Low-Cost, Fast, and Scalable Downstream Purification Process Development for a Clinical-Stage Retrovirus-Like Particle

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Abstract

Purification of enveloped viruses and virus-like particles presents several challenges due to their large size and complexity. Here we present a case study about resin screening, process development, and scale-up purification of a retrovirus-like particle. The resulting cGMP-compatible process required approximately 4 hours to purify 240 L of nuclease-treated bioreactor harvest and resulted in a 99% reduction in process volume and up to 65% recovery of virus particles with a final purity consistent with requirements for clinical trials. This protocol is readily scalable, fast, low cost, simple, and cGMP-compatible and can be used for the capture step in clinical-grade manufacture of retrovirus vectors.

Introduction

Retroviruses and retrovirus-like particles are increasingly being developed for use as gene transfer vectors for clinical applications (Ginn et al. 2018, Keeler et al. 2017). Their popularity stems in part from their ability to stably integrate up to 7.5 kb of genetic material into target cells and their relatively low immunogenicity (McTaggart and Al-Rubeai 2002).

As for any biologic intended for human use, downstream processing of retroviruses is required to remove upstream process components, such as medium constituents and induction agents, and upstream process contaminants, such as host cell proteins (HCP) and DNA, while maintaining high product retention and biological activity (International Conference on Harmonisation 1999). However, properties inherent to retroviruses and their production systems give rise to challenges not generally encountered in the downstream processing of most other biologics. These include the viruses' large size (~125 nm), their high heterogeneity with respect to surface protein and carbohydrate structure, and their susceptibility to damage by fluid shear conditions, interfacial stress, nonphysiological pH, and surfactants.

Methods for the purification of viruses, including retroviruses, have been reported, and include the use of ultracentrifugation, ultrafiltration, and chromatography (Segura et al. 2005, van der Loo and Wright 2016). Some of the published methods are impractical at scale (for example, size exclusion chromatography and ultracentrifugation) while others are challenging with respect to cGMP/CMC (for example, heparin affinity chromatography). However, ion exchange chromatography methods are generally scalable, and several such methods have been published (Fitchmun et al. 2015, McNally et al. 2014, Rodrigues et al. 2007).

Presented in this case study are data from the purification media screening, endonuclease treatment, and process scaling for the downstream processing of a retrovirus-like particle intended for use in human subjects.

Materials and Methods

Production of Virus Feedstream

HEK 293T–derived packaging cells, which constitutively shed engineered retrovirus-like particles, were grown in suspension bioreactor cultures. Mammalian producer cell supernatant was then clarified with a 0.45 µm filter. The initial bioreactor working volume was 3 L. GMP production was performed at a working volume of 250 L.

Anion Exchange Media Screening

Four commercial ion exchange media were screened: DEAE Sepharose Fast Flow Resin (GE Life Sciences), Nuvia Q Resin (Bio-Rad Laboratories), Q Sepharose Fast Flow Resin (GE Life Sciences), and Sartobind Q Membrane Adsorber (Sartorius AG). All products were acquired from the manufacturers as preassembled or prepacked 1.0 ml devices.

Load material for resin screening was prepared by adding 840 µl of 1.25 M sodium phosphate (NaPhos), pH 6.75, to 20 ml of freshly thawed clarified bioreactor harvest. Each device was equilibrated at 5.0 ml/min with 30 ml of 1.0 M NaOH, 10 ml of 1.0 M NaCl, and 50 ml of 50 mM NaPhos, pH 7.0. Flow rates

were then reduced to 0.30 ml/min. The devices were loaded with 18 ml of the material described above, washed with 10 ml of 50 mM NaPhos, pH 7.0, and then subjected to elution with 10 ml of 50 mM NaPhos with 1.5 M NaCl, pH 7, and a post-elution strip with 10 ml of 50 mM NaPhos with 2.0 M NaCl, pH 7.0. Relative product recoveries were determined by RT-qPCR as described below.

Chromatography Buffer System Development and Load Optimization

Small-scale experiments were performed using an NGC Discover 10 Pro Chromatography System (Bio-Rad) and 1.0 ml Foresight Columns (Bio-Rad) at a flow rate of 0.30 ml/min (18 column volumes (CV)/hr) during chromatographic operations and 3.0 ml/min (180 CV/hr) during column equilibration, regeneration, sanitization, and preparation for storage.

To limit the nonideal effects of phosphate interaction with the stationary phase, a relatively high phosphate concentration of 50 mM and a pH of 7.0 were maintained during column equilibration, feedstream loading, pre-elution, and elution steps. To identify binding and eluting conditions for the virus and its contaminants, 18 ml of a solution comprising four parts clarified harvest and one part 250 mM NaPhos, pH 7.0, was loaded onto a column pre-equilibrated in 50 mM NaPhos, pH 7.0. Following a column wash with 50 mM NaPhos, pH 7.0, NaCl was introduced via a 10 ml linear gradient to 2.0 M (Figure 1). The gradient was then replaced by steps comprising a pre-elution of bound process components at 440 mM NaCl followed by product elution at 1,100 mM NaCl (Figure 2). Optimizing the load step entailed replacing the 50 mM NaPhos, pH 7.0, used at a 1:4 ratio in loading the column, with 1.0 M NaPhos, 1.2 M NaCl, pH 6.75, used at a 1:19 ratio. Also, the load volume was increased from 18 ml to 50 ml (Figure 3).

Virus Quantitation

RNA was first isolated from samples using the NucleoSpin RNA Virus Purification Kit (Takara Bio USA) and then treated with recombinant DNase I (Takara Bio USA). Samples were then assayed via RT-qPCR on a CFX96 Real-Time PCR Detection System (Bio-Rad) using primers and a probe specific to the retroviral Psi packaging signal (GeneLink) and the TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher Scientific). Samples were analyzed in triplicate and the average cycle thresholds were compared to those of a standard curve to calculate virus concentration.

Virus Activity

To determine virus activity, samples were diluted in cell medium and added to A375 cells (ATCC, CRL-1619) grown in 6-well tissue culture plates seeded at 2 x 10^5 cells per well the previous day. Cultures were incubated for 16 hours before harvesting cells and extracting genomic DNA using the QIAamp DNA Blood Mini Kit (QIAGEN). Virus DNA was then assayed in triplicate via qPCR and gene expression compared to a reference control, also measured in triplicate.

Nuclease Digestion

Initial nuclease digests employed *Serratia marcescens* extracellular endonuclease (Benzonase, Millipore Sigma). For initial experiments involving nuclease digests, two parts clarified bulk were mixed with one part purified water prior to the addition of nuclease to a final concentration of 22 units/ml and incubation at 37.0 ± 0.5°C for 30 minutes. Following the digest, load preparation and chromatography were performed as described (Figure 4). During process optimization, dilution with water was eliminated, endonuclease concentration was reduced to 15 units/ml, and incubations were conducted at 34.5 ± 2.5°C for 75 minutes. Also, during process optimization Benzonase Nuclease was replaced with DENARASE Endonuclease (Sartorius).

Column Scaling

Column volume was increased in four steps. First, column length was increased from 2.0 to 20.0 cm. Next, column diameter was increased from 0.8 to 2.6 to 6.2 cm, and finally to 17.8 cm. Flow rate and load volume were increased in proportion to CV, with the largest column having a CV of 4.9 L, a flow rate of 1.7 L/min, and a load volume of 240 L (Figure 5). Column regeneration, cleaning, and preparation for storage (not shown on chromatograms) comprised 4 CV of each of the following solutions, sequentially: (1) 50 mM NaPhos, 2.0 M NaCl, pH 7.0; (2) 1.0 M phosphoric acid, 8.0 M urea; (3) 1.0 M NaOH; (4) 200 mM NaPhos, pH 7.0; and (5) 20% ethanol.

Testing of Purity and Safety Characteristics

Residual HCP was quantified using the HEK 293 HCP ELISA Kit (Cygnus Technologies) and a SpectraMax M2E Multi-Mode Microplate Reader (Molecular Devices). Residual endonuclease was measured using the Benzonase ELISA Kit II (Millipore Sigma) and the SpectraMax M2E Microplate Reader. Host DNA concentration, residual adenovirus E1 DNA, and residual SV40 large T-antigen DNA in the purified product were measured by the BioReliance Corporation using a GMP protocol. Bioburden and endotoxin testing were performed via USP Compendial Methods <61> and <85>, respectively.

Results and Discussion

The choice of anion exchange resins was based on several considerations. Unlike cation exchangers, anion exchangers generally bind retroviruses under pH and ionic strength conditions consistent with those of most bioreactor feedstreams. This is both convenient and economical in that product purification and concentration can be accomplished concurrently. Affinity modalities were not considered because they tend to be costly from material, quality assurance, and regulatory standpoints. Several mixed-mode resins, including Capto adhere and Capto MMC (GE Life Sciences), were screened following the manufacturers' recommendations. However, none of the mixed-mode resins tested provided satisfactory bind/elute recoveries (data not shown).

High Product Recovery with Nuvia Q Resin

Quantitation of virus eluted from each medium by RT-qPCR revealed that product recovery was higher with Nuvia Q than with the other resins tested (Table 1). No significant virus was detected in any of the post-elution 2.0 M NaCl washes. Based on these data, the downstream purification process was developed using Nuvia Q.

Table 1. Screening of anion exchange resins.

Anion Exchange Resin	Recovery, %
DEAE Sepharose Fast Flow	34
Q Sepharose Fast Flow	42
Nuvia Q	47
Sartobind Q	20

Chromatography Buffer System Development and Load Optimization

Gradient elution chromatography was used to identify approximate NaCl concentrations that would result in absorption and desorption of the product and its contaminants. Fractions were analyzed via RT-qPCR and compared to the chromatographic data (Figure 1). Most UV-absorbing material passed through the column during the load and wash (a) and did not contain significant virus as measured by RT-qPCR (a1). During the gradient portion of the experiment, most of the absorbed material eluted between approximately 100 mM and 1,100 mM NaCl (c).

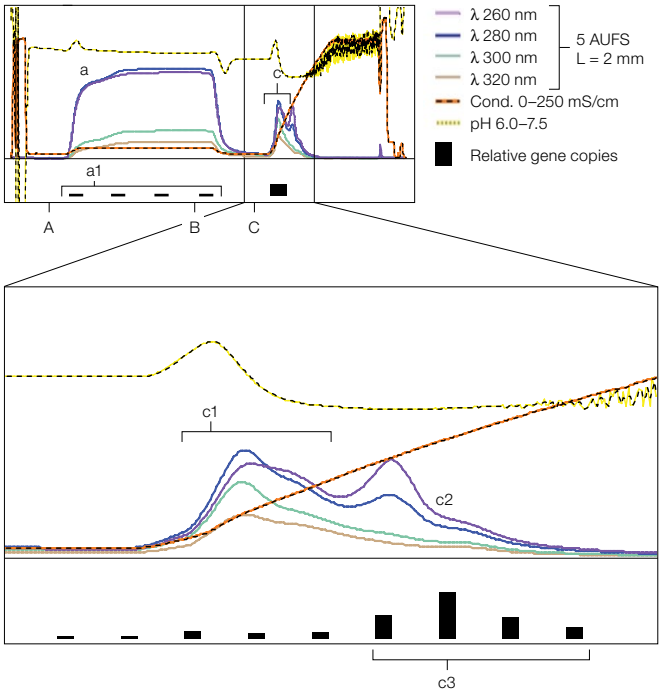


Fig. 1. Determination of approximate NaCl concentrations affecting the absorption/desorption behavior of product and contaminants.

The first material to elute contained little virus and absorbed more strongly at 280 nm than at 260 nm, consistent with a solution comprising more protein than nucleic acid (c1). The material that eluted around 1,100 mM NaCl absorbed more strongly at 260 nm than at 280 nm, consistent with a nucleic acid–rich virus (c2). These latter fractions contained 24% of the virus initially loaded onto the column (c3) but accounted for less than 2% of the overall UV absorbance, indicating that most of the contaminants present in the clarified bioreactor harvest did not coelute with the product.

Based on the above findings, the gradient was replaced with two steps: a pre-elution wash at 440 mM NaCl and a product elution at 1,100 mM NaCl (Figure 2, C and D). The wash peak (c) absorbed more strongly at 280 nm than at 260 nm, again, consistent with a higher level of protein than nucleic acid, while the product elution peak (d) absorbed more strongly at 260 nm than at 280 nm consistent with a nucleic acid–rich virus. The majority of virus gene copies and virus activity (d1: black and red bars) was associated with the second peak. No significant virus was found in the flowthrough and wash peaks (a1 and c1, respectively). Total product recovery was 48%.

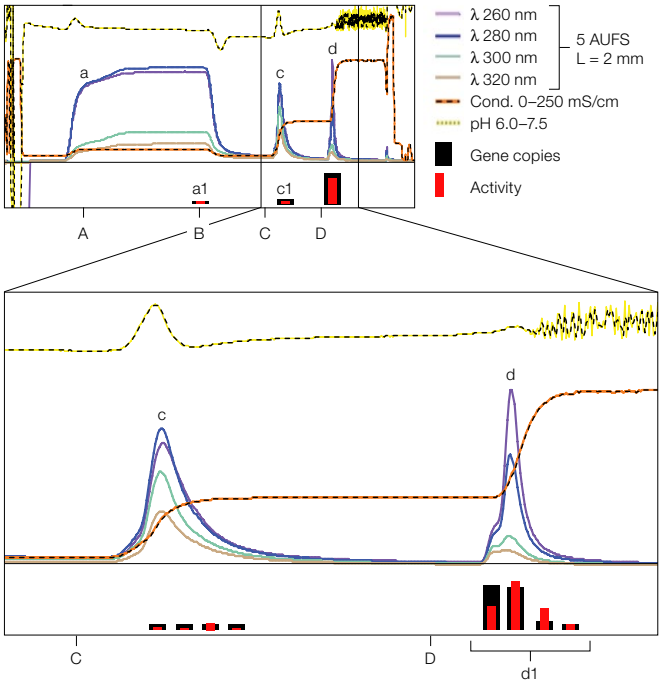


Fig. 2. Replacement of gradient elution with step elution. A single late flow-through fraction (a1) was analyzed to confirm that virus was not lost during the load and wash portions of the procedure (a). Forty-eight percent of the virus loaded, as measured by gene copy number, was recovered in the product rich peak (d), and there was good correlation between gene copy number, a measure of virus particle concentration, and virus activity measurements (d1).

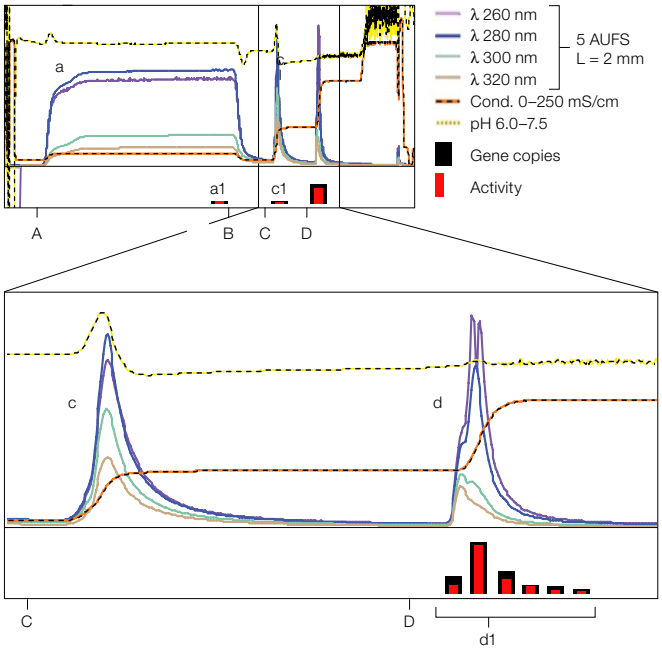


Fig. 3. Increasing the relative load volume and NaCl concentration improved product recovery. A single late flow-through fraction (a1) was analyzed to confirm that virus was not lost during the load and wash portions of the procedure (a). Seventy-one percent of the virus loaded, as measured by gene copy number, was recovered in the product rich peak, and there remained good correlation between gene copy number, as measured by RT-qPCR (black bars), and biological activity (red bars) (d1).

Next, the NaCl concentration in the feedstream was increased from approximately 110 to 170 mM in order to improve column capacity by reducing the absorption of charged contaminants. In anticipation of large volume processing, NaCl and NaPhos were introduced concurrently by adding 1.0 M NaPhos, 1.2 M NaCl, pH 6.75, to the clarified harvest at a 1:19 ratio. Also, the load volume was increased from 18 to 50 ml. In the aggregate, these process changes resulted in product recovery increasing from 48 to 71%. However, the principle features of the chromatogram (Figure 3) did not change significantly as a result of these changes, and again, no significant virus was found in the flow-through and wash peaks (a1 and c1, respectively).

Further increases in the load-to-CV ratio did not improve product recovery but would have increased the amount of time needed to complete the load. Therefore, the decision was made to stay with the 50:1 load ratio.

Endonuclease Digestion

Near pH 7, the presence of high molecular weight chromatin is known to cause significant amounts of both HCP and host DNA to elute from anion exchange resins. To reduce the chromatin's molecular weight, and thereby elute additional HCP and DNA during washing, the feedstream was treated with *S. marcescens* extracellular endonuclease prior to column chromatography. This resulted in a large reduction in the UV absorption of the product peak (Figure 4, d), without any associated reduction in product recovery, 72% as measured by RT-qPCR (d1), indicating a significant increase in product purity. This was accompanied by a corresponding increase in the size of the wash peak (c), presumably corresponding to additional HCP and DNA also being pre-eluted. No additional virus was detected in the flowthrough or washes (a1, a2, c1). As described in Materials and Methods, nuclease digestion conditions were optimized for anticipated GMP-compatible operations during the course of subsequent experiments.

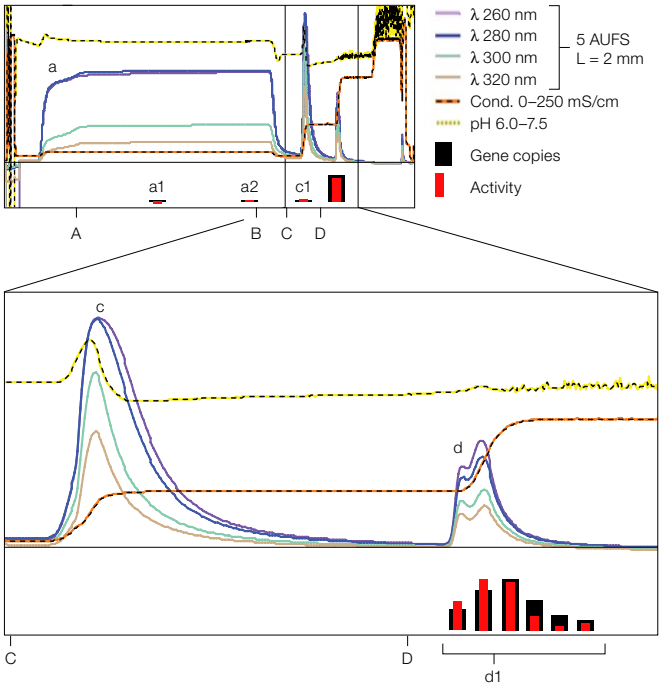


Fig. 4. Chromatogram after addition of nuclease. Compared to experiments performed without nuclease treatment, the contaminant rich peak at the 0–440 mM transition showed increased UV absorbance and an increased A260/A280 ratio (c), whereas the product rich peak at the 440–1,100 mM transition exhibited a corresponding decrease in UV absorbance (d). Fractions from the first peak were pooled and analyzed to confirm that virus was not being lost during the 440 mM NaCl wash portion of the procedure (c1). Seventy-two percent of the virus loaded, as measured by gene copy number, was recovered in the product rich peak, and there remained good correlation between gene copy number, as measured by RT-qPCR (black bars), and biological activity (red bars) (d1).

Scale-Up Studies

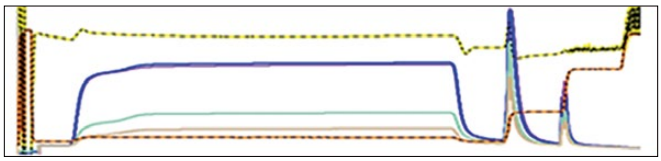
Process scale-up was performed as shown in Table 2. From the initial work outlined in column A to the GMP-scale production outlined in column F, bioreactor production volume was increased approximately 83-fold and the chromatography process volume was increased approximately 13,000-fold. Representative chromatography traces were comparable across the scale-up volumes (Figure 5).

Table 2. Process parameters during downstream process development.

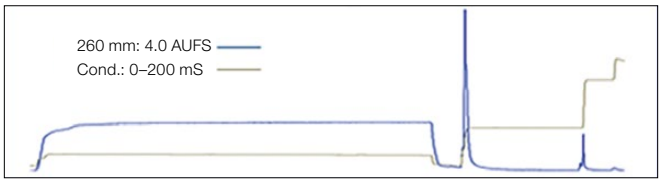
	A	B	C	D	E	F
Bioreactor volume, L	3	3	3	10	50	250
Load volume, L	0.018	0.050	0.5	5.3	33	240
Column diameter, cm	0.80	0.80	0.80	2.6	6.2	17.8
Column length, cm	2	2	20	20	20	20
Column volume, ml	1	1	10	105	605	4,900
Flow rate, ml/min	0.30	0.30	3.5	35	200	1,700
Relative flow rate, CV/hr	18	18	21	20	20	21
Relative load volume, CV	19	50	50	50	54	49
Linear flow rate, cm/hr	35	35	420	400	395	415
Scale factor (bioreactor)	1	1	1	3	17	83
Scale factor (chromatography)	1	3	28	295	1,800	13,000

Column A, buffer system development; B, relative load volume increase and introduction of nuclease treatment; C, scale-up via increase of column length and linear flow rate; D–F, scale-up via increase of column diameter.

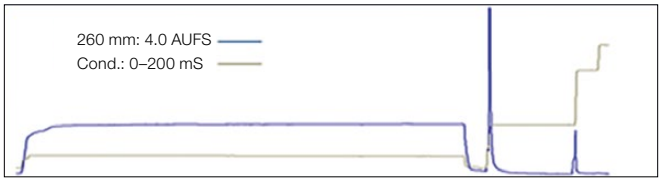
B. Bioreactor = 3 L; Column = 1 ml; Flow rate = 0.3 ml/min



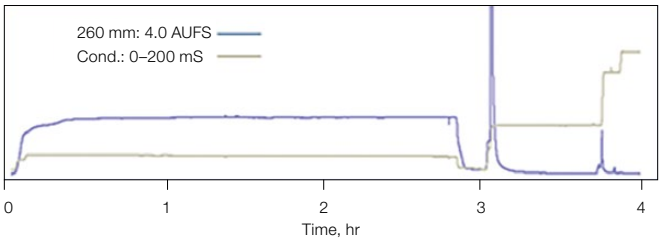
D. Bioreactor = 10 L; Column = 105 ml; Flow rate = 35 ml/min



E. Bioreactor = 50 L; Column = 600 ml; Flow rate = 200 ml/min



F. Bioreactor = 275 L; Column = 5,100 ml; Flow rate = 1,700 ml/min



Bioreactor/Column Scaling

	Reactor, L	Column, ml	Load, L	Flow, ml/min
B	3	1	0.06	0.3
D	10	105	6.4	35
E	50	600	36	200
F	250	5,100	275	1,700

Fig. 5. Scale-up for GMP manufacturing. Column volume was scaled from 1 to 10 ml, 105 ml, 0.6 L, and finally to 5.1 L. Corresponding flow rates were scaled from 300 µl/min to 1.7 L/min. A load volume-to-CV ratio of approximately 50:1 was maintained throughout. Representative chromatograms at the 1 ml (B), 105 ml (D), 600 ml (E), and 5.1 L scale (F) are shown. Letter designations correspond to the columns in Table 2.

At the time of harvest, HCP levels were determined to be approximately 66 µg/10^{10} viral particles (vp) and DNA levels were estimated to be approximately 420 µg/10^{10} vp. As described above, endonuclease was added to a level of 15 units/ml. These process contaminants were substantially removed during downstream processing, reductions being on the order of 600x for HCP, 700,000x for DNA, and greater than the limit of quantitation, 280x, for endonuclease (Table 3). Cell line–specific contaminants, genetic sequences coding adenovirus E1 and SV40 large T-antigen, were not detectable in the final batches. Endotoxin levels were well within the safe range for parenteral drug products, and the bioburden tests did not indicate the presence of viable organisms.

Table 3. Analytical test results.

Assay	Per 10^{10} vp
Potency, % relative to reference	127
Residual HCP, ng	111
Residual human DNA, ng	<0.6
Residual endonuclease, ng	<0.5
Residual adenovirus E1 DNA	Not detected
Residual SV40 large T-antigen DNA	Not detected
Bioburden (cfu) <61>	<0.6
Endotoxin (EU) <85>	0.4

Final viral recovery = ~65%

In summary, during the course of this project, process scale increased roughly 13,000-fold, from 18 ml for early resin screening and buffer system development to approximately 240 L for clinical-product manufacturing. The final process employed a single anion exchange chromatography step lasting a little over 4 hours, which resulted in a 99% reduction in process volume, a product recovery on the order of 65%, and a final product purity and quality consistent with requirements for clinical trials.

Conclusions

The process described here for retrovirus purification using Nuvia Q Resin constitutes an efficient single-step platform for low cost, fast, simple, and robust manufacture of retroviruses. This process is easily scalable and uses cGMP-compatible reagents.

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