CELL & GENE THERAPY INSIGHTS

DOWNSTREAM BIOPROCESSING

INNOVATOR INSIGHT

Enrichment of full rAAV capsids in a scalable, reproducible viral vector manufacturing platform

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Recombinant adeno-associated viruses (rAAV) are the gene transfer vector of choice for many *in vivo* gene therapies. These vectors are synthetic viral particles which can deliver a therapeutic gene to a patient or patients' cells to correct a genetic abnormality. These viral vectors can be produced in single-use bioreactors and purified using scalable single-use technologies. We evaluated the use of scalable, single-use filtration and chromatography technologies for downstream purification of an rAAV5 viral vector. In this testing, vector was produced in the Pall iCELLis® Nano bioreactor by polyethylenimine (PEI) mediated triple-plasmid transfection. The harvest material was clarified using direct flow filtration with a combination of Seitz-P grade depth and 0.2 µm sterilizing grade filters. The product was concentrated using 100 kDa Omega™ Membrane flat-sheet tangential flow-filtration (TFF) before primary purification with affinity chromatography. Affinity purified vector was polished using Mustang® Q membrane chromatography to enrich for full capsids. The rAAV5 product was then concentrated and diafiltered to the final formulation using 100 kDa Omega TFF membrane. The final product was sterile filtered using Pall's Supor® EKV validated sterilizing-grade filters. This manufacturing process was optimized and evaluated for vector yield, low contaminant profile and full capsid enrichment. We established feasibility of a near complete end-to-end manufacturing process using almost all materials available from Pall Corporation. This process resulted in a theoretical whole process yield of ~25% with a low contaminant profile (host cell protein [HCP] and [DNA]) and a ~5-fold enrichment of full capsids to total capsids. The purification process described here shows potential for a scalable, platformable process for rAAV products.

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Recombinantly produced adeno-associated viruses (rAAV) are now the predominant vector for *in vivo* gene therapies. These synthetic viruses can deliver a functional gene to correct a genetic defect and/or inhibit the cell from producing a defective version of the gene to restore normal function [1].

These medicines are a new class of biologics with the US FDA's first approval of an *in vivo* gene therapy in 2017. This viral vector was Spark Therapeutic's Luxturna® which is a treatment for biallelic *RPE65* mutation-associated retinal dystrophy [2]. Subsequently, the US FDA approved Novartis' Zolgensma® for spinal muscle atrophy (SMA) [3]. These treatments are literally lifesaving and can bring sight to the blind [4].

One of the biggest challenges in bringing these life-changing treatments to patients is their production. For products such as Luxturna where the disease indication is rare and the vector amount per dose is low (~1.5 x 10^{11} viral genomes per eye), there is a relatively low manufacturing burden. For more prevalent indications that require systemic administration with a high vector dose (>1 x 10^{14} vg/kg), such as Duchene's muscle dystrophy (DMD), manufacturing becomes a significant bottleneck [1]. Other viral vectors are in development such as Uniqure's AMT-061 (entranacogene dezaparvovec), a gene therapy treatment currently in clinical trials for Hemophilia B. This vector is based on adeno-associated virus serotype 5 (rAAV5) and utilizes a more moderate dose of 2 x 10^{13} vg/kg and has a moderate manufacturing burden [5].

Typical rAAV purification methods used in academic research are generally small scale and utilize ultracentrifugation for purification. This purification method results in very high purity product; however, these methodologies are not scalable [6].

The biopharmaceutical industry has decades of experience producing biologics such as recombinant proteins, predominately monoclonal antibodies, at industrial scale. These molecules are produced in single-use or stainless-steel bioreactors with batch sizes up to 20,000 L. The purification strategies for these moieties rely on technologies with scalable performance including depth and sterile filtration, affinity and ion-exchange chromatography and tangential flow filtration (TFF). These technologies are platformed into a common industrial strategy for the purification of monoclonal antibodies, via the following process steps: clarification by direct flow filtration (combination of depth, bioburden reduction, and sterile), affinity chromatography, ion-exchange chromatography polishing, concentration and diafiltration to final formulation using TFF and finally sterile filtration [7,8].

Here we evaluated the feasibility of applying a similar platform approach using many of the tools applied to mAb purification, for purification of a rAAV5 viral vector. The process was evaluated for product yield, purity and full capsid enrichment. In addition, specific unit operations were evaluated for process robustness.

MATERIALS & METHODS

rAAV5 Production in iCELLis® Fixed-bed Bioreactor

HEK293-T cells (American Type Culture Collection) were recovered from

cryopreservation and maintained in exponential growth using Dulbecco's Modified Eagle's Medium (DMEM, Thermo) supplemented with 10% fetal bovine serum (FBS, Thermo), 4 mM GlutaMax™ (Thermo) and 1X non-essential amino acids (NEAA, Thermo). Seed train biomass was propagated in T-flask and CellSTACK® (Corning) multi-layer trays.

Cell growth in the iCELLis® Nano bioreactor was monitored until the cell density reached ~150,000 cells/cm^2 at which point transient transfection was performed. The culture was continued for 5 days. The spent media was collected and treated with 25 U/mL Benzonase® (EMD Millipore) and 2 mM $MgCl_2$ for 1 hour at 37°C. The cells contained within the fixed bed were lysed with 10 mM tris-HCl, 2 mM $MgCl_2$, 1% Tween 20, and 25 U/mL Benzonase, pH 8.0 at room temperature overnight. After recovery of the lysate, the fixed bed was rinsed with 1 system volume of PBS. All harvest materials were brought up to a 500 mM total NaCl concentration. The final harvest material was a pool of the cell lysate, spent media and PBS rinse.

Clarification

Clarification of the harvest pool was performed using Seitz® P-grade PDK11 depth filters in series with Supor® EKV sterile filters. These are dual-layer cellulose depth filters (2–20 mm retention) and dual-layer polyethersulfone filters (PES, 0.65/0.2 mm), respectively. Depth filter work was undertaken in Supracap™ 50 capsules (22 cm^2) or 5" or 10" Supracap 100 capsules (0.025 and 0.05 m^2). Sterile filter work was undertaken in Mini Kleenpak™ Syringe filters (2.8 cm^2), Mini Kleenpak™ 20 capsules (20 cm^2) or Mini Kleenpak™ capsules (220 cm^2). In all cases capsule size was chosen based on the volumetric loading target. Prior to the clarification step operation, the filters were flushed with 20 L/m^2 of 1X PBS after which, the upstream holdup volume was drained from both filters. Turbidity of the crude harvest and clarified pools were measured by a Hach® 2100Q portable turbidimeter. Flux rates of 100 or 200 L/m^2/hr (LMH) were used on the depth filters, and 200-250 LMH on the sterile filters. Following loading, the filter trains were flushed with 1.5X holdup volumes with 1X PBS buffer.

Concentration

Pall Omega™ 100 kDa single-use TFF cassettes were used to concentrate the clarified pool. T01 (0.01 m^2) and T02 (0.02 m^2) cassettes were stacked in various combinations based on the volumetric loading target. Prior to use, the cassettes were flushed with water and equilibrated with 1X PBS buffer in accordance with their care and use procedures [9,10]. For the flux excursion work, the system was setup in full recycle and loaded with a clarified rAAV5 pool. An initial check showed stable flux over time for set crossflow and transmembrane pressure (TMP) conditions, indicating there was no significant fouling from the feed stream. For each crossflow condition the TMP was ramped up until the permeate flux levelled off. Membranes were depolarized between crossflow conditions by recirculating with the retentate valve open and permeate valve closed for >10 min.

Six concentration studies were performed with filters loaded between 178 and 206 L/m^2. A 10X volumetric concentration factor was targeted for each trial. Following concentration, the membrane was depolarized by recirculating with the retentate valve open and permeate valve closed for >10 min. The system was then drained into the retentate vessel. A 25 mL flush of 1X PBS (equivalent to ~1.5X holdup volumes) was added to the system, recirculated for 10 min with permeate closed, and drained. The recovery flush and concentrated pool were finally combined and filtered through a Supor® EKV sterilizing-grade filter.

Affinity chromatography

Post-TFF, rAAV5 harvest was loaded onto a Thermo Scientific™ POROS™ GoPure™ AAVX

Pre-packed Column, 0.8 x 10 cm, 5 mL. Affinity chromatography was performed on AKTA™ Avant system (Cytiva). To compensate for long loading times and mitigate pool instability from the recommended 3-minute residence time, the post-TFF pool was split into two fractions and loaded onto two columns (~400–600 mL/column). Columns were equilibrated with 50 mM Tris, 0.5M NaCl, pH 7.5. Wash buffer was the same as equilibration buffer. Elution buffer was 50 mM Citric Acid, pH 2.0. 1 mL of 1M Tris pH 8.5 per 5 mL of eluate was added to the collection tube for instant neutralization. The generic Thermo Scientific conditions for elution pH were modified to pH 2 instead of 3.

Membrane chromatography polishing

Post-affinity rAAV5 was diluted to ~1L with 20 mM Bis-tris propane (BTP) buffer at pH 9. Mustang Q chromatography was performed on AKTA™ Avant. After equilibrating the column, the diluted sample was loaded at 50 mL/min (10 MV/min) onto 5 mL Mustang® Q capsules, followed by wash with the equilibration buffer. The sample was eluted using a conductivity step gradient aiming for ~1 mS/cm increase per step, achieved by varying the percent amount of equilibration buffer to elution buffer (Table 1).

Formulation

Pall Omega™ 100 kDa single-use TFF cassettes were used to concentrate and diafilter the purified pool into formulation buffer. Prior to use, the cassettes were flushed with water and equilibrated with buffer in accordance with their care and use procedures [9,10]. The Mustang® Q elution pool was first concentrated to a target volumetric concentration factor of 10X, or until the retentate pool volume dropped to the TFF system holdup volume. The pool was then exchanged with seven diavolumes of 20 mM Tris (pH 8.0), 1 mM $MgCl_2$, 200 mM NaCl, 0.005% Pluronic F68 [11]. A crossflow rate of 7.5 L/m^2/min (LMM) and TMP of 15 psi was used for the concentration and diafiltration. For recovery the membrane was depolarized by recirculating with the retentate valve open and permeate valve closed for >10 min. The system was then drained into the retentate vessel. A 1.5X holdup volume flush of formulation buffer was added to the system, recirculated for 10 min with permeate closed, and drained. The recovery flush and concentrated pool were then combined before final filtration.

Sterile filtration

A Supor® EKV 0.65 / 0.2 µm sterilizing-grade filter was used for final filtration. The filtration was done using Mini Kleenpak™ Syringe capsules (2.8 cm^2) in constant flow mode with a flux target of 500 LMH. Feed and filtrate pools were analyzed for rAAV5 concentration to calculate virus transmission.

Analytical methods

In-process samples were collected and stored at -80 °C.

Viral vector physical titer was measured using droplet digital polymerase chain reaction (ddPCR) assay using the Bio-Rad QX200 AutoDG Droplet Digital PCR System. The PCR primer/probe (IDT) combination targeted an amplicon contained in the gene of interest of the rAAV transfer genome.

rAAV5 Capsid titer was measured using a commercially available AAV5 capsid ELISA (Progen). Host cell protein concentration was measured using a commercially available ELISA kit (Cygnus). dsDNA concentration was measured with Quant-It™ Picogreen™ Assay kit (Thermo).

SDS-PAGE was performed on a 10% Criterion™ XT Bis-Tris gel for 60 minutes at 150V. The gel was then stained using Sypro™ Ruby fluorescent stain and imaged on a Bio-Rad ChemiDoc.

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TABLE 1

The chromatography method used for rAAV5 full capsid enrichment.

Step	Buffers used/details	Membrane volumes used
0 – Priming of membrane	Equilibration (10 mL/min – upflow) Equilibration (10 mL/min – downflow) Conditioning (25 mL/min – downflow) Strip (25 mL/min – downflow) Equilibration (50 mL/min – downflow)	10 per step
1 – Equilibration of membrane	Equilibration buffer	20
2 – Application of sample	rAAV5 sample solution	N/A – Inject all sample using air sensor
3 – Washing of membrane	Equilibration buffer	10
4 – Step gradients	Buffer A – Equilibration buffer Buffer B – Elution buffer Step 1 = 10% Buffer B, 90% Buffer A Step 2 = 15% Buffer B, 85% Buffer A Step 3 = 20% Buffer B, 80% Buffer A Step 4 = 25% Buffer B, 75% Buffer A Step 5 = 30% Buffer B, 70% Buffer A Step 6 = 35% Buffer B, 65% Buffer A Step 7 = 40% Buffer B, 60% Buffer A Step 8 = 45% Buffer B, 55% Buffer A Step 9 = 50% Buffer B, 50% Buffer A Step 10 = 55% Buffer B, 45% Buffer A Step 11 = 60% Buffer B, 40% Buffer A Step 12 = 65% Buffer B, 35% Buffer A Step 13 = 70% Buffer B, 30% Buffer A Step 14 = 75% Buffer B, 25% Buffer A Step 15 = 80% Buffer B, 20% Buffer A Step 16 = 85% Buffer B, 15% Buffer A Step 17 = 90% Buffer B, 10% Buffer A Step 18 = 95% Buffer B, 5% Buffer A Step 19 = 100% Buffer B, 0% Buffer A	10 per step
5 – Strip	20 mM BTP, pH 9, 1M NaCl	10

RESULTS

Clarification

rAAV5 was produced in the iCELLis® Nano bioreactor as described in the methods section. Approximately ~60% of the rAAV5 virus is retained by the cells and ~40% secreted into the production media (data not shown). Functional rAAV vector is reported to be found both retained by cells and in the spend cell culture medium [12]. The crude harvest pool consisted of the fixed-bed lysate, spent media and a phosphate buffered saline (PBS) bioreactor rinse.

Multiple combinations of depth and sterilizing-grade filters were screened to evaluate clarification performance. The screening results showed the combination of Pall's P-grade PDK11 depth filter in series with a Supor® EKV sterilizing-grade filter resulted in the highest performance based on capacity, impurity reduction, and yield (data not shown).

The PDK11/EKV filter train was evaluated for robustness over the course of eight bioreactor harvests. The average titer of the crude harvest material was 7.82 x 10^9 gc/mL +/- 1.17 x 10^9. The turbidity of the crude harvest material ranged from 11 to 129 nephelometric turbidity units (NTU). A summary of the clarification performance is found in Table 2.

The data in Table 2 shows the filter train was able to consistently reduce the crude harvest turbidity down to less than 5 NTU with no significant product loss. Furthermore, we saw strong process robustness against feedstream turbidity with regards to clarified pool turbidity and step yield. A summary of the turbidity reduction is shown in Figure 1. Filter capacity was influenced by crude harvest

TABLE 2

rAAV5 clarification performance summary.

Attribute	Data points	Average	95% confidence interval
Feed turbidity	n=8	57.9 NTU	24.8 NTU
Pool turbidity	n=8	2.9 NTU	1.1 NTU
Yield (ddPCR)	n=8	104%	8.3%

turbidity, however all eight runs showed >250 L/m^2 throughput on the depth filter, and >450 L/m^2 throughput on the sterile filter.

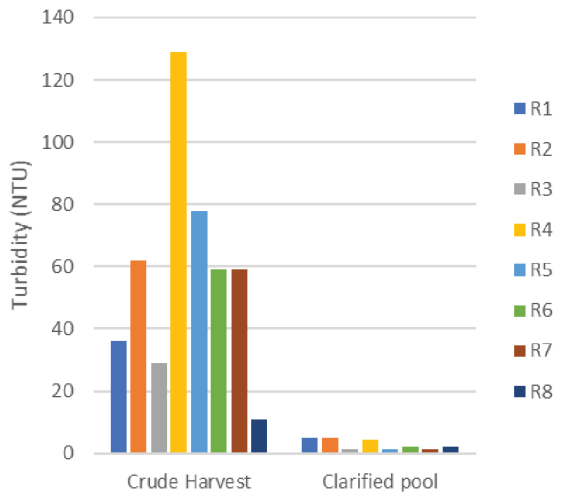
Trial number 4 (R4) showed a significantly higher turbidity at harvest than the other cultures. This culture showed similar cell densities at time of transfection and the resulting titer was comparable to the other harvests used during this testing. A root cause of the high turbidity observed in trial 4 was not found.

Concentration

Due to the ratio of harvest titer to the binding capacity of the affinity sorbent, direct loading onto affinity chromatography would require extended loading times. The clarified material was concentrated by TFF before purification. This reduced the affinity chromatography loading time from >20 hours to ~2 hours. For this process, we evaluated Omega™ 100 kDa PES single-use TFF membrane cassettes.

FIGURE 1

Clarification performance was assessed with eight bioreactor harvests (R1 through R8).



Ultrafiltration of rAAV products with 100 kDa pore size has been previously reported [13]. A flux excursion study was performed to identify the optimal crossflow and TMP process parameters. The results of this study are shown in Figure 2A & B and show a critical TMP at ~10–15 psi with a moderate benefit from increasing crossflow rate, resulting in limiting flux rates between 71 and 97 LMH. Note that after each TMP excursion, rAAV concentration was measured from the recirculating pool and the permeate line. Results from the three crossflow rate trials showed virus retentions of >99.7%. We also observed no significant trend to total gene copies in the recirculating pool, suggesting no significant virus loss due to shear at crossflow rates up to 7.5 L/m^2/min.

Six trials were performed to concentrate the clarified rAAV5 pool to a target volumetric concentration factor (VCF) of 10X. Across the trials, volumetric loading averaged 186 ± 9 L/m^2, virus loading averaged 1.3 x 10^{15} ± 2.6 x 10^{14} gc/m^2, and feed concentration averaged 7.3 x 10^9 ± 1.5 x 10^9 gc/mL. A summary of the filter performance is shown in Table 3 and includes an average vector step yield of 91% ± 8.0%. A representative plot of flux and VCF over time is shown in Figure 2B.

Chromatography purification

Affinity capture chromatography was performed and to speed up loading time, typically a single clarified harvest was purified on two columns simultaneously using two ÄKTA Avant chromatography systems. Across 16 total affinity chromatography purifications rAAV5 recovery was determined to be 68 ± 13% by capsid ELISA and 57 ± 30% by ddPCR method.

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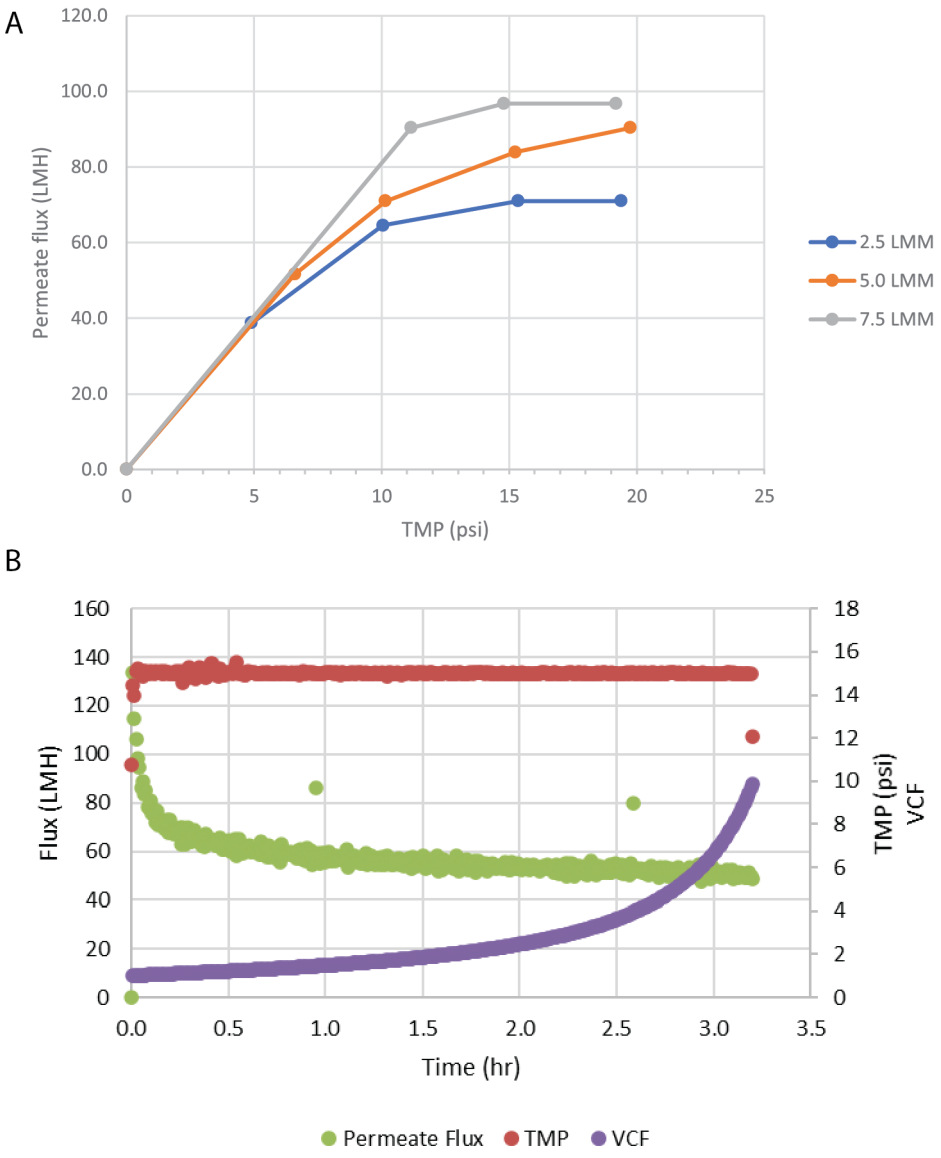
FIGURE 2

(A) Flux excursion evaluation of rAAV5 concentration. (B) Representative flux decay plot of rAAV5 concentration.



The affinity purified vector was polished to enrich for full capsids using Mustang® Q XT Anion Exchange membrane sorbent. The elution pool was diluted into Bis-tris propane (BTP) equilibration buffer to reduce the ionic strength of the material prior to polishing. After column washing, the virus was eluted from the membrane capsule using a ~1 mS conductivity step elution strategy. A representative elution profile is shown in Figure 3.

Elution fractions were analyzed for the gene of interest (the cargo) 'full capsids'

TABLE 3

Summary of rAAV5 concentration by ultrafiltration TFF.

Attribute	Data points	Average	95% confidence interval
Permeate flux	n=6	64 LMH	5.6 LMH
Yield (ddPCR)	n=6	91%	8.0%

using droplet digital polymerase chain reaction assay (ddPCR) and total capsids (AAV5 ELISA). Fractions that showed a higher absorbance at 280 nm than 260 nm correlated to low ratios of genome copies to total capsids, indicating a high percentage of empty capsids. The fractions with similar 280 nm and 260 nm signals showed a much higher ratio of genome copies to total capsids, indicating a higher proportion of full capsids.

When we calculate the mass balance from fraction pooling, we recover and carry forward close to 50% of the full capsids, but only retain 11% of the total viral particles. This results in close to a 5-fold enrichment of full capsids to total capsids.

Full capsid enrichment reproducibility

Mustang® Q polishing for full capsid enrichment was performed on five upstream batches, four of which were further analyzed via ddPCR and capsid ELISA. Figure 4 shows the normalized chromatograms for these five experiments.

Despite variation in the upstream conditions, we found the full capsid enrichment with Mustang® Q to be reproducible. Figure 5 plots the vector genomes (full capsids) for the load and the 5 peaks that were collected for analysis. Figure 6 plots the total number of capsids as determined by capsid ELISA for the load and the same 5 peaks of interest.

The combined peaks 1 and 2 reproducibly contain very little vector genomes (vg) (Figure 5), if any, and thus predominantly contain empty capsids. Peaks 3, 4 and 5 contain most of the genome content while the total number of capsids is low relative to combined peaks 1 and 2, indicating that these peaks are enriched for full capsids. To clearly demonstrate this phenomenon, we have divided the vg/capsid ratio of combined peaks 1 and 2 and combined peaks 3, 4 and 5, respectively, by the vg/capsid ratio of the load and plotted these in the Figure 7. The relative enrichment across processes is generally consistent and is consistent with differences in the UV traces.

Table 4 shows the capsid ELISA yield and vg yield of the empty (1+2) and full (3+4+5) peaks. The total capsid yield in the empty peaks (1+2) is 49 ± 10% while the genome (vg) yield is only 14 ± 8%. The capsid yield in the full peaks (3+4+5) is 19 ± 10% while the genome (vg) yield is 66 ± 13%. This clearly shows that combined peaks 3, 4 and 5 are enriched in full capsid relative the combined peaks 1 and 2.

FIGURE 3

Chromatogram of rAAV5 step elution from Mustang Q Membrane.

Step	LOAD	1	2	3	4	5	6
GC/mL	5.9E+10	1.0E+09	7.9E+10	6.1E+11	3.1E+11	6.8E+10	3.4E+10
Capsid/mL	4.5E+11	3.3E+11	5.0E+12	1.4E+12	4.8E+11	2.9E+11	5.4E+11
GC/Capsid	13%	0%	2%	44%	66%	23%	6%

Blue line represents OD280 absorbance (protein); red line represents OD260 absorbance (DNA); green line is the conductivity (mS/cm). rAAV5 titer based on genome copies (GC/mL) and capsid ELISA (Capsid/mL). The green box highlights elution peaks enriched for full capsids. Viral vector titers of each corresponding elution fraction are shown in the table below the chromatogram. The calculated full percent is also calculated.

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FIGURE 4

Superimposed chromatograms from elution fraction collection of individual Mustang® Q separation experiments.

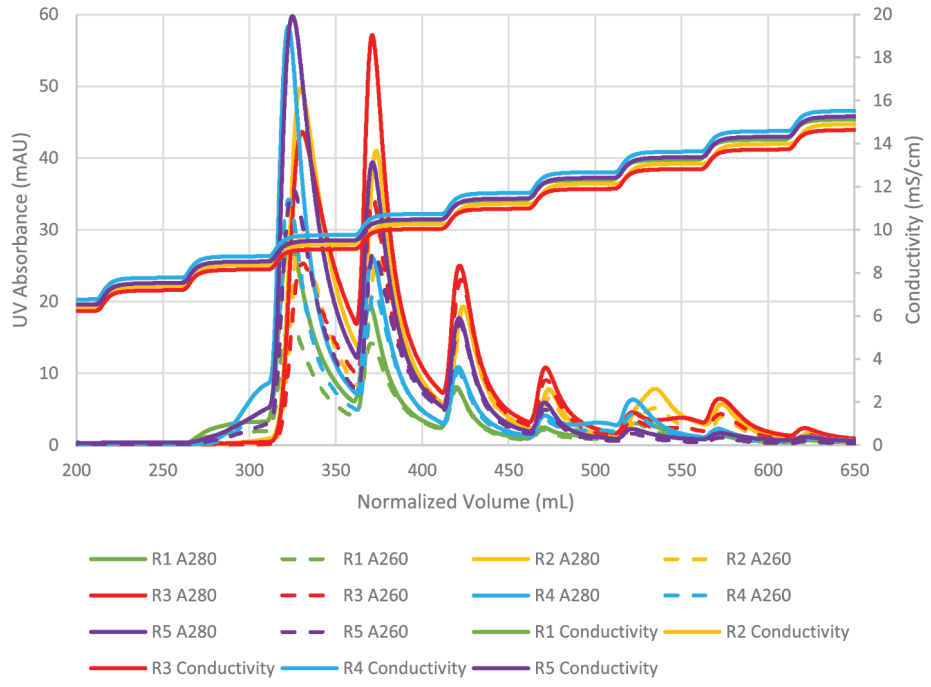
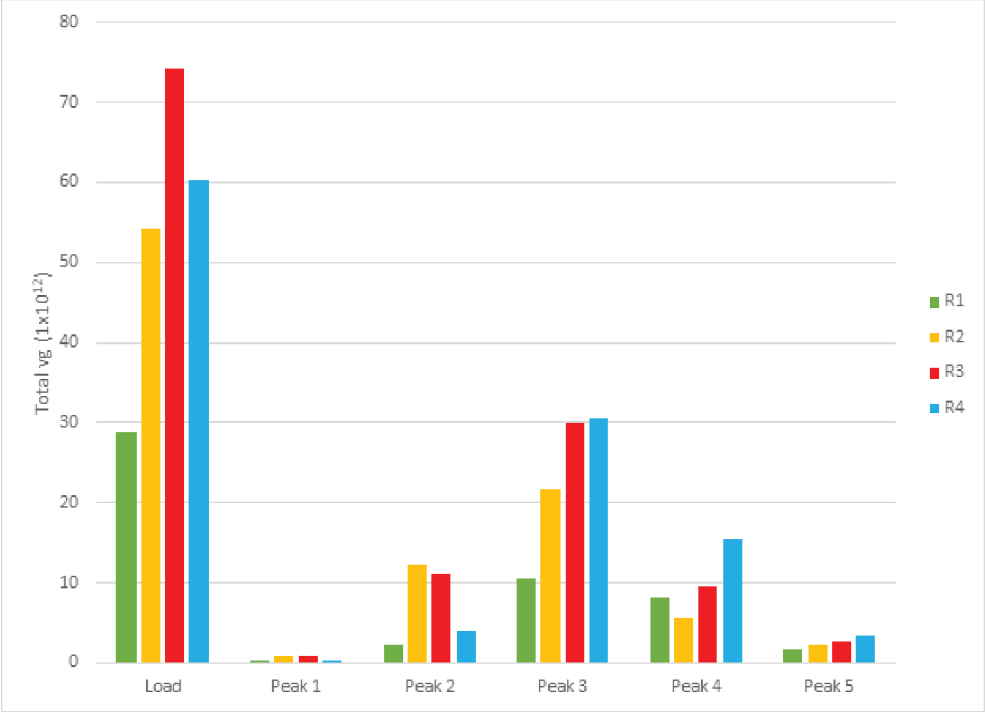


FIGURE 5

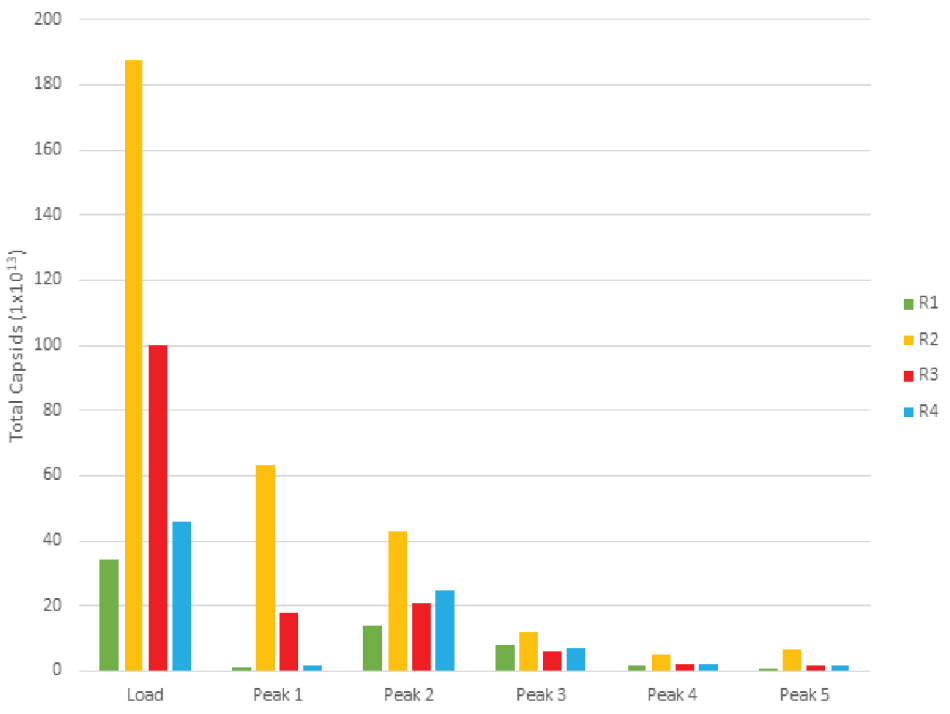
Mass balance of vector genomes during Mustang Q Polishing.



Total viral genome content in the Mustang® Q load material and distribution in each of the five elution peaks. Viral genome concentration determined by droplet digital PCR.

FIGURE 6

Mass balance of viral capsids during Mustang® Q Polishing.



Total viral capsid content in the Mustang Q load material and each of the five elution peaks. Viral capsid concentration determined by capsid ELISA.

Formulation

After Mustang® Q polishing, the next step in the process is to adjust the buffer and vector titer to the final formulation for clinical use. We evaluated the use of Omega 100 kDa single-use TFF filters for this ultrafiltration/diafiltration (UF/DF) step. The purified rAAV5 pool was concentrated to a targeted 10X volumetric concentration factor followed by a 7X diavolume buffer exchange into formulation buffer. The rAAV5 concentration/diafiltration was performed at a crossflow rate of 7.5 L/m^2/min and a TMP of 15 psi. Following diafiltration the filter was depolarized and drained, then flushed with 1.5X holdup volumes of formulation buffer.

Four UF/DF trials were completed using the process described above. Permeate flux measured throughout the concentration and diafiltration remained steady at ~200 LMH (concentration data shown in Figure 8). Virus concentrations were measured in the final permeate pools to measure virus retention. In three of four pools there was no virus detected in the permeate, the fourth pool was calculated at 99.9% virus retention. Virus yields averaged 89% over the four trials but were

TABLE 4

Average Mustang® Q polishing yield across processes.

Capsid yield				Vg yield			
	Empty peaks	Full peaks	Empty + full		Empty peaks	Full peaks	Empty + full
n	4	4	4	n	4	4	4
Average (%)	49	19	68	Average (%)	14	66	80
St dev (%)	10	10	14	St dev (%)	8	13	7

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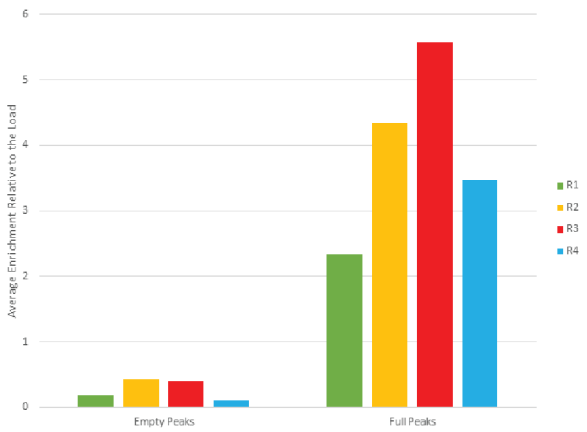
highly variable (95% confidence interval of 69%). We note that the virus and volumetric loading was relatively low due to the material available at these scales, averaging 2.4 x 10^{15} ± 8.9 x 10^{14} gc/m^2 and 11 ± 5 L/m^2 respectively. For a 2–3-hour process we would expect volumetric loading in the range of 150–250 L/m^2, and hypothesize that higher loading would reduce yield variability, but may also reduce permeate flux.

Final sterile filtration

The final step in rAAV manufacturing is to ensure patient safety by sterile filtration through a validated sterilizing-grade filter. We evaluated Supor® EKV for this final sterile filtration step. There was no significant pressure rise observed over the constant flow filtration experiments, though we note the loading was relatively low with the material available (<100 L/m^2). Virus concentration was measured in the feed and filtrate pools to calculate transmission. The results of 4 trials are shown in Figure 9 and Table 5 and show that high virus transmission (averaging 94%), can be achieved at final filtration with an EKV filter.

FIGURE 7

Full capsid enrichment by Mustang® Q Membrane.



Relative enrichment of the pooled elution fractions containing mostly empty capsids (Peaks 1 & 2) and those containing mostly full capsids (Peaks 3, 4, 5) normalized against the starting load material. Data collected from 4 separate bioreactor harvests (R1 – R4).

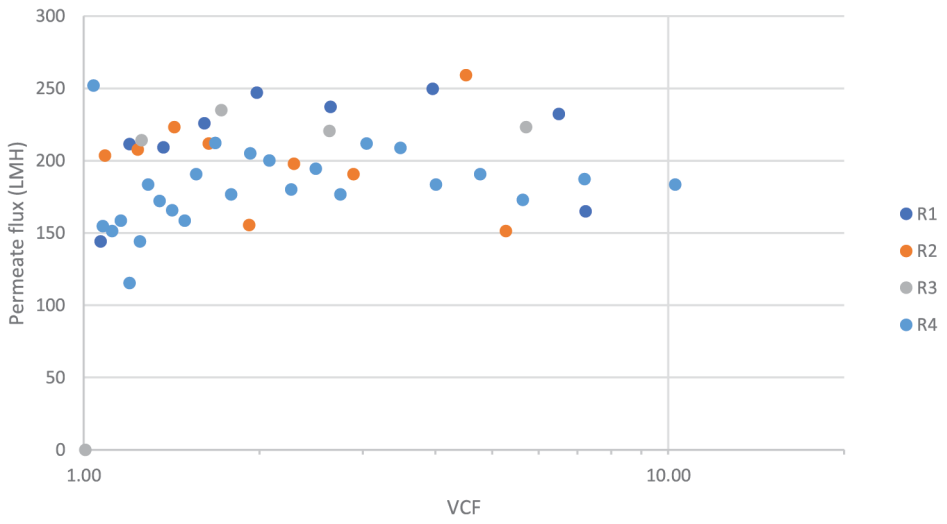
Total process yield & impurity removal

The step and cumulative vector yields from a representative run are shown in Figure 10.

In this run, we observed good vector yield in clarification, concentration, affinity purification and final sterile filtration. The yields observed during Mustang® Q purification

FIGURE 8

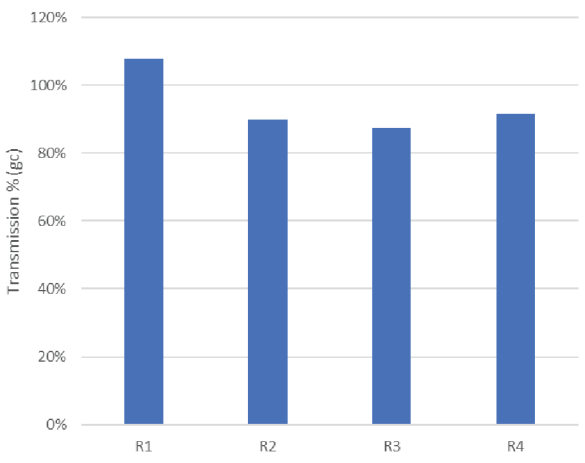
UF/DF of rAAV5, flux compared to VCF.



Correlation between permeate flux and volumetric concentration factor during 4 Ultrafiltration/Diafiltration experiments with rAAV5.

FIGURE 9

Transmission of rAAV5 during EKV sterile filtration.



Calculated transmission of rAAV5 during sterile filtration using EKV membrane filters.

show ~50% vector recovery; however, this loss was also accompanied by an 89% reduction of total capsids resulting in a ~5-fold increase in full capsid percentage.

Final formulation by UF/DF resulted in ~60% vector recovery. This less-than-ideal recovery was likely a result of this process being performed using an atypically low volume to surface area for this application. Based on the process flux measured here, final formulation by TFF could target a volume to surface area ratio of 250 L/m^2 in a 2–3 hour process. In this testing, we were limited by product volume and the resulting surface area to volume ratio was ~15 L/m^2. The pre-purification UF concentration of this vector resulted in an average yield >90% using the same filter, with a more appropriate volume to surface area ratio. We anticipate higher recoveries during scale-up as the relative impact of non-specific loss would be lowered.

The process was evaluated for contaminant removal including host cell proteins (HCP) and host cell DNA. Host cell protein concentration was determined by ELISA (Cygnus). Results are shown in Figure 11. Contaminant DNA was measured by Picogreen™ Assay (Thermo). Results are shown in Figure 12.

The data shown in Figures 11 & 12 show the contaminant HCP and DNA levels are both reduced to below assay limit of quantitation during Mustang® Q polishing.

The protein profile through the purification process was assessed by SDS-PAGE using Sypro™ Ruby fluorescent staining (see Figure 13).

The results from the SDS-PAGE gel show a complex protein mixture through clarification and TFF. As expected, affinity purification shows a substantial reduction in the number and intensity of contaminant proteins. There are only 3 significant bands observed in all samples after Mustang Q polishing. These three bands are the viral capsid VP1, VP2 and VP3 proteins. There are no other significant proteins found in these samples.

The results of the DNA, HCP and SDS-PAGE analysis show this purification scheme results in a very low contaminant profile.

CONCLUSIONS

We evaluated existing filtration and chromatography technologies commonly used in large scale recombinant protein purification, for the purification of a recombinant AAV vector (serotype 5). The purification strategy was based on the general platform process commonly utilized in large-scale industrial monoclonal antibody manufacturing including scalable clarification, affinity purification, ion-exchange polishing, tangential flow filtration and sterile filtration. Each unit operation was evaluated for vector yield based on droplet digital PCR, purity, and robustness.

TABLE 5

Summary of EKV sterile filtration performance for rAAV5.

Attribute	Data points	Average	95% confidence interval
Feed concentration	n=4	3.3 e11 gc/mL	1.5 e11 gc/mL
Transmission (ddPCR)	n=4	94%	9.1%

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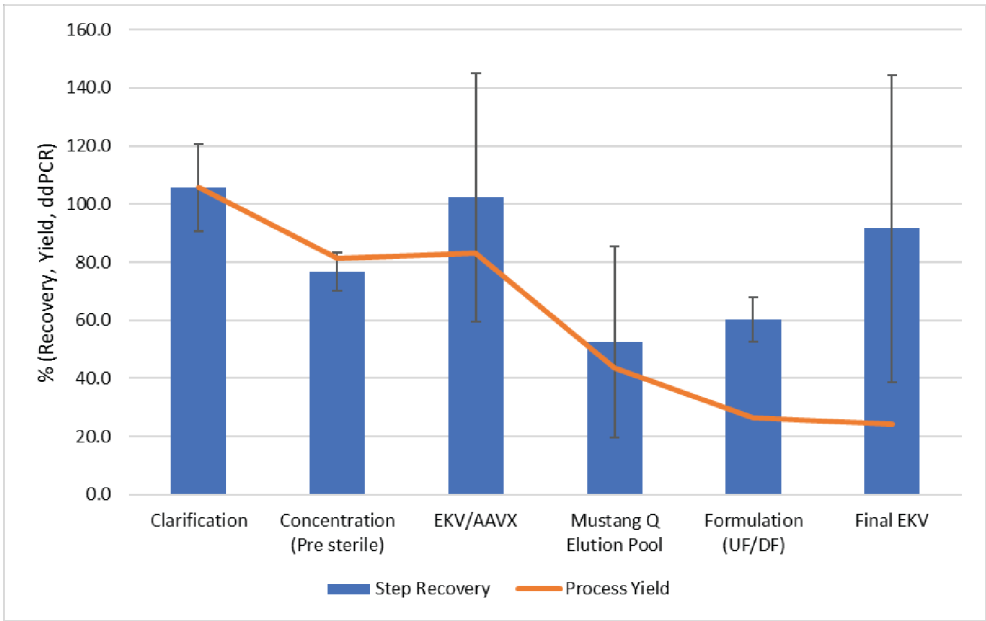
FIGURE 10

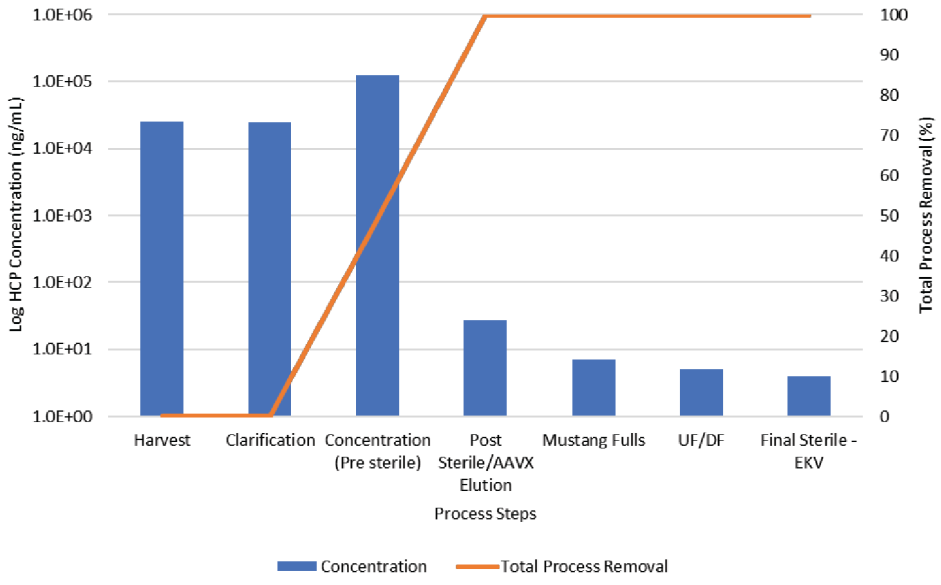
rAAV5 unit operation step recovery and cumulative process yield.



rAAV5 unit operation recovery as determined by ddPCR. Yellow trendline is the theoretical cumulative process yield of rAAV5 through described process scheme. Error bars represent 1 standard deviation.

FIGURE 11

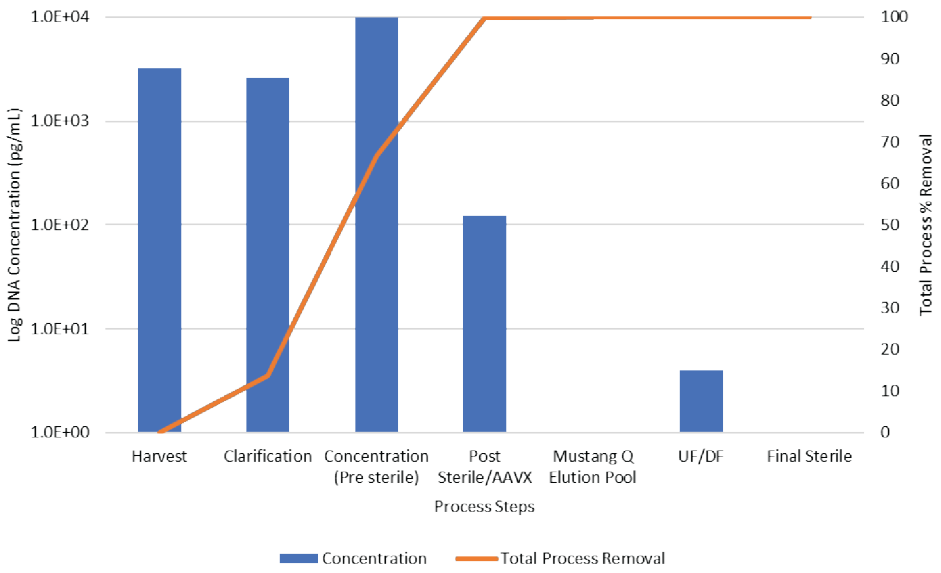
Host–cell protein ELISA results.



Host–cell protein contamination through rAAV5 purification. HCP concentration determined by Cygnus HCP ELISA.

FIGURE 12

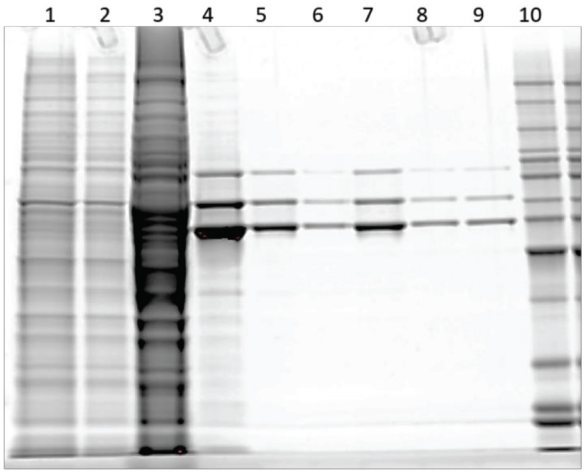
Contaminant DNA concentrations and cumulative removal.



DNA contamination through rAAV5 purification. DNA concentration determined by Picogreen assay

FIGURE 13

SDS-PAGE analysis of rAAV5 downstream purification.



Lane	Sample	VG/mL (ddPCR)
1	In-house AAV5 harvest	6.3E09
2	Post clarification	6.3E09
3	Post TFF	4.8E10
4	Post AAVX (Affinity)	1.0E12
5	Post Mustang Q (Empty Pool)	2.0E10
6	Post Mustang Q (Full Pool)	1.8E11
7	Post TFF/DF	3.2E11
8	Post Sterile (EKV)	3.0E11
9	Post Sterile (NGFF)	2.5E11
10	Ladder	N/A

10% Homogenous gel stained with Sypro Ruby fluorescent protein stain. Estimated viral genome loading calculated from ddPCR titer.

We observed an overall theoretical process yield of 25% full capsids (containing gene of interest) with a full capsid enrichment of ~5-fold compared to the total viral particles (total capsids including empty). This process resulted in rAAV material which had a very low HCP and DNA contaminant profile. Clarification, TFF concentration and final sterile filtration showed robust performance with average vector recovery >90%. The unit operations with the most vector loss were Mustang® Q polishing and UF/DF final formulation. We expect

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the total process yields to improve with further optimization, process understanding and scale-up.

TRANSLATIONAL INSIGHTS

The results presented here demonstrate feasibility of translating proven, scalable purification technologies used in recombinant protein manufacturing to the purification of viral vectors. Almost all the technologies employed are available from Pall Corporation. This enables a near complete end-to-end platform solution for recombinant adeno-associated viral vector manufacturing.

Next steps would likely include scalability of each unit operation, particularly for the final UF/DF and final filtration, where scale-up is required to further challenge the filters. To develop a full Quality by Design (QbD) manufacturing process would also require additional characterization of critical process parameters for each unit operation. A white paper outlining guidance on QbD manufacturing of rAAV products is available from Pall Corporation [14].

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AUTHORSHIP & CONFLICT OF INTEREST

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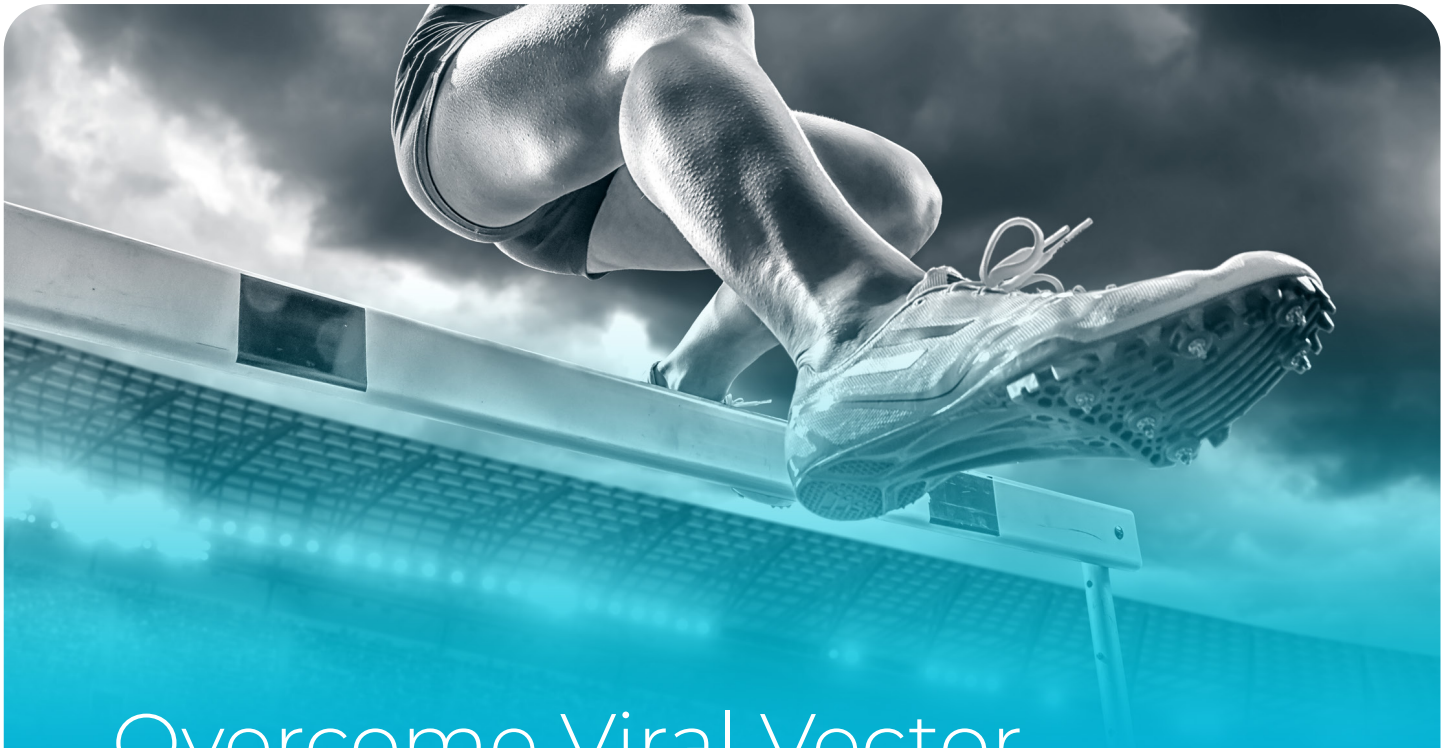

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