Development of an Efficient Manufacturing Process for Adenovirus

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Tech Note

Process Separations

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Abstract

Large-scale downstream processing of viruses for clinical applications poses challenges different from those for many other biotherapeutics. These challenges mostly arise from the size and complexity of the virus. Here, we present purification results of a process developed for the manufacturing of a recombinant adenovirus. This two-column process results in an adenovirus preparation comparable to clinical grade products, with high yield and very low host cell protein (HCP) and DNA contamination. It is readily scalable, simple, rapid, and efficient and thus well-suited for the production of clinical grade viral vectors.

Introduction

Adenovirus vectors are effective tools for the transfer of genetic material into mammalian cells. They offer several advantages, including the capacity to accommodate up to 37 kb of foreign genetic material, very high infection efficiency, the ability to infect a wide variety of both dividing and nondividing cell types, lack of integration into the host chromosome, and production systems capable of generating high virus titers. These and other qualities have led to adenoviruses being the most used gene transfer vectors in experimental therapies, accounting for 25% of all gene therapy trials; as of 2014, they had been used in almost 500 clinical trials.

Large-scale downstream processing of viruses for clinical applications poses challenges that arise, in part, from the viruses' large size and complexity. In the case of adenovirus, one intact virus particle (vp) contains more than 2,700 protein subunits, has a mass of approximately 165 MDa, and has a diameter of approximately 0.1 µm. The complexity of the particle gives rise to thousands of charge variants, making it difficult to establish well-defined binding and elution conditions on charged separation resin. In addition, adenoviruses tend to be acid labile, which further increases the complexity of process development.

We developed a two-column efficient purification process for a recombinant adenovirus after screening five chromatography resins. We show that the final process yields an active, concentrated virus product with purity, HCP levels, and DNA contamination comparable to clinical-grade products. The process is readily scalable and is sufficiently simple, rapid, and efficient to be used for the production of clinical-grade viral vectors.

Materials and Methods

Virus

We used a recombinant human adenovirus, Ad5-E1+GFP, serotype 5, in which the E1a gene was replaced with DNA coding for *Aequorea victoria* green fluorescent protein (GFP). The virus was expanded in HEK 293 cells grown in DMEM + 2% fetal bovine serum. The virus was harvested 36 hr after inoculation by adding a cell permeation agent (Somatek Inc.) to the culture to release the virus from the host cells.

Initial resin screening

Initial resin screening was performed using two 1 ml prepacked 8 x 20 mm Foresight™ Columns in series.

UNOsphere™ S and Nuvia™ S Cation Exchange Columns (Bio-Rad Laboratories, catalog #732-4730 and #732-4720, respectively) were equilibrated with 25 mM sodium phosphate, pH 6.8. The crude harvest was buffer-exchanged into equilibration buffer prior to column loading and virus was eluted with 25 mM sodium phosphate, 1 M NaCl, pH 7.4.

A Nuvia™ cPrime™ Hydrophobic Cation Exchange Column (Bio-Rad, catalog #732-4722) was equilibrated with 25 mM histidine, pH 6.5. The crude harvest was buffer-exchanged into equilibration buffer prior to column loading and virus was eluted stepwise with buffers of increasing pH and NaCl concentration.

UNOsphere Q and Nuvia Q Anion Exchange Columns (Bio-Rad, catalog #732-4732 and #732-4721, respectively) were equilibrated with 25 mM Tris, 250 mM NaCl, pH 8.1. Crude harvest was loaded directly onto the columns and virus was eluted stepwise with buffers of increasing NaCl concentration.

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Virus quantitation

Focus-forming units (FFU) were determined after tenfold serial dilutions of virus samples were applied in quadruplicate to 7 x 10^4 HEK 293 cells grown in 48-well plates. Transgene expression of GFP was determined by fluorescent spectroscopy after threefold serial dilutions of virus samples were applied in triplicate to 60–90% confluent HEK 293 cells grown in 96-well plates. Total virus concentration was determined using the following equation:

Number of particles =
A_{260} * dilution factor * 1.1 x 10^{12} particles/A_{260} unit

These total virus measurements were used only when both A_{260}/A_{280} = 1.3 ± 0.1 and A_{260} = 0.7 ± 0.2 unit.

Purity assessment

SDS-PAGE analysis was performed using 4–20% Criterion™ Tris-HCl Gradient Gels stained with Bio-Safe™ Coomassie Stain. HCP levels were determined using a HEK 293 HCP ELISA Kit (Cygnus Technologies). DNA was extracted from test samples and subjected to quantitative PCR analysis using primers and probes directed against a 63 bp region of the human 18S ribosomal RNA gene. Readouts were compared to a quantitative HEK 293 DNA standard curve.

Final process characterization

Data for both the final mass capture and the final anion exchange operations were generated using 5 ml prepacked 8 x 100 mm Foresight Columns.

The Nuvia cPrime Column (Bio-Rad, catalog #732-4742) was equilibrated with 25 mM histidine, pH 6.0. The culture supernatant was diluted 1:3 with equilibration buffer prior to column loading. The loaded column was washed with equilibration buffer and virus was eluted with 75 mM Tris, 525 mM NaCl, pH 8.5.

The Nuvia Q Column (Bio-Rad, catalog #732-4741) was equilibrated with 75 mM Tris, 250 mM NaCl, pH 8.0. Eluate from the Nuvia cPrime Column was diluted 1:1 with 75 mM Tris, pH 8.0, prior to column loading. The loaded column was washed with equilibration buffer and then with 75 mM Tris, 440 mM NaCl, pH 8.5. Purified virus was eluted with 75 mM Tris, 1 M NaCl, pH 7.5.

Results

Process development

Mass capture

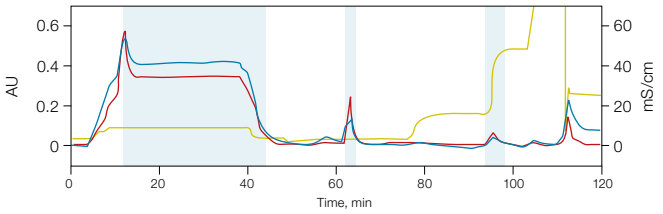
Initial screening showed that of the five resins screened, three had potential for use in a mass capture process — Nuvia cPrime (hydrophobic cation exchange), UNOsphere Q (anion exchange), and Nuvia Q (anion exchange) — since virus was not detected in either the flow-through or wash fractions (Table 1). Nuvia cPrime was selected for capturing the adenovirus as it offered the best clearance of feedstream contaminants. Eluate from this hydrophobic cation exchange resin could be loaded onto the subsequent column following a simple pH adjustment. Fouling of anion exchange chromatography resins in process manufacturing (Close et al. 2013, Drevin et al. 1989) and difficulties in their regeneration (Ng and McLaughlin 2007) have been reported due to the binding of excessive amount of impurities and ineffective cleaning. Therefore, the Q resins are more suitable for polishing purification. Between the two strong anion exchange resins, Nuvia Q was chosen for its higher binding capacity for target virus even in the presence of high NaCl concentrations.

Table 1. Results from the initial resin screening and their implications.

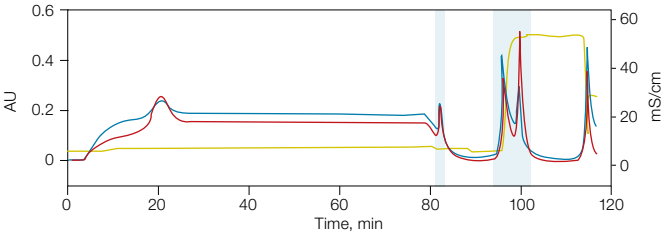
Column Type	Virus in Flowthrough/ Wash	Virus in Eluate	Notes/Implications
UNOsphere S (CEX)	+++	++	Poorly suited for virus purification in both bind-and-elute and flowthrough modalities
Nuvia S (CEX)	+++	++	Poorly suited for virus purification in both bind-and-elute and flowthrough modalities
Nuvia cPrime (MM)	–	++++	Partial elution in 125 mM NaCl, pH 6.5; hence, dilution of crude harvest required prior to column loading
UNOsphere Q (AEX)	–	++++	Could be considered for direct mass capture
Nuvia Q (AEX)	–	++++	Could be considered for direct mass capture

CEX, cation exchange; MM, mixed mode; AEX, anion exchange

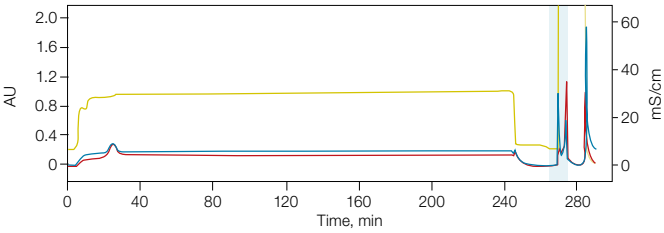
We developed the mass capture step using Nuvia cPrime by first replacing the buffer exchange step used in the screening study with a dilution step. This was carried out since virus partially eluted from the column when 125 mM NaCl was used. In addition, a nuclease digestion step was introduced prior to column loading to aid in nucleic acid clearance. Initial mass capture experiments were focused on reducing feedstream volumes and recovering virus. The chromatograms in Figure 1 illustrate the early progression of these experiments and indicate when significant transgene expression was detected.



Feed diluted 1:1 with 75 mM histidine, pH 6.0
Wash = 75 mM histidine, pH 6.0
Elution 1 = 75 mM Tris, pH 8.0
Elution 2 = 75 mM Tris + 125 mM NaCl, pH 8.0



Feed diluted 1:3 with 75 mM histidine, pH 6.0
Wash = 75 mM histidine, pH 6.0
Elution 1 = 75 mM Tris, pH 8.0
Elution 2 = 75 mM Tris + 525 mM NaCl, pH 8.5



Feed diluted 1:3 with 25 mM histidine, pH 6.0
Wash = 25 mM histidine, pH 6.0
Elution = 75 mM Tris + 525 mM NaCl, pH 8.5

Fig. 1. Iterative development of Nuvia cPrime capture. OD 260 (—); OD 280 (—); conductivity (—). Blue shading indicates detection of significant transgene expression. AU, absorbance units.

Anion exchange chromatography

Of the two anion exchange resins, UNOsphere Q and Nuvia Q, Nuvia Q was selected because it could adsorb virus at higher NaCl concentrations (Figure 2). It was therefore easier to work with downstream of the Nuvia cPrime capture step, where the eluate had an NaCl concentration of approximately 500 mM going on to the anion exchange column.

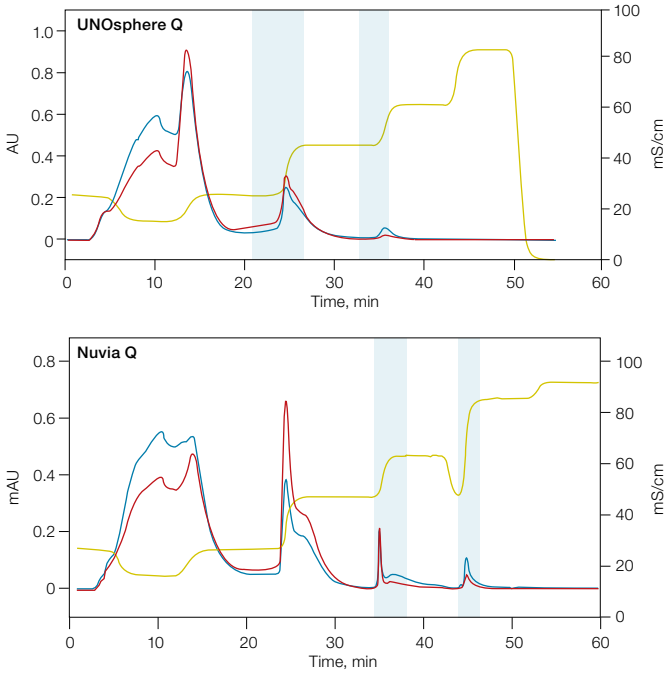


Fig. 2. Behavior of the crude harvest on the UNOsphere Q and Nuvia Q Columns. OD 260 (—); OD 280 (—); conductivity (—). Blue shading indicates detection of significant transgene expression. AU, absorbance units.

The anion exchange experiments with Nuvia Q were focused on attaining high product purity (Figure 3). As shown in the figure, increasing the pre-elution NaCl concentration from 400 to 440 mM prevented a suspected HCP contaminant from eluting with the product. Therefore, it would be possible to further improve the efficiency of this chromatography step by directly equilibrating the Nuvia Q Column with 75 mM Tris + 440 mM NaCl, pH 8.0.

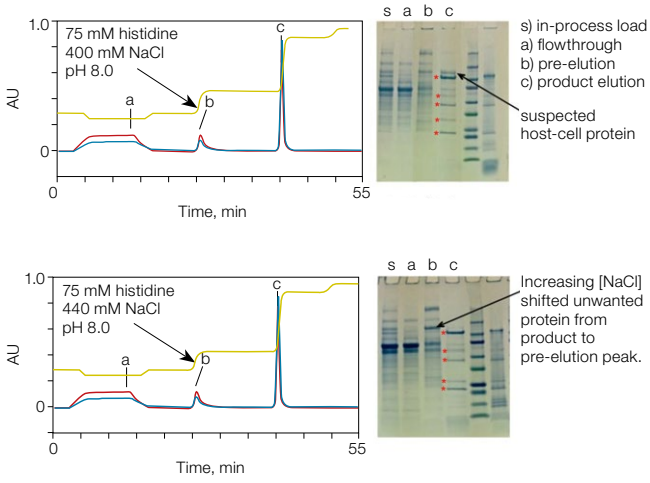


Fig. 3. Chromatograms and gels from anion exchange process development experiments. OD 260 (—); OD 280 (—); conductivity (—).
* Proteins presumed to be virus capsid components. AU, absorbance units.

Final process

Mixed-mode chromatography

Initial capture was accomplished using Nuvia cPrime Mixed-Mode Resin (Figure 4). This portion of the process achieved a tenfold reduction in processing volume and a significant reduction in feedstream contaminants (Figure 6, lanes 2–4).

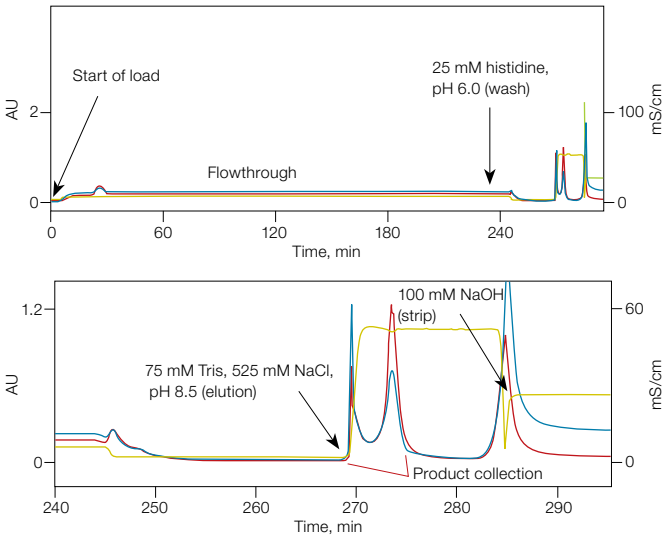


Fig. 4. Representative mixed-mode chromatogram. OD 260 (—); OD 280 (—); conductivity (—). AU, absorbance units.

A notable feature of the chromatograms generated using this method is a double peak following the application of the elution buffer. The first peak is concurrent with the salt front, while the second peak presumably corresponds to a pH shift. Both contained significant amount of virus. Therefore, product collection spanned both peaks. Increasing just the pH or ionic strength alone did not yield an eluate with a satisfactory amount of virus (data not shown).

Anonion Exchange Chromatography

Final virus purification was accomplished using Nuvia Q Resin (Figure 5). This portion of the process achieved an additional twofold reduction of product volume along with a significant improvement in product purity (Figure 6, lanes 4–7). Following this operation, nonvirus proteins were no longer evident by SDS-PAGE (Figure 6, lane 7).

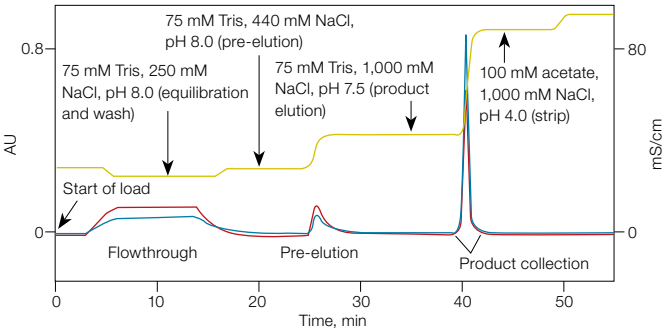


Fig. 5. Representative anion exchange chromatogram. OD 260 (—); OD 280 (—); conductivity (—). AU, absorbance units.

Analysis of in-process and final product

We used SDS-PAGE analysis to visualize the progressive reduction of contaminating proteins at each step of the purification process (Figure 6). Cell culture media components such as albumin and transferrin are clearly evident in the virus culture harvest, that is, the sample loaded onto the Nuvia cPrime Column (Figure 6, lane 2). These proteins are effectively separated from the viral protein components, as they are highly visible in the flow-through fraction from this column, under the current chromatography conditions (Figure 6, lane 3). Significantly fewer contaminants are seen in the sample loaded onto the Nuvia Q Column (Figure 6, lane 4). During the anion exchange process, the virus bound to the resin while contaminants either flowed through the column (Figure 6, lane 5) or were pre-eluted (Figure 6, lane 6). The five most prominent viral proteins — hexon, penton, core (V), hexon (VI), and core (VII) — are readily visible in the final purified product (Figure 6, lane 7).

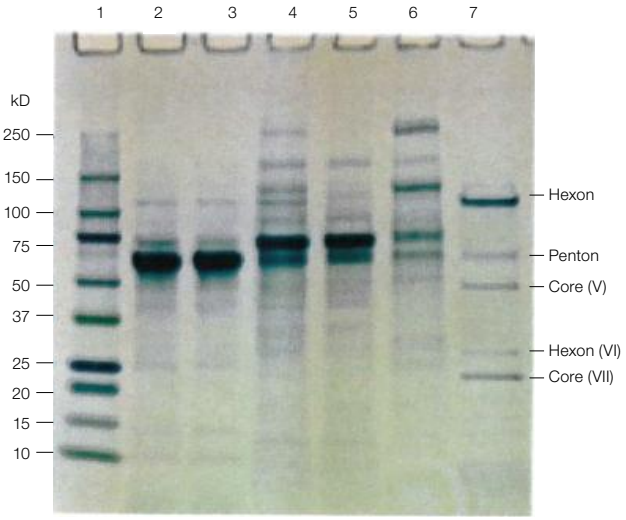


Fig. 6. SDS-PAGE of intermediates and the final product. Lane 1, MW marker; lane 2, Nuvia cPrime load; lane 3, Nuvia cPrime flowthrough; lane 4, Nuvia cPrime elution/Nuvia Q load; lane 5, Nuvia Q flowthrough; lane 6, Nuvia Q pre-elution; lane 7, Nuvia Q product.

Virus concentration and DNA levels were evaluated at select points along the downstream process (Table 2). The data demonstrate an overall recovery of virus particles of approximately 54%, with DNA levels below detection and HCP at 2 ng/10^{10} particles. These values are well within current guidelines for clinical and perhaps commercial use.

Table 2. Viral particle recovery and impurity clearance.

		Impurity Levels (ng/10^{10} Particles)	
Sample	Total Virus ($x10^{11}$ Particles)	DNA	HCP
Bulk harvest	30.6	3,144	n/d
Nuclease-treated harvest	31.8	30	3,022
Nuvia cPrime eluate	18.4	n/d	58
Nuvia Q eluate	16.4	<0.02	2

n/d, not determined.

Conclusions

The final process yields an active, concentrated virus product with purity, HCP, and DNA levels comparable to clinical grade products. While the purification methods presented here were developed using the Ad5-E1+GFP model virus, they are expected to be applicable to recombinant adenoviruses in general and to constructs derived from serotype 5 virus in particular. The process is readily scalable and uses procedures and reagents compatible with cGMPs. Also, it is sufficiently simple, rapid, and efficient to be used for the production of clinical grade virus-based gene therapy products and vaccines.

References

Close EJ et al. (2013). Fouling of an anion exchange chromatography operation in a monoclonal antibody process: Visualization and kinetic studies. Biotechnol Bioeng 110, 2425–2435.

Drevin I et al. (1989). Column performance of Q-Sepharose HP in analytical- and preparative-scale chromatography. J Chromatogr A 477, 337–344.

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