continuously circulating through its three dimensional mesh packed-bed substrate. Automated control of multiple processes is provided by an integrated touchscreen human-machine interface (HMI) with adaptable process parameters. With the Ascent FBR system, HEK293 attachment, expansion, and harvest can provide consistent cell quantities without the need for cryo-recovery passages in standard two dimensional (2D) cultureware.

Direct inoculation of the bioreactor can be accomplished with cryopreservation vials or bags depending on reactor size, concentration of the cell bank, and desired seed density. In some cases, removal of DMSO via centrifugation is not necessary, further shortening the process[1,2]. For more sensitive cell lines, resuspension in fresh medium to remove cryoprotectant prior to inoculation can also be employed.

Materials and Methods

HEK293 cells (ATCC® CRL-1573™) were cultured in DMEM (Dulbecco s Modified Eagle Medium; Corning 15-018-CM) supplemented with 10% FBS (Corning 35-10-CV), 4 mM L-glutamine (Corning 25-005-CI), and 1X penicillin-streptomycin (Corning 30-002-CI). A large volume cell bank was created by scaling cells in a Corning CellSTACK® 10-chamber culture vessel (Corning 3320), harvested with Accutase® (Corning 25-058-CI), and rinsed with DPBS (Corning 21-031-CM). After centrifugation at 250 x g for 10 minutes in 500 mL centrifuge tubes (Corning 431123), the cells were enumerated using the NC-200™ NucleoCounter® (Chemometec) and resuspended at a final concentration of 2 x 10^7 cells/mL in cryopreservation media (90% FBS plus 10% DMSO (Corning 25-950-CQC)). Cryopreserved cells (4.5 mL) were aliquoted into 5 mL cryogenic vials (Corning 430656), placed and frozen using a Corning CoolCell® container (Corning 432005) at -80°C for 24 hours prior to storage in liquid nitrogen vapor phase.

For bioreactor inoculation, four cryogenic vials were thawed in a 37°C water bath, pooled into a 50 mL centrifuge tube (Corning 430921) and pre-warmed complete media was added to a final volume of 50 mL. Cells were then centrifuged at 250 x g for 5 minutes, the supernatant aspirated, and the cell pellet resuspended in another 50 mL of pre-warmed media. This inoculum was enumerated and diluted to the required cell density in a total of 150 mL media up to 15 minutes prior to inoculation.

Reactor Setup and Inoculation

Bioreactor culture was performed in 3 replicates with a Corning Ascent FBR System 5 (Corning 6970) equipped with a Corning Ascent FBR 1 m^2 bioreactor (Corning 6971). At least 2 hours prior to inoculation, 2L of DMEM media with 10% FBS, 4 mM L-glutamine, and 1X penicillin-streptomycin was added to a 3L Erlenmeyer flask (Corning 6982) in a biosafety cabinet (BSC), aseptically connected to the media addition line, and 550 mL of complete media was transferred to the Ascent media conditioning vessel (MCV). The Media Conditioning program was used during the batching process, which allows the media to reach the desired temperature (37°C), dissolved oxygen (100%) to stabilize, and inline sensors to properly hydrate, prior to sensor calibrations (Figure 1).

Following calibration, the Media Maintenance program values were set according to Table 1, and the system allowed to equilibrate for a minimum of 1 hour prior to inoculation. The cells were prepared as described previously and added to a Corning Ascent 500 mL storage bottle (Corning 6980) at a concentration of 1.47 x 10^6 cells/mL in a total volume of 150 mL in a BSC. Inoculum was then aseptically connected to the MCV and the entire volume transferred using an air pressure bulb. This resulted in a seeding density of 22,000 cells/cm^2 with a total system volume of 700 mL (0.07 mL/cm^2). The Inoculation Phase program was used with attachment flow rate set to 60 mL/min. for 5 hours then, over the course of an hour, reduced to 50 mL/min. During the Inoculation Phase, media samples were taken every 15 minutes for the first

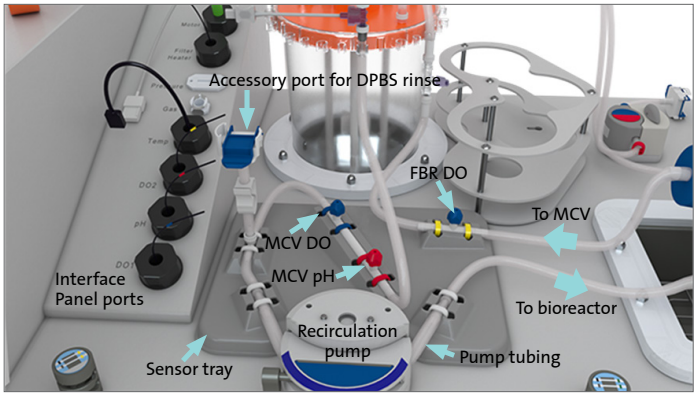

Figure 1. Connection point for additional DPBS rinse during harvest. *Actual instrument component appearance may differ from image.*

Table 1. Media Maintenance Phase set-up.

MCV TIC Setpoint	37°C
BRV TIC Setpoint	37°C
MCV DO Setpoint	100%
BRV DO Setpoint	20%
Min CO_2%	4%
pH Setpoint	7.2

hour, then every 30 minutes thereafter until >60% of cells were attached. After 4 days, the Media Addition Phase program was initiated to automatically add an additional 400 mL of media to the bioreactor, resulting in a system volume of 1100 mL (0.11 mL/cm^2). Samples were taken daily to measure nutrients, metabolites, and pH for a total culture duration of 7 days. Duplicate TC-treated T-75 flasks (Corning 430641U) were inoculated as controls using the same seeding stock and density as the FBR in 15 mL of complete media.

Harvest

On the final day of culture, an Ascent Harvest Consumable with 1L wash bottle (Corning 6986) was prepared by adding 500 mL of Accutase® cell detachment solution (Corning 25-058-CI) into the Harvest solution bottle and 300 mL of DPBS (Corning 21-031-CM) with 25 U/mL Benzonase (MilliporeSigma 71205-3) in the harvest wash bottle in a BSC. The filled bottles were then aseptically connected to the Ascent FBR while the tubing was loaded into the appropriately color-matched pinch valves on the front of the instrument. The outlet of the Ascent Harvest Consumable was connected to a Corning Ascent 2L roller bottle (Corning 6984) via the connect/disconnect port. A 500 mL accessory bottle (Corning 6980) was filled in the BSC with 300 mL of DPBS and connected to the aseptic port on the Ascent sensor tray (Figure 1).

To prepare for cell harvest, the fluid path was switched from the MCV-FBR recirculation loop to DPBS accessory bottle attached to the sensor tray. 300 mL DPBS was flushed through the FBR and back into the MCV in preparation for the introduction of Accutase to the mesh substrate. The automated harvest process was then initiated using the Harvest Prep Phase program on the HMI. Clamp and tubing configuration was switched to open the flow path through the FBR to the Harvest Consumable kit. To avoid dilution of the harvest reagent, 150 mL of Accutase was pumped through the FBR to the harvest waste bottle using the Harvest Wash Phase program whereby the Accutase pushes out the remaining volume of DPBS rinse. Next, the Cell Release Phase program was initiated with a flow rate of 150 mL/min. for 40 minutes to allow Accutase to flow through the FBR and dissociate the cells from the mesh substrate.

Cells released from the mesh substrate during Accutase recirculation were collected via pressurized air using the automated Cell Removal Phase program. During this step, the cell-saturated harvest reagent, along with any loosely attached cells on the mesh, are flushed out of the FBR and into the harvest collection bottle. Following collection of the Accutase fraction, the FBR was rinsed with 300 mL DPBS containing 25 U/mL Benzonase from the harvest wash bottle at a flow rate of 150 mL/min. for 15 minutes. Cell Removal Phase was repeated and the DPBS + Benzonase fraction collected into the same harvest collection bottle. Total harvest volume was measured, and cells were enumerated to calculate final cell density (cells/cm^2) and population doubling times.

Harvest efficiency was calculated as the percentage of cells removed by the automated process vs. cells harvested manually from mesh disks removed from the FBR after auto-harvest was completed. To manually harvest disks, two mesh disks were removed from various sections (top, middle, and bottom) of the FBR and placed in 100 mm cell culture dishes (Corning 430591), each containing 15 mL of Accutase. Dishes were incubated for 30 min. at room temperature with gentle agitation followed by pipetting of the Accutase across the surface of the disks to dislodge any remaining cells. The harvested cells were transferred to a 50 mL conical tube, then combined with 10 mL of DPBS used for a final rinse of the mesh prior to enumeration. Cell density was calculated using 75.85 cm^2 as the approximate surface area, based on computer modeling, of each disk and extrapolated to the 1 m^2 surface area of the FBR to estimate total remaining cells following automated harvest. High efficiency and viability in the automated harvest process are critical to maximize yields and reduce operator time in cell recovery for downstream processes.

Results and Discussion

Cell attachment in the Ascent FBR system ranged between 60 to 75% of the inoculation density within a 200-minute period and is calculated by the difference between the initial cell density and the measured density at each timepoint. Attachment in 2D controls averaged 50% after 24 hours despite 98% viability upon thaw. Unattached cells, in both 2D and the Ascent FBR system, remained suspended in the media.

Cell density upon harvest in the Ascent FBR system was 120 ± 12.6K cells/cm^2 compared to the 2D control at 154 ± 21.8K cells/cm^2 (Figure 2A). Doubling time in the Ascent FBR system was calculated to be 66.6 ± 4.0 hours compared to the 2D control at 58.3 ± 4.2 hours (Figure 2B) using the inoculation density of 22,000 cells/cm^2, cell density at harvest, and duration of culture. The difference between Ascent FBR system and 2D controls is not statistically significant for both cell density and doubling time ($p>0.05$). Viability on harvest was 96.5 ± 2.4% in the Ascent FBR system and 81.2 ± 3.7% in the control flasks, with the lower flask viability attributed to over-confluence (Figure 2C). The average automated cell harvest efficiency was 99.7% in the Ascent FBR system.

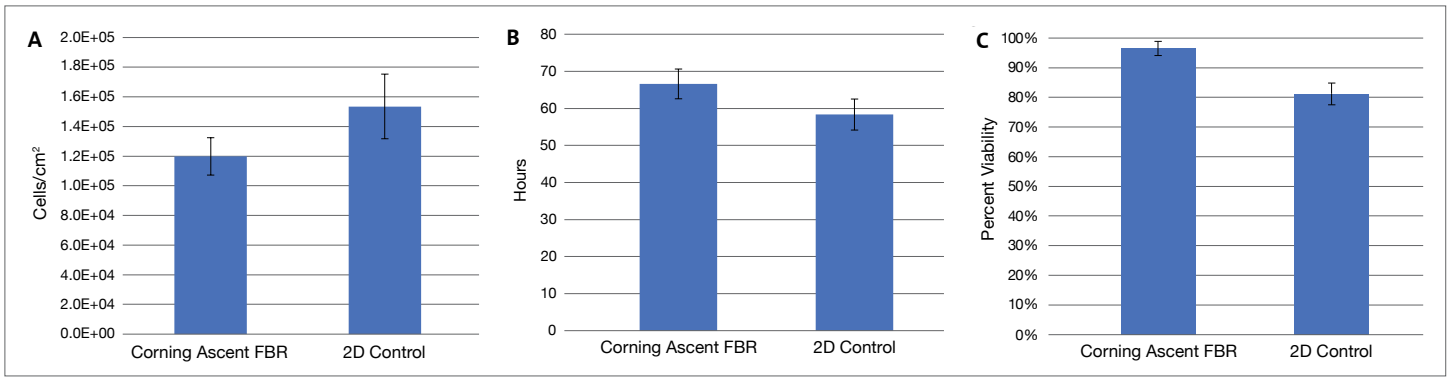

Figure 2. Cell yield on day 7 harvest (A), doubling time (B), and viability (C). Error bars are standard deviation with n=3.

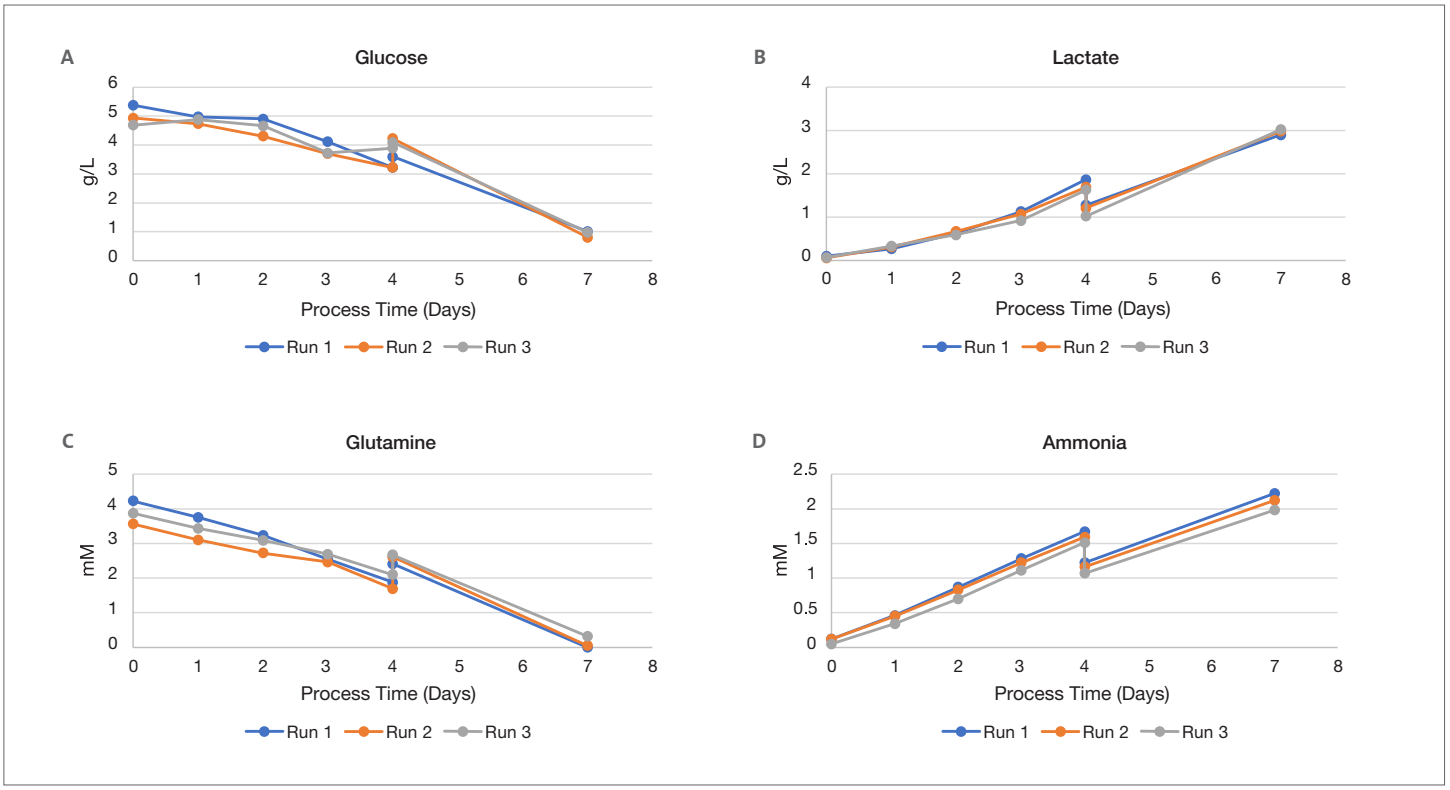

Figure 3. Measured metabolite values. **NOTE:** Day 4 post-feed timepoint was calculated for Run 2.

Several metabolites (glucose, lactate, glutamine, and ammonia) were measured and closely matched for each run (Figure 3). While glucose was maintained near 1 g/L on harvest day, glutamine was near depletion, indicating that if the duration culture were to be extended, additional feed would be required to maintain the cells in the Ascent FBR system. Lactate and ammonia remained within commonly allowable concentrations for each replicate. System pH was maintained at the setpoint and DO trends were similar for all replicates, where MCV DO was held stable at 100% and FBR DO gradually approached the 20% setpoint as cell density increased.

Conclusions

The Corning® Ascent® FBR System 5 supports expansion of HEK293 directly from cryopreserved cell banks with similar cell yield compared to 2D controls. Aided by the automated process steps built into the HMI, the Ascent FBR System 5 facilitates ease of use and consistency between replicate experiments. The process described herein shortens seed train timelines, increases consistency in cell yield and with the automated features offered by the Ascent FBR System 5, makes direct inoculation from cryopreserved cell banks an attractive option for experiments, seed trains, or production batches.

References

1. Heidemann R, et al. A new seed-train expansion method for recombinant mammalian cell lines. Cytotechnology 38.1 (2002): 99-108.
2. Lünse S, Heidemann R, and Zhang C. Optimization and characterization of the process for large volume cell banking in bags, cells, and culture. Springer, Dordrecht, 2010. 665-668.

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