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An investigative case study of the removal of host cell DNA from HEK detergent lysates using 3M™ Harvest RC Chromatographic Clarifier

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Highlights

- 3M™ Harvest RC Chromatographic Clarifier was used to clarify HEK detergent lysates and provided >90% AAV recovery and >95% turbidity reduction for model lysates containing AAV2, AAV5, and AAV6.
- In this case study, HEK AAV detergent lysate fluids clarified with 3M™ Harvest RC Chromatographic Clarifier contained comparable residual levels of DNA in both the presence and absence of nuclease treatment, as confirmed by qPCR and ddPCR.
- 3M™ Harvest RC Chromatographic Clarifier effectively removed HEK host cell DNA irrespective of DNA fragment sizes in this case study.

Key words

- Gene therapy
- AAV manufacturing
- Residual host cell DNA
- 3M™ Harvest RC Chromatographic Clarifier

Introduction

Adeno-associated viruses (AAV) are widely used vectors for the delivery of gene therapies to treat various human diseases. As of March 2024, 5 AAV-based therapies have been approved by the United States FDA (Food and Drug Administration), and over 200 clinical trials are ongoing.[1] Methods for the production of AAV include transient triple transfection in host cells such as HEK293, baculovirus-based expression systems, and helper virus-free approaches such as the herpes simplex virus (HSV) system.[2] The AAV particles are often intracellular and the AAV purification process typically begins with the release of the viruses from the host cells by detergent lysis; this cell lysis process also frequently releases hcDNA to the fluids. The presence of hcDNA in the final product poses substantial safety concerns for treated patients, given its potential infectivity and oncogenicity.[3] Consequently, the FDA enforces stringent regulations regarding residual hcDNA concentration and fragment sizes.[4]

Current hcDNA removal strategies often rely on nuclease digestion before clarification. Endonucleases such as Benzonase® endonuclease or DENARASE® endonuclease hydrolyze internal phosphodiester bonds in DNA and RNA and cleave them into smaller fragments.[5] However, the use of nuclease has significant limitations. 1) Complete DNA digestion requires high nuclease concentrations (40–100 U/mL), which constitute a substantial portion of the cost of goods in AAV production[6]. 2) Nuclease addition introduces impurities, including the nuclease

itself and contaminants from its production, that complicate downstream purification[7]. 3) Nuclease digestion may generate problematic impurities, such as (i) short DNA fragments that are undetectable by PCR but potentially infectious and oncogenic and (ii) cytotoxic histones released during DNA digestion.[8]

In contrast to enzymatic digestion of hcDNA by nucleases, in this study, the efficacy of 3M™ Harvest RC Chromatographic Clarifier in clarifying and removing hcDNA from model crude HEK cell lysates containing AAV2, AAV5, or AAV6 was investigated. These lysates were either (i) passed directly through 3M™ Harvest RC Chromatographic Clarifier or (ii) incubated with nuclease prior to clarification with 3M™ Harvest RC Chromatographic Clarifier. The quantities of host cell DNA (hcDNA) in the various model fluids were measured using qPCR and ddPCR, and additional DNA analysis techniques were used to assess hcDNA fragment size and oncogene E1A.

Methods and materials

AAV production by triple transfection in HEK cells

AAV2, AAV5, and AAV6 were produced in HEK293 cells (Viral Production Cells 2.0, Gibco™) via transient triple plasmid transfection using pALD-AAV Rep/Cap Plasmids from Aldevron, or AAVpro packaging plasmid sets from Takara with FectoVIR®-AAV from PolyPlus. The suspension host cells were cultured in Viral Production Media (Gibco™) in a 10 L bioreactor (Eppendorf, BioFlo 320) and were transfected when the cell density reached 2E+6 cells/mL. Harvesting was performed 72 hours post-transfection.

Cell culture detergent lysis and nuclease treatment at harvest

At harvest, Tween20 (final concentration: 0.5%) and DENARASE® endonuclease (c-LEcta GmbH) at varying concentrations (0 U/mL, 10 U/mL, or 40 U/mL) were added to the bioreactor during HEK cell lysis. The culture was then incubated in the bioreactor at 37°C with agitation (145 rpm) for 2 hours. After incubation, 5 M NaCl solution was added to adjust lysate conductivity to 25 mS/cm.

Lysate clarification using 3M™ Harvest RC Chromatographic Clarifier

Prior to clarification, 20 mL of salt-adjusted cell lysate were pulled from the bioreactor and centrifuged at 3200 xg for 10 minutes. The resulting supernatant was aliquoted and immediately stored at -20°C; this fluid was used as the lysate baseline for AAV recovery and hcDNA removal assessment.

Before use, the 3M™ Harvest RC Chromatographic Clarifier capsules were flushed with 1X PBS at a flow rate of 100 LMH, and the residual PBS in the capsule head space was emptied. The flushed 3M™ Harvest RC Chromatographic Clarifier capsules were used to clarify the crude AAV lysates. During clarification, a 3M™ Harvest RC Chromatographic Clarifier capsule was connected to the bioreactor harvest line, and the crude lysate was passed through 3M™ Harvest RC Chromatographic Clarifier at a flow rate of 100 LMH using a peri pump. Clarification was terminated once the capsule inlet pressure reached 5 psi. Under the experimental conditions in this case study, the throughputs of nuclease-free lysates (average of 96 L/m^2, ranging from 60–120 L/m^2) were overall lower than the throughputs of nuclease-treated lysates (average of 180 L/m^2, ranging from 130–230 L/m^2).

The resulting clarified fluids were aliquoted and promptly stored at -20°C as clarified samples for AAV recovery and DNA removal analysis. Turbidity measurements were conducted for both the crude lysate and clarified fluid using a turbidity meter.

AAV and hcDNA quantification

AAV2, AAV5, and AAV6 capsid titers were assessed using AAV Xpress ELISA kits (Progen). HEK hcDNA extraction was performed using resDNASEQ™ HEK293 DNA Sample Preparation Kits (Applied Biosystems), followed by quantification via qPCR using resDNASEQ™ Quantitative HEK293 DNA Kits, as per the manufacturer's instructions. HEK hcDNA fragment sizes were characterized using ddPCR with Vericheck ddPCR HEK293 Residual DNA Size Kits and a Bio-Rad ddPCR system. E1A fragments were extracted and quantified using resDNASEQ™ Quantitative E1A DNA Fragment Length Kits.

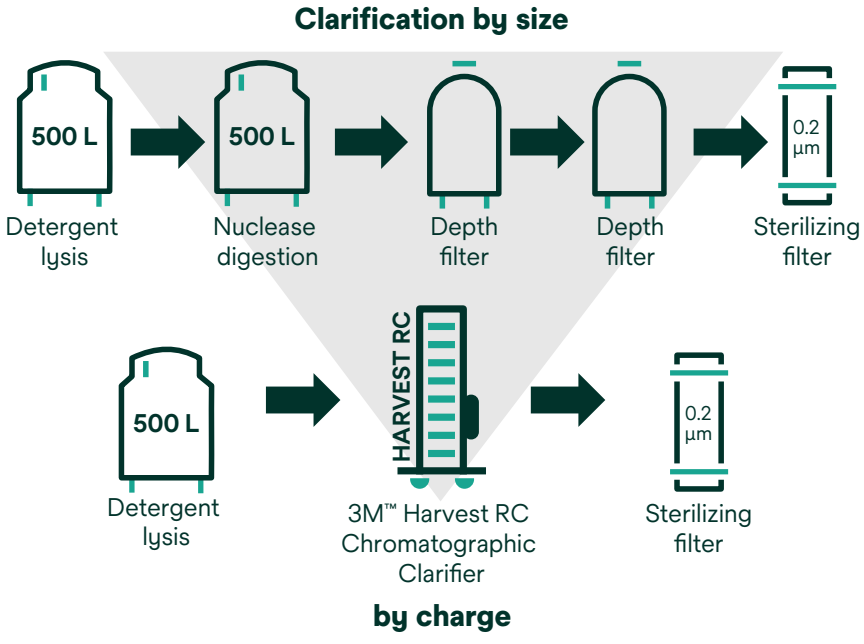
Results and discussion

AAV clarification by 3M Harvest RC chromatographic clarifier in presence or absence of hcDNA-digesting nuclease prior to clarification

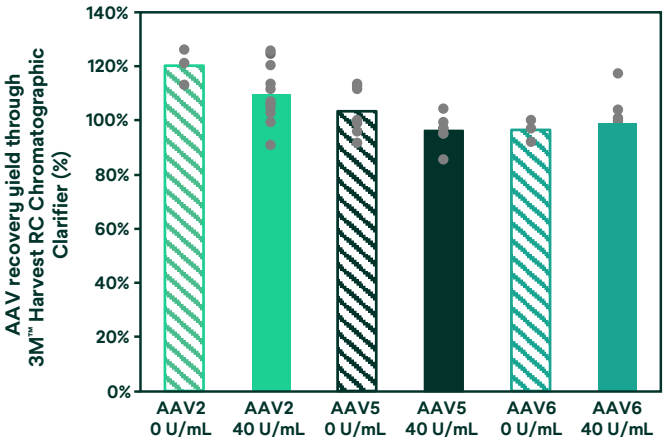
3M™ Harvest RC Chromatographic Clarifier has demonstrated efficacy in increasing product yields and streamlining recombinant protein processes by replacing centrifugation and/or depth filtration steps[9]. However, AAV bioprocessing faces challenges in harvesting AAV particles due to concurrent hcDNA release during cell lysis, often necessitating expensive nucleases for pre-clarification removal. 3M™ Harvest RC Chromatographic Clarifier, employing a charge-based mechanism with synthetic fibrous material containing quaternary amine grafted fibers, potentially removes more DNA than traditional depth filters[10] and may reduce the need for pre-clarification nucleases (see Fig. 1A). This study investigated 3M™ Harvest RC Chromatographic Clarifier's application for AAV harvest and clarification using serotypes AAV2, AAV5, and AAV6. Investigators at Regeneron have reported broadly similar results for serotypes AAV8 and AAV9 using the same 3M™ Harvest RC Chromatographic Clarifier product.[11]

3M™ Harvest RC Chromatographic Clarifier was used to clarify lysates containing the AAV2, AAV5, and AAV6 serotypes. In this study, HEK cell culture either contained no DENARASE® endonuclease (0 U/mL) or were treated with 40 U/mL of DENARASE® endonuclease during detergent cell lysis. The conductivity of each crude lysate was subsequently adjusted to 25 mS/cm with NaCl solution. Each conductivity-adjusted crude lysate was then processed using 3M™ Harvest RC Chromatographic Clarifier. In this study, the presence of nuclease did not have a significant effect on the recovery of AAV following clarification using 3M™ Harvest RC Chromatographic Clarifier; average recovery of AAV was >90% for all clarified lysates, as illustrated in Fig. 1B. The nuclease-treated lysates were characterized by increased turbidity, presumably due to DNA fragments generated by nuclease treatment. Despite the differences in initial turbidity, the clarified fluids were characterized by similarly low turbidities of <10 NTU irrespective of whether nuclease had been added to the lysate (Fig. 1C). Overall, 3M™ Harvest RC Chromatographic Clarifier was effective in AAV recovery and reducing crude lysate turbidity in this case study, irrespective of DENARASE® endonuclease treatment or AAV serotypes. Utilizing 3M™ Harvest RC Chromatographic Clarifier for AAV clarification in the absence of the nuclease hcDNA digestion step could potentially impact various aspects of AAV manufacturing such as cost of goods sold, AAV quality, and residual hcDNA removal. Subsequent sections of this study will investigate its impact on DNA removal; an additional case study (A case study for understanding impact of nuclease elimination on bioprocess cost and downstream AAV5 quality using 3M™ Harvest RC Chromatographic Clarifier Application Note, Solventum, 2024) and a publication from Regeneron[11] discuss the impacts on bioprocess design and the potential to reduce the cost-of-goods for the harvest step in AAV manufacturing when utilizing 3M™ Harvest RC Chromatographic Clarifier.

1A



1B



1C

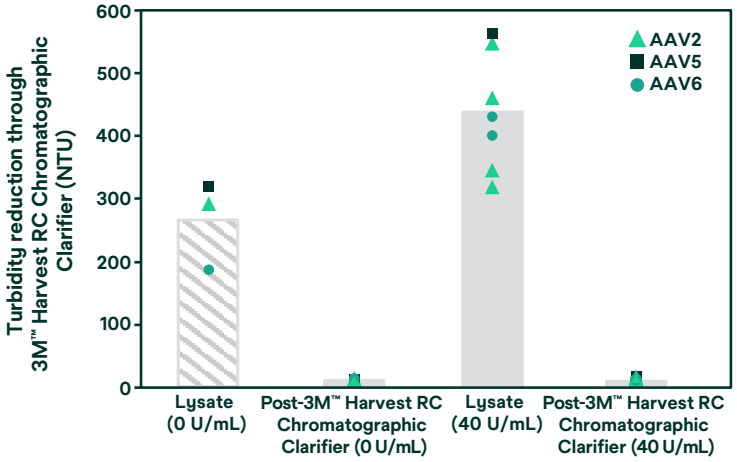


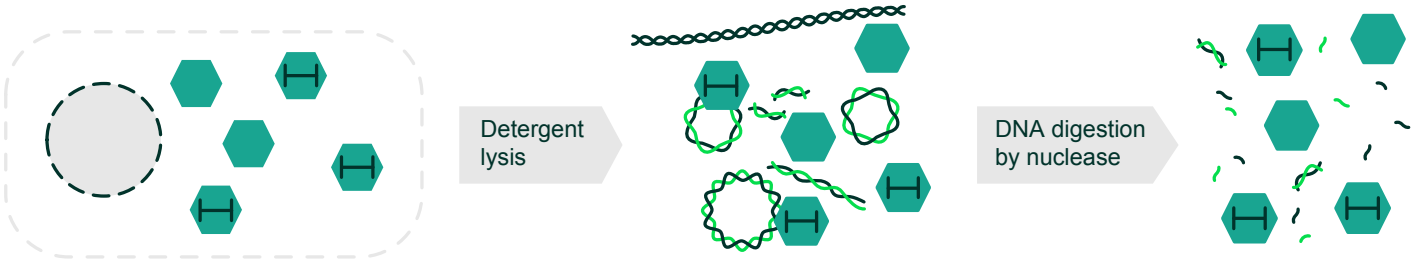
Figure 1. AAV clarification by 3M™ Harvest RC Chromatographic Clarifier in presence or absence of hcDNA-digesting nuclease. (A) A single-stage and nuclease-free AAV clarification approach using 3M™ Harvest RC Chromatographic Clarifier. (B) AAV2, AAV5, and AAV6 recoveries using 3M™ Harvest RC Chromatographic Clarifier to clarify nuclease-free (0 U/mL) or nuclease treated (40 U/mL) crude lysate. (C) Turbidity reduction of AAV crude lysate through 3M™ Harvest RC Chromatographic Clarifier. Note: Bars indicate mean values; dots indicate individual clarification runs using 3M™ Harvest RC Chromatographic Clarifier.

Evaluation of the removal of HEK hcDNA from AAV lysates with and without nuclease using 3M™ Harvest RC Chromatographic Clarifier

3M™ Harvest RC Chromatographic Clarifier comprises synthetic fibers that are functionalized with quaternary amine moieties, and the 3M™ Harvest RC Chromatographic Clarifier device has previously been shown to remove DNA from clarified fluids containing recombinant proteins.[10] This study interrogated the characteristics of the DNA in each of the clarified fluids to probe 3M™ Harvest RC Chromatographic Clarifier's ability to remove DNA from AAV-containing fluids. The charged fibers are characterized by positive charge under the neutral pH conditions in cell lysates and are thus able to bind the negatively charged hcDNA via electrostatic interactions (Fig. 2B). 3M™ Harvest RC Chromatographic Clarifier, a charge based fibrous AEX chromatography device, comprises synthetic fibrous material with quaternary amine grafted fibers. A micrograph of these fibers is provided in Fig. 2C; the underlying functional fibrous layers can capture small-sized impurities, including hcDNA, which may be too diminutive for traditional size-based depth filters.

In order to quantify HEK hcDNA removal by 3M™ Harvest RC Chromatographic Clarifier, crude lysates treated with 0 U/mL or 40 U/mL DENARASE® endonuclease were processed by 3M™ Harvest RC Chromatographic Clarifier, and hcDNA levels in the lysates and clarified fluids were quantified via qPCR. In this case study, the concentrations of hcDNA in the clarified fluids were comparable (Fig. 2D), regardless of whether nuclease had been added prior to passing the fluid through the 3M™ Harvest RC Chromatographic Clarifier device.

2A



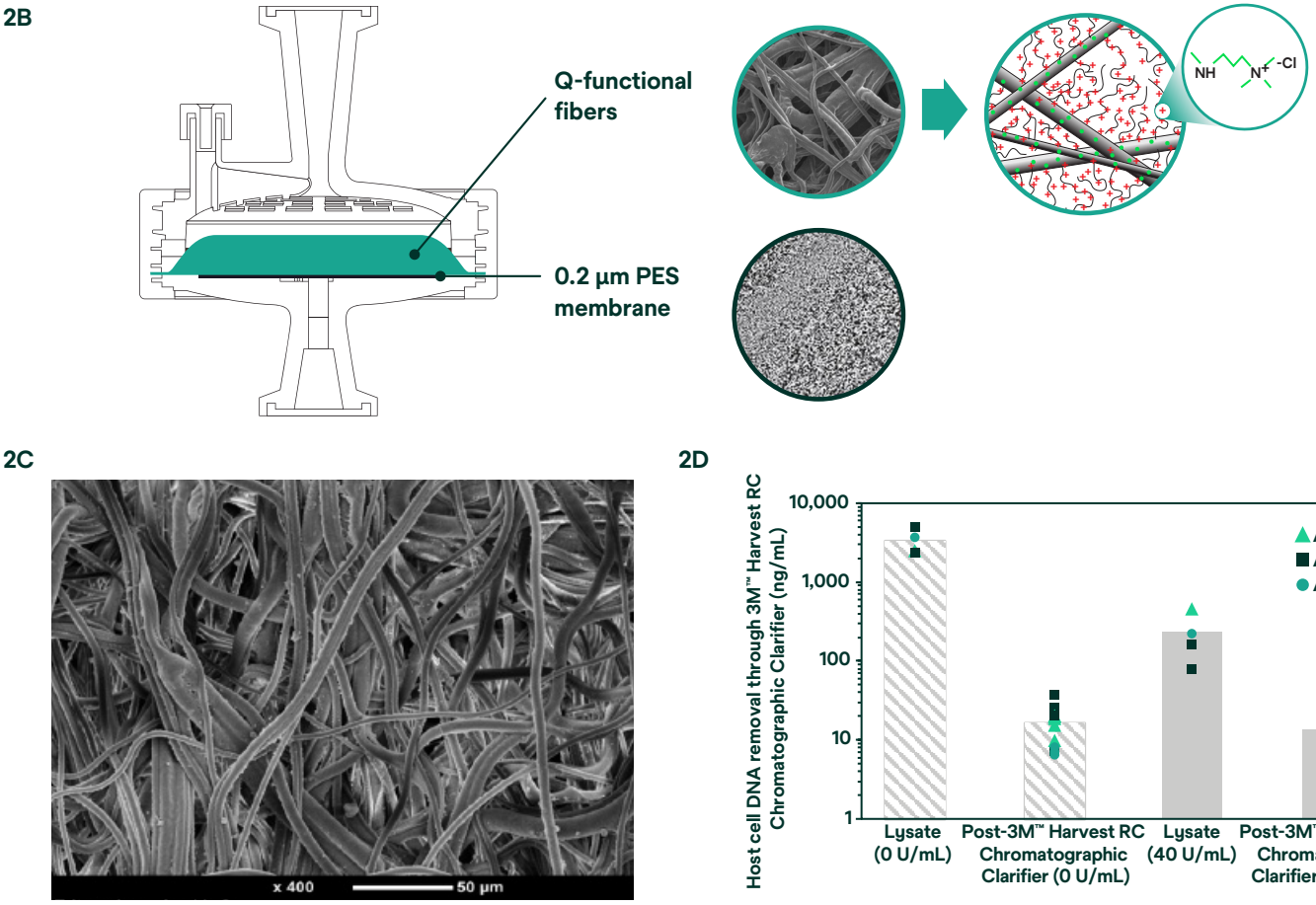


Figure 2. HEK hcDNA was removed by a synthetic fibrous material containing quaternary amine grafted fibers. (A) HEK hcDNA release and fragmentation by nuclease in a crude AAV lysate. (B) 3M™ Harvest RC Chromatographic Clarifier devices comprise multiple Q-functional fibrous layers on the top and a PES 0.2 µm sterile membrane on the bottom. Under neutral pH, the Q-functional group is positively-charged. (C) Negatively-charged impurities (including hcDNA) are captured by the positive-charged fibers. (D) HEK hcDNA removal by 3M™ Harvest RC Chromatographic Clarifier from lysates with 0 U/mL DENARASE® endonuclease or 40 U/mL DENARASE® endonuclease.

Study of the impact of 3M™ Harvest RC Chromatographic Clarifier AAV clarification on HEK hcDNA fragment sizes

In addition to the overall concentration, an important characteristic of hcDNA is the distribution of fragment sizes that comprise the hcDNA population. Due to the complexity of AAV and limitations in analytic methods, gene therapy manufacturers are currently permitted to establish their own hcDNA specification limits for final products based on risk assessments to ensure patient safety.[4] This approach contrasts with the one used in mAb manufacturing, where quantifiable limits are set for hcDNA concentrations, typically at a level of 10 ng/dose with DNA fragments smaller than 200 base pairs (bp).[12] Studies have shown that longer hcDNA fragment sizes can increase oncogenicity/infectivity risks.[13] In this study, we utilized an advanced ddPCR method to characterize hcDNA size profiles in both the crude lysates and clarified fluids. The data for the fluids containing AAV5 and either 0 U/mL, 10 U/mL, or 40 U/mL of nuclease are presented in the left side of Fig. 3A. In these experiments, the addition of 10 U/mL of nuclease led to a reduction in the number of long DNA fragments but an increase in the amount of short DNA fragments that need to be removed in downstream steps. This change in size distribution is the result of cleavage of the longer DNA fragments into smaller sized fragments.[14] When the DENARASE® endonuclease concentration is increased to 40 U/mL, the short DNA fragments are presumably further digested to sizes undetectable by ddPCR. These three crude lysates with differing hcDNA levels were clarified using 3M™ Harvest RC Chromatographic Clarifier. In all three examples, the quantities of both long and short DNA

fragments were reduced by at least 40x following clarification with 3M™ Harvest RC Chromatographic Clarifier. Furthermore, the clarified fluids are characterized by similar hcDNA levels and fragment size profiles across the three different DENARASE® endonuclease concentrations used in this study.

The same experiments and analyses were conducted for serotypes AAV2 and AAV6. The results of these three experiments are presented in Fig. 3B and are consistent with 40 U/mL of DENARASE® endonuclease effectively cleaving most long DNA into short fragments. In these experiments, the quantities of long chain DNA segments were comparable following either the addition of 40 U/mL of nuclease or clarification with 3M™ Harvest RC Chromatographic Clarifier. It is noteworthy that clarified fluids of AAV2 contained more of the long hcDNA fragments than clarified fluids of AAV5 or AAV6, (green triangles on the second bar in Fig. 3B). This observation aligns with the known tendency of AAV2 to be hard-to-handle and prone to aggregation.[15] We hypothesize that the long DNA fragments were carried through the 3M™ Harvest RC Chromatographic Clarifier device by AAV2-DNA complexes or aggregates[16] and consequently escaped capture by the charged fibers. In contrast, for short DNA fragments as shown in Fig. 3C, 3M™ Harvest RC Chromatographic Clarifier exhibited consistent DNA removal performance on both nuclease-free and nuclease-treated lysates across all three serotypes. Furthermore, in these model experiments, the quantities of short DNA fragments were approximately 10-fold lower in the fluids clarified using 3M™ Harvest RC Chromatographic Clarifier than they were in the fluids that were only treated with 40 U/mL of nuclease, as shown in Fig. 3C.

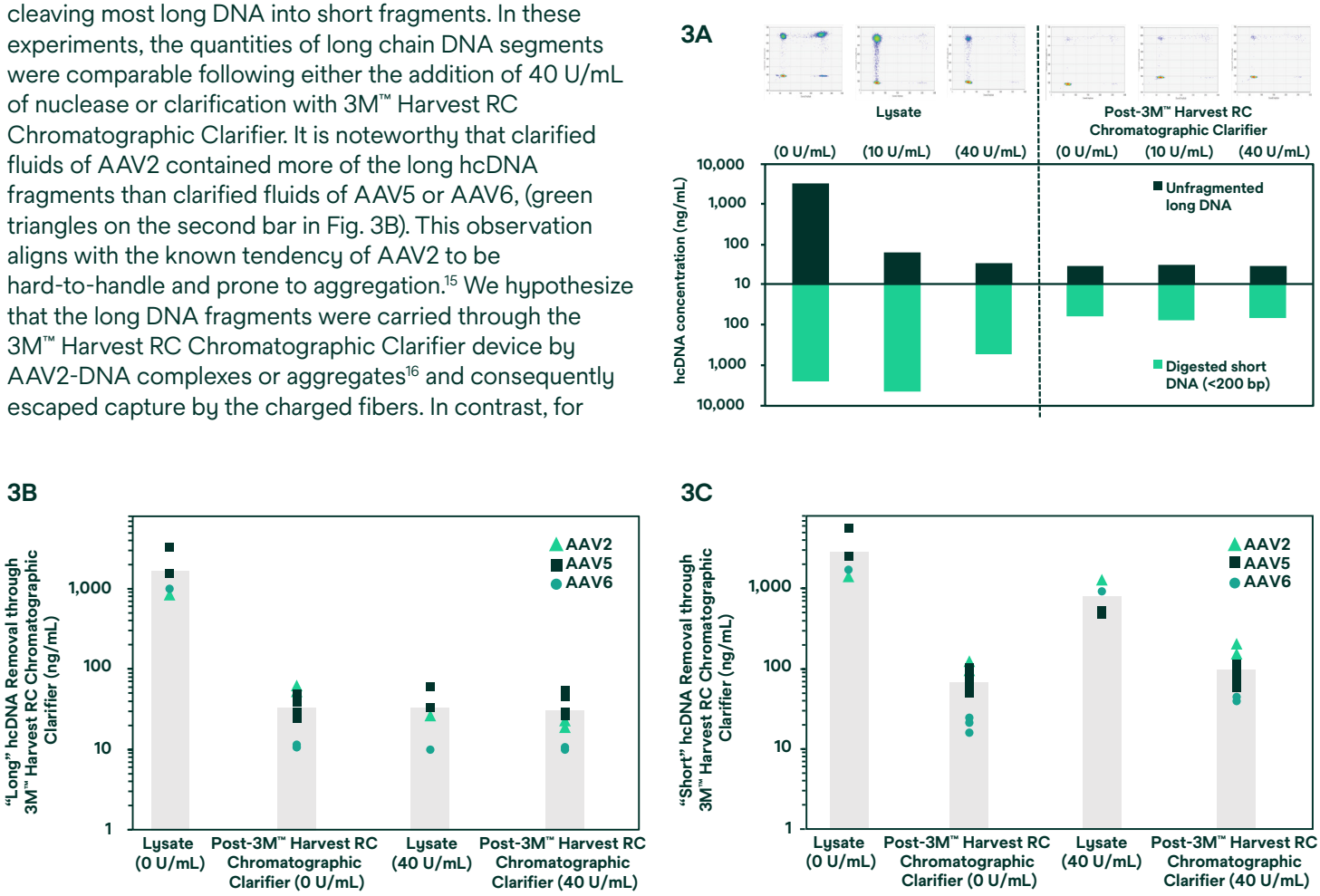


Figure 3. Analysis of HEK hcDNA fragment sizes in crude lysates and fluids clarified using 3M™ Harvest RC Chromatographic Clarifier (3A) Analysis of HEK hcDNA fragment sizes in AAV5 crude lysates (following incubation with 0 U/mL, 10 U/mL, or 40 U/mL DENARASE® endonuclease) and 3M™ Harvest RC Chromatographic Clarifier — clarified fluids. Top: 2D graphs from ddPCR analysis using Vericheck ddPCR HEK293 Residual DNA Size kits. x-axis: FAM channel, y-axis: HEX channel. (FAM+, HEX+) droplets represent unfragmented long DNA; (FAM-, HEX+) droplets represent short DNA fragments (<200 bp); (FAM+, HEX-) droplets represent DNA fragments between 200 bp–2000 bp; (FAM-, HEX-) droplets indicate negative droplets without detectable DNA. Bottom: Concentrations of unfragmented "long" DNA (dark green) and digested "short" DNA (light green), estimated based on FAM and HEX channel concentrations respectively (Bio-Rad bulletin 3305). (3B) Removal of "long" HEK hcDNA fragments by 3M™ Harvest RC Chromatographic Clarifier from crude lysates treated with 0 U/mL or 40 U/mL DENARASE® endonuclease. (3C) Removal of "short" HEK hcDNA fragments by 3M™ Harvest RC Chromatographic Clarifier from crude lysates treated with 0 U/mL or 40 U/mL DENARASE® endonuclease.

Evaluation of the removal of E1A oncogene from HEK detergent lysates clarified using 3M™ Harvest RC Chromatographic Clarifier

Typically, AAV production cell lines like HEK293 are engineered to express E1A and E1B genes to facilitate recombinant virus replication within host cells. However, their propensity to induce extensive cellular DNA replication renders them oncogenic and studies have demonstrated that they may pose significant safety risks to patients.[17] Consequently, concentrations and sizes of E1A are monitored closely during gene therapy biopharmaceutical manufacturing. In this study, we assessed the quantities of the E1A gene from the following AAV5-containing fluids: nuclease-free crude lysate, lysate treated with 40 U/mL of nuclease, and versions of the aforementioned fluids that were subsequently clarified using 3M™ Harvest RC Chromatographic Clarifier. The data from these experiments are presented in Fig. 4. The addition of 40 U/mL of nuclease to this model crude lysate effectively removed >95% of E1A through DNA digestion. The quantities of E1A in the fluids clarified with 3M™ Harvest RC Chromatographic Clarifier were even lower than the quantity in the fluid treated with only nuclease, as illustrated in Fig. 4B.

In this case study, the data from HEK hcDNA quantification (Fig. 2), HEK hcDNA size analysis (Fig. 3), and E1A concentration/size analysis (Fig. 4) collectively demonstrate that in the presence or absence of DENARASE® endonuclease hcDNA digestion prior to clarification with 3M™ Harvest RC Chromatographic Clarifier, the clarified fluids exhibit comparable hcDNA levels. Eliminating nucleases from the bioprocess could potentially reduce clarification costs, simplify upstream processing, and avoid challenges associated with nuclease and related impurity removal during downstream purification and polishing steps.

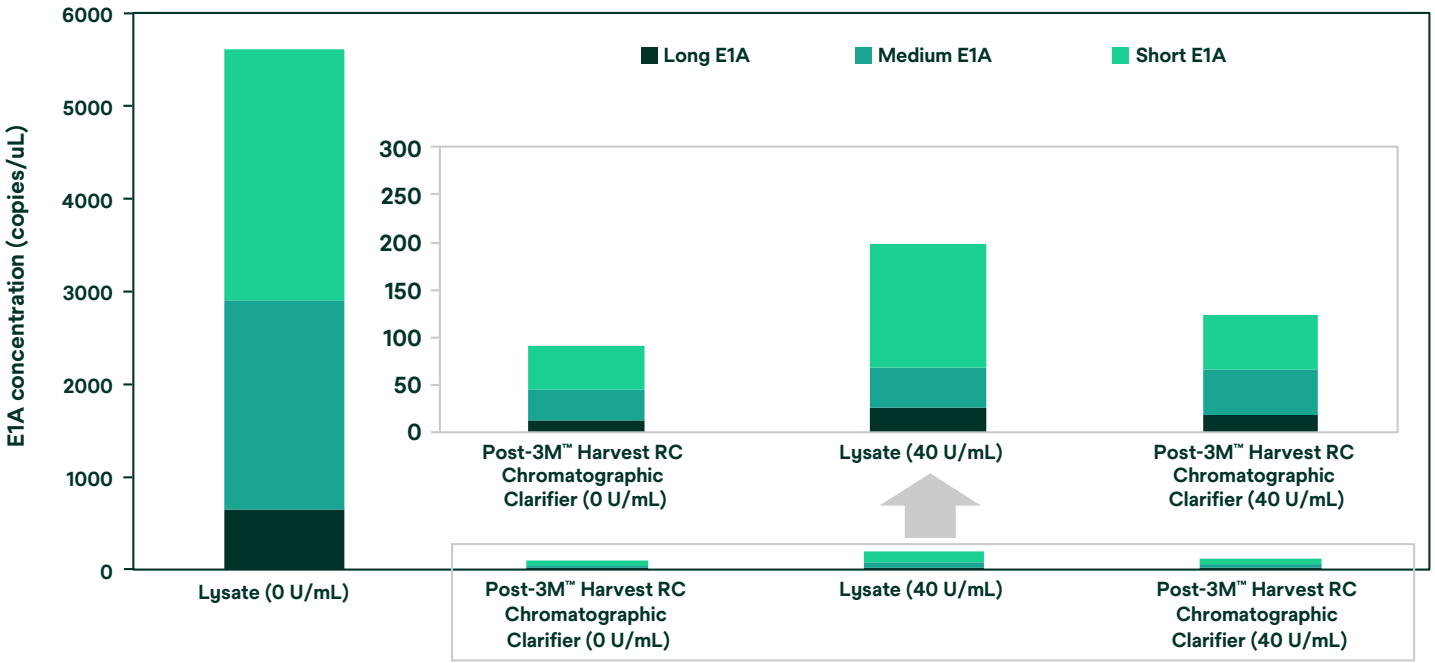


Figure 4. Analysis of E1A fragment size and concentration in crude lysates and in fluids clarified using 3M™ Harvest RC Chromatographic Clarifier. (A) Concentrations of long, medium, and short E1A fragments in crude lysates (treated with 0 U/mL or 40 U/mL DENARASE® endonuclease) and in fluids clarified using 3M™ Harvest RC Chromatographic Clarifier. (B) Zoomed-in view of the low DNA concentrations in DENARASE® endonuclease-treated lysate (40 U/mL) and in fluids clarified using 3M™ Harvest RC Chromatographic Clarifier (treated with 0 U/mL or 40 U/mL of DENARASE® endonuclease). Data collected via qPCR and analyzed using the resDNASEQ™ Quantitative E1A DNA Fragment Length Kits.

Study of the HEK hcDNA removal by different sizes of 3M™ Harvest RC Chromatographic Clarifier for AAV clarification

The 3M™ Harvest RC Chromatographic Clarifier product family comprises laboratory products (CT15 conical tube, WP6 well plate, BT500 bottle top, BC4, BC25), pilot capsules (BC340, and BC1020), and production capsules (BC2300 and BC16000). Ensuring process consistency from laboratory-scale to manufacturing is crucial for bioprocess development, with speed and efficiency being vital for high-throughput screening at the research scale. In this study we evaluated the efficacy of the CT15 conical tube, WP6 well plate, BT500 bottle top, BC4, BC25, and BC340 3M™ Harvest RC Chromatographic Clarifier devices in clarifying AAV5-containing lysate. No nuclease was added to the fluids that were clarified by the various devices. The recovery of AAV5 was >85% and the removal of hcDNA was >94% in all of these experiments, regardless of the size of the 3M™ Harvest RC Chromatographic Clarifier device used to clarify the fluid. The fraction of DNA removed was higher when the capsule devices were used (>99%) than when the BT500 (>98%), WP6 (>94%), or CT15 (>94%) devices were used. Scalable performance was demonstrated for capsules BC4, BC25 and BC340 with serotypes AAV2 and AAV6 (A case study for understanding impact of nuclease elimination on bioprocess cost and downstream AAV5 quality using 3M™ Harvest RC Chromatographic Clarifier Application Note, Solventum, 2024)

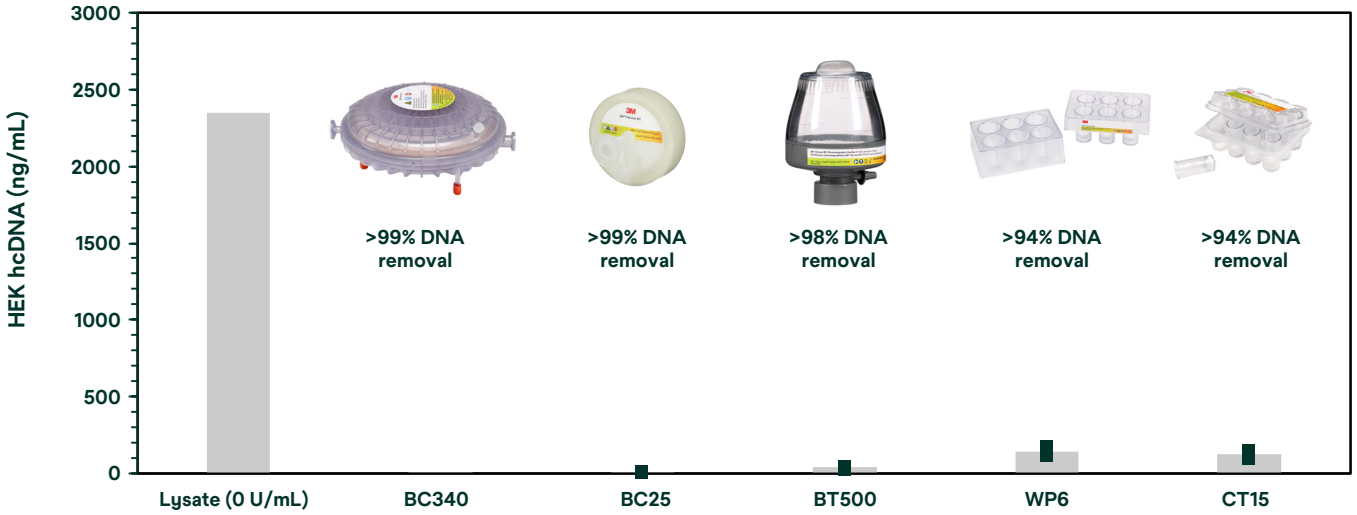


Figure 5. Removal of HEK hcDNA by different sizes of 3M™ Harvest RC Chromatographic Clarifier products from crude AAV5 lysate; no DENARASE® endonuclease was added to digest DNA in these samples. The 3M™ Harvest RC Chromatographic Clarifier devices include BC25 and BC340 capsules (with surface areas of 25 cm^2, EMP301HRC2FA, and 340 cm^2, EMP513HRC2FA), BT500 (500 mL bottle top, EMP051HRC2FA), WP6 (15 mL 6-well plate, EMP006HRC2FA), and CT15 (15 mL conical tube, EMP015HRC2FA). Data were collected using qPCR and analyzed following the instructions of the resDNASEQ™ Quantitative HEK293 DNA Kits.

Summary

In this case study, both crude and endonuclease-treated AAV-containing lysates were clarified using 3M™ Harvest RC Chromatographic Clarifier. The clarified fluids contained comparable residual DNA levels (with or without endonuclease addition), as evaluated by multiple analytical methods including HEK hcDNA quantification (qPCR), HEK hcDNA size characterization (ddPCR), and E1A size characterization (qPCR). Additionally, in this case study 3M™ Harvest RC Chromatographic Clarifier, via Q-functional nonwoven media, reduced HEK hcDNA regardless of DNA fragment sizes. The data in this case study suggest that 3M™ Harvest RC Chromatographic Clarifier could provide a scalable, single-stage, and nuclease-free AAV clarification approach from laboratory research to manufacturing. This strategy may enable reduced costs,[11] streamlined clarification procedures, and accelerated AAV bioprocess development.

Abbreviations

AAV: adeno-associated virus
HEK293: human embryonic kidney 293 cell line
hcDNA: host cell DNA
ELISA: enzyme-linked immunosorbent assay
qPCR: quantitative polymerase chain reaction
ddPCR: droplet digital polymerase chain reaction
NTU: nephelometric turbidity units

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