Development of a cGMP-Ready Purification Process for Adenovirus Purification

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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. For example, the adenovirus vector comprises 25% of all gene therapy clinical trials due, in part, to its ability to infect both nondividing and dividing cells with persistent expression. The vector contains over 2,700 protein subunits and has a mass of approximately 165 MDa and a diameter of approximately 0.1 µm.

Here, we present purification results of a cGMP-ready process developed for a recombinant adenovirus. The two-step column process results in an adenovirus preparation with high yield and very low host cell protein (HCP) and DNA contamination, comparable to clinical grade products.

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 efficiencies, 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 virus's large size and complexity. In the case of adenovirus, one intact virus particle (vp) contains over 2,700 protein subunits, has a mass of approximately 165 MDa, and has a diameter of approximately 0.1 µm. The complexity gives rise to thousands of charge variants, making it difficult to establish well-defined binding and elution conditions. Further complexity is attributed to the fact that adenoviruses tend to be acid labile.

We developed a two-column cGMP-ready purification process for a recombinant adenovirus after screening five Bio-Rad 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 considered 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 hours 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 1 ml prepacked 8 x 20 mm Foresight™ Columns.

UNOsphere™ S and Nuvia™ S Cation Exchange Columns 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 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 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.

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} x dilution factor x 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, which were stained with Bio-Safe™ Coomassie Stain.

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 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 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 8.0. Purified virus was eluted with 75 mM Tris, 1 M NaCl, pH 7.5.

Results

Process Development

Initial Screening Results

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

Mass Capture

As shown in Table 1, three resins had potential for use in a mass capture process — Nuvia cPrime, UNOsphere Q, and Nuvia Q. Of these, Nuvia cPrime was selected because its requirement for virus binding (low salt and low pH) made it poorly suited for use *after* an anion exchange capture operation (where the resulting feedstream would be expected to have high ionic strength and perhaps high pH).

Developing the mass capture step using Nuvia cPrime involved replacing the buffer exchange step used in the screening study with a dilution step. Also, a nuclease digestion was introduced prior to column loading. Initial mass capture experiments were focused on reducing feedstream volumes and recovering virus. Example chromatograms, overlaid with the results of the transgene expression assay, illustrate the early progression of these experiments (Figure 1).

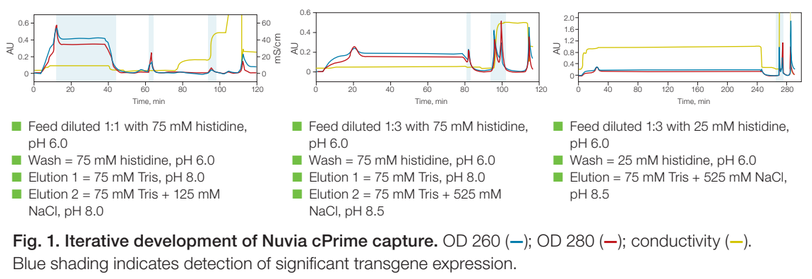

Fig. 1. Iterative development of Nuvia cPrime capture. OD 260 (—); OD 280 (—); conductivity (—). Blue shading indicates detection of significant transgene expression.

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 a NaCl concentration of approximately 500 mM going on to anion exchange.

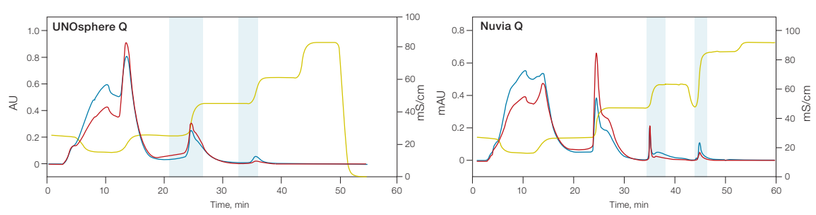

Fig. 2. Behavior of the crude harvest on the UNOsphere Q and Nuvia Q Columns. OD 260 (—); OD 280 (—); conductivity (—). Blue shading indicates detection of significant transgene expression.

Anion exchange experiments with Nuvia Q were focused on attaining high product purity (Figure 3). In one late-stage experiment, a suspected HCP contaminant was prevented from eluting with the final product by increasing the pre-elution NaCl concentration.

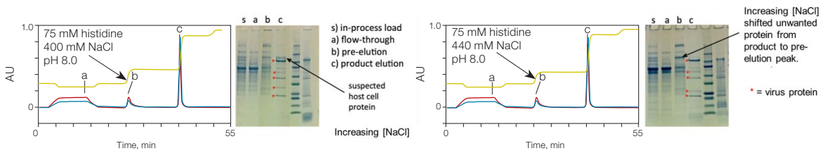

Fig. 3. Chromatograms and gels from anion exchange process development experiments. OD 260 (—); OD 280 (—); conductivity (—). * Proteins presumed to be virus capsid components.

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).

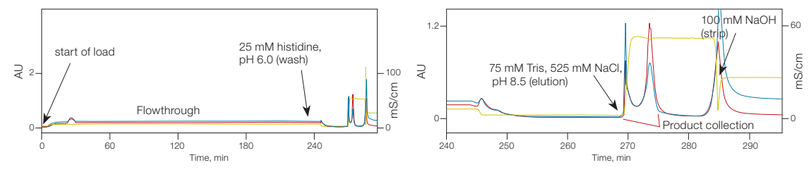

Fig. 4. Representative mixed-mode chromatogram. OD 260 (—); OD 280 (—); conductivity (—).

Anion 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 through 7). Following this operation, nonvirus proteins were no longer evident by SDS-PAGE (Figure 6, lane 7).

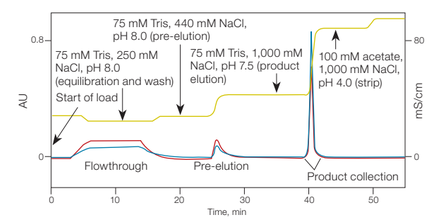

Fig. 5. Representative anion exchange chromatogram. OD 260 (—); OD 280 (—); conductivity (—).

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). 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).

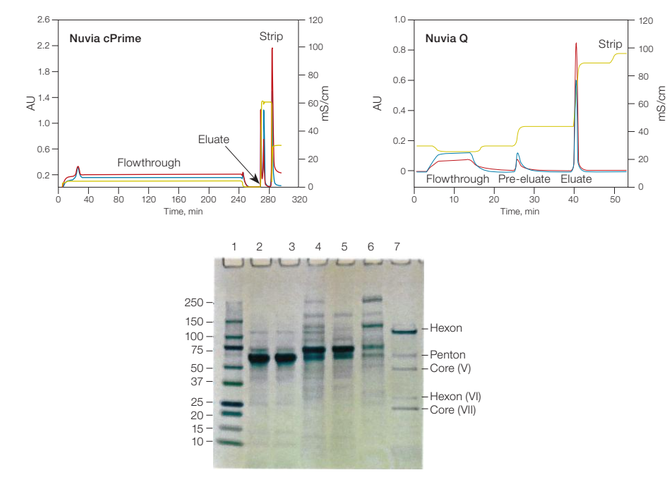

Fig. 6. SDS-PAGE of intermediates and the final product. Lane 1, MW marker; lane 2, Nuvia cPrime load; lane 3, Nuvia cPrime flowthrough; lane 4, Nuvia cPrime elution/Nuvia Q load; lane 5, Nuvia Q flowthrough; lane 6, Nuvia Q pre-elution; lane 7, Nuvia Q product. OD 260 (—); OD 280 (—); conductivity (—).

The data in Table 2 demonstrate an overall recovery of virus particles of approximately 54%, with DNA levels below detection and host cell protein 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.

Sample	Total virus (x10^{11} particles)	Impurity levels (ng/10^{10} 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 viruses, 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.

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