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Large-Scale Production and Purification of a Lentiviral Vector Reference Material

By Aziza Manceur, Anja Rodenbrock, Danielle Jacob, Mehul Patel, Sushma G. Puttaswamaiah, July Dorion-Thibaudeau, Julia Transfiguracion, Krishnaraj Tiwari, and Martin Loignon

Abstract

Lentiviral vectors (LVV) are widely used in an increasing number of approved cell and gene therapies, and benchmarking is important in evaluating production processes and products. We have produced a lentiviral vector reference material (LVV-RM) that expresses the common marker gene (green fluorescent protein [GFP]) to enable data comparisons and support LVV research programs. To generate that reference material, we have applied our previously developed fed-batch process to a 100 L production using an inducible LVV-producer cell line. The LVV material was harvested three days post-induction from a 200 L single-use bioreactor. A downstream purification process was also developed and scaled-up to meet production requirements: It consisted of nuclease digestion, clarification by depth filtration, chromatography capture using CIM-QA monolith anion exchangers, and ultrafiltration/diafiltration using a hollow fiber membrane prior to bulk-filling the final product. A total of 4.4 L of diafiltered and concentrated LVV product was obtained (9.2×10^{10} particles/mL, 3.6×10^{7} TU/mL) and stored at -80°C. Nearly 8000 vials are now available to the LVV community via the American Type Culture Collection (ATCC).

INTRODUCTION

Reference materials are used for the calibration of internal standards and analytical methods used in several laboratories, and to enable comparisons between different groups and organizations developing lentiviral vectors (LVV).[1,2] This is especially needed for viral vectors due to their complex nature. Even though the biological assays used to measure the functionality and potency of LVV are prone to high variability, a reference material was not available yet for purchase. To address this issue, a working group that met at the ISBioTech annual meeting in 2018 issued a formal request for proposal for the production and purification of an LVV reference material (LVV-RM). This initiative comes after similar accomplishments for the generation of reference materials for adenovirus 5 (Ad5)[3], adeno-associated virus 2 (AAV2), and adeno-associated virus 8 (AAV8).[4] All consumables and raw materials used during production and purification were donated by suppliers (listed in acknowledgments), whereas the National Research Council (NRC) Canada contributed labor and expertise and led the process development, production, and purification of the reference material using NRC's proprietary platforms. ISBioTech and BioProcessing Journal were central to soliciting donations, posting information, and coordinating meetings and conference calls.

The original goal of the LVV-RM project was to generate enough material to fill thousands of vials at 0.5 mL volume containing 0.5×10^{8} transducing units (TU)/mL. To meet the requirements of the project, a production process was developed and optimized at small scale in benchtop bioreactors.[5] The final process parameters obtained were used for the 100 L-scale production. Briefly, we have generated a suspension producer cell line that grows in serum-free media. The LVV production is triggered by the addition of two inducers (cumate and doxycycline hyclate [DOX]) and does not require transfection, thereby reducing costs and process complexity. The addition of two boluses of feed resulted in a 3 to 5-fold increase in titer in bench-scale bioreactors, and was therefore applied to the large-scale production described here. To purify and concentrate the harvest, a downstream process was also developed. Our two main selection criteria for the different unit operations were recovery and scalability. Downstream processing of LVV has undergone significant advancement in recent years, resulting in increasing product yield and purity, consistent with the needs for both *in vivo* and *ex vivo* LVV applications.[6] Depth filtration in clarification is becoming increasingly utilized, generally with good throughput, turbidity reduction, and infectious titer recoveries.[6,7] Chromatographic purification unit operations have largely replaced preparative centrifugation methods, with anion-exchange (AEX)-based membranes and monoliths representing the dominant, scalable capture chromatography technology for LVV purification (reviewed in [8]). Polishing chromatography using resin beads has also proven successful in small-scale screening experiments where buffer pH, buffer ion chemistry, and elution salt concentration have all shown to be important variables that require optimization for maximum product recovery.[9] Scale-up of chromatography resins for LVV purification could prove more challenging, however, where process chromatography time scales are longer and where the product would be exposed to higher ionic strength over longer periods. High-recovery sterile filtration of even concentrated LVV (4.5×10^{12} particles/mL) has been reported, demonstrating that there are now workable strategies and materials available to overcome this challenge.[10]

Therefore, the downstream processing (DSP)-scalable process selected was comprised of four steps and consisted of nuclease digestion, clarification by depth filtration, chromatography capture, and ultrafiltration/diafiltration (UF/DF) using a hollow fiber (HF) membrane.

In this publication, the upstream and downstream processes used to generate the LVV-RM are described. Following bulk fill, the material was shipped to the American Tissue Type Collection (ATCC) for sterile filtration, vialing, storage, and distribution. Several other organizations and laboratories will be involved in the characterization of the material, which will be subsequently published.

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.[11] The production of an LVV expressing green fluorescent protein (LVV-GFP) is triggered by the addition of 50 μg/mL 4-Isopropyl benzoic acid (cumate, Arkpharm) and 1 μg/mL DOX, MilliporeSigma), which activates a tetracycline and a cumate switch.[5, 12] LVV-GFP production is further boosted by the addition of 7 mM sodium butyrate (NaB, MilliporeSigma) one day post-induction (dpi).[12]

Seed Train

Clone 18-8 was grown and maintained in HyCell TransFx H media (Cytiva) supplemented with 4 mM L-glutamine (MilliporeSigma) and 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 and TriForest) at 37°C with an agitation of 120 rpm using orbital shakers (Infors HT, 25 mm orbital diameter) and 5% CO_2 with a cell density range of $0.2–1.8\times10^6$ cells/mL.

The final amplification steps were carried out in a 50 L Cellbag (Cytiva), which represents the n-1 vessel. The Cellbag was initially seeded at 0.29×10^6 cells/mL with a total volume of 8.0 L and grown for three days (37°C, 23 rpm rocking speed, 8° angle, 0.3 L/min, air/5% CO_2 overlay). After reaching 1.61×10^6 cells/mL, the culture was diluted to 0.62×10^6 cells/mL in a final volume of 18.8 L and grown for two more days before reaching 1.64×10^6 cells/mL (37°C, rocking speed of 27–30 rpm, 8° angle, 0.4–0.5 L/min, air/5% CO_2 overlay).

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).

Fed-Batch Production in Bioreactor

Upstream production of the LVV-RM was carried out in an Excellerex XDR-200 single-use bioreactor (Cytiva) using an XDR-200 Dev bag, with a final process volume of 100 L. Temperature (37°C), pH (7.10 ± 0.05), and dissolved oxygen (DO, 40%) were measured and controlled with appropriate sensors (Hamilton). Control of pH was achieved via base addition (Bioreactor pH adjustment solution, MilliporeSigma) or CO_2 addition through the microsparger. The agitation rate was adjusted to maintain volumetric power inputs of 113 W/m³ at inoculation (day 0) and 152–155 W/m³ between day 1 and harvest, which was equivalent to agitation rates of 140 rpm and 155–194 rpm, respectively. A higher volumetric power input, compared to our pilot-scale runs[5], was required in the XDR-200 in order to maintain similar hydrodynamic characteristics as those described at smaller scale by Kreitmayer *et al.* (2023)[13]

The bioreactor was seeded at 55 L (or 57% of the final working volume) with a target seeding density of $0.5–0.6\times10^6$ cells/mL. After reaching a cell density of $\geq 1.5\times10^6$ cells/mL, the culture was diluted 1.75-fold with fresh media for a total of 90% of the final working volume. Culture media were supplemented with a HyClone antibiotic/antimycotic 100X solution (Cytiva). Two feed additions of HEK FS (Xell, now Sartorius) were carried out, based on the post-dilution culture volume—the first one on the day following dilution (4% v/v) and the second one after reaching $\geq 3.5\times10^6$ cells/mL (6% v/v). Induction was performed immediately after the second feed by the addition of cumate and DOX, followed by supplementation with NaB 20 ± 4 hours post-induction (hpi), as described above. (All reagent additions were based on the post-feeding culture volume).

Nucleic Acid Digestion

At 3 dpi (or 72 ± 4 hpi), the culture liquid was treated with 10 U/mL Benzonase nuclease (MilliporeSigma) in the presence of $MgCl_2$ (2 mM) at 37°C for 1–2 hours in the bioreactor with constant mixing followed by harvest. The process is summarized in **Figure 1**.

Clarification

The Benzonase-treated harvest was applied to the filtration train consisting of primary depth filters (Clarisolve 60HX; 2×0.55 m², pore size 60 µm, MilliporeSigma) followed by a secondary depth filtration step using Polysep II (4×1.36 m², pore size 1.0/1.2 µm, MilliporeSigma) at a flow rate of 150 L/m²/h. Pre-wetted primary depth filters, using 100 L/m² of Super-Q water (SQW, MilliporeSigma) at 600 L/m²/h were connected to secondary filters in series and wetted with SQW at 150 L/m²/h for an additional ten minutes before equilibrating with buffer (20 mM Tris, 2 mM $MgCl_2$, 150 mM NaCl, pH 7.0) and processing the feed. A post-use buffer flush of 50 L was collected with the clarified material.

Anion-Exchange Chromatography

AEX chromatography was performed using two CIMmultus QA (CIM-QA) 800 mL, 6 µm monoliths in parallel (BIA Separations, Sartorius). An ÄKTA Pilot chromatography workstation (Cytiva) was used to monitor a portion of the column effluent flow in order to record absorbance at 280 nm, conductivity, and pH using its flow cell and in-line probes. The remainder of the flow path was directed to vessels to collect waste or wash fractions, with the eluted bulk product from each monolith collected separately in bioprocessing bags. A conditioned, clarified lysate volume of 144 L was processed as 2×72 L volumes over two cycles of chromatography. The monoliths were initially sanitized with 1 M NaOH, neutralized with SQW, regenerated using high-salt buffer (20 mM Tris, 2 M NaCl, pH 7.0), and then equilibrated in start buffer (20 mM Tris, 2 mM $MgCl_2$, 150 mM NaCl, pH 7.0). Clarified harvest was pumped onto the monolith columns at a flow rate of 3 L/min using an external peristaltic pump. Following loading, the columns were washed with ten times the column volume (10×CV) of wash buffer (20 mM Tris, 2 mM $MgCl_2$, 150 mM NaCl, pH 7.0), followed by elution with 10×CV of elution buffer (20 mM Tris, 2 mM $MgCl_2$, 1.4 M NaCl, 6% [w/v] sucrose, pH 7.0). In order to reduce the ionic strength after elution, the collected 6×CV eluate was immediately diluted 6× into buffer (20 mM Tris, 2 mM $MgCl_2$, 6% [w/v] sucrose, pH 7.0). The monoliths were then stripped using a high salt buffer (20 mM Tris, 2M NaCl, pH 7.0). The eluted product was stored overnight at 2–8°C prior to further processing the next day.

Concentration and Diafiltration

Following overnight storage, the purified product underwent concentration by UF/DF followed by a final UF concentration step (UF/DF/UF). A tangential flow filtration (TFF) system comprised of an in-house prepared flowpath with separate feed, recirculation, and permeate

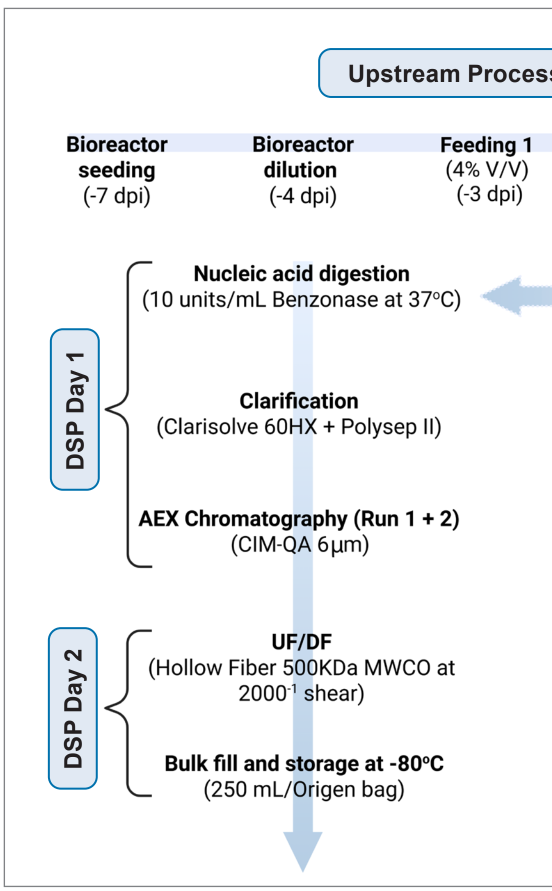


FIGURE 1. Overview of the processes for upstream and downstream (DSP) used for the generation of the LVV-RM.

pumps were utilized in the UF/DF setup. A Repligen KrosFlo HF cartridge (PES, 500 kDa MWCO, 0.5 mm lumen i.d., 2.6 m^2 surface area) was used for processing the material at a shear of 2000 s^{-1}. Pre-use HF wetting (SQW), sanitization with NaOH, and equilibration in buffer (20 mM Tris, 2 mM $MgCl_2$, 6% [w/v] sucrose, pH 7.0) was performed prior to the initial UF (4× concentration factor), followed by DF (5× diafiltration volumes [DV]) and final UF (4×). For the DF step, the formulation buffer consisted of 20 mM HEPES, 75 mM NaCl, 2.5% (w/v) sucrose, pH 7.0. A final membrane rinse was performed using the same buffer. A total of 4.4 L product was dispensed as 250 mL volumes into bioprocessing bags (OriGen Biomedical) and frozen at -80 °C.

Metabolite Measurements

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* during the growth and production phases. Metabolite concentrations were photometrically measured using the Cedex Bio Analyzer (Roche Custom Biotech).

Total LVV Particle Titer by Anion-Exchange HPLC

An AEX high-performance liquid chromatography (AEX-HPLC) method was used for the quantification of LVV particles (VP/mL) according to an internally developed method.[14] Total VP/mL, as mentioned here, consists of infectious and non-infectious particles as well as the minimal presence of extracellular vesicles (EVs). It was reported that EVs are also produced during LVV production, and some co-elute with LVV due to similarities in physico-chemical properties using AEX-HPLC.[15] Therefore, this method was optimized to minimize the EV co-elution to less than 10 %.[14] The method was used as an in-process monitoring tool, and only a single injection of samples was performed.

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.

Intact Viral Particle Titer by p24 ELISA

The 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 2000 molecules of p24 in one LVV particle:[16]

$$2000 \times 24 \times 10^3 / (6 \times 10^{23}) \text{ g of p24} = 8 \times 10^{-5} \text{ pg of p24}$$
$$\text{or } 1 \text{ ng p24} = 1.25 \times 10^7 \text{ LPs}$$

Infectious Virus Titer by Gene Transfer Assay (GTA)

Titers were measured with a flow cytometry–based assay.[11, 17, 18] LVV samples were serially diluted in Dulbecco's Modified Eagle Medium (DMEM) plus 5 % fetal bovine serum (FBS) 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.

Dynamic Light Scattering (DLS)

For DLS measurements, a Zetasizer Nano (Malvern Instruments) was used. LVV samples were diluted ten times in PBS, and 800 µL were transferred into a microcuvette. The sample was equilibrated to 20°C and three readings were performed.

Residual DNA by PicoGreen Assay

Lambda DNA stock (1 µg/mL, Invitrogen) was diluted in Tris-EDTA (TE) buffer to generate a standard curve (6.25–400 ng/mL). Four sample dilutions were prepared in TE buffer for measurement. Lambda DNA served as a positive control. PicoGreen dye concentrate (Thermo Scientific) was diluted 200-fold into TE buffer to prepare a working stock solution. Volumes of 100 µL sample and of dye/working stock solution were added to a 96-well plate, incubated for 15 min at room temperature, and the fluorescence signal (excitation wavelength = 480 nm, emission wavelength = 520 nm) were measured using a Synergy H1 BioTek plate reader (Agilent). Reagent blank fluorescence values were subtracted from measurements and the DNA concentration was determined relative to the Lambda DNA standard curve.

Residual Host Cell Proteins

An ELISA kit (Cygnus Technologies) specific to HEK293 cells was used to measure residual proteins according to manufacturer's instructions.

RESULTS AND DISCUSSION

Upstream Process

In order to build the cell inoculum for the 200 L bioreactor, an n-1 Cellbag was used with a working volume of 20 L. After five days of growth with a doubling time of 27–35 h, cells were transferred to the 200 L bioreactor (starting volume of 55 L). After inoculation, the bioreactor was sampled to confirm the seeding cell density (0.57×10^{6} cells/mL in 55 L culture volume). A VCC (**Figure 2A**) of 1.68×10^{6} cells/mL was reached after three days of growth and the bioreactor was diluted to 0.92×10^{6} cells/mL by transferring 41 L of fresh medium supplemented with 1% antibiotic/antimycotic solution. HEK FS feed was added 23 hours later at 1.30×10^{6} cells/mL (4%) as well as four days post-dilution (6%), after reaching $\geq 3.5\times10^{6}$ cells/mL. Following the second feed addition, induction was performed by transferring 50 µg/L cumate and 50 µg/L DOX (final concentration) to the culture. A post-induction VCC of 3.90×10^{6} cells/mL was measured. To boost virus production, a final concentration of 7 mM NaB was added 21.2 hpi. After induction, no further cell growth was observed and the VCC decreased to 3.12×10^{6} cells/mL prior to harvest. At 3 dpi or 70.7 hpi, 10 U/mL Benzonase was added to the culture. Harvest was initiated following an incubation period of 85 min, at 72.2 hpi. Bioreactor controllers remained active during the Benzonase treatment and harvest steps.

Culture viability in the bioreactor (**Figure 2A**) was maintained at 96–99% before induction, followed by a decline to 87% at 3 dpi, before harvest. A functional virus titer of $1.59\pm0.36\times10^{7}$ TU/mL was measured at 3 dpi (sample retained at harvest before Benzonase treatment). Intact p24 capsid particles and total viral particles reached $2.93\pm0.02\times10^{9}$ LP/mL and $2.72\pm0.04\times10^{10}$ LP/mL, respectively. The ratio between total lentiviral particles measured by p24 ELISA, and active particles measured by GTA, is correlated with the material potency. This ratio for the 100 L LVV-RM production at harvest was 184 LP/TU (**Figure 2B**), a value indicative of a vector preparation of good quality.[8]

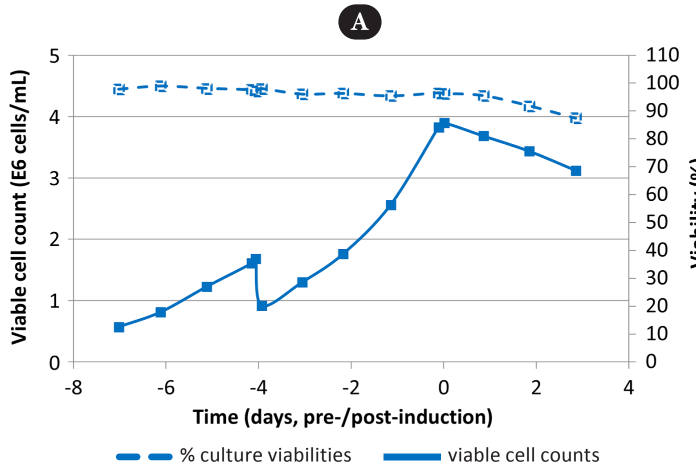
Metabolite concentrations (**Figure 2C**) measured throughout the 100 L production were comparable to data obtained at smaller bioreactor-scale.[5] The glc concentration was maintained above 20 mM until harvest while lac, NH_3, and LDH reached maximum concentrations of 47 mM, 2.4 mM, and 576 U/L, respectively.

The osmolality remained stable between days 0 and 4 (-7 to -3 dpi) with 312–308 mOsm/kg. While each feed addition did not have a significant impact on the osmolality, an increase to a 388 mOsm/kg was observed prior to harvest on day 10 (3 dpi), coinciding with base addition to the bioreactor between days 6–10 (-1 to 3 dpi). The total base volume added was 1.42 L.

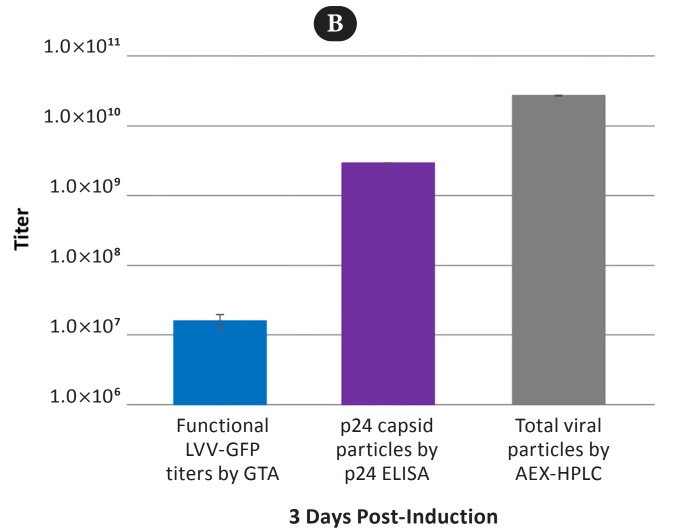
Downstream Process

The ultimate goal of the DSP process was to deliver sufficiently pure LVV product of 0.5×10^{8} TU/mL to prepare at least 3000 vials of reference material. A fit-for-purpose downstream process was developed based on the donated material consisting of: nucleic acid digestion using Benzonase nuclease, clarification using primary and secondary depth filtration, capture chromatography using a CIM-QA monolith, followed by UF/DF/UF using a HF cartridge to formulate

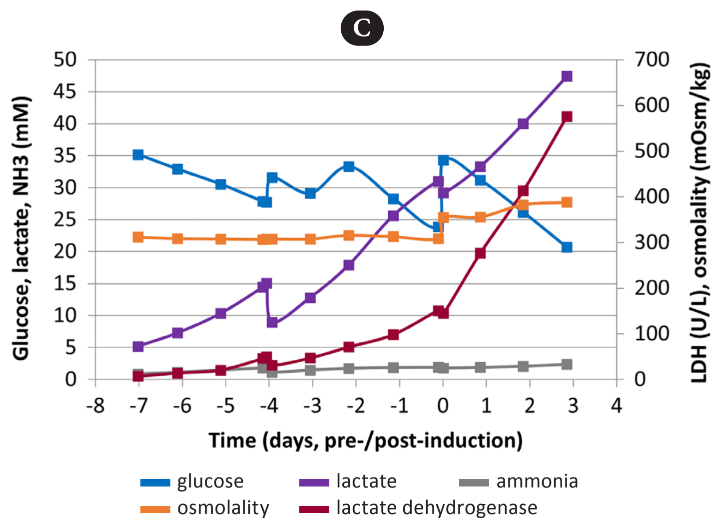
FIGURE 2.
LVV-RM fed-batch production in a 200 L bioreactor.



(A) Cell count and viability.



(B) LVV-GFP titers. Standard deviations are based on technical replicates and were calculated on at least two technical replicates.



(C) Metabolite concentrations.

the final product prior to bulk filling and storage at -80°C (**Figure 1**). The DSP process and related process parameters used for each operation were based on a series of scaled-down, pilot DSP runs performed using material produced at the 10 L-scale (data not shown). These pilot-scale DSP runs showed overall infectious titer recoveries (prior to sterile filtration) of 10–20%. A comparison of the recoveries based on functional titer between a 10 L-scale production and the 100 L LVV-RM production is shown in **Figure 3**, demonstrating that the downstream purification developed is scalable. More details about each step are provided below.

As part of the DSP process development work, several important process parameters required optimization at the pilot-scale (10 L production volume) and were key to successful overall process performance at the 100 L-scale. Optimization of Benzonase nuclease digestion conditions showed that 10 U/mL for 1 h at 37°C resulted in 91% depletion of DNA. With longer incubation times of up to 3 h, or higher nuclease doses up to 30 U/mL, no further improvement was offered. For the 100 L production, we observed a similar trend with an overall clearance of 99.5% DNA in the final TFF retentate sample (**Table 1**). Setting a maximum pressure threshold of 3 psi for primary and secondary depth filtration operations was necessary in order to minimize product aggregation and turbidity breakthrough during the clarification step with the filter train used. Following the Clarisolve 60HX primary depth filter (capacity of 123 L/m²), Polysep II secondary filter (5.5 m² total area with a capacity of 22 L/m²) was used in order to achieve the required clarification performance at the 100 L-scale. We obtained total LVV particle (83%) and infectious titer (57%) step recoveries from the clarification operation (**Figure 4**), the latter within the expected infectious titer step recovery range of 53–69%, based on pilot-scale runs (**Figure 3**).

With the AEX purification step, the CIM-QA monolith capacity was established in pilot runs to be ~90 mL of clarified product per mL of CV. On this basis, the decision was made to perform two cycles of chromatography using 2 × 800 mL CIM-QA monoliths in parallel, representing ~50% of total capacity for the 72 L volume, in order to process all of the clarified material. Use of the convective interaction media for chromatography permits high loading flow rates and fast mass transfer, which may be important, considering the recent observation of a negative correlation between recovery and contact time seen using membrane absorbers such as Sartobind-Q.[19] In order to minimize the effect of high salt concentration (1.4 M in elution buffer) on the LVV product infectious titer, a 6-fold dilution (<15 mS/cm conductivity target) in buffer containing 6% (w/v) sucrose was performed immediately after product elution. Infectious

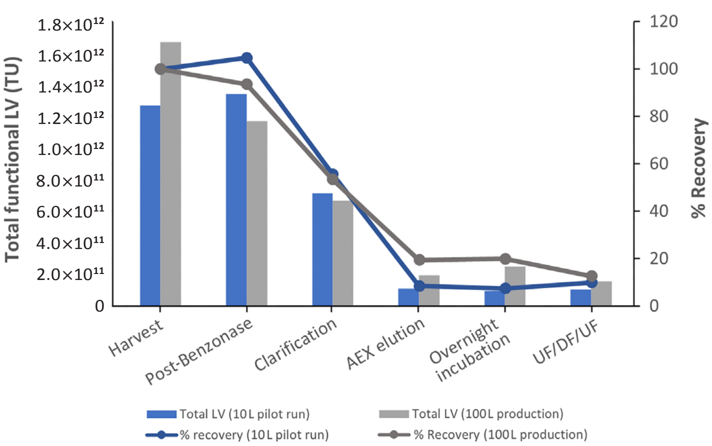


FIGURE 3. Step and overall recovery after each downstream purification step based on functional titer. Comparison between a representative 10 L pilot run and the 100 L LVV-RM production run.

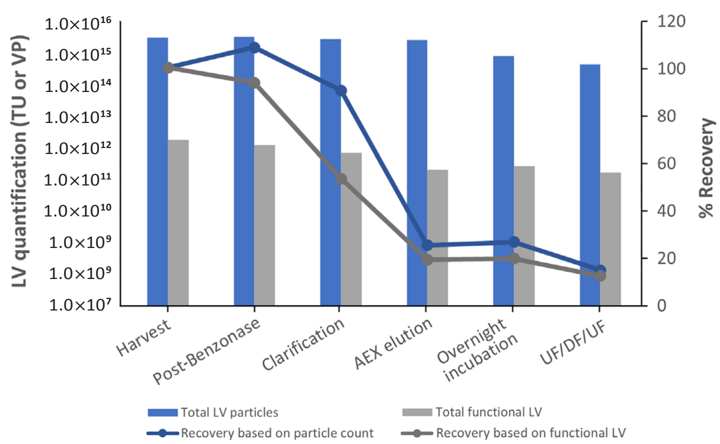


FIGURE 4. Step and overall recoveries for the 100 L LVV-RM production run calculated based on total particle count (measured by AEX-HPLC) or functional active LVV (measured by GTA).

TABLE 1. Host cell DNA analysis of samples retained during the purification process.

Sample Description	Volume (L)	Concentration (ng/mL)	Concentration Total (µg)	Step Clearance (%)	Overall Clearance (%)
Bioreactor Harvest	105.0	6907.0	725235.0	0.0	—
Harvest Post-Benzonase	106.0	1925.0	204050.0	71.9	—
Post-AEX Chromatography	30.8	1142.8	35175.3	38.5	—
Post-TFF	4.4	730.0	3212.00	95.34	**99.53**

titer measurement of the AEX eluant indicated ~40% step recovery, higher than that typically determined for pilot-scale runs (32–33%, n=2) (**Figure 4**). These functional step recoveries are in the range of expected values (30–80%) based on a large number of LVV AEX purification studies and reported in Moreira *et al.* (2021).[8] A subsequent overnight hold of the eluted product at 2–8°C revealed no decrease in infectious titer.

On an operational note, in order to load the column at 3 L/min, a separate pump was required as this flow rate exceeded the maximum possible flow rate of the ÄKTA Pilot system, which is 0.8 L/min. At the same time, in order to collect the chromatogram, it was necessary to collect the absorbance at 280 nm, pH and conductivity signals from the column outlet. The solution to this was to divert a portion of the outlet fluid stream to the ÄKTA Pilot system, while the main process fluid was being pumped by the external pump. A schematic of the homemade setup is shown (**Figure 5A**). The chromatogram reveals a peak at 38 min, as expected based on previous pilot runs (**Figure 5B**).

Formulation of the bulk product was achieved by TFF in a UF/DF/UF sequence. The first UF was performed (3.5–3.8×) followed by 5 DV of buffer exchange into the final formulation buffer (20 mM HEPES, 75 mM NaCl, 2.5% [w/v] sucrose, pH 7.0). A final UF step (5×) was then performed to achieve the final product concentration. Even though this unit operation routinely resulted in high recovery of infectious product titer in pilot-scale runs (92–108%, **Figure 3**), the recovery here was only 64% for unknown reasons. Therefore, the overall recovery prior to sterile filtration is between 13 and 15% (**Figure 4**). The overall ratio of total to infectious LVV particles is consistent through different process steps and is on the order of 1×10^3, a value indicative of a vector preparation of good quality.[8]

Sterile filtration post-purification of LVV can pose risks and yield variable product recovery and capacity, depending on the purity state of the product, including aggregate level prior to filtration, as well as the process conditions used for processing.[10] One important conclusion from the work of Vogel *et al.* (2024)[10] is that residual DNA content, and possibly DNA size, can impact the sterile filtration step, highlighting the importance of post-harvest Benzonase treatment. Our pilot-scale studies also high-lighted the importance of not exceeding the 3 psi pressure limit during clarification (with the filtration train used in this study), beyond which LVV aggregate formation could result and negatively impact sterile filtration performance. When the aggregate content of purified products is low, as revealed by methods such as dynamic light scattering (DLS), filtration using 0.45 µm and 0.22 µm filters can result in high (>80%) product recoveries (data not shown). The LVV analysed by DLS prior to sterile filtration has a Z-average of 155 ± 78 nm (**Figure 6**), which suggests that the viral vector is not aggregated and suitable for sterile filtration.

The DSP workflow described here shares some common features with other published scalable DSP processes used for LVV products produced using adherent cells or in suspension culture.[8,20] Depth filtration trains are being used for clarification, AEX membranes and monoliths for chromatographic capture, and product concentration and diafiltration is being performed by TFF. This type of workflow can be considered as a general platform approach for scalable LVV DSP, with the possibility of adding additional steps such as a UF/DF step prior to capture chromatography, or an additional polishing chromatography step prior to formulation, as required for the specific process. While conventional size-exclusion

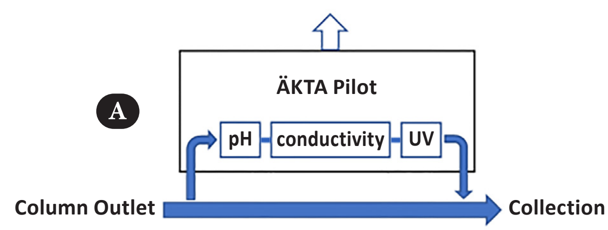
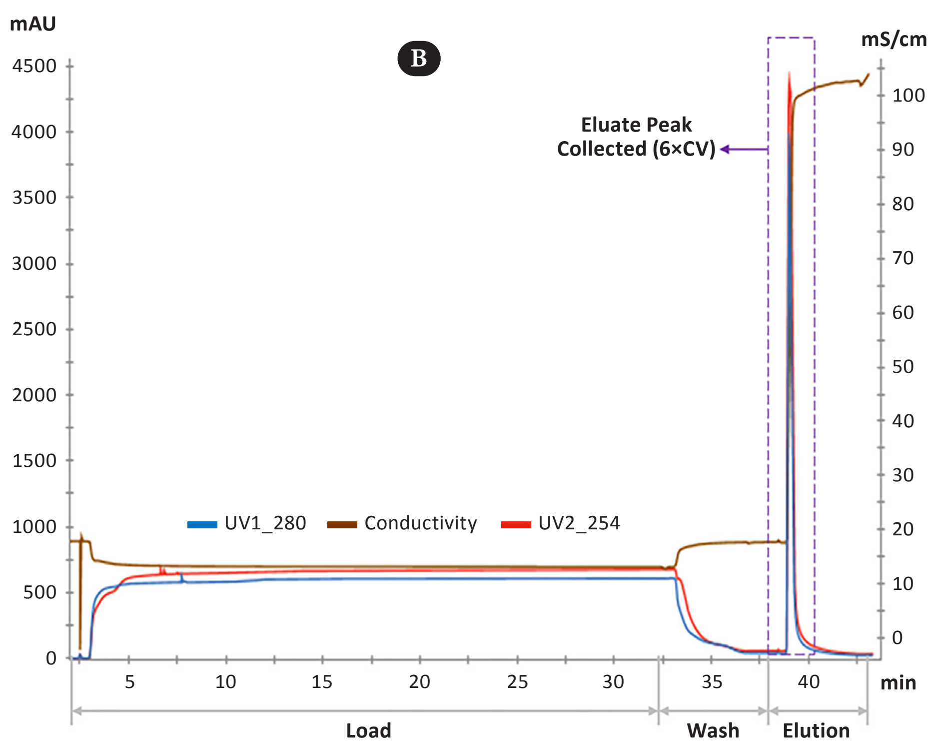


FIGURE 5. (A) Schematic diagram showing a portion of the flow path from the column outlet diverted to the ÄKTA Pilot to measure UV, pH, and conductivity values in-line, while the bulk of the eluant is collected; and **(B)** AEX chromatogram depicting the absorbance at 280 nm signal for the product elution peak.



chromatography (SEC) is sometimes used as a polishing step, core shell mixed-mode resins with large molecular weight cut-offs could offer better purification performance and would be a more scalable option for manufacturing purposes.

CONCLUSIONS

The biomanufacturing process implemented to produce a LVV-RM involved the use of a suspension stable-producer cell line in a large-scale bioreactor. The production was performed in serum-free media and mirrored a process developed at smaller-scale.[5] Using a producer cell line reduced the costs significantly since transfection reagents were not required, and simplified the process. A DSP process was also implemented for the 100 L production run based on unit operations developed using prior 10 L pilot-scale batches. The process was successfully executed from clarification to bulk fill of the final product. Sufficient purified LVV-RM was obtained to prepare nearly 8000 vials (9.2×10^{10} particles/mL, 3.6×10^{7} TU/mL). The bulk product was shipped to ATCC for sterile filtration, vialing, storage, and distribution. The LVV-RM described here is not meant to be used as a stand-alone standard, but to provide a means for validating internal reference materials using different methods. Therefore, NIST (U.S. National Institute of Standards and Technology) will coordinate the characterization of the final product with a consortium of organisations from academia, government agencies, and industry. Data pertaining to infectious titer, particle concentration, and size measured by quantitative polymerase chain reaction (qPCR), droplet digital PCR (ddPCR), different p24 ELISA kits, and other techniques will be subsequently published.

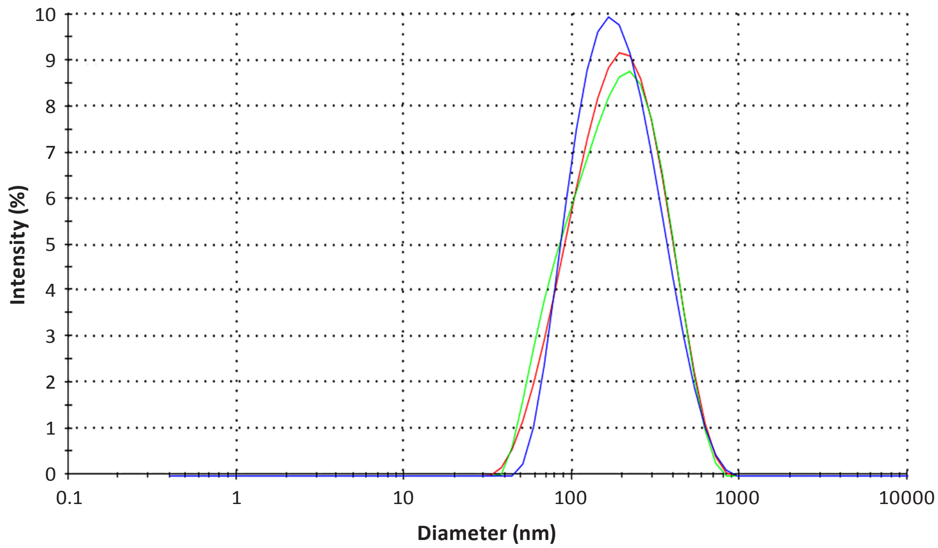


FIGURE 6. Size distribution (by intensity) of the LVV post-TFF by DLS. Each color corresponds to a reading for a total of three readings.

Author Disclosures

No competing financial interests exist.

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About the Authors

Aziza Manceur*[1], Anja Rodenbrock[1], Danielle Jacob[1], Mehul Patel[1], Sushma G. Puttaswamaiah[2], July Dorion-Thibaudeau[3], Julia Transfiguracion[1], Krishnaraj Tiwari[1], and Martin Loignon[1]

1. Bioprocess Engineering Department, Human Health Therapeutics Research Center, National Research Council Canada, 6100 Royalmount Avenue, Montreal, QC, H4P 2R2 Canada
2. Novavax, 700 Quince Orchard Road, Gaithersburg, Maryland 20878 USA
3. Groupe Pharmaceutique Duchesnay, 950 Boulevard Michèle-Bohec, Blainville, QC, J7C 5E2, Canada

****Corresponding Author:***

Aziza Manceur, PhD, Research Officer, National Research Council Canada

Phone: 1-438-988-1289 | Email: aziza.manceur@nrc-cnrc.gc.ca