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Development of a Scalable Fed-Batch Bioreactor Process for High-Titer Production of Lentiviral Vector Using an Inducible HEK293 Producer Cell Line

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Abstract

The rapidly growing interest for cell and gene therapies demands the development of robust, scalable, and cost-effective bioprocesses for viral vector production. For the production of lentiviral vector (LVV) at high titers, we have developed an inducible packaging system in suspension HEK293 cells from which we can also generate stable producer cell lines, in serum-free conditions. To evaluate the potential of this platform, we have generated a stable cell line that produces an LVV encoding a green fluorescent protein (GFP) and obtains 1×10^7 to 1×10^8 transduction units (TU)/mL at the 4 L, 10 L and 50 L scales. Functional LVV titers were maintained across all scales in bioreactors with different configurations and geometries indicating process robustness. Further, the addition of 10% feed increased the volumetric productivity by 3.5-fold in comparison to batch production, making our platform suitable for large-scale LVV production and showing a real potential for commercial manufacturing.

INTRODUCTION

An increasing number of genetic diseases and cancers are treated with cell and gene therapies. Many pre-clinical applications and treatments rely on stably inserting a gene in disease model cell lines or patients' cells. Because lentiviral vectors (LVVs) are extremely efficient, self-integrating vectors, they have become the focus of a growing number of research programs.[1,2] LVVs are a tool of choice because long-term expression of large transgenes can be achieved in dividing and non-dividing cells. In addition, LVVs are typically pseudo-typed with VSV-G, an envelope protein conferring a wide tropism to the vector.[3]

Significant improvements, in terms of safety and efficacy, have been achieved since the first generation of LVVs in 1996[4], promoting their use in the field. Since 2017, ten LVV-based treatments have been approved by the FDA, demonstrating the enthusiasm for these life-saving therapies.[5,6]

However, one of the main limitations encountered in clinical trials or commercial manufacturing is the difficulty to produce LVVs at large scale. For instance, an estimated annual vector requirement of 3.5×10^{12} transduction units (TU) would be needed to treat 5,000 pediatric cancer patients, which would require hundreds to thousands of liters of LVV, depending on the final titer.[7] Current methods are difficult to scale-up mainly because adherent cells are used as a production platform, which requires the multiplication of 2D-planar plasticware or the use of a fixed-bed reactor (FBR). Even though FBRs represent an improvement compared to 2D-planar culture systems, they have several inherent limitations (reviewed in[7]). Adherent cells, growing in the presence of serum, require intense labor, adding to production costs.

LVVs are mainly produced using a multi-plasmid transfection process. This entails the use of three to four plasmids corresponding to LVV accessory genes (*gag-pol*, *rev*, and VSV-G) and the gene of interest, along with a costly transfection reagent. According to a recent study, the plasmids and transfection reagent account for 40% of the cost of raw material.[8] To reduce these costs, LVV producer cells have been engineered based on two strategies.[9] First, the cytotoxic VSV-G and *rev* are placed under the control of inducible systems working in ON or OFF mode that requires adding or removing inducer(s) respectively to initiate LVV production. The second strategy consists of reducing the cytotoxicity of VSV-G and *rev* to allow for constitutive LVV production. While these advancements have reduced production costs, only two of the 17 engineered producer cell lines grow in suspension, including ours.[9]

Our approach to improving scalability and reducing production costs was to develop LVV stable producer cell lines that grow in suspension, in serum-free media.[10–13] Suspension cultures dramatically decrease the complexity of the production process and allow for simplified scale-up,

minimizing efforts and timelines, and most effectively reducing the overall costs of manufacturing.[7,8] In pursuing the goal of reducing production costs, we have also shown that adding 16% of a chemically defined feed at specific time points led to a 3.5-fold improvement in titer in shake flask suspension cultures[13] while a 10-fold increase was measured in a perfusion culture.[12,14] Because the perfusion mode is typically complex to set up and requires specialized equipment, we sought to optimize and scale-up a fed-batch process to increase titers and keep the process relatively simple. Here, we expand on our previous small-scale process[13] by testing different feed regimens and by scaling up to the 50 L scale in bioreactors to establish a biomanufacturing-ready process using a producer cell line. The cell line used produces LVVs encoding green fluorescent protein (GFP) under the control of inducible promoters (*i.e.,* LVV-GFP).[12] Using this cell model, we have obtained reproducible titers of about 7×10^7 TU/mL at harvest in fed-batch mode in bioreactors at scales ranging from 4 L to 50 L.

MATERIALS AND METHODS

HEK293-Derived Cell Line and Lentivirus Production

A stable producer clone, HEK293SF-LVP-GFP #18-8 (also referred to as clone 18-8), was generated from NRC's proprietary cell line HEK293SF-3F6 following the same process as published previously.[12] The production of a lentivirus vector expressing GFP (LVV-GFP) is based on induction and controlled by tetracycline and cumate switches. Once the target cell density for induction has been reached, 50 µg/mL 4-Isopropyl benzoic acid (cumate, Arkpharm) and 1 µg/mL doxycycline hyclate (DOX, MilliporeSigma) are added to the culture. While cumate prevents binding of the cumate repressor (CymR) to the cumate operator (CuO), DOX promotes binding of the reverse tetracycline transactivator (rtTA2s-M2) to the CymR promoter (TR5).[10] LVV-GFP production is further boosted by the addition of 7 mM sodium butyrate (MilliporeSigma) one day post-induction (dpi).[11]

Cell Culture Conditions in Shake Flask

Clone 18-8 was grown and maintained in HyCell TransFx H media (Cytiva) supplemented with 4 mM L-glutamine (MilliporeSigma) as well as 0.1% Kolliphor P 188 (MilliporeSigma), a shear protectant for high-shear stress environments. Cells were cultivated in suspension in polycarbonate Erlenmeyer shake flasks (Corning or TriForest) at 37°C with an agitation of 120 rpm using orbital shakers (Infors HT, 25 mm orbital diameter) and 5% CO_2. For the fed-batch conditions, the culture was supplemented with HEK FS feed (Sartorius). Cell counts were performed with an automated cell counter (Cedex HiRes, Innovatis) and trypan blue stain (Life Technologies) to determine culture viability. For more accurate cell counts, cell samples were treated with a cell dissociation agent (Accumax, Innovative Cell Technologies) prior to cell counting. Cells were maintained between 0.2 and 2.0×10^6 cells/mL for both cell maintenance and expansion. Cell counts reported in this publication refer to viable cell counts (VCC).

Screening of Different Fed-Batch Regimens in Shake Flasks

Based on previous work performed by our group[13], the HEK FS feed was selected and three different fed-batch regimens were tested in shake flasks side-by-side. The batch process was included as the control experiment to confirm the increase in infectious titer for each fed-batch condition, and to choose the best process for bioreactor scale-up.

To maintain identical starting conditions, all tests in **Table 1** were initiated from the same shake flask seeded at 0.24×10^6 cells/mL on day 0 and split on day 3 after dilution with fresh medium. Each feed regimen was then tested in duplicate flasks, each with a culture volume of 25 mL (125 mL shake flask). Even though not required for shake

TABLE 1. Fed-batch and batch processes tested in 125 mL shake flask.

Day	Fed-Batch Regimen 1 (FB R1)	Fed-Batch Regimen 2 (FB R2)	Fed-Batch Regimen 3 (FB R3)	Batch Control
0	Cell seeding[1]			
3	2× dilution[2]			
4	Feed 1 (4%)[3]	Feed 1 (4%)[3]	—	—
5	—	Feed 2 (6%)[3]	Feed 1 (6%)[3]/Induction[4]	Induction
6	Feed 2 (6%)[3]/Induction[4]	Feed 3 (6%)[3]/Induction[4]	Booster[5]	Booster[5]
7	Booster[5]	Booster[5]	—	—
8	—	—	3 dpi sampling	3 dpi sampling
9	3 dpi sampling	3 dpi sampling	4 dpi sampling	4 dpi sampling
10	4 dpi sampling	4 dpi sampling	—	—

[1] Culture volume of 1×130 mL with a seeding cell density of 0.24×10^6 cells/mL

[2] Culture diluted 2-fold and then split into 8×25 mL (duplicate flasks per condition)

[3] Feed additions calculated as % v/v and based on the post-dilution culture volume (25 mL)

[4] Subsequent additions of cumate (50 µg/mL final) followed by DOX (1 µg/mL final); day of induction defined as day 0 (pre-/post-induction)

[5] 7 mM sodium butyrate (final concentration)

flask screening, a dilution step was included in the process schedule. The dilution step is of interest particularly for bioreactor scale-up since it reduces the required volume of cell inoculum needed for bioreactor seeding. All additions of feed and other reagents were calculated based on the post-dilution culture volume (25 mL). LVV-GFP production was induced by the addition of cumate followed by DOX, as per **Table 1**, followed by the addition of sodium butyrate 16 – 24 hours post-induction (hpi) to boost the production of LVV-GFP.[11] Flasks were sampled daily to monitor cell growth, viability, and metabolites. Functional LVV-GFP titer was measured at 2, 3, and 4 dpi by gene transfer assay (GTA). The four conditions tested in shake flasks are summarized in **Table 1**.

Fed-Batch Production in Bioreactor

The process transfer and scale-up from shake flask to bioreactor production was carried out in 4 L (BioBLU 3c, Eppendorf), 10 L (BioBLU 10c, Eppendorf), and 50 L (DynaDrive, ThermoFisher) single-use vessels with final process volumes of 3.8 L, 10 L, and 30 L, respectively. Appropriate sensors were installed to measure and control temperature (37°C), pH (7.10 ± 0.05), dissolved oxygen (DO, 40%), and agitation. Control of pH was achieved via base addition (Bioreactor pH adjustment solution, MilliporeSigma) or CO_2 addition through the headspace (4 L and 10 L bioreactor) or macrosparger (50 L bioreactor). The agitation rate was adjusted to maintain volumetric power inputs of 10 – 59 W/m³ across scales, which is equivalent to rates of 100 rpm at 4 L scale (10 W/m³), 80 – 95 rpm at 10 L scale (11 – 19 W/m³), and 130 – 150 rpm at 50 L scale (28 – 59 W/m³). A higher volumetric power input was required in the 50 L bioreactor in order to maintain similar hydrodynamic characteristics created at smaller scale.[15]

Bioreactor seeding was done at a low fill (45% of the final working volume) and then diluted 2-fold with fresh media after reaching a target cell density of at least 1.5×10^6 cells/mL for dilution and 3.5×10^6 cells/mL for induction. For the 50 L production, seed train amplification was completed in the bioreactor vessel. The bioreactor was seeded at 7 L (n-1), then diluted to the process starting volume (15 L; $n = 0$), and finally diluted a second time to reach the final process volume (30 L). The additional 10% increase in volume was related to the feeding post-dilution and pre-induction.

Metabolite Analyses

Glucose (glc), lactate (lac), ammonia (NH_3), and lactate dehydrogenase (LDH) analyses were carried out on spent media samples after centrifugation for five minutes at 300×*g*. Metabolite concentrations were photometrically measured using the Cedex Bio Analyzer (Roche Custom Biotech).

Titration Using a Gene Transfer Assay

Titers were measured using a flow cytometry–based assay.[12, 16, 17] LVV samples were serially diluted in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 8 µg/mL polybrene. Transduction was performed by adding the diluted LVV to HEK293A (adherent) cells and incubating for 72 h at 37°C. GFP-expressing cells were quantified by flow cytometry using an LSRFortessa (BD Biosciences). Standard deviations (SD) were calculated on at least two technical replicates.

An in-house LVV standard was used in all measurements to validate interassay variability.

p24 ELISA for Intact LVV Particle Quantification

The LVV particle concentration (LP/mL), based on intact p24 capsid, was quantified using a commercially available kit from Cell Biolabs. The manufacturer's assay procedure was followed. All LVV samples were diluted in 20 mM Tris-HCl, pH 7.5, and analyzed in duplicate unless otherwise specified. Readouts were performed by absorbance at 450 nm and 630 nm (reference wavelengths). The concentration of intact p24 (ng/mL) in the unknown sample(s) was calculated based on a p24 standard curve. The LP/mL was then calculated based on the relationships that there are 2,000 molecules of p24 in one LVV particle:[18]

$$2000\times24\times10^3/(6\times10^{23})\text{ g of p24} = 8\times10^{-5}\text{ pg of p24}$$
$$\text{or } 1\text{ ng p24} = 1.25\times10^7\text{ LPs}$$

Anion-Exchange HPLC for Total LVV Particle Quantification

An anion exchange high-performance liquid chromatography (AEX-HPLC) method was used for the quantification of LVV total viral particles (VP/mL) according to a method developed in-house.[19] Total viral particles (VP/mL), as mentioned here, consists of infectious and non-infectious particles as well as the minimal presence of extracellular vesicles (EVs). It is widely known that EVs are also produced during LVV production, and some co-elute with LVV due to similarities in physico-chemical properties using AEX-HPLC. This method was optimized to minimize the EV co-elution to less than 10%. The method was used as an in-process monitoring tool, and only a single injection of samples was performed, except for the 50 L production run, which was injected in duplicates.

Briefly, 50 µL of 0.45 µm filtered LVV samples were injected into a UNO Q polishing column (0.16 mL, Bio-Rad Laboratories) followed by column washing with 20 mM Tris-HCl, pH 7.5. LVV was then eluted using a step gradient at 420 mM NaCl in 20 mM Tris-HCl, pH 7.5. The column was regenerated with 1.5 M NaCl, then re-equilibrated with 20 mM Tris-HCl, pH 7.5, for the next sample injection. The output stream was monitored by both fluorescence and UV detection at excitation and emission wavelengths of 290 and 335 nm and absorbance at 260 nm and 280 nm, respectively.

RESULTS AND DISCUSSION

Fed-Batch Screening in Shake Flasks

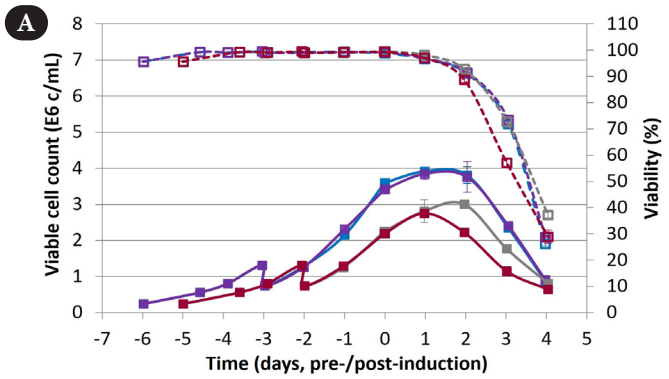
A first series of experiments was performed in shake flasks to test three different fed-batch regimens. Results for cell growth, viability, and LVV-GFP titer by GTA for fed-batch regimens 1 to 3 (referred to as FB R1 to FB R3) compared to batch production are shown in **Figure 1**. Since all conditions were started from the same flask, cell counts and viability profiles were identical until day 3 (corresponding to –3 dpi for FB R1 and FB R2, or –2 dpi for FB R3 and the control batch due to the shorter process duration; refer to **Table 1**).

Following dilution on day 3 (–3 dpi or –2 dpi, respectively), cell growth profiles (**Figure 1A**) were comparable for FB R1 and FB R2, reaching a VCC of 3.4 – 3.6×10^6 cells/mL before induction and a maximum VCC of 3.9×10^6 cells/mL at 1 dpi. Cell growth profiles for FB R3 and the control batch were almost identical post-dilution although pre-induction and maximum VCCs were 35% and 25% lower than FB R1 and FB R2. The addition of a total of 16% (v/v) HEK FS feed (FB R2) did not improve cell growth compared to 10% (FB R1). However, a single addition of 6% (FB R3) resulted in slower and lower overall cell growth and did not offer any advantage over the control batch. Culture viability (**Figure 1A**) profiles were comparable for all conditions tested except that a faster decline in viability was observed for the batch condition. Functional LVV-GFP titers (**Figure 1B**) were highest at 3 dpi with 1.78×10^8 TU/mL for FB R1 and 1.84×10^8 TU/mL for FB R2 compared to 7.01×10^7 TU/mL for the batch process, representing a 2.5 – 2.6-fold increase in fed-batch production mode. This is in line with our previous results where a 3.5-fold increase in titer was obtained with a fed-batch regimen similar to FB R2 using a different producer clone.[13] FB R3 resulted with 1.08×10^8 TU/mL in an approximately 30% titer increase compared to batch mode.

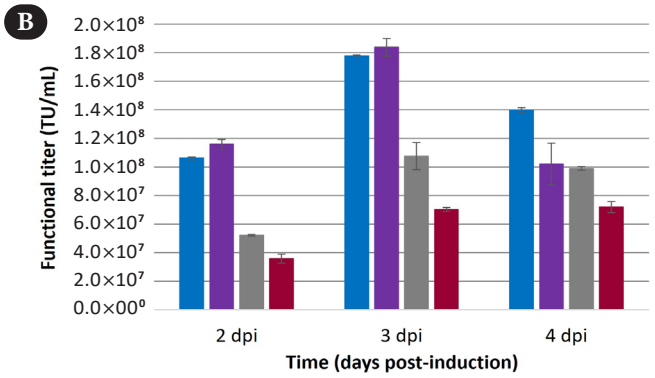
Metabolite analyses were carried out to evaluate the impact of the feeding strategies on glc and LDH concentrations, as well as the waste products lac and NH_3 in the culture, compared to batch cultivation. On days of feeding, glc concentrations were only measured pre-feeding, which means that the peak concentrations referred to below in **Figure 2** are those recorded the day following the feed.

Since all conditions were started from the same flask,

FIGURE 1. Screening of fed-batch conditions versus batch in 125 mL shake flasks—cell growth, viability, and functional virus titer.

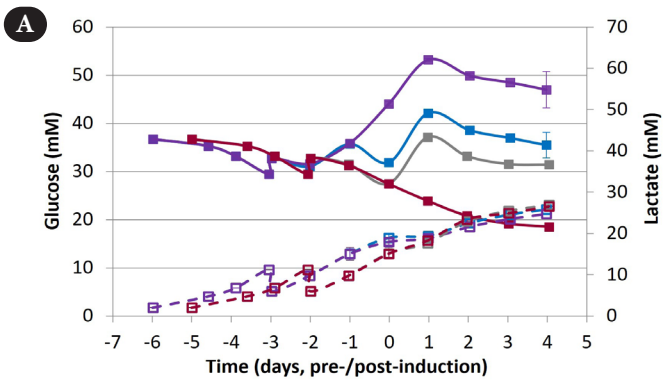


(A) VCC in solid symbols/solid lines and culture viabilities in empty symbols/dotted lines for FB R1 (blue), FB R2 (purple), FB R3 (grey) compared to batch (red); SDs are based on counts for duplicate flasks.

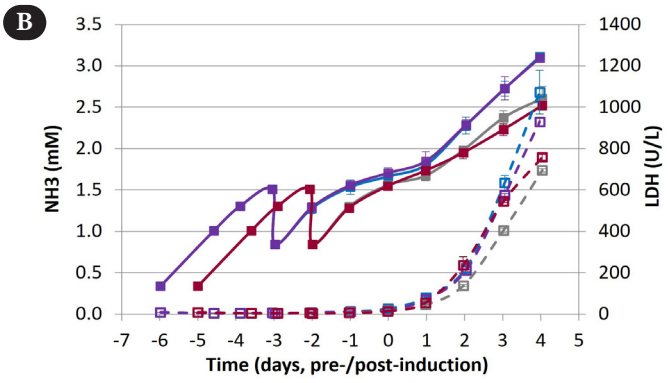


(B) Functional LVV-GFP titers by GTA for FB R1 (blue), FB R2 (purple), FB R3 (grey) compared to batch (red) at 2, 3, and 4 dpi; SDs are based on GTA results for duplicate flasks.

FIGURE 2. Screening of fed-batch conditions versus batch in 125 mL shake flasks—metabolite profiles.



(A) Glc in solid symbols/solid lines and lac in empty symbols/dotted lines for FB R1 (blue), FB R2 (purple), and FB R3 (grey) compared to batch (red).



(B) NH_3 in solid symbols/solid lines and LDH in empty symbols/dotted lines for FB R1 (blue), FB R2 (purple), and FB R3 (grey) compared to batch (red).

metabolite concentrations were identical until dilution with fresh media. Due to the feed additions, glc concentrations were higher overall for all fed-batch conditions compared to batch, and directly linked to the amount of feed added. None of the flasks dropped below 27 mM before induction or 19 mM at 4 dpi, which suggests that glc concentrations were sufficient in all test conditions.

Lactate, a waste product from glc consumption via glycolytic pathway[20,21], followed an inversely proportional profile compared to the glc concentration (**Figure 2A**). This was expected due to their metabolic relationship. In contrast to the glc profile, lac was less impacted by the number and concentration of feed additions, and measured concentrations were comparable for all conditions tested. This may be caused by a metabolic shift to lac consumption in fed-batch mode, which has also been observed in other cell culture processes.[22]

Ammonia concentrations (produced from glutamine consumption) were not significantly affected by feeding. LDH, typically an indicator for cellular damage[23,24], showed a correlation with feeding and higher glc concentrations, reaching higher final maximum concentrations for FB R1 and FB R2 compared to FB R3 and the batch process (**Figure 2B**). The differences observed are likely due to the different maximal VCC reached under each condition (**Figure 1A**).

Based on the results of this experiment, FB R1 and a harvest date of 3 dpi were chosen for bioreactor scale-up since this was when a peak in titer was observed. The functional LVV-GFP titer was comparable to FB R2 at 3 dpi while requiring 6% less feed.

Similar growth profiles and titers were obtained in 2 L shake flasks with 600 mL culture volume for FB R1 and the control batch (data not shown).

Comparison of Batch and Fed-Batch Production in 4 L Bioreactor

A summary of the final bioreactor process schedule for fed-batch and batch production is shown in **Figure 3**. The batch process, from seeding to harvest, spans over eight days while the fed-batch process lasts ten days. The day of inoculation is defined as day 0.

To ensure identical starting conditions for bioreactor scale-up of both fed-batch (FB R1) and batch cultures, two 4 L single-use bioreactors were seeded in parallel using the same cell inoculum.

After inoculation at 0.3×10^6 cells/mL on day 0 (–6 dpi for the fed-batch process and –4 dpi for the batch process, respectively) with a starting volume of 1.75 L, both bioreactors reached a VCC of 2.0×10^6 cells/mL before dilution on day 3 (–3 dpi or –1 dpi, respectively), as shown in **Figure 4A** on the following page. Following dilution to 3.40 L, the fed-batch bioreactor was fed with HEK FS at –2 and day 0 (pre-induction) as per **Table 1** (FB R1). The feed addition increased the cell growth to 5.5×10^6 cells/mL while the batch process only reached 1.8×10^6 cells/mL at day 0 (pre-induction). Cell growth was overall higher for the fed-batch bioreactor process compared to shake flask, while no significant difference was observed between production systems for batch mode.

Culture viability (**Figure 4A**) was comparable for both processes up to 2 dpi (≥ 97%) and 6% lower for the batch process at 3 dpi (88% vs. 94%).

LVV-GFP functional titers (**Figure 4B**, following page) reached 6.91×10^7 TU/mL for the fed-batch and 2.10×10^7 TU/mL for the batch processes, representing a 3.3-fold improvement in titer. This ratio was higher than the previously obtained 2.5-fold increase in the shake flask screening experiment (**Figure 1B**). Satellite shake flasks were performed in parallel to the bioreactors (25 mL working volume in 125 mL shake flasks; data not shown). The titer of the batch satellite flask was 1.62×10^7 TU/mL, almost identical to the bioreactor titer, demonstrating good linearity from shake flask to bioreactor. The titer for the fed-batch satellite flask was 3.31×10^7 TU/mL, representing a 2-fold increase in titer compared to batch, similarly to **Figure 1B**. Therefore, the feed-dependent increase in titer is more pronounced in bioreactors than in shake flask and likely due to the bioreactor's controlled culture conditions (pH, DO, etc.).

The physical titer in the supernatants collected at 3 dpi for the fed-batch and batch processes, using two methods,

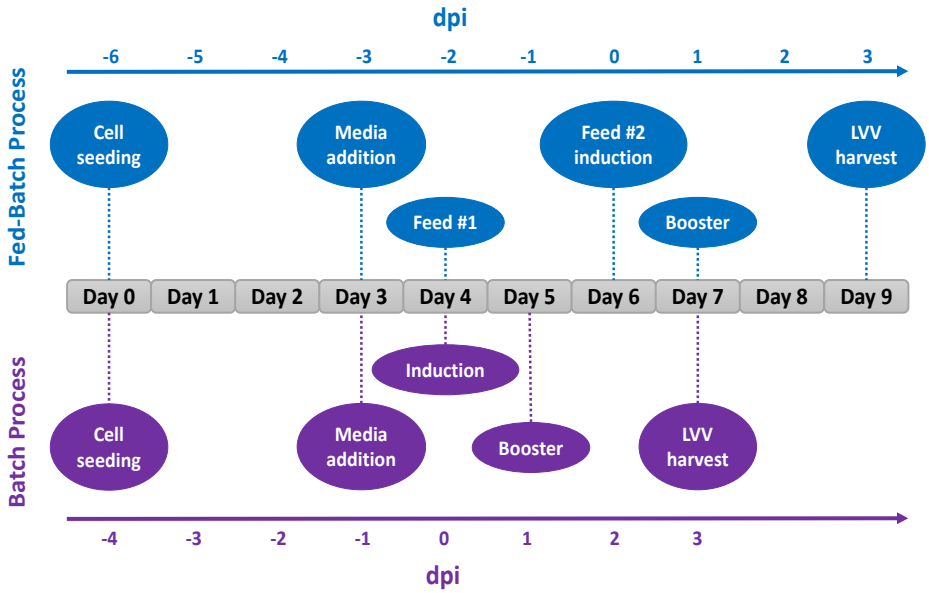


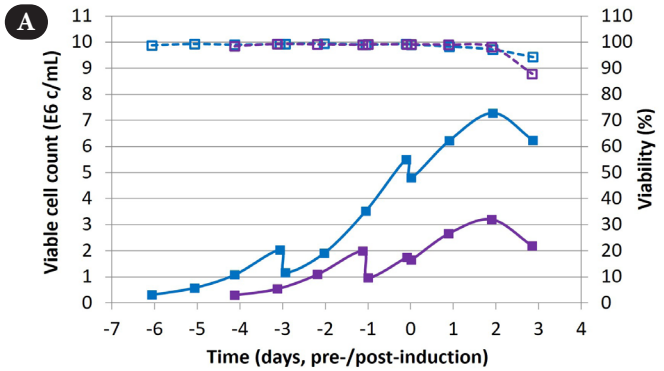
FIGURE 3.
Process flow chart for LVV bioreactor production in fed-batch and batch modes.

is shown in **Figure 4C**: p24 ELISA (LP/mL) and AEX-HPLC (VP/mL). With both methods, the particle count was higher for the fed-batch compared to the batch production. For the fed-batch process, particle concentrations of 6.44×10^{9} LP/mL (by p24 ELISA) and 1.83×10^{10} VP/mL (by AEX-HPLC) were obtained, while the batch resulted in 9.14×10^{8} LP/mL (by p24 ELISA) and 1.26×10^{10} VP/mL (by AEX-HPLC). However, the extent of this increase in titer in fed-batch mode is different, depending on the method used. While we observed an increase in titer of 3.3-fold by GTA, the p24 ELISA suggests an increase of 7-fold, and the AEX-HPLC results in an increase of 1.5-fold. Regarding the physical titer, it is difficult to conclude since the cells were induced at different cell densities in batch and fed-batch mode (**Figure 3A**), which results in different EV content and might interfere with the measurements. Interestingly, we have also observed an important discrepancy between the AEX-HPLC and the p24 ELISA results, especially for the batch bioreactor (a 13.7-fold difference), which might indicate a higher content of exosomes and EVs in batch mode compared to fed-batch.

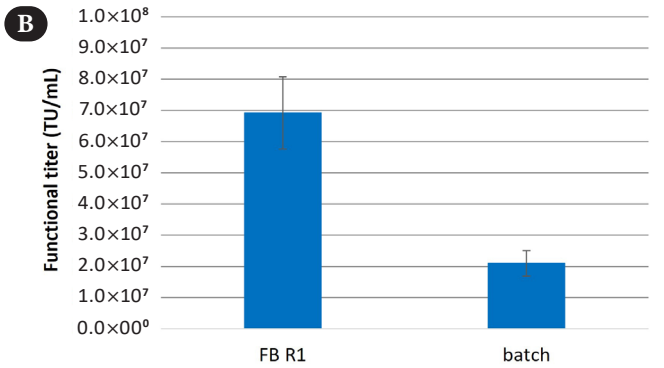
The ratio of physical titer by p24 ELISA to the functional titer by GTA determines the quality of LVV between the two production processes. Here, we observed ratios of 93 LP/TU for FB R1 and 44 LP/TU for the batch process.

In addition to cell growth and viral titer assessment of both production modes, samples were taken to monitor glc, lac, NH_3, and LDH concentrations (**Figure 5**). Glucose profiles (**Figure 5A**) followed the same trends as observed in shake flask (glc concentrations were higher for the fed-batch condition compared to batch). The overall glc concentrations for both conditions were, however, slightly lower in the 4 L bioreactors compared to shake flasks (**Figure 2A**). This could be due to higher VCCs and possibly higher glc consumption rates caused by higher shear stress in the bioreactor culture. Similar lactate profiles (**Figure 5A**) were observed for both runs, but concentrations were overall higher compared

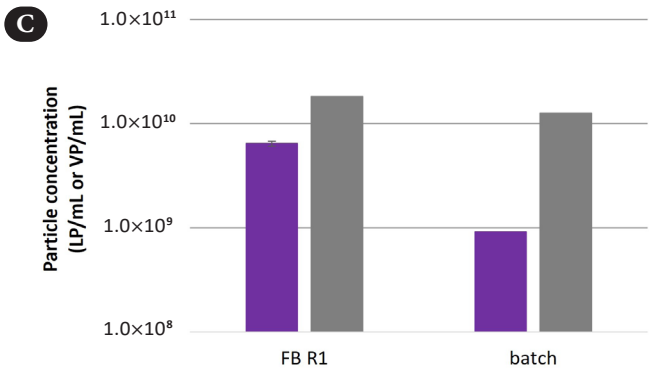
FIGURE 4. Comparison of fed-batch (FB R1) versus batch production in 4 L bioreactor—cell growth, viability, and virus titers.



(A) VCC in solid symbols/solid lines and culture viabilities in empty symbols/dotted lines for FB R1 (blue) compared to batch (purple).

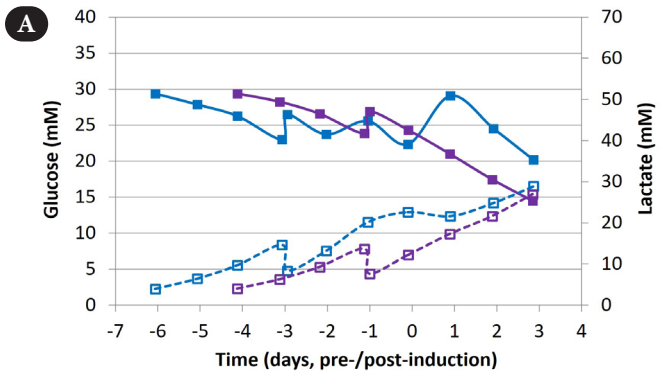


(B) Functional LVV-GFP titers by GTA for FB R1 compared to batch at 3 dpi; SDs are based on technical replicates.

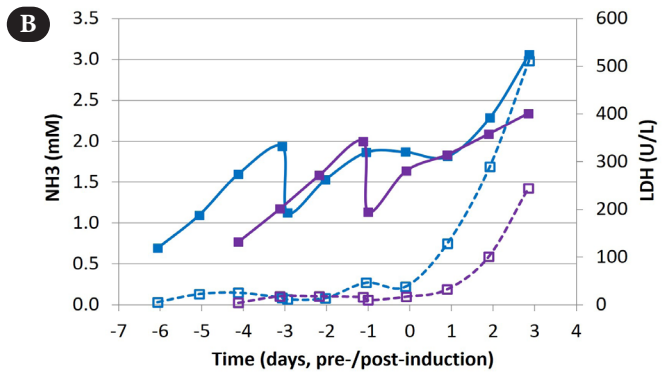


(C) Particle concentration by p24 ELISA in LP/mL (purple) and by AEX-HPLC in VP/mL (grey); p24 ELISA results are mean and SD of duplicate analyses while HPLC results are from a single analysis.

FIGURE 5. Comparison of fed-batch (FB R1) versus batch production in 4 L bioreactor—metabolite profiles.



(A) Glc in solid symbols/solid lines and lac in empty symbols/dotted lines for FB R1 (blue) compared to batch (purple).



(B) NH_3 in solid symbols/solid lines and LDH in empty symbols/dotted lines for FB R1 (blue) compared to batch (purple).

to shake flasks due to the higher glc consumption in bioreactor. Ammonia profiles in the bioreactors (**Figure 5B**) were similar to the shake flask productions (**Figure 2B**). As observed in shake flask, LDH concentrations reached higher concentrations at harvest in fed-batch compared to batch production mode.

Scale-Up of Fed-Batch Production to 10 L Bioreactor and Process Reproducibility

Following the process comparison at 4 L scale, the fed-batch process was transferred to the 10 L bioreactor and executed four times to assess cell growth and production of functional LVV-GFP, as well as process reproducibility and robustness.

Bioreactors were seeded at 0.30 – 0.33×10^6 cells/mL (except for run 3: 0.25×10^6 cells/mL) in a culture volume of 4.6 L and reached 1.4 – 1.7×10^6 cells/mL before dilution to 9.0 L (–3 dpi) (**Figure 6A**). Feed additions of 4% and 6% were done as per **Table 1** (FB R1). Runs 1 and 4 grew to VCCs of 4.9×10^6 cells/mL before induction and were therefore comparable with the 4 L production while runs 2 and 3 were induced at 3.6×10^6 cells/mL (0 dpi). Peak VCCs of 6.0 – 7.0×10^6 cells/mL, comparable to the 4 L fed-batch process, were observed for runs 1, 3, and 4 while run 2 reached a maximum of 4.9×10^6 cells/mL. Run 2 was the only production that was inoculated from a 10 L Cellbag (Cytiva) (cultivated at 37°C, 22 rpm, 9° angle, 0.1 SLPM overlay with 5% CO_2) whereas the cell inoculum was built using shake flasks for all other runs. The cell doubling times were under 30 hours in shake flasks, while it reached 37 hours in the Cellbag, demonstrating that the growth conditions were sub-optimal and required further optimisation. Once inoculated from the Cellbag, doubling times initially remained above 30 h in the bioreactor (data not shown), resulting in lower pre- and post-dilution as well as induction cell densities.

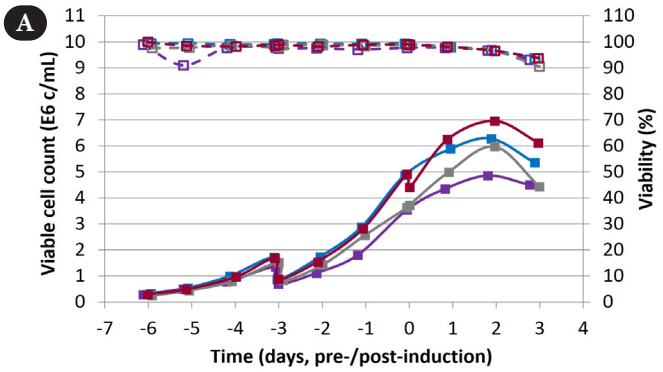
Similar culture viability (**Figure 6A**) was observed for the four runs with a slight decrease starting after 1 dpi and reaching 91 – 94% at 3 dpi, as with the 4 L fed-batch process.

Figure 6B shows that the functional titers obtained for four runs at the 10 L scale were between 5.81×10^7 and 8.88×10^7 TU/mL, with an average of 6.99×10^7 TU/mL (SD of 20%), which was comparable to 4 L bioreactor fed-batch production. Lower titers were likely related to lower VCC at induction as well as lower peak VCC reached before harvest.

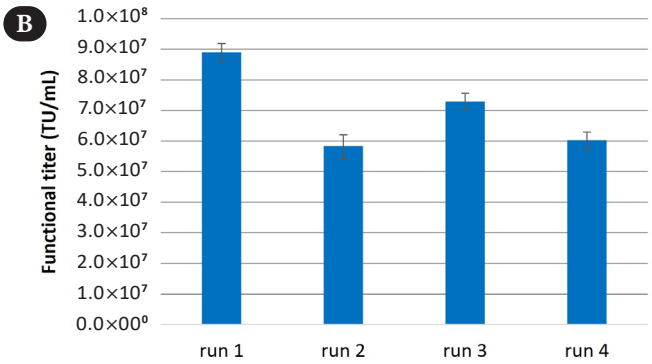
Figure 6C shows that the particle concentration by p24 ELISA and AEX-HPLC followed a similar trend as the functional titers (**Figure 6B**). The particle count by p24 ELISA ranged between 3.73×10^9 and 7.35×10^9 LP/mL while it was between 1.99×10^{10} and 2.81×10^{10} VP/mL by AEX-HPLC. The p24 ELISA/GTA ratios were in the same range for all the runs (83, 81, 97, and 62 LP/TU) and similar to what was measured for the 4 L bioreactor (93 LP/TU), indicating that the process is reproducible and robust.

Metabolite analyses (data not shown here) revealed overall comparable profiles with the 4 L fed-batch production.

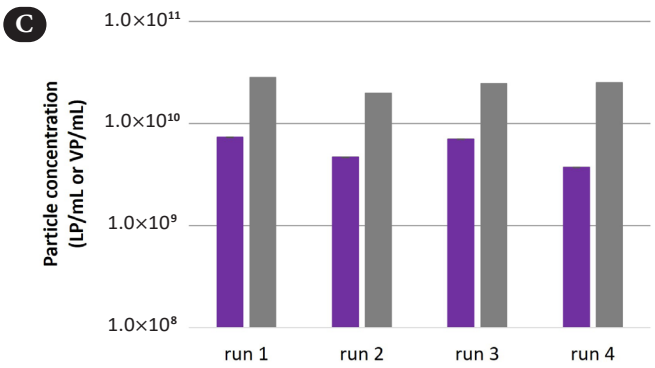
FIGURE 6. Comparison of four fed-batch productions (FB R1) at 10 L scale—cell growth, viability, and virus titers.



(A) VCC in solid symbols/solid lines and culture viabilities in empty symbols/dotted lines for fed-batch runs 1 (blue), 2 (purple), 3 (grey), and 4 (red).



(B) Functional LVV-GFP titers by GTA for fed-batch runs 1, 2, 3, and 4 at 3 dpi; SDs are based on technical replicates.



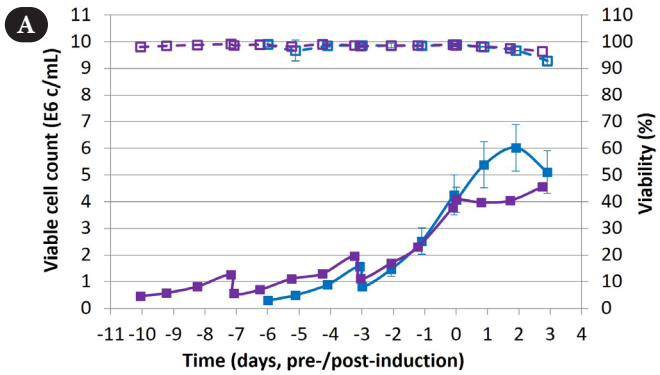
(C) Particle concentration by p24 ELISA in LP/mL (purple) and by AEX-HPLC in VP/mL (grey) for fed-batch runs 1, 2, 3, and 4; p24 ELISA results are mean and SD of duplicate analyses while HPLC results are from a single analysis.

Scale-Up of Fed-Batch Production to 50 L Bioreactor

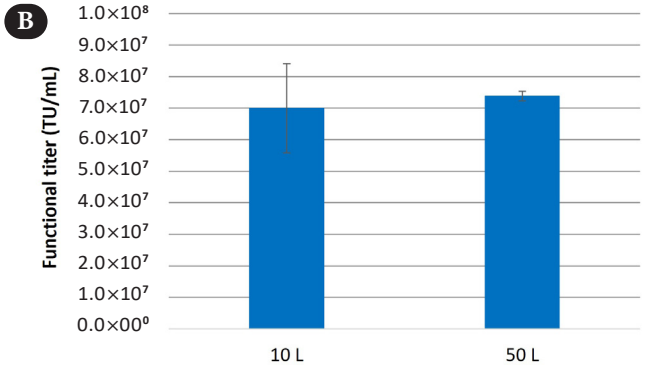
To mimic the last step of the seed train, the DynaDrive bioreactor was inoculated at 0.45×10^6 cells/mL and 7.0 L culture volume. This step replaced the seed train amplification typically carried out in shake flasks for 4 – 10 L bioreactors, or a Cellbag for larger bioreactors. Agitation was initially set to 130 rpm (28 W/m^3) during the seed train phase followed

by an increase to 140 – 150 rpm for the production phase (15 – 30 L; 45 – 59 W/m³). Due to the slower growth rate at this scale compared to bench-scale bioreactors, the fed-batch process spanned over 11 days instead of ten days. Key steps (dilution, feeding, and induction) were performed only when target cell densities were reached (1.5×10^6 cells/mL for dilution and 3.5×10^6 cells/mL for induction).

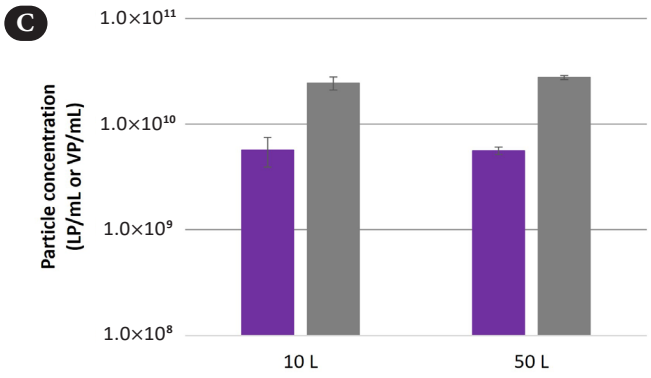
FIGURE 7. Comparison of fed-batch productions (FB R1) at 10 and 50 L scale—cell growth, viability, and virus titers.



(A) VCC in solid symbols/solid lines and culture viabilities in empty symbols/dotted lines for the average of four 10 L (blue) and one 50 L (purple) fed-batch productions.



(B) Functional LVV-GFP titers by GTA for 10 L (average of four runs) and 50 L (single run) fed-batch productions at 3 dpi; SDs are between individual 10 L productions or based on technical replicates for the 50 L run.



(C) Particle concentration by p24 ELISA in LP/mL (purple) and by AEX-HPLC in VP/mL (grey) for 10 L (average of four runs) and 50 L (single run) fed-batch productions at 3 dpi; SDs are based on the average of four runs (10 L) or technical replicates (50 L).

As for all other bioreactor fed-batch productions, culture viability (**Figure 7A**) remained high despite slower cell growth than observed in the small-scale bioreactor and slightly decreased at 2 and 3 dpi, reaching 96% at the end of the run.

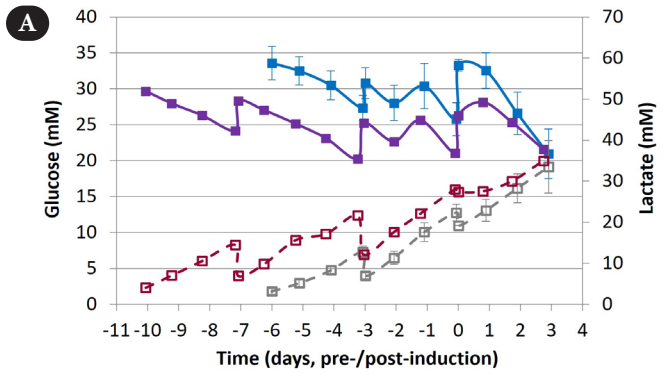
The functional viral titer (**Figure 7B**) of 7.38×10^7 TU/mL was comparable to the average obtained at 10 L scale.

A particle concentration by AEX-HPLC of 2.75×10^{10} VP/mL obtained with the 50 L run was in close agreement with the average titer of 2.44×10^{10} VP/mL obtained with the 10 L runs (**Figure 7C**). Likewise, results obtained by p24 ELISA were comparable between the 50 L run and the average of the 10 L runs with titers of 5.70×10^9 LP/mL and 5.61×10^9 LP/mL, respectively, resulting in a similar p24 ELISA/GTA ratio for both scales (average of 81 LP/TU for the 10 L runs versus 76 LP/TU for the 50 L run).

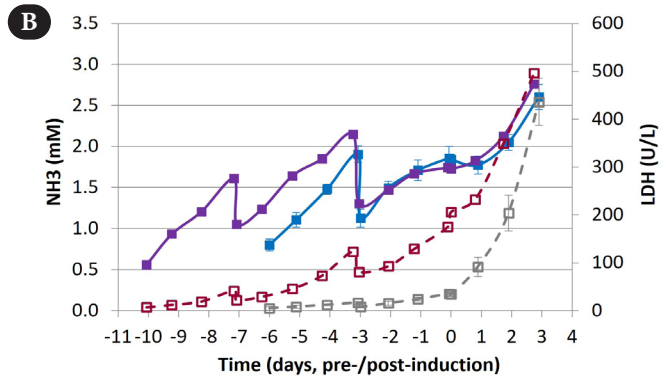
Metabolite profiles (**Figure 8**) were again comparable to 4 L and 10 L bioreactor productions.

In summary, to develop a bioreactor fed-batch, process conditions were selected in shake flask experiments and then transferred to bioreactor while scaling up from 4 L to 50 L scale (3.8 L to 30 L working volume). Functional titers remained stable across the bioreactor scales with

FIGURE 8. Comparison of fed-batch productions (FB R1) at 10 and 50 L scale—metabolite profiles.



(A) Glc in solid symbols/solid lines and lac in empty symbols/dotted lines for the average of four 10 L (blue, grey) and one 50 L (purple, red) fed-batch productions.



(B) NH_3 in solid symbols/solid lines and LDH in empty symbols/dotted lines for the average of four 10 L (blue, grey) and one 50 L (purple, red) fed-batch productions.

TABLE 2. Summary of functional viral titer and specific productivity for fed-batch versus batch across bioreactor production scales (based on GTA analysis).

Process and Bioreactor Scale	Titer		Specific Productivity[1]	
	$\times 10^7$ TU/mL	-fold[2] increase	TU/cell	-fold[2] increase
Batch, 4 L ($n = 1$)	2.10	—	12.7	—
Fed-batch, 4 L ($n = 1$)	6.91	3.3	14.4	1.1
Fed-batch, 10 L ($n = 4$)	6.99 ± 1.41[3]	3.3	16.9 ± 2.5[3]	1.3
Fed-batch, 50 L ($n = 1$)	7.38	3.5	18.0	1.4

[1] Specific productivity = (GTA titer at 3 dpi) / (VCC at induction)

[2] -fold increase of fed-batch versus batch at 4 L bioreactor scale

[3] SD between four runs

6.91×10^7 TU/mL at 4 L scale, 6.99×10^7 TU/mL at 10 L scale (average), and 7.38×10^7 TU/mL at 50 L scale, respectively (**Table 2**). Therefore, a 3.3 to 3.5-fold increase in functional LVV titer was achieved compared to 4 L batch production. Previous data generated by our group show that the specific productivity decreases with increasing cell density at induction in batch mode.[13] Here, the use of a fed-batch process alleviates this limitation and even improves the specific productivity at higher cell densities. Compared to the 4 L batch process, the specific productivity in fed-batch mode was increased by up to 40% (**Table 2**). Overall, the higher functional viral titer in bioreactor fed-batch versus batch production was due to a combined increase in cell density and specific productivity.

CONCLUSIONS

We have successfully designed a stable inducible producer cell line growing in suspension for the production of LVV-GFP. This cell line was used to develop a fed-batch process, and we successfully scaled-up the process up to 50 L bioreactor while maintaining the volumetric LVV-GFP functional titer. The HEK FS feed, selected after screening a dozen of different feeds and additives, increased the cell density and specific virus productivity in fed-batch culture, resulting in an improvement of the functional titers by about 3-fold compared to batch mode. Three orthogonal methods were used to quantify LVV in this project: a GTA in order to measure the functional titer, a p24 ELISA in order to measure intact particles, and an AEX-HPLC method in order to measure total particles. The AEX-HPLC method leads to an overestimated particle count compared to the p24 ELISA due to the presence of other vesicles, but represents a valuable in-process quantification method since results can be obtained within 15 minutes. The functional titer demonstrates that the process developed with clone 18-8 is scalable up to at least pilot scale. The ratio of p24/GTA was between 76 and 97 LP/TU across the different bioreactor scales, which indicates that the process is robust and reproducible. Metabolite profiles were similar at all scales tested. The simple biomanufacturing process described here is therefore scalable, improves the yield by 3-fold, and can be applied to other producer cell lines expressing clinically-relevant transgenes.

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Author Disclosures

No competing financial interests exist.

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