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# Impact of N-1 Seed Train Intensification on Viral Vector Production

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## Introduction

The demand for gene therapy viral vectors requires manufacturing processes to scale up to 2000 L and, preferably, to use single-use technologies. To produce viral vectors at this scale, a 9- to 12-day seed train using two to three seeding bioreactors (i.e., 20, 50, 100, and 200 L) is needed from the cell bank to the final production bioreactor, leading to increased risk of failure and high cost of manufacturing.

Perfusion-based cell culture intensification techniques are proven to shorten and simplify (fewer seeding bioreactors) the seed train for production of recombinant proteins and monoclonal antibodies. This results in significant time and cost savings, enabling the production of more batches per year, using less equipment and smaller size seed bioreactor volumes. The intensification of viral vector production will provide the same benefits for gene therapy (Rene Gantier, 2021).

In this study, Repligen TFDF® (Tangential Flow Depth Filtration) Technology is used in perfusion mode to intensify the N-1 seed culture of HEK293 cells to two and three times higher than the normal viable cell density. The intensified N-1 seed cultures were used to seed standard batch production-stage cultures to determine the effect on cell growth, viral production, and product quality. The standard batch cell cultures were used to produce lentivirus (LV) and adeno-associated virus (AAV, serotypes 8 and 9) particles. The quantity and quality of the virus produced from the intensified process were compared to those from a standard batch process with a control N-1 cell culture. TFDF® Technology functions as a single-use, gamma-irradiated, closed cell retention device. The technology is scalable from 2 L to 2000 L for the N-1 application and has a small manufacturing footprint of approximately 1 square meter. TFDF® Technology is recommended for production stage intensification and clarification of viral vectors and for N-1 seed train intensification, thus enabling one technology for multiple steps in an intensified upstream manufacturing process.

## Materials and methods

### Cell line, media, and seed expansion

Expi293F™ cell lines (Thermo Fisher, cat# A14527) were used for the expression of three viral vectors: AAV8, AAV9, and lentivirus. This cell line is commonly used for viral vector cell culture and maintains cells in log phase in batch mode at 3 – 5 x $10^6$ cells/mL VCD. This study will push the VCD seen with this cell line using perfusion to keep viability high. The cells were thawed and sub-cultured every three to four days for at least three passages, until the doubling time was stable and consistent at ≤ 24 hours before transfection. Expi293™ Expression Media (Thermo Fisher, cat# A1435102) was used for cell passaging, and incubator conditions were set at 37° C, 8% $CO_2$, 125 ± 5 rpm for shaker diameter of 19 mm. Seeding density targeted 0.3 - 0.5 X $10^6$ viable cells/mL. Target density was 3.0 - 5.0 X $10^6$ viable cells/mL at > 95% viability (≤ 5.0 X $10^6$ viable cells/mL is required to stay in log-phase growth). Cell counting and metabolites testing were performed using a BioProfile® Flex 2 Analyzer (Nova® Biomedical), and cell viability using a Vi-CELL™ XR Cell Viability Analyzer (Beckman Coulter).

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### N-1 seed train bioreactor, culture conditions, and production cultures

BioBLU® 3c Single-Use Vessels with macrosparger and two pitched blade impellers (Eppendorf, cat# 1386121000) were used with BioFlo® 320 Controllers (Eppendorf) for cell cultivation. 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% air saturation 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. Daily sampling to monitor VCD, viability, and metabolites was performed through day of harvest.

The bioreactor was harvested at three time points and at targeted cell densities: 5 X $10^6$ (control), 10 X $10^6$ (2X intensified) and 15 X $10^6$ (3X intensified) cells/mL VCD to inoculate 125 mL shake flasks (Corning®, cat# 431143) for lentivirus and AAV8 (n=3) production at 0.15 X $10^6$ cells/mL. For AAV9, two BioBLU® 3c Single-Use Vessels with macrosparger and two pitched blade impellers (Eppendorf, cat# 1386121000) were used in conjunction with BioFlo® 320 Controllers (Eppendorf) for production. Bioreactors were inoculated to a seeding density of 0.15 X $10^6$ cells/mL using two different VCDs: 1) 5 X $10^6$ VCD (control) and 2) 15 X $10^6$ (3X intensified) cells/mL VCD. The AAV9 production bioreactor conditions were the same as noted above for the N-1 vessel. The non-intensified N-1 seed train was harvested on Day 2 post inoculation to represent a typical, low VCD batch process. The second and third seed train collections were intensified using perfusion starting on Day 2. This was 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 rate was set at one vessel volume per day (vvd) throughout the remainder of the run with fresh Expi293 Expression Media being added and spent media being removed at the same rate. The reactor was harvested on Day 3 for the 10 X $10^6$ cells/mL VCD sample and Day 4 for the 15 X $10^6$ cells/mL VCD.

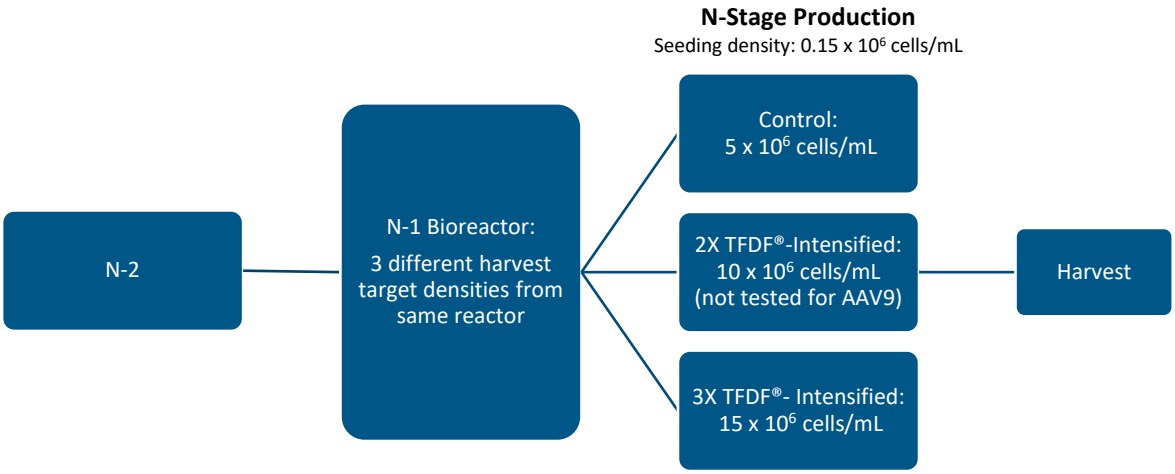


**Figure 1.** Study design for N-1 seed train intensification with batch N-stage production. Executed for 3 viral vectors: lentivirus (shake flasks), AAV8 (3x shake flasks), and AAV9 (bioreactors).


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### Transfection and harvest of production cultures

Production cultures grew to 2 X $10^6$ cells/mL prior to transfection. On day of transfection, VCD was used to calculate the amounts of pDNA and transfection reagents needed. The transfection complex was prepared per manufacturer instructions (Lentivirus: prototype reagent; AAV8 and AAV9: Trans-It VirusGEN® (Mirus®, cat# MIR 6705) with DMEM, high glucose, GlutaMAX™ Supplement (Thermo Fisher, cat# 11960044)). The following plasmid DNA were used for each transfection:

- Lentivirus: pALD-LentiEGFP-K RG, pALD-Rev-K RG, pALD-GagPol-K RG, pALD-VSV-G-K RG (Aldevron®)
- AAV8: pDNA AAV8-GFP, AAV8 Rep/Cap, pHelper (VectorBuilder)
- AAV9: pDNA AAV9-GFP, AAV9 Rep/Cap, pHelper (VectorBuilder)

The complex was mixed for 15 ± 5 minutes prior to adding to production vessels. For the benchtop bioreactors, the gassing strategy was switched to overlay prior to the addition of the transfection complex to avoid the negative effect of micro bubbles on transfection efficiency. After three hours, sparging resumed and continued for the duration of the production run.

Cell density, viability, and metabolites were tested throughout the production culture. Lentivirus was harvested 48 hours (Day 2) post-transfection. AAV8 was harvested 96 hours (Day 4) post-transfection. Production vessel supernatants, containing lentivirus and AAV8 viral vectors, were sampled. AAV9 was harvested 96 hours (Day 4) post-transfection and both the supernatant and cell pellet were sampled since ≥20% of AAV9 virus is intracellular. Cell lysis was performed by directly adding lysis buffer (0.5M Tris-HCl, pH 8.0, 20 mM magnesium chloride, 1% Tween®20) at a 1:10 dilution to the bioreactor and incubating at 37° C with agitation at 200 rpm for one hour.

### Methods for virus analysis

### Transduction Unit (TU) Assay - Lentivirus

Transduction unit titration for lentivirus was performed via a cell-based potency assay. HEK293A cells were seeded into a 24-well plate at 1 x $10^5$ cells/mL, and viral dilutions were added to the plates. A spin inoculation was performed via centrifugation at 2500 rpm for 90 minutes. The cells were incubated for 48 hours post-transduction and harvested for Fluorescence-Activated Cell Sorting (FACS) cytometry using a CytoFLEX Flow Cytometer (Beckman Coulter). The cell populations were gated for green fluorescent protein (GFP)-positive cells. The percentage of GFP-positive cells was calculated and used to back-calculate titer (TU/mL) with the following equation:

$$Titer\left(\frac{TU}{mL}\right) = \frac{\%\ of\ GFP\ positive\ cells\ \div 100}{volume\ of\ virulent} \times dilution\ factor \times cell\ number\ at\ time\ of\ transduction$$

### Droplet Digital Polymerase Chain Reaction (ddPCR) - AAV8 and AAV9

Genome titer for AAV was determined using ddPCR on a QX200™ Droplet Digital PCR System (Bio-Rad Laboratories). The samples were diluted, digested by DNase I endonuclease enzyme, prepped for PCR using master mix, and emulsified into individual water-oil based droplets. PCR was performed using a thermocycler, and droplets were read via QX200™ plate reader. The raw data was analyzed by QuantaSoft™ software from Bio-Rad. Titer was calculated by multiplying raw instrument readings by dilution factors.


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### ELISA methods - AAV8 and AAV9

AAV8 and AAV9 titration ELISA kits (Progen cat# PRAAV8XP and PRAAV9) and protocols were used to measure capsid titers. Color intensity was measured with a SpectraMax® i3x photometer (Molecular Devices cat# i3x) at a wavelength of 450 nm. Titers were extrapolated based on a four-parameter logistic (4PL) curve fit and calculated using the 4PL equation generated from the curve.

## Results and discussion

### Intensified N-1 seed train for lentivirus production

Growth, viability, titer, and productivity data (Figure 2) for lentivirus production shake flasks were inoculated from the three N-1 VCDs with either non-intensified, 2X, or 3X TFDF-intensified cells. Prior to transfection on Day 4, the control and 2X intensified cells followed a similar growth trend. A slight lag in cell growth in cells that were inoculated with 15 X $10^6$ cells/mL was from Day 0 to Day 4. Post-transfection growth data are only available for cells from the control and the 3X TFDF-intensified cultures. These data show the control decreasing in VCD and having a steep decline in viability during the production phase. The culture seeded with 3X TFDF-intensified cells (15 X $10^6$ cells/mL VCD) continued growth and maintained high viability through day of harvest (Figure 2B).

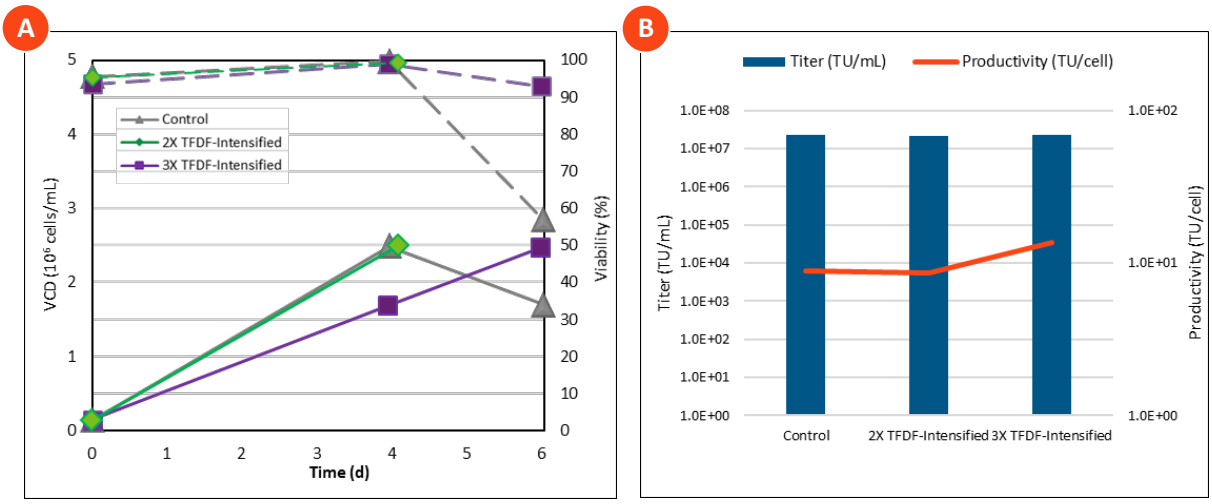


**Figure 2.** Lentivirus N-Stage Production data (A) VCD (solid line) and viability (dotted line) and (B) titer and productivity.

These data suggest that, for lentivirus production, cells from TFDF-intensified N-1 bioreactor provided comparable titers and productivity compared to cells from non-intensified N-1 control bioreactor. A slight lag in cell growth was observed for intensified cells from the 3X TFDF-intensified N-1 reactor. Titer and productivity from this production vessel were not impacted by the lag in cell growth since 3X TFDF-intensified cells also show comparable high productivity to both 2X TFDF-intensified and control cells.

### Intensified N-1 seed train for AAV8 production

Growth, viability, titer, and productivity data (Figure 3) were collected for AAV8 production shake flasks inoculated in triplicate from three N-1 VCDs with either non-intensified, 2X, or 3X TFDF-intensified cells. Prior to transfection on Day 4, the cells followed a similar growth trend. These data show comparable cell viability trends from the control, 2X-, and 3X-intensified cultures during the production phase through day of harvest.


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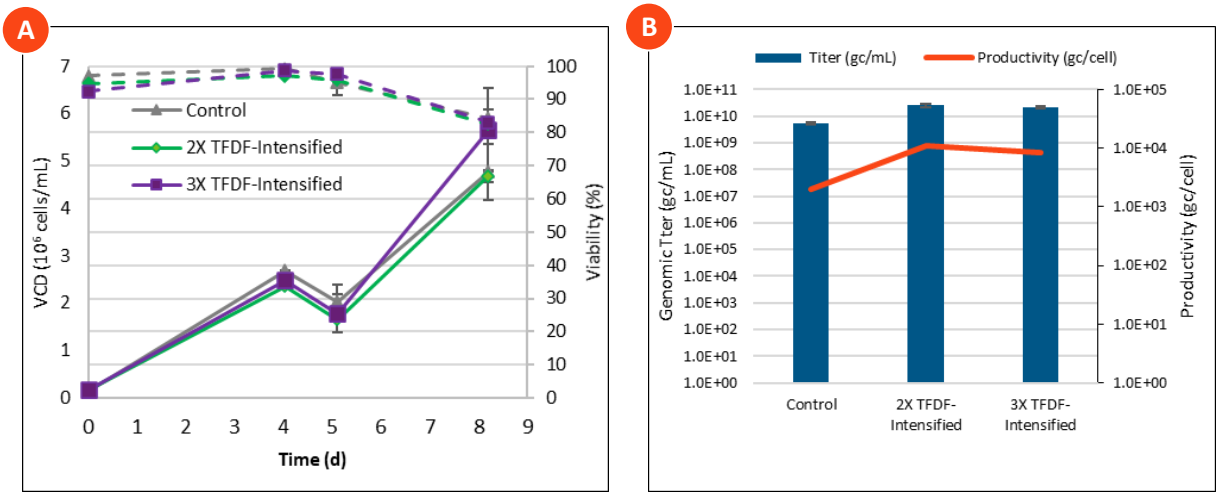


**Figure 3.** AAV8 N-Stage Production average data (A) VCD (solid line) and viability (dotted line) and (B) titer and productivity (n=3).

AAV8 titer and productivity data (Figure 3B) for Day 4 (96 hours) post transfection suggest that cells from TFDF-intensified N-1 bioreactor produced comparable titers and productivity compared to control. Both 2X and 3X TFDF-intensified cells show higher titer and productivity compared to control cells, but differences measured are within variation generally reported in literature, which denotes the difficulty to completely control all process parameters for consistent production of viral vectors. Many factors impact transfection, including DNA/transfection reagent complexation, DNA/cell ratio at transfection, and reagents volumes and concentrations. The overall data presented here suggest similar titer and productivity data between non-intensified cells (batch) in production and show normal variability.

## Intensified N-1 Seed Train for AAV9 production

HEK293 cells were cultivated to 15 X $10^6$ cells/mL VCD in N-1 bioreactors using TFDF® Technology to intensify the step using perfusion. When used to seed a production vessel, the TFDF-intensified N-1 cells produced comparable lentivirus and AAV8 titers compared to the control. These results suggest that 3X TFDF-intensified cells and control cells have similar virus production capacity. To support that hypothesis on a larger scale and with another viral vector, AAV9 viral production was explored in a 2 L bioreactor compared to the 125 ml shaker flasks. The cells were transfected once cell density reached 2 X $10^6$ cells/mL VCD on Day 4 with similar doubling time (~25 hours) and cell viability (> 95%), to produce AAV9. Trends in VCD and viability of cells from both reactors were similar (Figure 4B).

Day 4 post-transfection cell pellet and supernatant were analyzed for AAV9 production titers for both conditions (Figure 4B). The data shows similar capsid performance between the two VCD N-1 cells used for production inoculation. Capsid titer data also suggest that the 3X TFDF-intensified bioreactor and the control produced similar titers in bench scale bioreactors. The genomic titers for cells from the 3X TFDF-intensified and control bioreactors were also similar (5.0 x $10^{10}$ gc/mL and 4.5 x $10^{10}$ gc/mL, respectively), indicating the full capsid ratio remained comparable. These data indicate that cells from TFDF-intensified and non-intensified N-1 bioreactors have similar AAV9 virus production capacity and virus quality.


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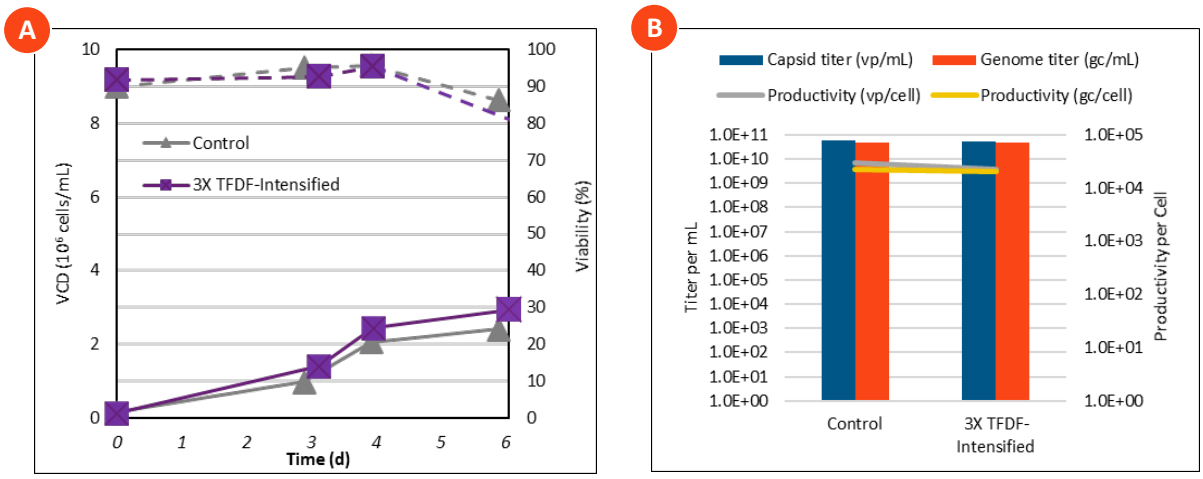


**Figure 4.** AAV9 N-Stage Production data (A) VCD (solid line) and viability (dotted line) and (B) titer, capsid quality, and productivity.

## Advantages of N-1 seed train intensification at 2000 L viral vector manufacturing scale

Data supporting the successful N-1 TFDF-intensified seed train and viral production data for three viral vectors (LV, AAV8 and AAV9) suggest that transferring this application into commercial manufacturing scale (i.e., 2000 L) is feasible and will provide many benefits. The advantages of using intensified N-1 seed train at the manufacturing scale include the reduction of capital due to fewer seeding vessels and smaller bioreactors. This allows for a smaller manufacturing footprint and reduces operation expenditures for consumables, including cell culture media. N-1 intensification also allows for faster seed train processing, reducing time in manufacturing and allowing for more batches to be made in the same equipment. Removing seed train process steps also minimizes risk of failure throughout the process.

A 2000 L non-intensified manufacturing process inoculated at 0.15 x $10^6$ cells/mL requires three seed train vessels (Figure 5). Over 100 L of media is required to reach the N bioreactor over 13 days of production.

A TFDF-intensified process requires only two seed train vessels to achieve the same level of production. The N-1 vessel, cultivated to 15 x $10^6$ cells/mL uses 3.3X less inoculum than the non-intensified process. The media volume, 58 L, is approximately 1.7X less for the intensified process. Seed train duration is reduced 25% to 9.5 days. Alternatively, the N-1 step could be run at the traditional scale of 100 or 200 L and intensified to 15 x $10^6$ cells/mL VCD. The N-stage production vessel, inoculated at 5 - 10X seeding density, decreases duration of the production phase. Following optimization of the transfection process, pre-transfection cell density could be increased more while maintaining seed train duration (data not shown).


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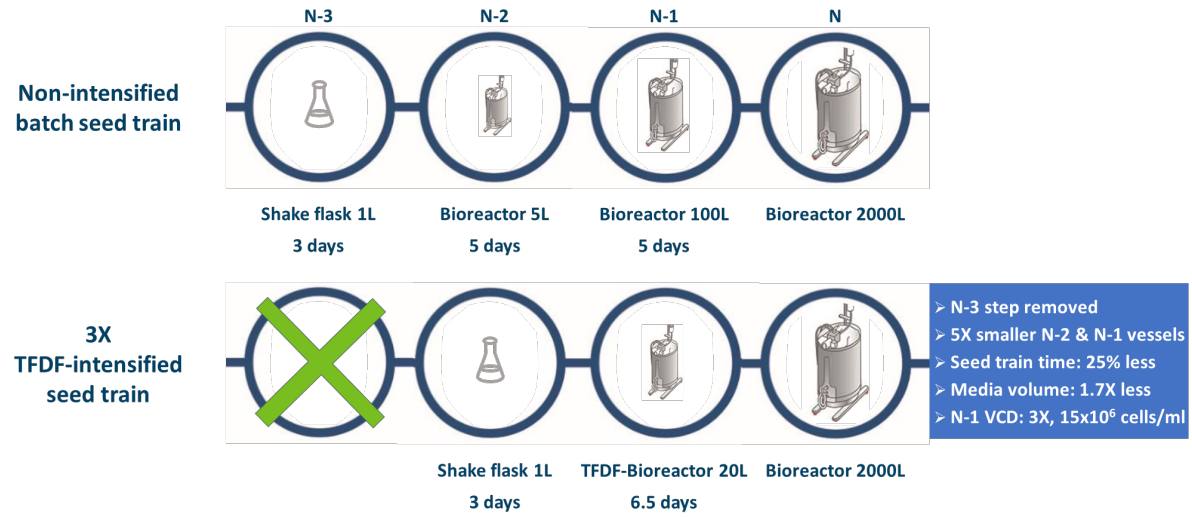


**Figure 5.** Batch vs TFDF-Intensified Seed Train scheme for a 2000 L viral vector manufacturing process.

# Conclusion

The purpose of this study was to explore the feasibility and applicability of process intensification on the N-1 bioreactors for large-scale manufacturing for several viral vectors using TFDF® Technology. The goal was to simplify and shorten the seed train process, increase the seeding VCD to achieve the same seeding density with a smaller volume, and reduce risk in manufacturing. TFDF® Technology increased the viable cell density 3X, from 5.0 x $10^6$ to 15 x $10^6$ cells/mL. TFDF-intensified N-1 cells maintained high viability throughout production phase through day of harvest compared to non-intensified N-1 batch cells. Overall, the data suggest similar performance for cell growth, viral yield, and viral quality in LV and AAV processes using TFDF-intensified N-1 cells at the production stage. This seed train intensification application can be utilized for viral vector production at manufacturing scale with many advantages.

Key benefits using intensification for the seed train process in manufacturing:

- **Simplify process steps:** TFDF-intensified N-1 process only requires two seed train vessels (1 L shake flask, 20L bioreactor), while the non-intensified process requires three bioreactor vessels.
- **Increase VCD 3-Fold:** N-1 inoculum volume required to inoculate at the 2000 L scale is reduced 3.3X due to intensification of cell density to 15 X $10^6$ cells/mL.
- **Decrease reactor volume and media use:** Required media volume is reduced approximately 1.7X with TFDF® intensification versus the traditional batch process.
- **Improve plant capacity utilization:** Seed train duration is reduced 25%, allowing production of additional batches.

# Reference

Rene Gantier, “Gene Therapy Manufacturing 2.0”, 2021, www.repligen.com/resources/library

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