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# Optimization of midstream cell lysis and virus filtration steps in an adenovirus purification process

This application note describes screening of detergents for release of adenovirus from HEK293 host cells to replace the traditionally used detergent Triton™ X-100, now on the authorization list (Annex XIV) of registration, evaluation, authorization, and restriction of chemicals (REACH). In addition, screening of clarification filter pore size was performed in small scale with ULTA™ filter discs using the ÄKTA™ pure 25 system, and results were confirmed in larger scale with ULTA filter capsules. For concentration and buffer exchange, hollow fiber filters for ultrafiltration/diafiltration (UF/DF) with different nominal molecular weight cut-off (NMWC) were compared using ÄKTA flux 6. Consumables and conditions that provided the highest virus purity and recovery were selected as a suggestion for a midstream process for adenovirus recovery from a 10 L cell culture, to provide process material ready for the subsequent downstream purification step.

## Introduction

Adenovirus (AdV)-based vectors are widely evaluated as vaccine delivery system in preclinical and clinical studies for various infectious diseases. AdV has also been extensively explored as a viral vector for oncolytic use. Manufacturing of a safe and efficacious clinical-grade virus relies on a scalable and cost-effective production process.

Non-lytic viruses, such as the adenovirus, require methods for the release of the virus from its host cell. Traditionally, Triton X-100 has been used as detergent for cell lysis and has provided sufficient overall yield in viral vector purification processes. Triton X-100, however, is on the authorization list (Annex XIV) of REACH and can only be used in limited amounts for research purposes. Common detergents used for cell lysis and their REACH status are listed in Table 1.

An alternative detergent or method for cell lysis should not affect the virus infectivity and stability, nor should it

interfere with downstream process steps, and an assay for analysis of residual detergent is required. Repeated freeze-thawing is a detergent-free mechanical lysis method, however, more suitable for smaller scales for practical reasons. Non-ionic detergents, similar to Triton X-100, are likely to be good candidates. In this study, harvest material from adenovirus-infected HEK293 cells grown in suspension was used. Alternative detergents for cell lysis as well as filters for clarification, concentration, and buffer exchange were screened, and consumables and conditions providing the highest virus purity and recovery were selected. The results provide a suggestion for a midstream process in adenovirus production and purification. This work is part of a project for development of an adenovirus production process, ranging from upstream production to a purified sterile-filtrated bulk product.

**Table 1.** Common detergents for cell lysis

Detergent	Properties	REACH status
Brij™-35	Non-ionic	✘
CHAPS	Zwitterionic	✔
IGEPAL™ CA630 (Nonident NP-40)	Non-ionic	✘
Octyl glycoside	Non-ionic	✔
Sodium deoxycholate	Ionic (-)	✔
Tergitol™ NP-40 (NP40)	Non-ionic	✘
Triton X-100	Non-ionic	✘
Tween™ 20	Non-ionic	✔
Tween 80	Non-ionic	✔
Zwittergent™ 3-14	Zwitterionic	✔

✔ = low risk of being added to authorization list ✘ = high risk for being added to authorization list  
 ✘ = on authorization list

## Materials and methods

### Cell culture and infection

Shake flasks (1 L) and bioreactor (10 L) cultures of HEK293 suspension cells (HEK-293.2sus, ATCC), infected with E1/E3-deleted recombinant AdV serotype 5 (AdV5) coding for green fluorescent protein (GFP), were performed as previously described (1).

### Detergent screening

For detergent screening, 10% (w/v) solutions of Triton X-100 (reference treatment), Tween 20, Tween 80 (Thermo Scientific), and Zwittergent 3-14 (Calbiochem) were used. A 2 mL cell harvest sample was incubated with each detergent at varying concentration under slow rocking motion for 1 h at 37°C. After incubation, the sample was either analyzed directly for cell viability and remaining intact cells or centrifuged at 5000 × g for 5 min and analyzed for virus protein, infectious virus titer, total protein, and gDNA in the supernatant. DNA fragmentation was not performed.

For freeze-thawing (reference treatment), sample was frozen in dry ice/ethanol ice bath for 10 min, followed by thawing in warm water (37°C). This procedure was repeated three times. After the last thawing, the sample was centrifuged at 5000 × g for 5 min and the supernatant was collected.

Filter capacity for the different detergents was measured as feed volume (L/m<sup>2</sup>) passing through a filter (47 cm<sup>2</sup> filter disc) at 150 L/m<sup>2</sup>/h until the pressure reached 0.5 bar.

### Screening of filters for harvest clarification

Screening of clarification filters for removal of cell debris was performed using 47 cm<sup>2</sup> ULTA filter disks for normal flow filtration (NFF). The filters were mounted in a filter holder (Millipore) that was connected to an ÄKTA pure 25 system. A 1 L culture harvest sample was subjected to cell lysis using 0.5% Tween 20 and DNA fragmentation using 10 U/mL Benzonase™ (Merck), after which the sample was incubated at slow mixing for 2 h at 37°C. Thereafter, the sample was filtered through either a single filter or a filter train (separate filtrations in sequence) at 150 L/m<sup>2</sup>/h until pressure reached 1 bar.

#### ULTA Disc single filters

2.0 µm glass fiber (GF)

0.6 µm GF

0.6 µm/0.2 µm polyethersulfone (PES)

#### ULTA Disc filter trains

2.0 µm GF + 0.6 µm GF

2.0 µm GF + 0.6 µm/0.2 µm PES

5.0 µm GF + 0.6 µm/0.2 µm PES

5.0 µm GF + 1 µm GF

### Screening of hollow fiber filters for concentration and buffer exchange

A 10 L Xcellerex™ XDR-10 bioreactor culture was subjected to cell lysis by addition of 0.5% Tween 20 and DNA fragmentation using 20 U/mL Benzonase (Merck) and 1 mM MgCl<sub>2</sub>, after which the culture was incubated for 4 h at 37°C under mixing at 200 rpm in the bioreactor.

The sample was clarified using filters selected from the screening: ULTA Capsule 2.0 µm GF (10 inch, 0.52 m<sup>2</sup> surface area) and ULTA Capsule 0.6 µm GF (6 inch, 0.22 m<sup>2</sup> surface area). The filters were connected in series to a Watson-Marlow 520S pump (Watson Marlow Fluid Technology). A flow rate of 350 mL/min was applied, corresponding to a flux of 40 L/m<sup>2</sup>/h for the 10 inch filter and 96 L/m<sup>2</sup>/h for the 6 inch filter.

Duplicate 1 L samples of clarified harvest were subjected to tangential flow filtration (TFF) using ÄKTA flux 6 and hollow fiber filters, comparing three filters with the same surface area (1400 cm<sup>2</sup>) but with different NMWC: M<sub>r</sub> 300 000 (UFP-300-C-4XM2A), 500 000 (UFP-500-C-4X2MA), and 750 000 (UFP-750-C-4X2MA).

The samples were concentrated 5 times and subjected to a 5-fold diafiltration into 20 mM Tris, pH 8 + 300 mM NaCl. A shear rate of 3000 sec<sup>-1</sup> was used for all three filters, resulting in a transmembrane pressure of 0.3 for the 300-C and 500-C filters and 0.07 bar for the 750-C filter. To monitor the filtration process, samples were taken for analysis of impurity level at 1.25-, 1.7-, 2.5-, and 5 times concentration and thereafter at 1.25-, 1.7-, 2.5- and 5-fold diafiltration.

The 300-C hollow fiber filter was selected and the process was verified in larger scale, where a 6 L sample of clarified harvest was subjected to TFF using ÄKTA flux 6 at a shear rate of 3000 s<sup>-1</sup>, for a 15 times concentration and a 10-fold diafiltration into 20 mM Tris, pH 8.

### Analytical assays

Trypan blue exclusion method was used for measuring number of intact cells and cell viability using Vi-CELL™ cell counter and cell viability analyzer (Beckman Coulter), respectively.

Total and virus protein (hexon) concentrations were analyzed by SDS-PAGE and Western blot. A polyclonal anti-Adv5 primary antibody (Abcam), a Cy<sup>TM</sup>3-labeled secondary antibody, and human Adv5 (ATCC) as standard were used for analysis of hexon protein. For total protein, Cy5-prelabeling was employed, using Amersham™ QuickStain prelabeling kit. Signal for detection was obtained by the Amersham WB image analysis software.

Total virus titer was analyzed in triplicate samples by qPCR using PureLink™ Viral RNA/DNA Mini Kit, TaqMan™ Universal PCR Master mix, and forward and reverse primers for hexon DNA and TaqMan MGB 6-FAM probe on the StepOnePlus™ Real-Time PCR System (all Applied Biosystems). Human Adv5 DNA (3.1 × 10<sup>7</sup> copies/mL) (ViraPur) was used as standard.

Infectious virus titer was analyzed in triplicate samples by automated fluorescence microscopy of live cells using the IN Cell Analyser, and the images were analyzed for GFP signal (coded by virus) with a methodology similar to TCID<sub>50</sub>.

A HPLC method was used for analysis of intact virus particles, using a 1 mL Tricorn™ 5/50 column packed with Q Sepharose™ XL. Elution was performed with a gradient of NaCl in 20 mM Tris, pH 7.5 at a flow rate of 1.5 mL/min (2). Group separation by size exclusion using 70 mL Sepharose 4FF was used to confirm presence of intact virus particles only in the virus peak. Sample load was 10% of column volume (CV), flow rate was 1.67 mL/min (flow velocity 50 cm/h).

A BCA assay kit (Thermo Scientific) with an albumin standard was used for analysis of total protein concentration, and total DNA was determined with Quant-iT™ PicoGreen™ dsDNA Reagent (Invitrogen). Analyses were performed in duplicate.

Concentration of genomic DNA (gDNA) was determined in triplicate samples by qPCR (reagents from Applied Biosystems) using primers for GAPDH (Invitrogen). Samples were prepared using PrepSEQ™ Residual DNA Sample Preparation kit and MagMax™ Express 96 purification instrument (Life Technologies) with purified HEK293 DNA used as standard.

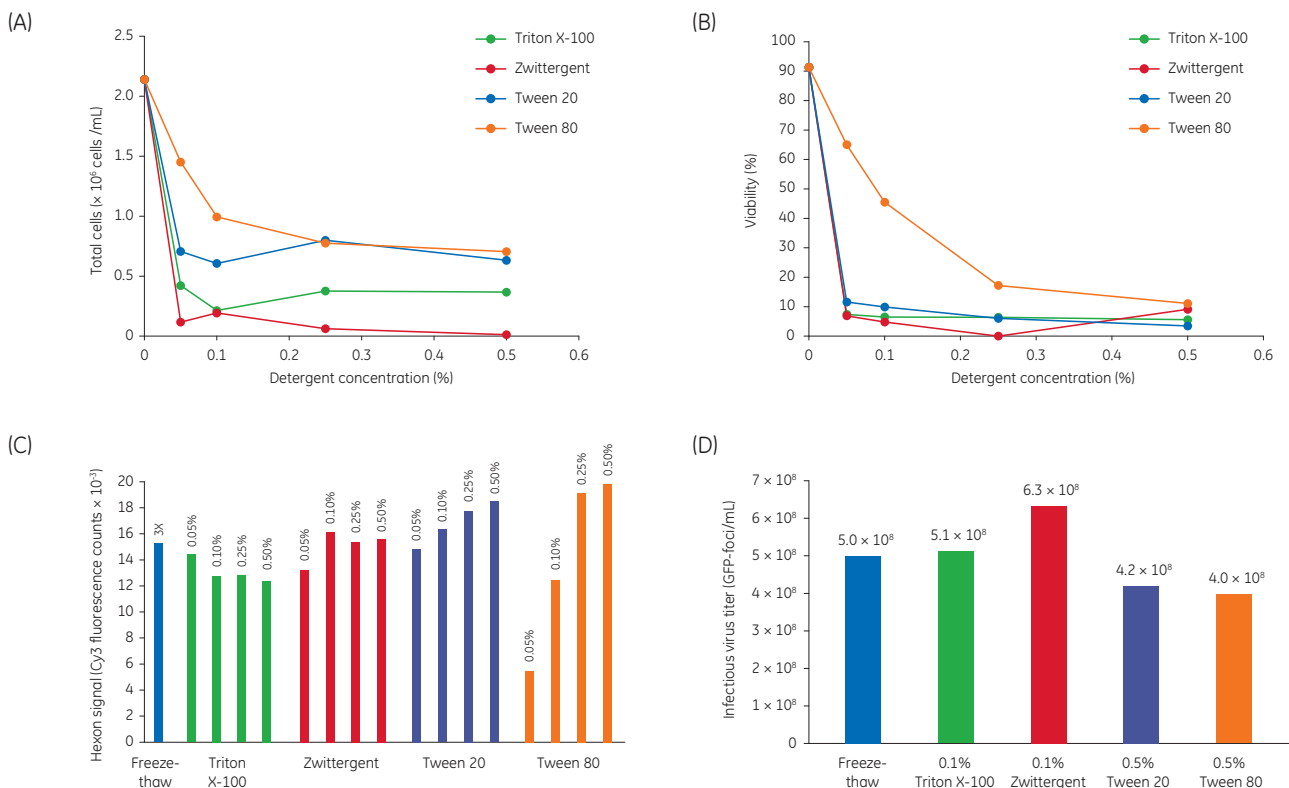
Residual Tween 20 was determined by liquid chromatography-mass spectrometry (LC-MS) as described previously (3). A UPLC system (Waters) with an Oasis™ MAX 2.1 × 20 mm Online

Cartridge Column 30 μm (Waters) was used to separate the Tween 20 peaks that were further analyzed with a Q-TOF MS (Waters). Flow rate was 0.5–1 mL/min and injection volume 5 μL. A gradient of 0.2% formic acid in MQ water and 0.2% formic acid acetonitrile was used.

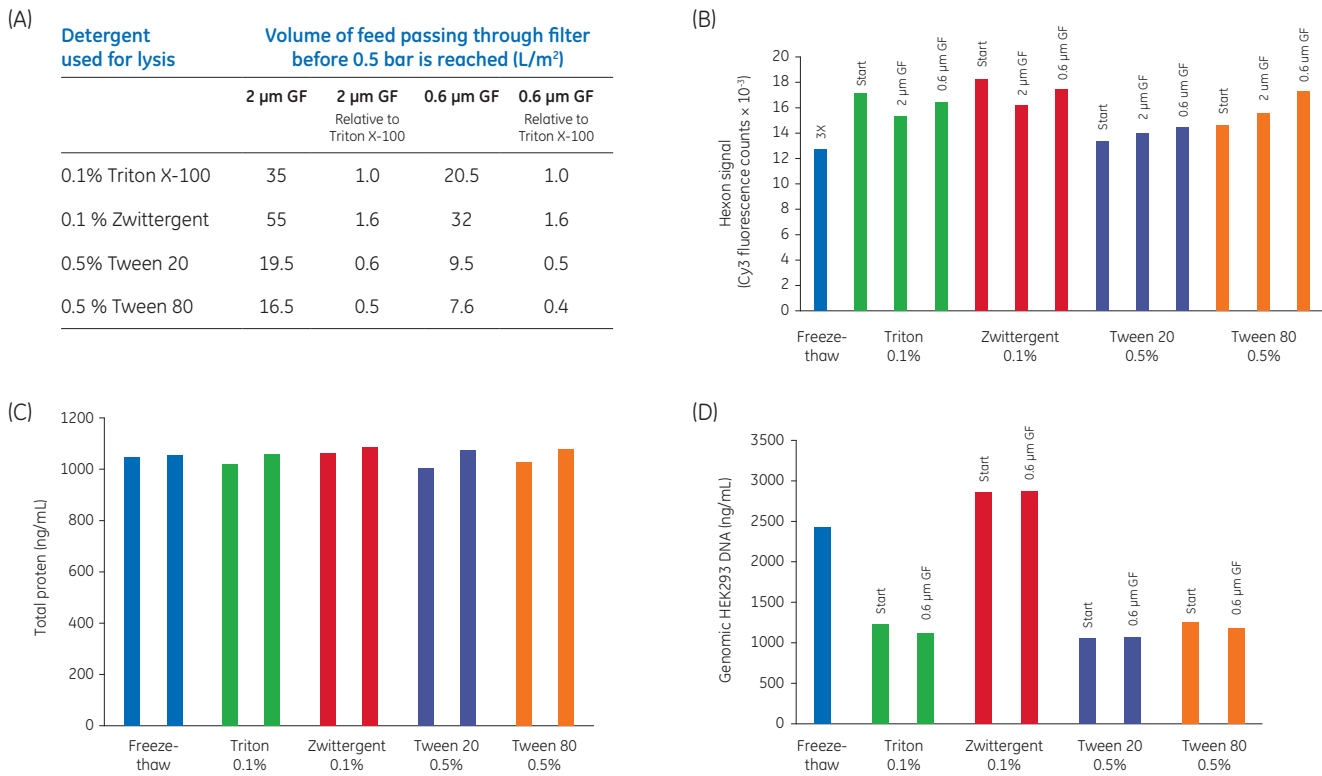
## Results

### Detergent screening

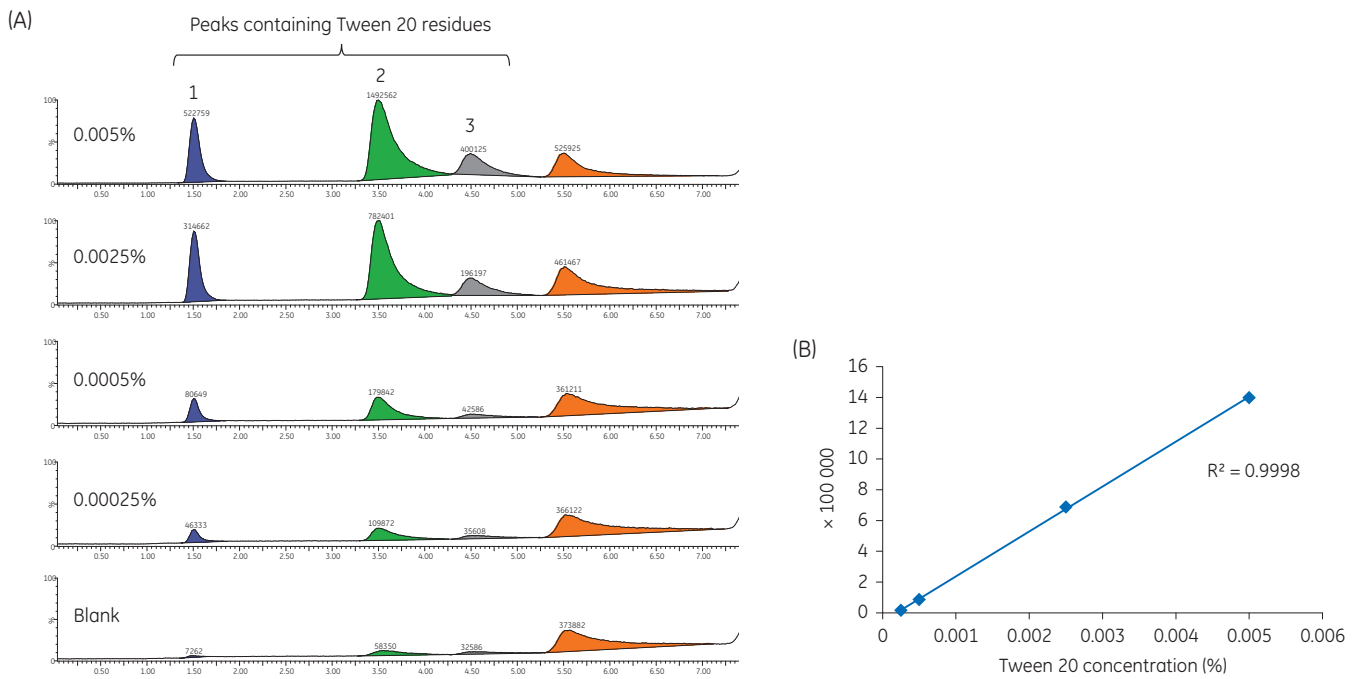
Lysis was monitored to assess remaining intact cells and cell viability after treatment with the different detergents. Lysis using 0.5% Tween 20 and Tween 80 resulted in more intact cells, and viability was higher for Tween 80 at lower concentrations (Fig 1A and B). Determination of released hexon protein (major capsid protein of AdV5) and infectious virus titer indicated similar virus release as the control treatments using Triton X-100 and freeze-thawing (Fig 1C and D). No further lysis of cells was seen after 2 h of incubation, and efficiency of lysis was not affected for cell densities below  $5 \times 10^6$  cells/mL (data not shown). Known differences in critical micelle concentration (CMC) for the detergents support the observation that Triton X-100 and Zwittergent with lower CMC require lower concentrations for sufficient virus release, whereas treatments with Tween 20 and Tween 80 with higher CMC benefit from the use of higher detergent concentrations (Fig 1C).



**Fig 1.** HEK293 cells infected with adenovirus were lysed using three candidate detergents and by using Triton X-100 and freeze-thawing as reference treatments. (A) Number of intact cells, (B) cell viability, (C) levels of released hexon protein, and (D) infectious virus titers.



**Fig 2.** Clarification of detergent-treated feed (no DNA fragmentation) using 2  $\mu$ m and 6  $\mu$ m GF filters. (A) Filter capacity for the different detergents as well as release of (B) virus protein, (C) total protein, and (D) HEK293 cell gDNA after lysis.



**Fig 3.** (A) Analysis of residual Tween 20 identified three peaks that contained Tween 20 residues. (B) Tween 20 between 0.00025% and 0.005% could be detected with a linear response.

Clarification was shown to be affected by the detergent used. For Tween, the higher number of intact cells reduced the filter capacity (Fig 2A), although the release of hexon protein was shown to be similar between the detergents as well as before and after the filtration step (Fig 2B). A larger filter area is needed when Tween 20 and Tween 80 are used for lysis compared with when using Triton X-100. Total protein release was similar between the detergents (Fig 2C). Release of gDNA, however, was higher for lysis by freeze-thawing and by the use of 0.1% Zwittergent and lower for Tween 20 and Tween 80 (Fig 2D).

Residual Tween 20 could be analyzed down to 0.00025% by LC-MS (Fig 3). Tween 20 was separated into three peaks with different degrees of hydrophobicity. Heating of samples to 80°C (for virus inactivation) or presence of virus and proteins did not affect the peak shape or the analysis results (data not shown).

All detergents tested in this study showed good performance relative to the reference treatments with Triton X-100 or by freeze-thawing. However, treatment with Zwittergent released gDNA to a higher degree and has zwitter-ionic properties that can have a negative impact on downstream steps. The use of Tween 20 and Tween 80 reduced the capacity of the clarification filter, but gDNA removal in the clarification step was higher for these detergents compared with the use of Zwittergent. Based on the overall results, 0.5% Tween 20 was selected as replacement for Triton X-100 in the lysis step of our adenovirus purification process. It should be noted, however, that Tween 20 might cause precipitation under certain hold point conditions (buffer components, temperature, time).

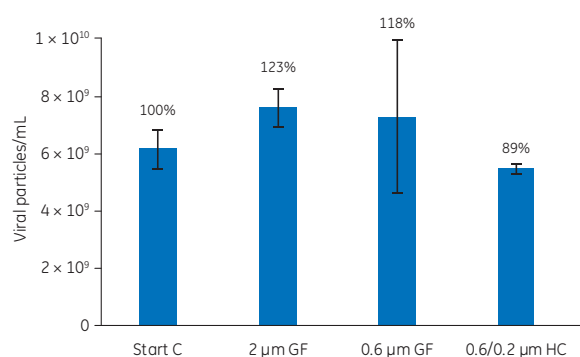
### Screening of clarification filters

Filter pore size screening was performed with 47 cm<sup>2</sup> filter discs for NFF. For single filters, the 2 µm filter reduced turbidity (data not shown) and impurity levels, whereas the capacity was high until filter fouling increased the pressure. For the 0.6 µm and 0.6/0.2 µm filters, impurities were reduced to a higher degree, especially for gDNA. The capacity, however, was reduced by approximately 50% or more with little effect on virus concentration in the filtrate (Table 2 and Fig 4). Using filter trains, with the first filter having the larger pore size, resulted in an increased impurity removal and filtration capacity compared with single filters (Table 3 and Fig 5). When using the 0.6/0.2 µm filter as the second filter in the train, the virus recovery was reduced. In two cases (2 µm + 0.6 µm and 5 µm + 1 µm), the feed volume was consumed over the second filtration step, but the pressure was stable at 0.5 bar and did not increase further at the end of filtration. The volume before the pressure reached 1 bar was therefore estimated to be higher than for the filter trains using 0.6/0.2 µm as the second filter. Based on the overall results for capacity, impurity removal, virus

recovery, and turbidity level, a combination of the 2 µm and 0.6 µm filter was selected. Using this combination in the larger scale, with 10 L feed and ULTA capsules, the achieved recoveries of total and infectious virus titer were both 100%. After this clarification step, the sample is ready for the next downstream processing step.

**Table 2.** Summary of results from single filter screening

Sample	Total protein reduction (%)	gDNA reduction (%)	Vmax (L/m <sup>2</sup> )
2 µm GF	14	28	42
0.6 µm GF	27	80	18
0.6/0.2 µm HC	30	97	7

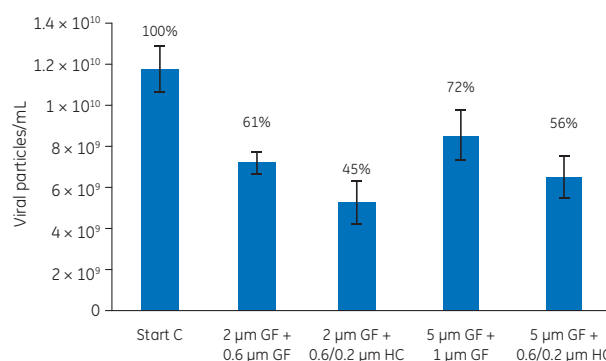


**Fig 4.** Total virus titer, as determined by qPCR, after filtration in the screening of single filters.

**Table 3.** Summary of results from filter train screening

Sample	Total protein reduction (%)	gDNA reduction (%)	Vmax (L/m <sup>2</sup> )
2 µm GF + 0.6 µm GF	37	88	> 77*
2 µm GF + 0.6/0.2 µm HC	42	98	95
5 µm GF + 1 µm GF	30	85	> 60*
5 µm GF + 0.6/0.2 µm HC	38	97	62

\* Feed volume was consumed over second filtration step, but the pressure was stable at 0.5 bar at the end of the filtration.



**Fig 5.** Total virus titer, as determined by qPCR, after filtration in screening of filter trains.

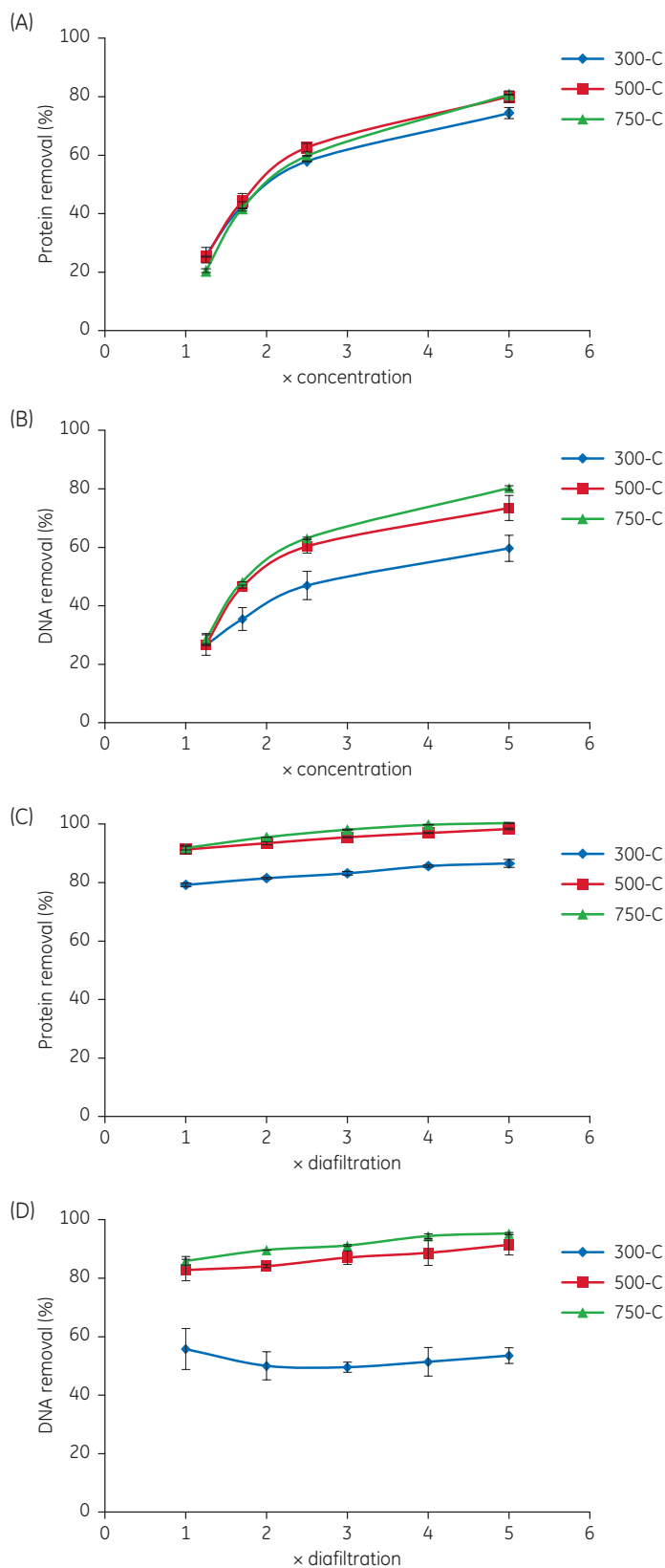
## Screening of hollow fiber filters

TFF with hollow fiber filters is used for concentration and buffer exchange by UF/DF. After a 5 times concentration, a 5-fold diafiltration into 20 mM Tris, pH 8 + 300 mM NaCl was conducted, as this buffer is compatible with anion exchange chromatography that is the next step in the purification process. The concentration and DF factors were selected for maximized impurity removal with complete buffer exchange (data not shown).

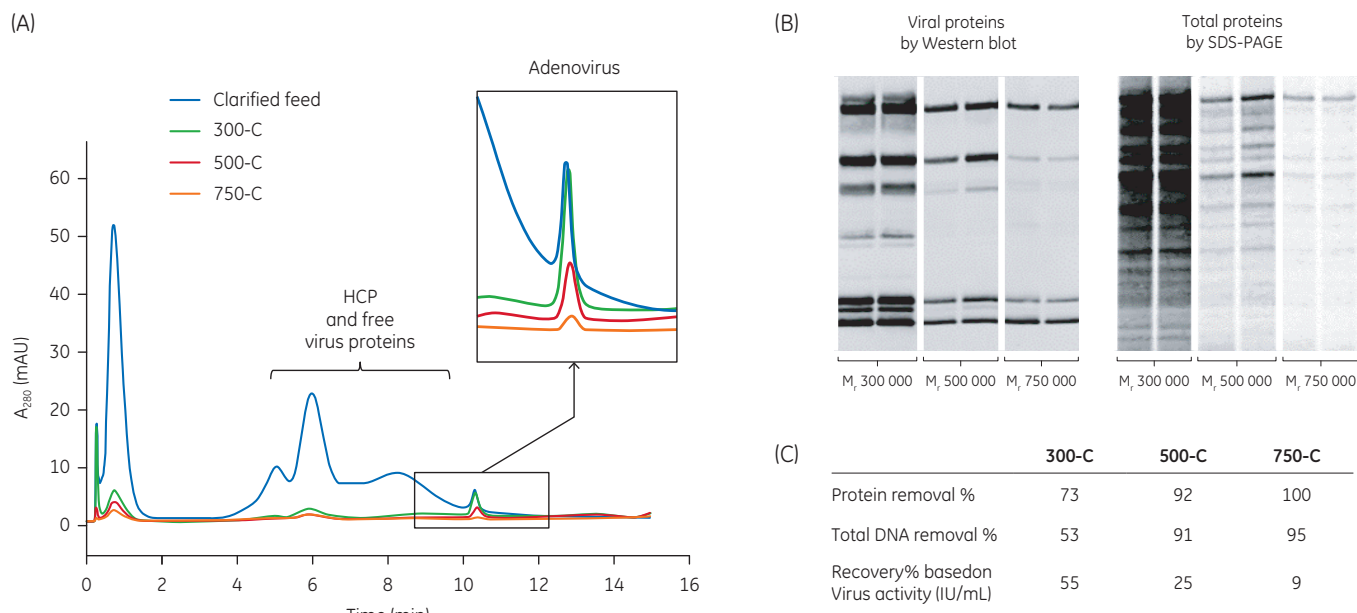
In the hollow fiber filter screening, filters with NMWC of  $M_r$  300 000, 500 000, and 750 000 were evaluated. After a 5 times concentration, protein and DNA reduction reached a plateau, with no further reductions observed after the 5-fold diafiltration (Fig 6). Using the 300-C filter, more intact virus retained in the retentate than for the 500-C and 750-C filters (Fig 7A). Higher level of virus protein was also observed with the 300-C filter (Fig 7B), however, the signal could have been enhanced by the presence of more free virus protein in the sample from the 300-C filter than from the 500-C and 750-C filters.

Analysis of total and infectious virus titers in the retentate showed higher virus titers, but also higher impurity levels with the 300-C and 500-C filters, while reductions in both virus and impurity levels were observed with the 750-C filter (Fig 7C). Using the 300-C filter, virus was not detected in the permeate. For the 500-C and 750-C filters, virus was observed in the permeate, indicating that these pore sizes were too large, allowing for virus to pass through the filter (data not shown). Recovery of infectious virus particles was 55% using the 300-C filter with 1 L feed, which is considered a low load for the 1400 cm<sup>2</sup> filter area. However, recovery can be improved further by filtration of larger volumes and by flushing the system as a last step, thereby reducing the amount of virus that is trapped in the system.

In process verification at larger scale, using the 300-C filter with 6 L clarified feed, the achieved recoveries of total virus titer and infectious virus titer were 92% and 118%, respectively. The removal of protein and total DNA was 80%, whereas full length gDNA was not reduced further. Based on these results, a hollow fiber filter with a NMWC of  $M_r$  300 000 can be recommended for the UF/DF step. Using the described process, impurities were reduced, and the sample was concentrated and conditioned (buffer exchanged) to be ready for the next step in the downstream purification process.



**Fig 6.** Impurity removal by UF/DF using hollow fiber filters 300-C, 500-C, and 750-C. Total protein was analyzed by BCA and total DNA was analyzed by the PicoGreen assay. (A) Protein and (B) DNA removal in the concentration step. (C) Protein and (D) DNA removal in the diafiltration step.



**Fig 7.** (A) Intact virus, (B) hexon protein and total protein levels in retentate, detected using Cy3-labeled antibody and Cy5-prelabeling, respectively, as well as (C) virus recovery and removal of total protein and DNA by UF/DF using hollow fiber filters 300-C, 500-C, and 750-C (mean value of two runs). Total protein was analyzed by BCA and total DNA was analyzed by the PicoGreen assay.

## Conclusion

This work aims to enable suggestion for consumables and conditions for virus release and filtration in midstream steps of an adenovirus production process. The described process allows for preparation of AdV5 from 10 L upstream cell culture for further downstream purification. A screening of detergents for HEK293 host cell lysis showed that treatment with 0.5% Tween 20 for a minimum of 2 h at 37°C under mixing is a good alternative to Triton X-100 that is on the authorization list (Annex XIV) of REACH. An LC-MS method can be used for determination of residual Tween 20. A combination of 2 µm and 0.6 µm GF filters are suggested for removal of cell debris and initial impurity reduction. UF/DF using hollow fiber filter with NMWC of  $M_r$  300 000 provide further reduction of impurities, while preparing the adenovirus-containing sample for subsequent downstream purification steps.

## Ordering information

Product	Size	Product code
ULTA Disc GF: 5.0 µm GF	47 cm <sup>2</sup>	DGF-A-05-470
ULTA Disc GF: 2.0 µm GF	47 cm <sup>2</sup>	DGF-A-02-470
ULTA Disc GF: 1 µm GF	47 cm <sup>2</sup>	DGF-A-01-470
ULTA Disc GF: 0.6 µm	47 cm <sup>2</sup>	DGF-A-96-470
ULTA Disc HC: 0.6 µm/0.2 µm PES	47 cm <sup>2</sup>	DMP-HC92-470
ULTA Capsule GF, 2.0 µm, 10 inch	0.52 m <sup>2</sup>	KGF-A-0210TT
ULTA Capsule GF, 0.6 µm, 6 inch	0.22 m <sup>2</sup>	KGF-A-9606GG
Hollow fiber cartridge UFP-300C-4X2MA	1400 cm <sup>2</sup>	56-4102-16
Hollow fiber cartridge UFP-500-C-4X2MA	1400 cm <sup>2</sup>	56-4102-18
Hollow fiber cartridge UFP-750-C-4X2MA	1400 cm <sup>2</sup>	29-0110-52
ÄKTA flux 6	NA	29038438
ÄKTA pure 25	NA	29018225
IN Cell Analyzer 2200	NA	29027886
Tricorn 5/50	NA	28406409
Q Sepharose XL	300 mL	17507201
Sepharose 4 Fast Flow	1 L	17014901
Amersham WB goat anti-rabbit Cy3	150 µg/vial	29038276
Amersham QuickStain	1 kit	RPN4000

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- Blanche *et al.* An improved anion-exchange HPLC method for the detection and purification of adenoviral particles. *Gene Therapy* **7**, 1055–1062 (2000).
- Shi *et al.* A highly sensitive method for the quantitation of polysorbate 20 and 80 to study the compatibility between polysorbates and m-cresol in the peptide formulation, *J Anal Bioanal Tech* **6**, 3 (2015).



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GE Healthcare Bio-Sciences AB  
Björkgatan 30  
751 84 Uppsala  
Sweden