Intensified AAV Production With TFDF®-based Perfusion Cell Culture

Application Note

Summary

Previous attempts failed to increase the production of adeno-associated virus (AAV) by increasing the cell density at transfection (or infection) with batch cell culture.

This study demonstrates that 3-fold intensified perfusion cell cultures, using the Tangential Flow Depth Filtration (TFDF®) cell retention technique, lead to 3 to 10-fold increased production of AAV6, 8, or 9 compared to batch cell cultures.

Increasing the AAV production through the implementation of TFDF-based process intensification will help meet the high global demand and reduce the cost for viral vectors and enable high accessibility to next generation gene therapies.

Introduction

The demand for adeno-associated virus (AAV) viral vectors has been steadily increasing due to their widespread use in gene therapy and other biomedical applications. Efficient and scalable production methods are crucial to meet these growing needs. Current low cell density cell culture processes (1 – 3 x 10^6 cells/mL at time of transfection, induction, or infection) provide low virus yield that is not sufficient to meet the global demand. Cell culture intensification has been proposed as a solution to increase virus production, with the assumption that having more cells will lead to more virus produced. Several studies have shown that intensified cell cultures, where high cell density at time of transfection (or infection) is used, lead to decreased cell specific virus productivity (Bernal *et al.*, 2009; Le Ru *et al.*, 2010; Lavado-Garcia *et al.*, 2020). It is still unknown if this phenomenon, called the cell density effect (CDE), is affecting virus production as a result of cell nutrient deprivation, accumulation of toxic metabolites, or both (Lavado-Garcia *et al.*, 2022). Perfusion cell culture techniques, providing continuous addition of nutrients and removal of waste metabolites, could prevent or at least limit the CDE. With filtration-based perfusion cell culture techniques, the cells continuously circulate from the bioreactor to a filter with pore size allowing for cell retention, and back to the bioreactor. Fresh media is continuously added to the bioreactor, while the spent media is collected on the permeate side of the filter, at the same flow rate as the fresh media addition. These techniques have been successfully used to intensify cell cultures to produce recombinant proteins and monoclonal antibodies for decades. Viral vector production also benefits from these process intensification methods.

In this application note, we present a study where a perfusion-like cell culture in 25 – 30 mL working volume shake flasks was first used as a small-scale model to investigate the role of perfusion for AAV8 and 9 productions at high cell densities. AAV production was obtained from cultures at 9 x 10^6 cells/mL with a daily complete media change (CMC) performed before & after transfection, or before transfection only. The virus production was compared to that of batch low cell density (3 x 10^6 cells/mL). To further confirm the effect of perfusion on virus production, the process was then transferred to 1.5 – 2 L TFDF-based perfusion cell cultures in single-use bioreactors to produce AAV6, 8 and 9 at high cell densities (9 x 10^6 cells/mL). The TFDF technology utilizes a 2 – 5 µm pore size depth filter, operated in tangential flow mode, to retain cells recirculating in the bioreactor and remove waste metabolites and secreted vector from the cells' environment. The TFDF-based perfusion process was used before and after transfection or before transfection only. The study compared the virus production achieved from each perfusion strategy to that of a low cell density (3 x 10^6 cells/mL) batch cell culture process.

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Materials and Methods

Batch, Perfusion-Like Shake Flask and Perfusion Bioreactor Cell Cultures

Expi293F™ cell lines (Thermo Fisher, Cat# A14527) were thawed and sub-cultured every three to four days for at least three passages, until the viability & doubling time were stable with viability ≥95% and doubling time consistent at ≤24 hours before performing experiments. Expi293 expression medium (Thermo Fisher, Cat# A1435102; shake flasks experiments) or BalanCD HEK293 medium (Fujifilm Irvine Scientific, Cat# 94137-10L; bioreactor runs) was used for cell passaging, seed train, cell expansion, and virus production. Viable cell density (VCD), cell viability, and metabolites testing were performed using a BioProfile® Flex2 Analyzer (Nova® Biomedical).

The inoculum cell culture and perfusion-like CMC studies were conducted in a shake flask with incubator conditions set at 37°C, 8% CO_2, 125 ± 5 rpm for a shaker diameter of 19 mm. Seeding density targeted 0.3 – 0.5 x 10^6 viable cells/mL for inoculum preparation. The target density for perfusion-like shake flask studies was ~0.4 x 10^6 for batch or ~1.1 x 10^6 viable cells/mL with >95% viability. Daily perfusion-like CMC started on Day 2 after inoculation when VCD reached ~4.0 x 10^6 cells/mL. Briefly, cells were centrifuged at 500 g for five minutes, media was removed, and fresh media was added to resuspend the cells. Daily CMC continued for the entire process for perfusion-like CMC in before & after transfection shake flasks or CMC was stopped for perfusion-like CMC in before transfection only shake flasks. Shake flask sampling for virus production was performed at the end of the production process.

BioBLU® 3c Single-Use Vessels with macrosparger and two pitched blade impellers (Eppendorf®, Cat# 1386121000) were used with BioFlo® 320 Controllers (Eppendorf, Cat# 1379963011) for bioreactor cell expansion and virus production. Agitation was set at 200 rpm, temperature at 37°C, and pH at 7.0 ± 0.2 controlled with CO_2. Dissolved oxygen (DO) was set at 50% and was supplemented by macrosparging using the 3-gas auto mixture of air, O_2, and CO_2. The total constant gas flow rate was set at 0.3 L/min. Bioreactors were inoculated to a seeding density of 0.4 X 10^6 cells/mL for the batch runs and 1.1 X 10^6 cells/mL for TFDF runs to perform the transfection on the same day. The TFDF-based perfusion runs were achieved using a KrosFlo® TFDF Lab System (Repligen, Part Number TFDFLP2S2F1T0NCFRS) equipped with a TFDF-30 ProConnex® TFDF Flow Path (Repligen, Cat# STFDFCL15546S). The perfusion process started at 1.0 vessel volume per day (vvd) on Day 2 after inoculation when VCD reached ~4.0 X 10^6 cells/mL. The perfusion was performed either both before & after transfection or before transfection only. Daily sampling to monitor VCD, viability, metabolites, and virus titer was performed throughout the process.

Transfection

A triple transfection to produce AAV was performed on day 3 after inoculation, at VCD of 3.0 X 10^6 cells/mL for the batch run and 9.0 x 10^6 cells/mL for the TFDF-intensified/perfusion-like CMC runs, using the following plasmids, DNA: 1 x 10^6 cells, and DNA: transfection reagent ratio:

- AAV6: pDNA AAV6-GFP, AAV6 Rep/Cap, pHelper (VectorBuilder)
- AAV8: pDNA AAV8-GFP, AAV8 Rep/Cap, pHelper (VectorBuilder)
- AAV9: pDNA AAV9-GFP, AAV9 Rep/Cap, pHelper (VectorBuilder)
- DNA (µg): 1 x 10^6 cells (mL) = 0.75
- DNA (µg): transfection reagent (µL) = 1

The DNA was mixed with either (i) VirusGEN® SELECT AAV kit transfection solutions (Mirus® cat# MIR 6770) for AAV8 and 9 production or (ii) DMEM (Gibco™, Ref# 10566-016) and FectoVIR® AAV (Polyplus® cat# 101000022) for AAV6 virus production. The complex was mixed for 15 – 30 minutes prior to adding to cell culture vessels. For perfusion before and after transfection runs, after adding transfection complex, perfusion was stopped for 2 hours and continued for the duration of the viral vector productions.

Monitoring of Virus Production

The production of AAV was monitored daily for 3 – 4 days post-transfection by taking samples from inside the shake flask or bioreactor (batch and perfusion or perfusion-like CMC runs), and from the TFDF-permeate line and harvest bottle (perfusion runs

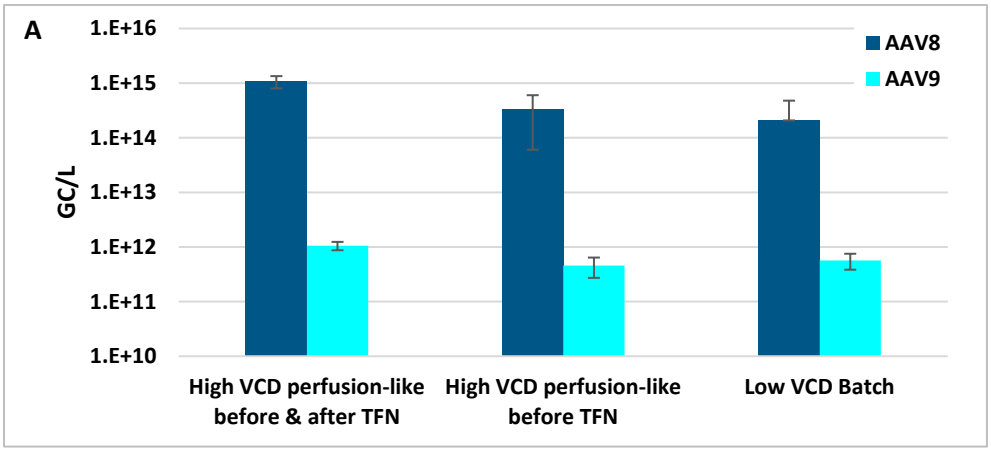
only). Samples were centrifuged for 10 minutes at 500 g and the supernatants were kept for analytical assays for AAV8. For AAV6 and AAV9, samples were lysed with 50 mM Tris (Corning™, Cat# 77-86-1), 1% Tween 20 (VWR Cat# 9005-64-5) and 2 mM of $MgCl_2$ (VWR, Cat# E525-500ML) for 1 hour at 37°C.

Genomic titrating for AAV was obtained via ddPCR using QX200™ Droplet Digital PCR System (Bio-Rad Laboratories) and following the vendor's instructions.

Results and Discussion

Role of Perfusion

A perfusion-like cell culture using a CMC strategy in shake flasks or TFDF perfusion bioreactors was used to investigate the role of perfusion in AAV8 and AAV9 production. Virus production results from shake flasks and bioreactors are presented in Figure 1. In shake flasks, perfusion-like CMC enabled intensified cell growth to 3-fold higher viable cell density (9.0 x 10^6 VCD) before transfection compared to a batch control (3.0 x 10^6 VCD). The CMC strategy, with the daily replacement of used media by fresh media, prevented nutrient deprivation, removed potential growth inhibitory factors, and, therefore, kept the cell culture in the logarithmic growth phase. High cell density perfusion-like daily CMC performed before and after transfection produced 1.1 x 10^{12} AAV9 genome copy (GC) per liter in shake flask while high VCD perfusion-like CMC conducted before transfection only, and low VCD batch control produced 4.6 x 10^{11} and 5.7 x 10^{11} GC/L AAV9, respectively (Figure 1A). Similarly, AAV8 production in high cell density perfusion-like CMC before & after transfection was 1.1 x 10^{15} GC/L, but high cell density perfusion-like CMC before transfection only and low cell density batch control were 3.3 x 10^{14} GC/L and 2.1 x 10^{14} GC/L, respectively (Figure 1A). High cell density perfusion-like daily CMC before and after transfection enabled an increase of 2-fold AAV9 and 4-fold AAV8 production compared to a batch control. A similar cell specific productivity (GC/cell) was therefore achieved at high cell density compared to low density batch, which indicates the media replacement limited CDE. But perfusion-like CMC before transfection only showed a batch comparable AAV production indicating CDE was not prevented, as reflected by lower cell specific virus productivity (GC/cell). To maintain the cell specific productivity at high cell density the process needs fresh nutrients and removal of waste metabolites before and after transfection.



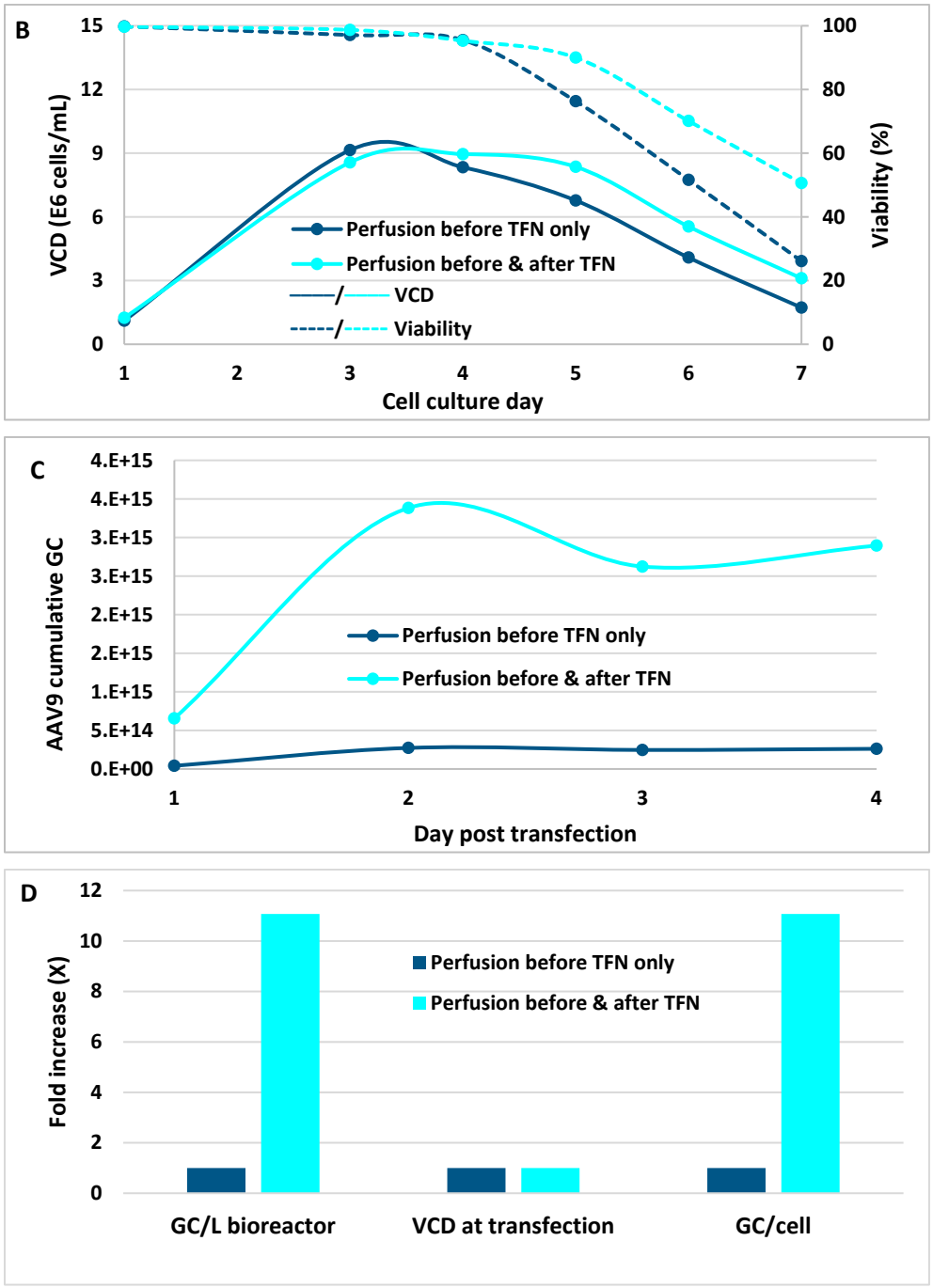


Figure 1. AAV Production in Perfusion-Like CMC Shake Flasks and TFDF Perfusion Bioreactors. A) AAV8 and AAV9 GC production per liter of media (in shake flask). B) HEK293 cell growth and viability in bioreactor for AAV production using perfusion before & after transfection vs perfusion before transfection only. C) Cumulative AAV9 production in bioreactor using perfusion before & after transfection vs perfusion before transfection only. D) Compared performance of AAV9 production in bioreactor from perfusion before & after transfection vs perfusion before transfection only. TFN: transfection, Data are average from three shake flask runs. Error bars: +/- standard deviation.

To confirm the shake flask perfusion-like CMC study observation, the virus production achieved from TFDF perfusion bioreactors of perfusion before transfection only or perfusion before and after transfection were compared. Figure 1B shows the cell growth and viability data for AAV9 production from two bioreactors seeded at 1.1 x 10^6 cells/mL with >95% viability. Perfusion before

transfection only was applied for one bioreactor run, while the perfusion process was before and after the transfection for the other bioreactor. Perfusion increased the cell density to ~9.0 x 10^6 cells/mL for both bioreactors on Day 3 of cell culture, when the transfection was performed. Post transfection VCD and viability decreased sharply for the bioreactor with perfusion before transfection only compared to the one with perfusion before and after transfection. At the end of the virus production process, the VCD was 1.7 x 10^6 cells/mL with 26.1% viability for the bioreactor with perfusion before transfection only, while the perfusion before and after bioreactor had 3.1 x 10^6 cells/mL VCD and 50.6% viability. These data suggest that perfusion before and after transfection through the addition of fresh media and/or the removal of waste metabolites better maintains VCD and viability, even as cells start producing cytotoxic products (van der Loo JC and Wright JF, 2016). This cytotoxicity is not well understood, but the higher cell density and viability observed when perfusion is maintained after transfection suggest that combining continuous nutrient addition and removal of waste metabolites can limit cell death during virus production. The cumulative AAV9 GC production for perfusion before and after transfection bioreactor at the end of the run was 2.9 x 10^{15} GC while perfusion before transfection only yielded 2.6 x 10^{14} GC **(**Figure 1C).

Data from both daily CMC in shake flasks and TFDF perfusion in bioreactors strongly indicate that perfusion cell culture can intensify the AAV vector production, but a perfusion before and after transfection is required to provide such an achievement (Figure 1D).

AAV Production With Optimized TFDF-Intensified Compared to Batch Cell Culture in Bioreactor

After defining that perfusion before and after transfection provides optimum benefits, additional TFDF perfusion runs were conducted and compared to batch cell culture runs to produce AAV9, 8 and 6 viral vectors.

Production of AAV9 From TFDF-Intensified and Batch Cell Cultures in Bioreactor

Cell growth and viability data for AAV9 production from batch and TFDF-intensified bioreactors are presented in Figure 2. Bioreactors were seeded at 0.4 x 10^6 and 1.3 x 10^6 cells/mL with >95% viability. TFDF perfusion culture enabled a higher VCD (8.6 x 10^6 cells/mL) compared to batch (3.3 x 10^6 cells/mL) on Day 3 of cell culture (Figure 2A). At that time, with cell viability >95% for both bioreactors, the triple plasmids transfection for AAV9 production was performed. After transfection, VCD slightly increased and then started decreasing for both batch and TFDF-intensified processes. The viability started decreasing right after transfection for both bioreactors, but the perfusion before and after transfection with the TFDF-intensified process enabled a higher cell viability (41%) than batch cell culture (33%) at the end of the runs (Day 6 of cell culture, 3 days after transfection).

The cumulative GC production of AAV9 at the end of processes was 6.7 x 10^{14} GC and 2.3 x 10^{15} GC for batch and TFDF-intensified bioreactors, respectively (Figure 2B). The TFDF-intensified process yielded a 3-fold increase in total GC production compared to batch bioreactor, which resulted from a 3X VCD increase and a similar cell-specific GC productivity (Figure 2C).

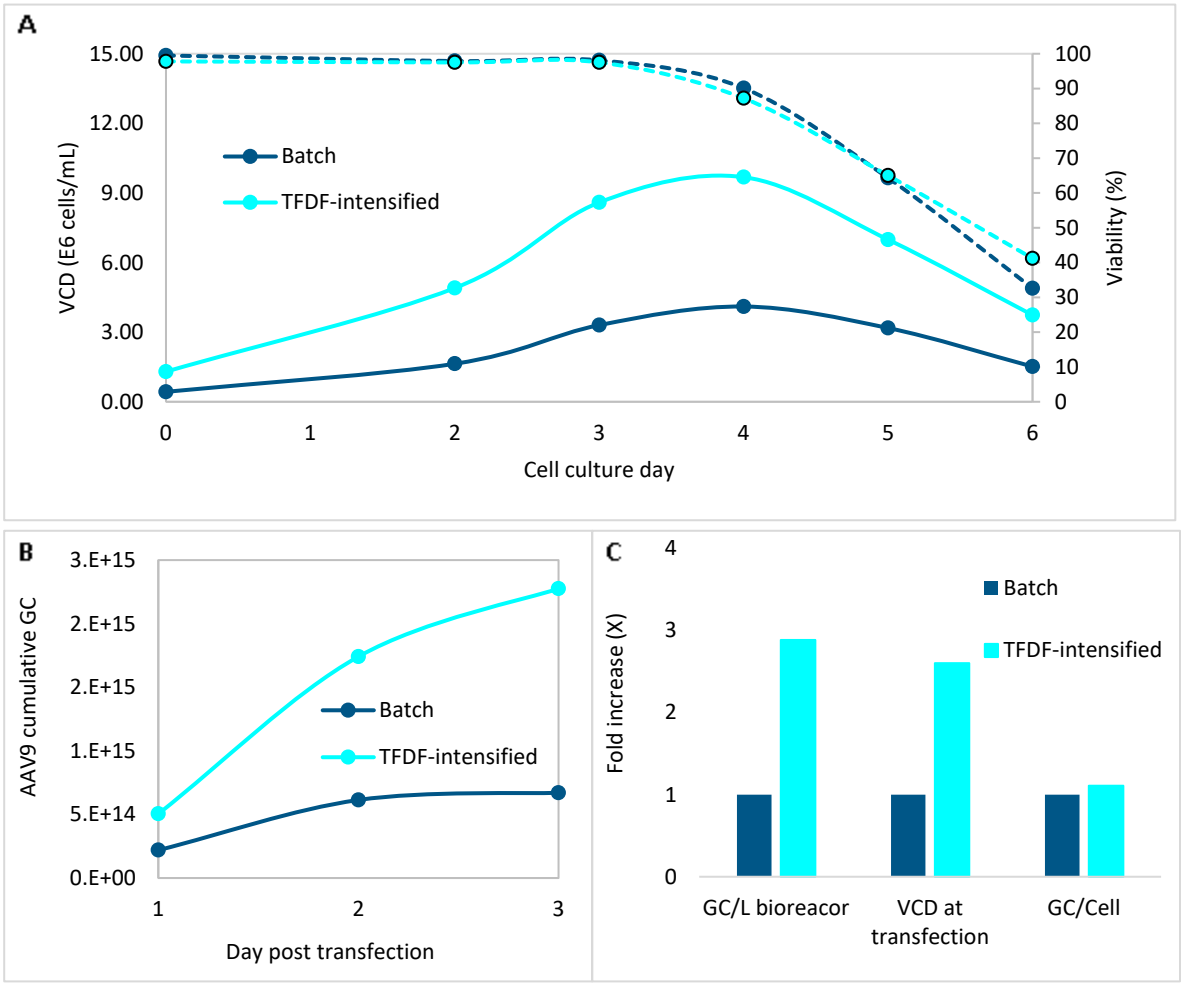


Figure 2. Production of AAV9 From TFDF-Intensified and Batch Cell Cultures in Bioreactor. A) HEK293 cell growth and viability for AAV9 production in batch vs TFDF-intensified bioreactor. B) Compared AAV9 cumulative GC production from batch vs TFDF-intensified bioreactor runs. C) Compared performance of AAV9 production from batch vs TFDF-intensified bioreactor runs.

Production of AAV8 From TFDF-Intensified and Batch Cell Cultures in Bioreactor

Three batch and three TFDF-intensified cell culture AAV8 production runs were performed, following the same process as for AAV9 production, resulting in a 3-fold TFDF-intensified process compared to batch. Figure 3A shows the average batch and TFDF perfusion bioreactors cell growth and viability data for AAV8 production. After transfection, conducted on Day 3 of cell culture, the batch VCD and viability trended similarly to that observed for AAV9 production. For the TFDF-intensified runs, both VCD and viability dropped one day after transfection, but then remained almost stable until the end of the virus production phase (10 x 10^6 cells/mL VCD and 71% viability at the end of the process). These data demonstrate that, for AAV8 production, the perfusion process was even better at preventing cell death than for AAV9 production.

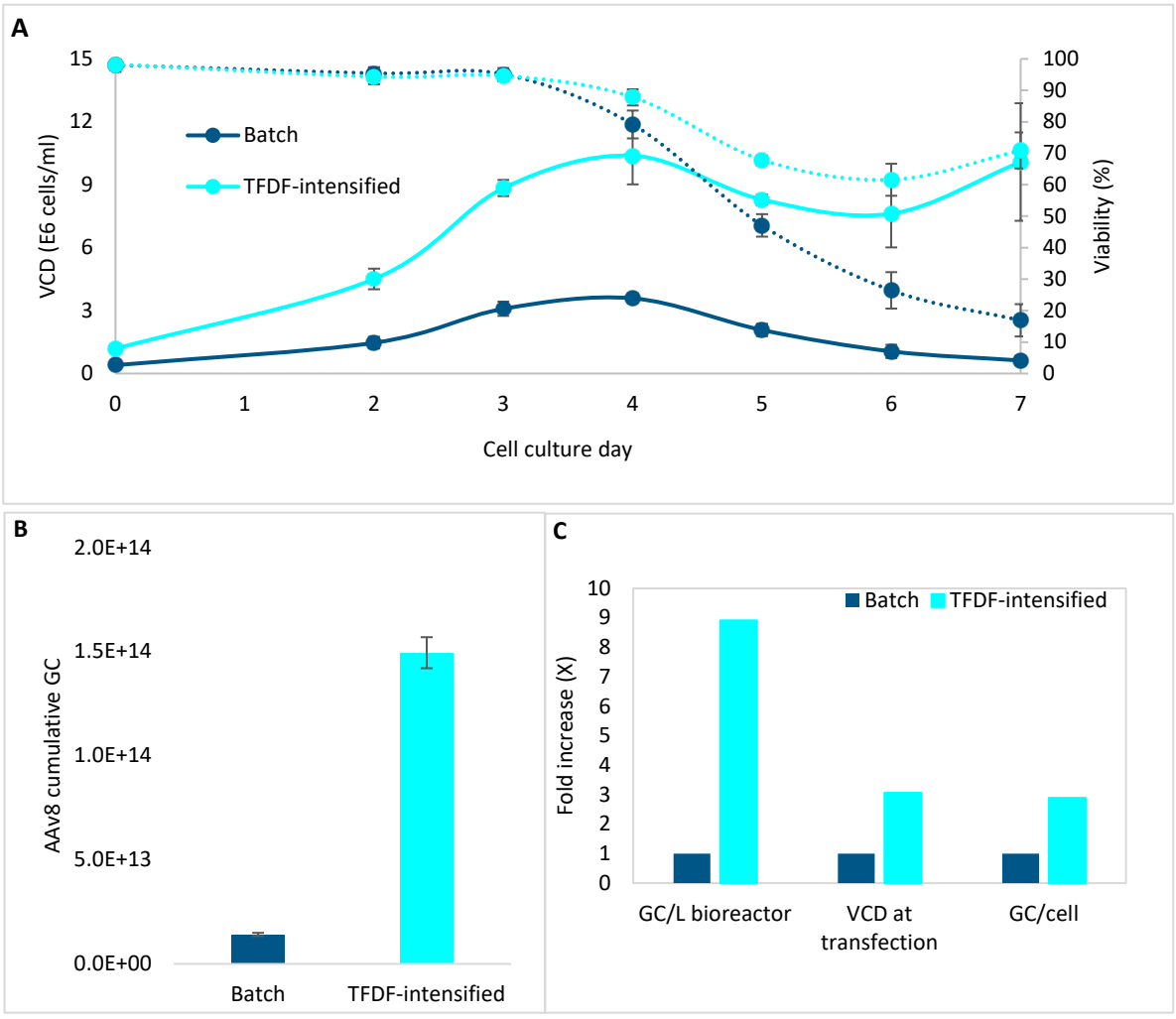


Figure 3. Production of AAV8 From TFDF-Intensified and Batch Cell Cultures in Bioreactor. A) HEK293 cell growth and viability for AAV8 production in batch vs TFDF-intensified bioreactor. B) Compared AAV8 cumulative GC production from batch vs TFDF-intensified bioreactor runs. C) Compared performance of AAV8 production from batch vs TFDF-intensified bioreactor runs. Data are average from three runs +/- standard deviation (error bars).

The cumulative AAV8 GC production at the end of the process was 1.4 x 10^{13} GC and 1.5 x 10^{14} GC for batch and TFDF-intensified bioreactors, respectively (Figure 3B). TFDF-intensified bioreactors generated a 10-fold increase in total GC production, which resulted from the three times higher VCD and a higher than 3-fold increase in cell specific virus productivity (Figure 3C).

Production of AAV6 From TFDF-Intensified and Batch Cell Cultures in Bioreactor

Figure 4A shows the batch and TFDF perfusion bioreactors cell growth and viability data for AAV6 production. As for AAV9 and 8 productions, the batch and TFDF perfusion bioreactors were seeded at 0.4 x 10^6 and 1.1 x 10^6 cells/mL, respectively, with >95% viability. TFDF perfusion culture enabled a 2.5-fold higher VCD (7.1 x 10^6 cells/mL) compared to batch (2.8 x 10^6 cells/mL) with >95% viability on transfection day (Day 3 of cell culture, Figure 4A). After transfection, both VCD and viability started dropping for batch and TFDF-intensified processes in a similar way to those of AAV9 production runs. At the end of the run, VCD (1.8 x 10^6 cells/mL) and viability (47%) for batch were higher than measured for AAV9 production (1.5 x 10^6 cells/mL VCD and 33% viability). These data

indicate the production of AAV6 could have less impact on cells than AAV9 production. Still, the final VCD (1.8 x 10^6 cells/mL) and viability (71%) from the TFDF perfusion run were higher than for batch, confirming the positive impact of perfusion on cell health.

The cumulative AAV6 GC production at the end of production processes was 7.7 x 10^{13} GC and 2.8 x 10^{14} GC for batch and TFDF-intensified bioreactors, respectively (Figure 4B). The TFDF-intensified process produced a close to 4-fold increase in total GC production compared to batch bioreactor, resulting from a 2.5-fold VCD increase and a 1.4-fold increase of cell-specific virus productivity (Figure 4C). These data further confirm the ability to successfully intensify the virus production from a TFDF-based perfusion cell culture process.

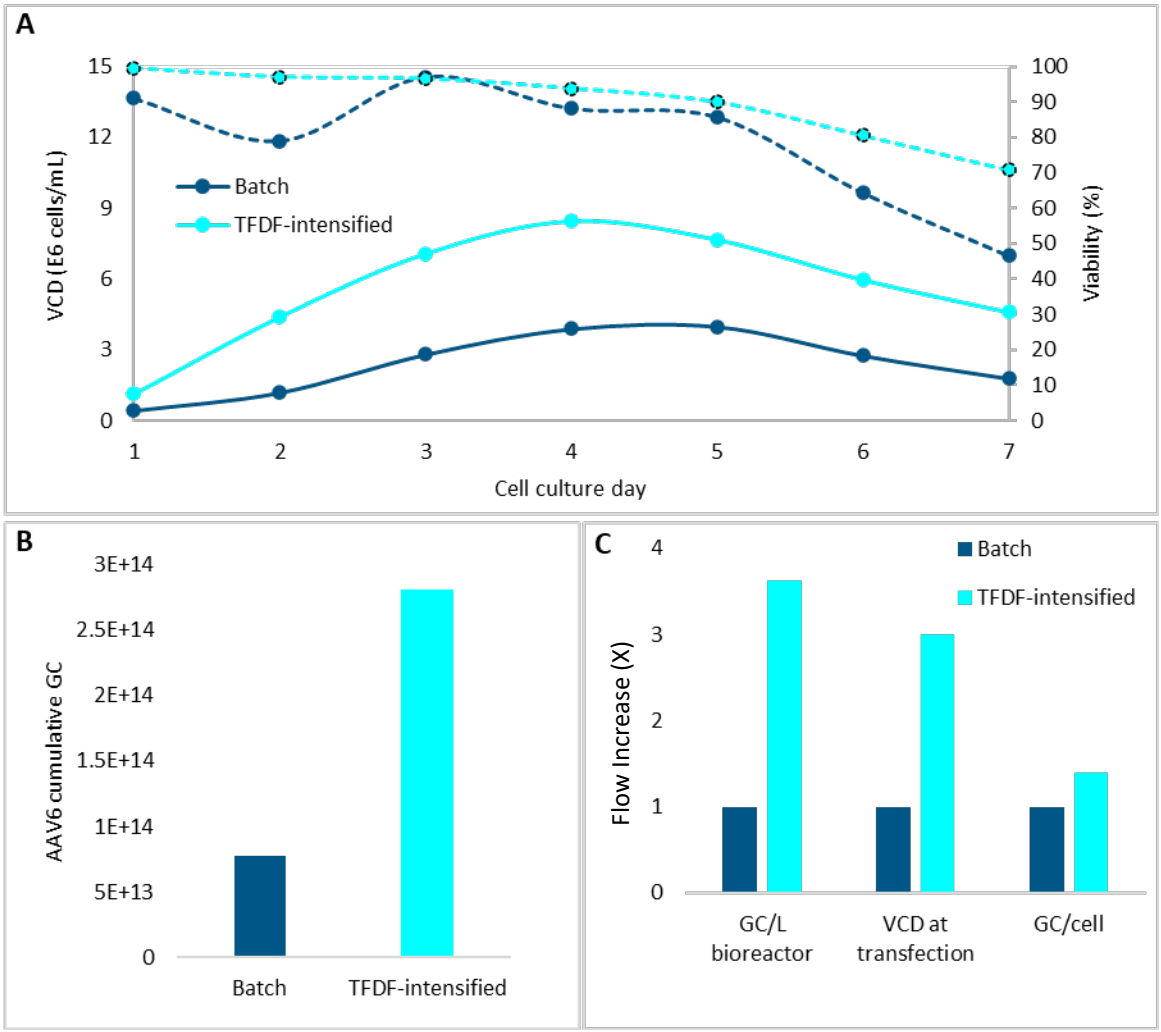


Figure 4. Production of AAV6 From TFDF-Intensified and Batch Cell Cultures in Bioreactor. A) Batch vs TFDF-intensified bioreactor VCD and viability data. B) Batch vs TFDF-intensified bioreactors AAV6 cumulative GC production. C) Batch vs TFDF-intensified bioreactor fold increase (X) of GC/L of bioreactor, VCD at transfection and GC/cell.

Conclusion

The implementation of TFDF-based perfusion cell culture for viral vector production can limit the cell density effect and, therefore, intensify the virus production compared to current batch processes. To achieve this, a perfusion process before and after transfection is required. TFDF-based perfusion enabled 3 to 10-fold AAV6, 8 and 9 viral vector production intensification compared to a batch culture.

The implementation of such TFDF-based perfusion processes can significantly boost the viral vector production and can help meet the global demand to make gene therapies accessible for a large patient population.

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Customer Service

Repligen Corporation
99 South St
Hopkinton, MA 01748

customerserviceUS@repligen.com

(508) 845-6400

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