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Downstream Processing of a Mid-Scale Lentiviral Vector Batch for the Generation of CAR-T Cells

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Abstract

Chimeric antigen receptor (CAR) T-cells – which are engineered to target malignant cells - have emerged as an important cancer immunotherapy. Lentivirus purification is a significant barrier to the productive manufacturing of genetically modified T cells, the backbone of CAR-T therapy. Currently, there is an absence of established technologies to support the downstream processing of lentiviruses. This study aimed to showcase the application of tangential flow filtration (TFF) systems for lentivirus downstream processing. Our results demonstrate that omitting capture chromatography and employing TFF systems for harvest, clarification, and ultrafiltration and diafiltration is an effective strategy for purifying midscale batches of lentiviral vectors.

Abbreviations

CAR	Chimeric Antigen Receptor
CPP	Critical Process Parameter
DF	Diafiltration
(ds)DNA	(Double-stranded) deoxyribonucleic acid
HEK	Human embryonal kidney cells
LV	Lentiviral vector
PES	Polyethersulfone
RNA	Ribonucleic acid
TFF	Tangential flow filtration
TU	Transducing units
UF	Ultrafiltration
VP	Viral particles

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Introduction

The production of therapeutic cells for chimeric antigen receptor (CAR) T-cell therapy relies on efficiently manipulating the cells' genetic information. Lentiviruses are used as vectors to introduce the genes of interest into the target cells.

One of the production bottlenecks in creating CAR-T cell therapies is the downstream processing of the lentiviral vectors. There is an absence of established and robust methods for purifying lentiviruses at midscale. Traditional chromatography methods result in poor recovery of the virus (typical yields are as low as 40%) and failure to meet purity and quality standards. Manufacturers are forced to rely on manual systems, which are labor-intensive and lack robustness and consistency. New innovations are required to maximize productivity in the downstream.

Our aim was to develop a new downstream process for the purification of lentiviral vectors to transduce T-cells for the production of CAR-T cell therapy. To obtain the purest possible virus while minimizing potential loss of infectivity, we omitted the capture chromatography step to purify the target molecule. Instead, we employed a lateral approach using tangential flow filtration (TFF) systems for harvest, clarification, ultrafiltration, and diafiltration.

Methods and Results

An overview of our approach is shown in Figure 1.

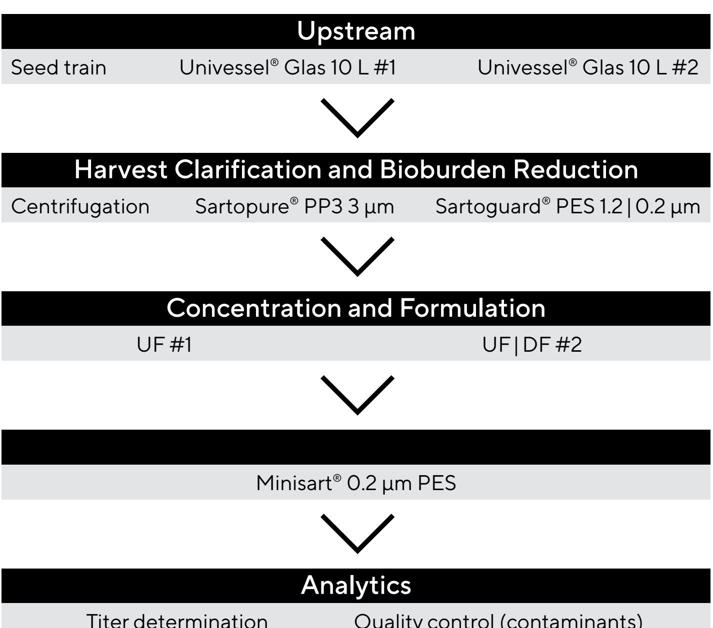

Figure 1: Overview of lentiviral vector batch production strategy

Upstream Processing

To express the lentiviral vector, suspension HEK293T cells were transiently transfected with four plasmids encoding the essential lentiviral genes, as described in detail by Labisch et al.[1]. We produced 20 L of lentivirus by cultivating two separate batches of host cells in 10 L Univessel® glass bioreactors. Using Sartorius process insights tool, we identified optimal process parameter setpoints for the 10 L bioreactor based on an established lentivirus production process in the Ambr® 250 Modular. Our scaling strategy was based on maintaining a constant tip speed, and gas flow rates were adjusted to the bioreactor volume.

Before harvesting the lentiviral particles, free nucleic acids were digested by the addition of a benzonase (10 U/mL Denarase) directly into the bioreactor, followed by incubation for one hour. Next, we harvested the viral particles and removed cells and cell debris by centrifugation at 800 × g for 5 minutes. The first 10 L virus batch (Batch A) was stored at -80 °C until the second batch (Batch B) was harvested under the same conditions. The viral particle and infectious virus titers were analyzed for each batch separately (Table 1).

LV batch	Particle titer p24-ELISA (vp/mL)	Infectious titer (TU/mL)
LV Batch A	3.38×10^8	3.02×10^5
Reference LV Batch A	5.76×10^8	
LV Batch B	3.59×10^7	5.62×10^4
Reference LV Batch B	3.89×10^8	

Table 1: Viral particle and infectious titer of the two 10 L lentivirus batches compared to reference shake flasks

The particle titer of the first 10 L bioreactor run (Batch A) was comparable to the reference shake flask. The titer of the second run (Batch B) was significantly lower than both its reference shake flask and Batch A. This difference was caused by reduced cell viability and growth.

Downstream Processing

The viral vector supernatants from both upstream batches were pooled in a 20 L Flexboy® bag before downstream processing.

Clarification

Clarification filtration was performed at a constant flow rate with the Sartoflow® Advanced TFF system and Sartorius single-use filters (Figure 2). Two filters (operating in a train) were used to achieve the desired virus clarification.
The first filter was a polypropylene fleece-based depth filter (Sartopure® PP3) with a pore size of 3 µm, employed to remove larger impurities like cell debris. The second filter (the asymmetric PES membrane filter, Sartoguard® PES) was used to further clarify the virus and remove smaller impurities. Size 0 capsule formats were used for both filters (0.3 and 0.52 m^2 membrane surface area, respectively). The filters were selected based on their excellent performance in previous small-scale optimization studies.

After installation of the filters, tubing, and the receiving bag (Sartoflow® Advanced Bag 20 L), the filters were pre-flushed with ~2 L of virus production medium (Freestyle 293), and the filtrate was discarded. The bag containing the viral vector supernatant was installed, and clarification was performed using the Sartoflow® Advanced system pump at a fixed speed. After clarification, the filters were flushed with 1.5 L of virus production medium.

To assess the clarification performance, samples of the virus were taken before and after the filtration and turbidity, viral particle concentration, and infectious viral particle concentration were analyzed. The volume of the virus solution was measured to calculate the mass balance.

We observed no increase in pressure or reduction in feed flow during filtration, indicating the capacity of the filters was sufficiently high (> 66 and > 38 L/m^2). The filtrate had a clear appearance, confirmed by analysis demonstrating that turbidity could be reduced from 50 NTU to less than 9 NTU, representing an 82% reduction (Table 2). The viral particle recovery was 57%.

Figure 2: Sartoflow® Advanced system and Sartorius filter used for the clarification filtration of the virus solution.

Turbidity (NTU)	Starting material	50.9
	Clarified material	8.85
Reduction of turbidity (%)		82.61
Feed volume (L)		20
Pump power (%)		10
Flow (L/h)		117
Time (min)		11
Max. pressure (bar)		0.02
Filter surface area (m^2)	Sartopure® PP3	0.3
	Sartoguard® PES	0.52
Capacity (L/m^2)	Sartopure® PP3	> 66.67
	Sartoguard® PES	> 38.46
Flux (L/h × m^2)	Sartopure® PP3	12.22
	Sartoguard® PES	7.05

Table 2: Process conditions and results for the clarification filtration of the lentivirus solution.

Tangential Flow Filtration (TFF)

Figure 3: Sartoflow® Advanced crossflow system with the ultrafiltration cassette used for the concentration of the virus solution.

Ultrafiltration (UF) 1

The clarified virus solution was concentrated to reduce the product volume using the Sartoflow® Advanced crossflow system (Figure 2). A Hydrosart® membrane-type cassette with a molecular weight cut-off of 300 kDa and a surface area of 0.7 m² (selected based on the results of a previous optimization study) was used for ultrafiltration (UF). We used flat sheet ultrafiltration modules for this study as Sartorius hollow fiber cassettes were not yet part of Sartorius portfolio at the time the study was conducted.. After installation, the cassette was flushed with WFI, PBS, and virus production medium (1 L).

Next, the bag containing the virus solution was installed on the feed inlet and retentate outlet of the crossflow system, and the procedure was started. The virus feed was circulated through the system and cassette at a low pump speed while the permeate valve was closed for two minutes. The permeate valve was then opened, and the concentration of the virus solution was initiated. The concentration process was operated at a constant pump power and was stopped once a final volume of 1 L retentate was obtained (20-fold concentration). Next, the cassette was flushed with ~1 L virus production medium, and the system was drained. The retentate mass was noted, and samples were drawn for virus titration.

Concentration was performed with the Sartoflow® Advanced crossflow system at a constant flow rate, and we observed no blocking of the cassette. However, because we flushed the cassette with a relatively large volume after the ultrafiltration (due to the large dead volume of the cassette and the cross-flow system), the virus was diluted again. The final concentration factor was 8.6.

Volume (L)	Start	21.15
	End	1
	Post Flush	2.459
Concentration factor		8.6
Membrane area (m²)		0.7
Capacity (L/m²)		> 30.21
Flow (L/h)		60
Time (min)		27
Flux (L/(h*m²))		67.14
TMP (bar)		0.35
dP (bar)		0.7
Pump speed | power (%)		15

Table 3: Process conditions and results of the first ultrafiltration of the lentivirus solution.

Ultrafiltration | Diafiltration (UF | DF) 2

The second ultrafiltration followed by a diafiltration to formulate the lentivirus was performed using the Sartoflow® Smart (Figure 3) and a Hydrosart® membrane-type cassette with a molecular weight cut-off of 300 kDa and a surface area of 0.02 m² (Sartocon® Slice 200 Eco).

After installation, the cassette was flushed with WFI, PBS, and virus production medium. The Flexboy® bag containing the virus solution was installed on the feed inlet and retentate outlet of the crossflow system.

The virus feed was circulated through the system and cassette at a low pump speed while the permeate valve was closed for 2 minutes. The permeate valve was then opened to initiate the concentration of the virus solution. The virus was concentrated to a defined volume to obtain a 22-fold concentration. The solution was then diafiltered five times with a formulation buffer (20 mM HEPES pH 7.0, 75 mM NaCl, 2.5% Sucrose). Next, the cassette was flushed with formulation buffer, and the system drained. The retentate mass was noted, and samples were drawn for virus titration. A total concentration factor of 22 was obtained.

A limitation of this step was the small surface area of the cassette, as the pore blockage increased steadily over time. This led to a significant decrease in flow rate during ultrafiltration and increased the overall process time. As lentiviral vectors are relatively unstable at room temperature and shear-sensitive, a loss of infectivity likely occurred during the second UF | DF process step. Due to the steadily decreasing flux and the increase in pressure needed to keep a certain flux, the ultrafiltration was stopped at 80 mL of retentate solution to minimize the damage to the virus solution. The cassette was subsequently flushed with formulation buffer, and the system was drained.

Initially, the process was run at a constant flow, but when the maximum dP value (2 bar) was reached, the pump speed was adjusted to maintain the process at this pressure.

Figure 4: Sartoflow® Smart crossflow system and Sartorius ultrafiltration cassette (slice format) used for the concentration and formulation of the virus solution.

Volume (L)	Start	2.459
	End	0.116
Concentration factor		21.2
Membrane area (m²)		0.02
Flow (L/h)		0.6 – 1.5
Time (h)		3.30
Flux (L/(h × m²))		37.26
TMP (bar)		0.75
dP (bar)		1.3 – 2
Pump speed | power (%)		15 – 23

Table 4: Process conditions and results of the second ultrafiltration and diafiltration step of the lentivirus solution.

Sterile Filtration

Sterile filtration (using a 0.2 µm filter) is typically a challenging step to perform during lentiviral purification due to their large size. However, sterility is essential when transducing T-cells for clinical purposes.

Figure 5: A Minisart® filter used for the sterile filtration of the lentivirus solution.

First, the virus solution was aspirated with a sterile syringe. Then, Minisart® syringe filter (Figure 5) was attached to the Luer-fitting of the syringe, and the virus was filtered through the membrane. After sterile filtration, the final solution was pipetted into 0.5 mL aliquots in cryo-preservation vials and stored at -80 °C.

Analytics

We performed several quality control assays to determine the physical and chemical properties of the final lentiviral vector product as well its intermediates during downstream processing.

The following analytical parameters were employed:

- Measurement of turbidity (Orion™ AQUAfast AQ3010 turbidity meter, Thermo Fisher Scientific)
- Total dsDNA concentration (Quant-iT™ PicoGreen™ Assay, Thermo Fisher Scientific)
- Total protein concentration (Pierce™ Coomassie Bradford protein assay kit, Thermo Fisher Scientific)
- Total LV particle titer (determined by a p24-ELISA, QuickTiter™ Lentivirus Titer Kit, Cell Biolabs)
- Infective lentiviral particle titer (determined by transduction of adherent HEK293T cells with virus samples and measurement of transgene expression (CD19-CAR construct) by flow cytometry with the iQue® Screener PLUS (Sartorius), as previously described[1]).

Table 5 gives an overview of the characteristics of the final product. Overall, 9.01% infectious viral particles were recovered during the downstream process. Each process step leads to a loss of 35 – 60% total viral particles (Figure 5).

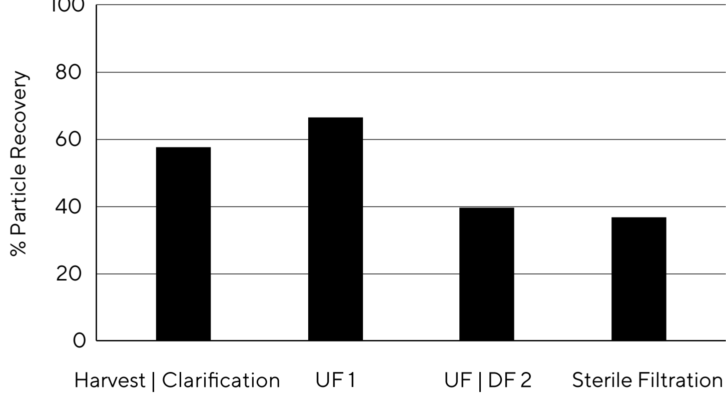

Figure 5: Total viral particle recovery of every step of lentivirus downstream processing (determined by p24-ELISA).

Even without purification by chromatography, the contaminating DNA was reduced by over 99% to a final concentration of 0.6 ng/µL. The highest amount of DNA was removed during the ultrafiltration steps (Figure 6). Furthermore, contaminating protein was reduced by over 80% without performing a purification step (Figure 7).

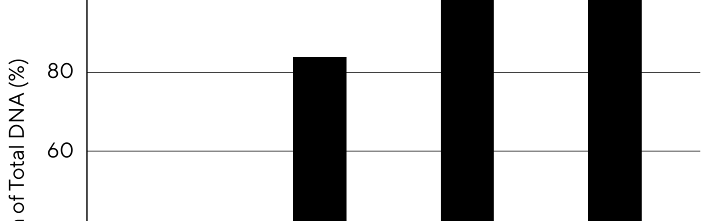

Figure 6: Percentage of contaminating DNA reduction after each step of the lentivirus downstream processing.

Total Protein Reduction

Figure 7: Percentage of contaminating protein reduction at each step of the lentivirus downstream processing.

Parameters	LV batch results
pH	7.11
Physical titer (VP/mL)	6.44×10^9
Infective titer (TU/mL)	1.35×10^7
Res. total DNA conc. (ng/µL)	0.60
Res. total protein (mg/mL)	19.24
Formulation	20 mM HEPES pH 7.0, 75 mM NaCl, 2.5% Sucrose

Table 5: Final lentiviral vector product characteristics.

A barrier to the efficient manufacturing of cell and gene therapies, such as CAR-T cell therapy, is in producing viral vectors of sufficient yield and purity while retaining their infectivity. Obtaining highly concentrated lentivirus solutions is challenging because concentration processes also cause impurities to accumulate and viral particles to aggregate. These factors create issues with ultrafiltration and final sterilizing filtration of lentiviral vectors. Additionally, commonly used chromatography technologies are not well-suited to purifying lentiviruses due to their size and fragility, leading to a low recovery.

In this proof-of-concept study, we demonstrated that, even in the absence of chromatography, it is possible to produce a good yield of lentiviral vector that fulfills all quality criteria, including removing contaminants. By minimizing the process steps, we achieve industry-standard yields of infectious viral particles (~10% recovery) while still eliminating contaminants.

The Sartoflow® Advanced crossflow system offers flexibility in bioprocessing and can be used for dead-end harvest clarification and bioburden reduction filtration in addition to tangential flow filtration for purifying medium batch scales of lentiviral vectors.

Acknowledgments
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References

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