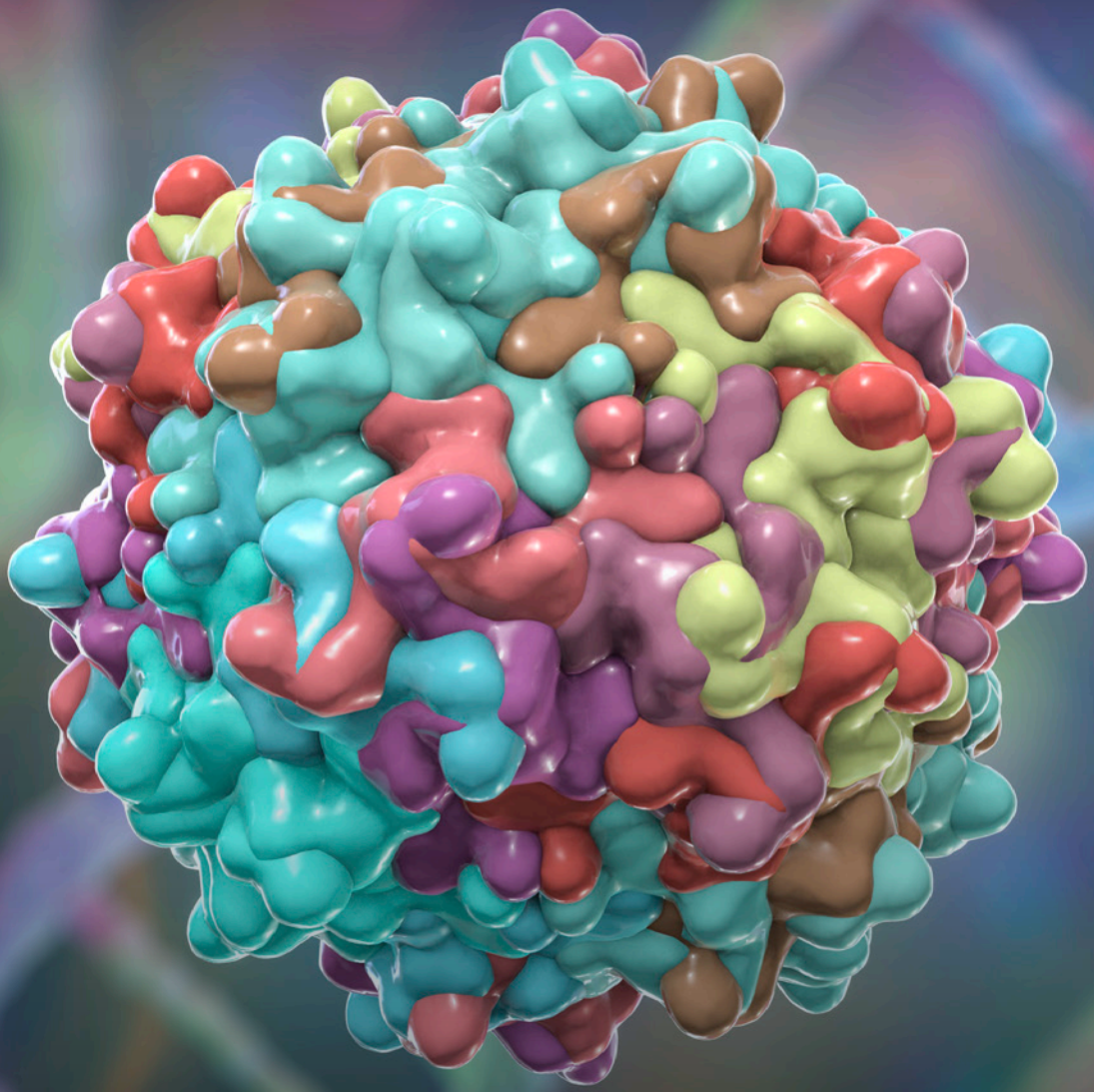
Technology Networks



AAV Purification and Scale Up

AAV Vector Manufacturing Workflow for Gene Therapy Products

Viral Safety in AAV Production: How Affinity Chromatography Effectively Contributes

Platform Optimization for Efficient AAV Purification: Insights from a CDMO

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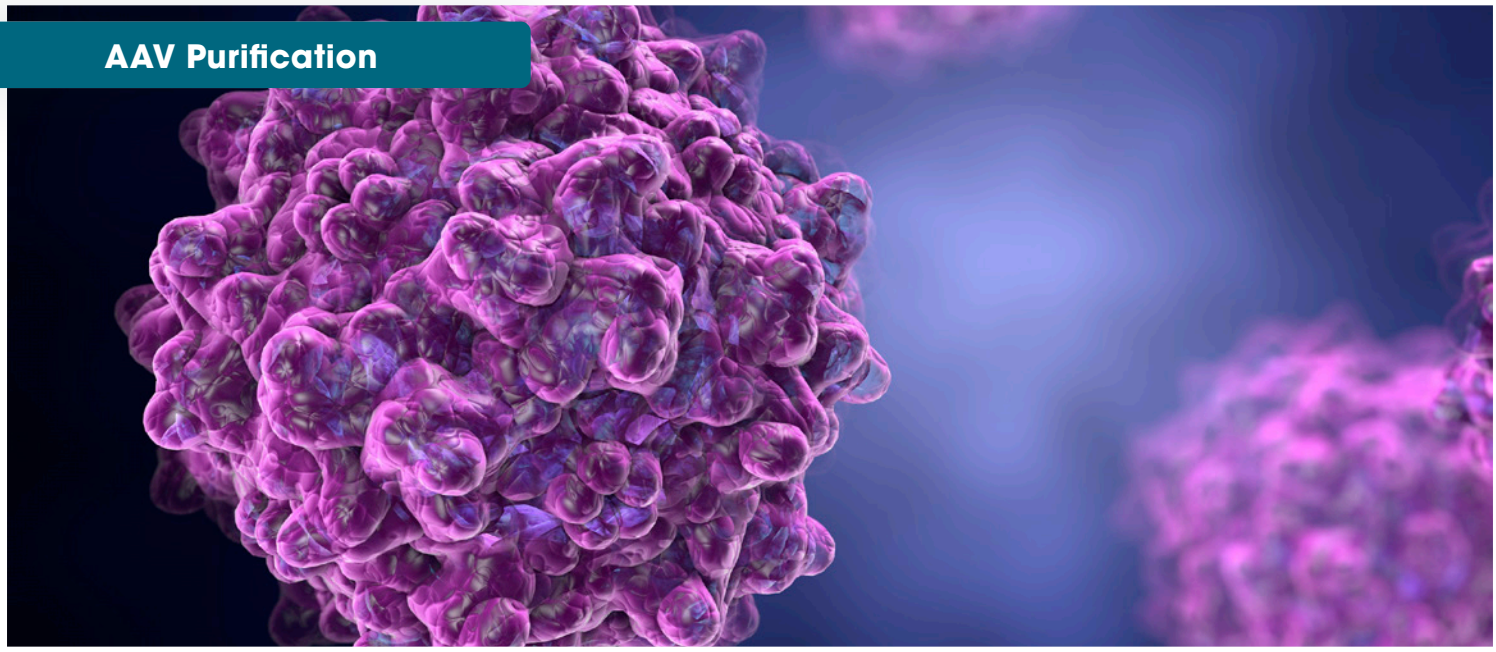
Contents

Foreword

Gene therapy is a rapidly evolving field that holds great promise for the treatment of a wide range of genetic conditions including cancer, inflammatory diseases, and neuromuscular disorders.

At the frontline of gene therapy delivery systems are adeno-associated viruses (AAVs), as they are considered one of the most effective and safe ways to deliver therapeutic genes to target tissues. Thus, as the demand of AAV-based gene therapy products steadily increases, so does the need for scalable, cost-effective manufacturing processes that meet clinical standards.

This eBook summarizes AAVs manufacturing workflow and presents innovative purification tools that help to ensure the optimization and standardization required to obtain high yield at lower costs.



AAV Production for Gene Therapy: An Introduction

Introduction

Gene therapy refers to the modification of genes or gene transcripts for the treatment of disease.[1] The aim is to replace or correct a defective gene with a functional copy. This is achieved through methods such as viral vector delivery, DNA editing, and/or gene transfer via nanoparticles or liposomes.[2,3,4,5] Therapeutic genes are designed and delivered for expression in target cells, leading to the production of functional proteins and correction of the genetic defect. Gene therapy can be administered directly to patients or transduced into cells ex-vivo.1 Applications include the treatment of cancer as well as blood, neuromuscular, and cellular storage disorders.[6,7,8,9]

What are AAVs?

Viral vectors are an effective way to deliver genes to living tissues, with adeno-associated viruses (AAVs) considered among the safest and most efficient vectors available.[2] AAVs are small, non-pathogenic viruses with a single-stranded DNA genome consisting of three functional genes (*Rep*, *Cap*, and *Aap*) surrounded by two inverted terminal repeats. These genes play a role in viral lifecycles, capsid formation, and scaffold assembly. However, AAVs are replication-defective and require the presence of a helper virus – usually an adenovirus – to replicate.

During AAV production, wild-type AAV genomes are replaced by recombinant plasmids containing a promoter, therapeutic gene of interest, and terminator. Hence, AAVs used in gene therapy are referred to as recombinant AAVs (rAAVs). With the demand for gene therapy increasing, efficient and high-yield rAAV production is crucial if researchers are to meet clinical standards.[10,11] Thus, rAAV production methods that focus on achieving high purity, yield and throughput can support scientists and healthcare professionals in the successful delivery of gene therapy.[12, 13]

How are recombinant AAVs produced?

AAVs are produced using multi-step workflows which can be broadly characterized into three stages: upstream, downstream and final fill and finish.[14,15] Upstream processing refers to the production of the rAAV vector itself; it includes the characterization of raw materials, plasmid development, cell culture expansion, transfection, and clarification. Downstream processing refers to the purification and preparation of products, including final formulation. It also includes viral clearance, concentration and filtration to obtain a high-quality, pure and consistent product(Figure 1).

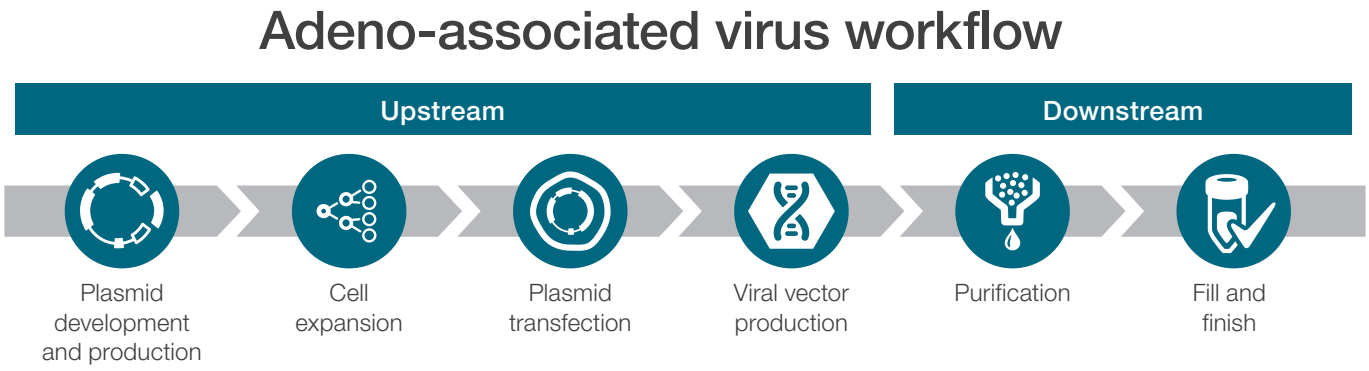


Figure 1. *AAV production workflow*

During the first steps of upstream production large amounts of plasmid DNA are produced. Plasmids containing the desired genetic sequence are then transfected into host cells for replication (Figure 2).[16] One of the most common techniques in AAV-based gene therapy production is the triple transfection technique. In this method, three plasmids are transfected into host cells for replication. The first plasmid contains the therapeutic gene with AAV inverted terminal repeats, the second has AAV *Rep* and *Cap* genes, and the third is a helper plasmid from an adenovirus that allows the recombinant AAV to replicate. Successfully transfected mammalian cells then transcribe and translate the supplied genetic material, giving rise to new, engineered rAAVs. Additional, specific rAAV properties can also be engineered to ensure desired properties such as tissue tropism.[17]

HEK293 is a common mammalian cell line used for plasmid production; while they are typically grown as an adherent monolayer, they can be adapted for suspension culture.[18] Suspension methods are increasingly preferred as they offer greater scalability. Likewise, transfection methods can also be optimized to suit the needs of the manufacturer. Transient transfection offers flexibility and speed in early product development, while stably transfected cell lines offer greater scalability and efficiency for large-scale production.[19] Intact rAAV vectors containing the therapeutic gene are then purified from lysed cell cultures. rAAVs are typically purified through column chromatography.[20] Affinity chromatography has become the preferred option for AAV vector capture due to its high efficiency and selectivity for multiple AAV serotypes.[21,22,23,24] The polishing step is usually performed using ion exchange chromatography.[25]

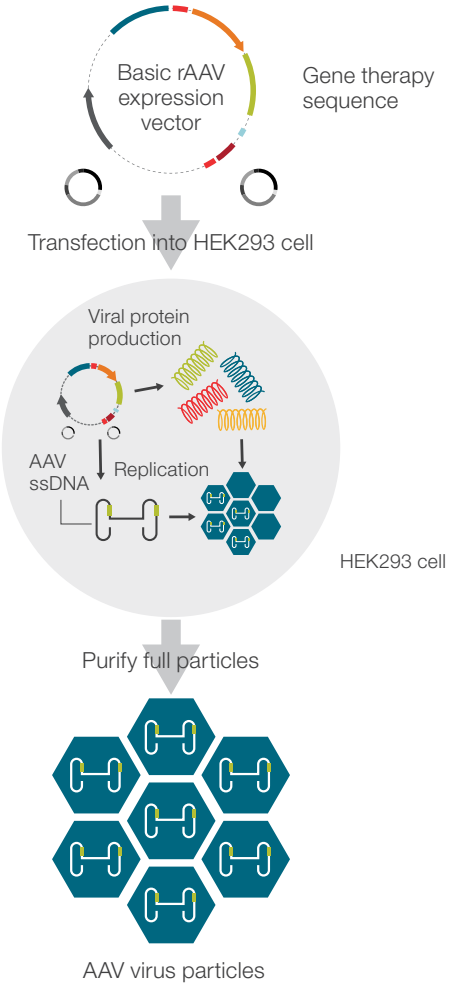


Figure 2. *AAV vector triple transfection methods in gene therapy production*

During affinity chromatography, rAAVs are separated from process-related impurities such as cell debris, protein fragments and DNA. This is done using an affinity resin containing ligands that bind to specific proteins on the rAAV capsid.[22] Hence, rAAVs are retained within the column while impurities are washed away. Bound rAAV particles are then released from the column using a specific elution buffer. In recent years, the development of affinity resins binding all AAV's serotypes has positioned affinity chromatography as the ideal purification tool.[26] Hence, it is now possible to optimize a single manufacturing workflow to produce multiple rAAV products using a single resin.

Further purification steps involve the separation of full rAAVs from empty or partially filled capsids, which are by-products of the AAV manufacturing process. During this process, ion exchange (IEX) chromatography is commonly used because it can exploit the minor differences in charge between full and partially filled or empty AAV particles.[27,28,29] However, high-resolution chromatography methods and optimization are needed to obtain a successful separation.[30] Elution is performed using a salt or pH gradient which will elute the full and empty particles in two separate peaks.

Current challenges in rAAV downstream processing

As the demand for gene therapy increases, the efficient and high-yielding production of rAAVs becomes ever more important to meet clinical demands.[31] Contaminant carry over from cell cultures can significantly impede downstream production of AAV-based gene therapy products leading to costly delays in product development and manufacture. Mycoplasma – a group of bacteria that commonly infects *in vitro* cell cultures - has been shown to negatively impact transfection efficiency.[32] Additionally, due to its potential pathogenicity in humans, the US Food and Drug Administration (FDA) requires that all gene therapy products must be free of mycoplasma contamination.[33] To support efficient and uninterrupted rAAV production, regular, rapid and accurate testing must be carried out to ensure that cell cultures remain mycoplasma free.

After their harvest during upstream processing, rAAV capsids represent less than 0.1% of the total proteins generated by the cells. The remaining 99.9% are impurities, such as host cell proteins, DNA/RNA, plasmid DNA, cell culture medium components and supplements, that reduce the efficacy and safety of the gene therapy product.[26,34] In order to avoid delays in production and regulatory approval, effective methods, such as affinity chromatography, must be used to remove these product impurities.[26] Another important challenge is to ensure the product is free of endogenous or adventitious viruses. An effective viral clearance program has three pillars: prevention, detection and removal. Prevention can be achieved by adopting sterile, single-use technology to minimize the risk of adventitious contamination.[35] Detection tests during different stages of the manufacturing process (cell banks, harvest, and final product) are also critical but have limitations. Thus, it is essential that the purification process remove residual viruses. This can be effectively done implementing a two-step approach that uses affinity capture chromatography first followed by anion exchange polishing.[26] Efficient separation of full and empty particles without hampering the final yield is yet another critical step in the purification process.[36] The ratio of empty to full capsids after the affinity capture and polishing steps can be determined by high-performance liquid chromatography (HPLC). These results provide a baseline for further downstream purification.

While rigorous purification processes are important in the development of safe and effective gene therapy products, so is the optimization of high-yielding workflows. During purification, a proportion of the rAAV vector may be lost or degraded, leading to a reduction in the overall yield and workflow efficiency.[37] Additionally, each purification process may require multiple steps to achieve the required quality standards. Not only does this further the potential loss of product, but it also contributes to the high costs of gene therapy applications and limits their throughput production.

The future of AAV-based gene therapy

AAV-based gene therapy is a rapidly evolving field that holds great promise for the treatment of a wide range of genetic diseases.[6,7,8,9] As the applications of viral vector-based therapeutics continue to increase, rAAV production workflows require greater flexibility and scalability than ever before. The requirement for higher yield, lower costs workflows and standardization across the industry has driven the development of platform manufacturing tools that can be optimized and repurposed. Hence, not only does reducing the number of required purification steps help to optimize efficiency and return on investment, it also supports scientists in meeting clinical demands.[21] Not only will improved purification tools enable the development of safer, more effective, scalable gene therapy solutions, but they may also support an accelerated route to regulatory compliance and approval. Overall, efficient and effective rAAV purification is crucial for the future of AAV-based gene therapy.

Learn more about the latest AAV purification solutions

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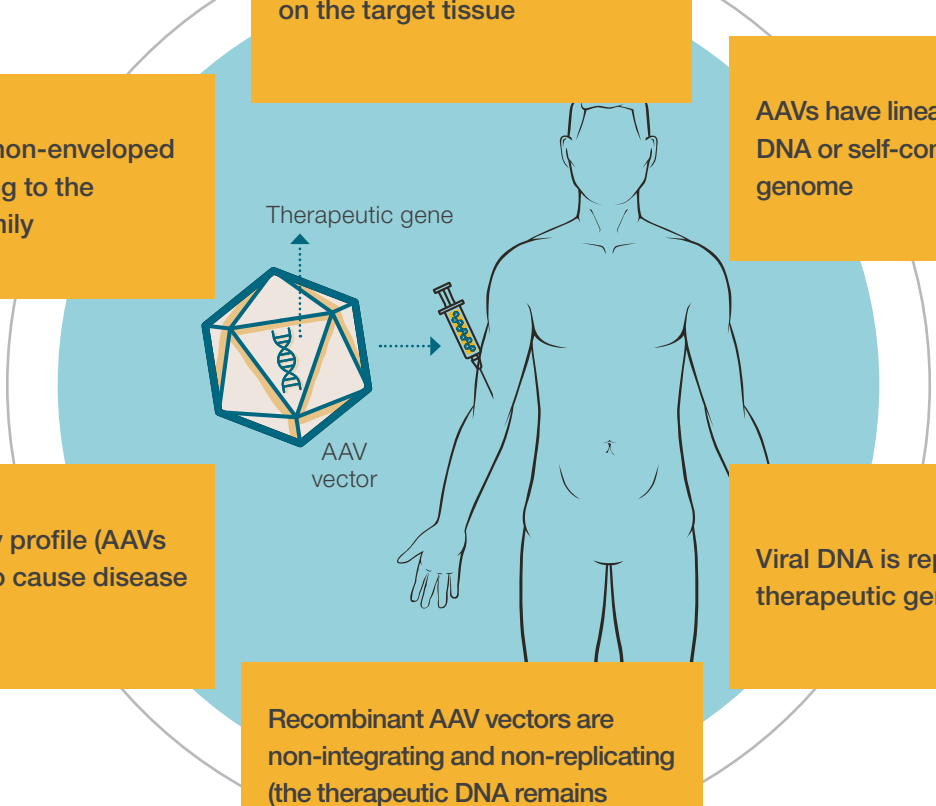
AAV Vector Manufacturing Workflow for Gene Therapy Products

Adeno-associated viruses (AAV) are the leading gene delivery platform for the treatment of many diseases. As the field of gene therapy expands, so does the need for scalable, cost-effective viral vector manufacturing processes that meet clinical standards.[1,2] This infographic outlines AAV vector manufacturing processes and analytics for gene therapy applications.

How does AAV-based gene therapy work?

In AAV-based gene therapy, AAVs are transformed into a delivery platform for the therapeutic gene. This is achieved by replacing the viral DNA with the therapeutic gene. The AAV vector is then used to deliver the therapeutic gene to the right tissues or organs in the body.



Different serotypes depending on the target tissue

AAVs are small non-enveloped viruses belonging to the *Parvoviridae* family

AAVs have linear single-stranded DNA or self-complementary genome

Favorable safety profile (AAVs are not known to cause disease in humans)

Viral DNA is replaced by a therapeutic gene

Recombinant AAV vectors are non-integrating and non-replicating (the therapeutic DNA remains separate from the human genome)

How are AAVs manufactured?

The AAV production workflow is divided into an upstream and a downstream process. During the upstream process, plasmids with the encoding genes are generated and transfected into the selected cell line. The cells which are now producing the AAV vectors are expanded in bulk sizes. The downstream process starts with harvesting and clarification of the vectors, followed by various purification and chromatography steps. When the AAV vectors are purified, final lot-release testing is performed and formulation and filling of the final drug product takes place.

UPSTREAM

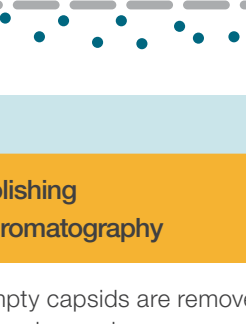
Plasmid development and production

Production of three plasmids carrying:

- the therapeutic gene
- Adenovirus genes
- AAV Cap and Rep genes

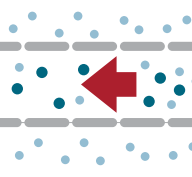
Cell expansion

A suitable cell line is selected and expanded.



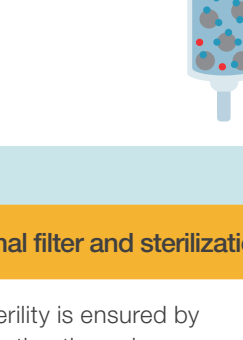
Plasmid transfection

Plasmids containing viral backbone and therapeutic gene components are introduced into the cells



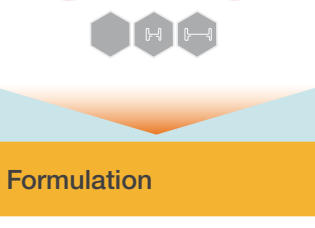
Viral vector production

Transfected cells are cultured in a bioreactor



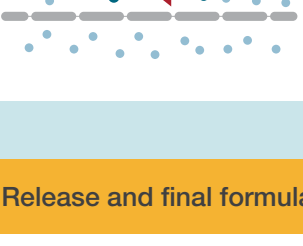
Cell lysis

The cell membrane is destroyed to release the viral vectors from the host cells



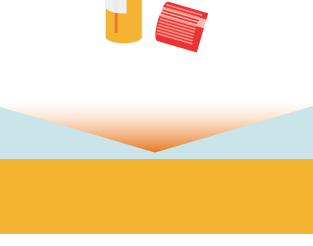
Clarification

Cell debris are removed by centrifugation and/or microfiltration



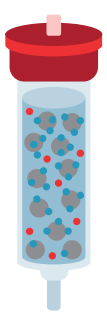
Concentration

Volumes are reduced by tangential flow filtration or/ and ultracentrifugation



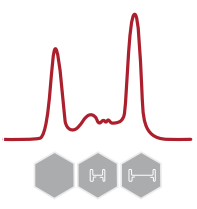
Capture chromatography

Process-related impurities are removed using affinity chromatography



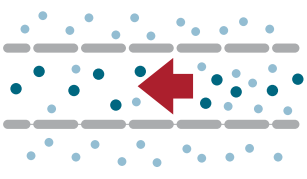
Polishing chromatography

Empty capsids are removed using ion exchange chromatography



Formulation

The product is further concentrated and formulated using tangential flow filtration



Final filter and sterilization

Sterility is ensured by filtration through a 0.2 µm filter

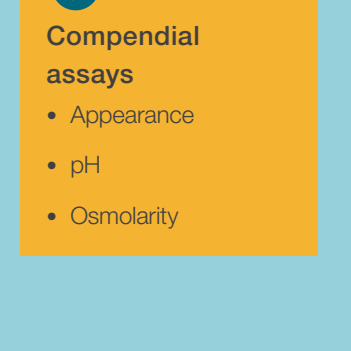
FILL AND FINISH

Release and final formulation

The final product is verified and packed in a sterile environment

ANALYTICS

Analytical testing is performed for raw material and product characterization. Safety and environmental testing is also done throughout the process.



Identity
- Capsid/serotype ID
- Transgene ID

Potency
- Infectious titer
- Functional analysis

Compendial assays
- Appearance
- pH
- Osmolarity

Strength
- Viral genome titer
- Total viral yield

Purity
- Host cell protein/DNA
- Residual impurities (BSA, endonucleases, plasmids, reagents, etc.)
- Genome integrity
- Protein purity and aggregation
- Empty/full capsid ratio

Safety
- Absence of viruses, mycoplasma and endotoxins
- Sterility

Learn more about **ThermoFisher AAV production solutions**

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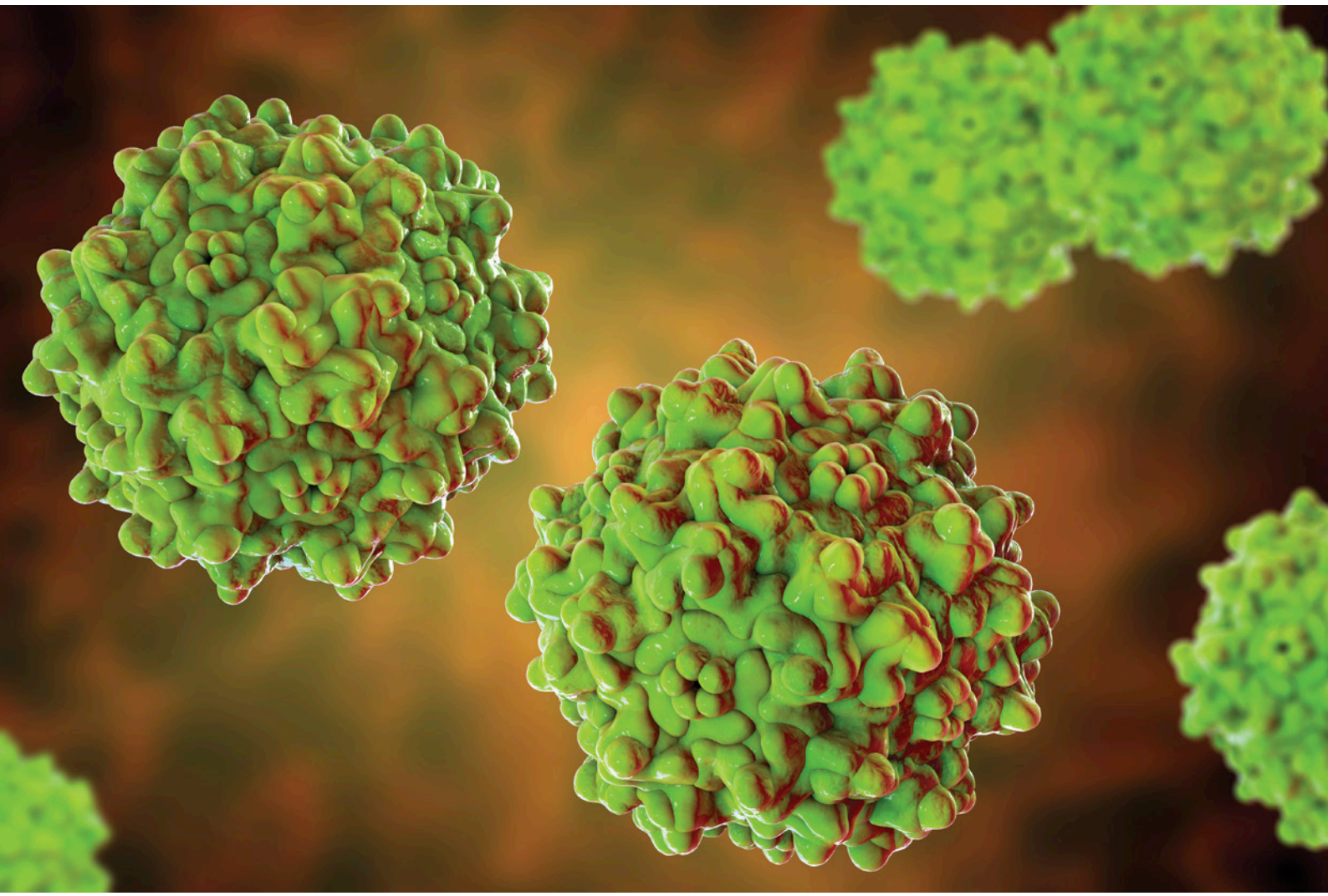
Viral Safety in AAV Production: How Affinity Chromatography Effectively Contributes



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Viral Safety in AAV Production: How Affinity Chromatography Effectively Contributes

An efficient and scalable affinity purification solution, POROS™ CaptureSelect™ AAVX affinity chromatography resin contributes to the overall viral clearance in a process.

Adeno-associated virus (AAV) vectors, the workhorse for gene therapy delivery, have been administered in an estimated 250-300 clinical trials.[1] To date, two gene therapy medications are approved–Luxturna for patients with confirmed biallelic *RPE65* mutation-associated retinal dystrophy and Zolgensma for children less than 2 years old with spinal muscular atrophy (SMA). An anticipated surge in AAV-enabled gene therapy clinical trials is expected to drive increased demand for vectors along with the need for reliable and scalable manufacturing platforms.

Affinity resins, such as POROS™ CaptureSelect™ AAV resins, represent some of the more efficient and scalable purification tools available to purify AAV vectors. These resins reduce the number of purification steps, maximize productivity, and offer scalability and process consistency at commercial scale.

POROS™ CaptureSelect™ AAV8 resin and POROS™ CaptureSelect™ AAV9 resin are serotype-specific to AAV8 and AAV9, respectively, while the POROS CaptureSelect AAVX resin is a universal capsid affinity resin covering all naturally occurring serotypes known to date, as well as engineered and chimeric versions tested.

"The POROS CaptureSelect AAVX resin enables a paradigm shift in viral vector downstream purification," said Nicolas Laroudie, Senior Field Application Specialist, Purification, Thermo Fisher Scientific. "The versatility of this chromatography resin opens opportunities for its use as a platform product." In addition, free conjugated ligands in biotinylated and HRP formats are available for the development of in-house total capsid quantification ELISAs.

Decomplexifying Downstream Purification

The process of disrupting production cells for vector release, combined with the multiplicity of upstream production systems, results in a large range of impurity profiles in the initial feedstock and increases purification complexity. AAV capsids are less than 0.1% of the total proteins generated by the cells.

To make AAV purification more straightforward, affinity chromatography is often used as the first chromatography step in the downstream process. POROS CaptureSelect AAVX resin is a robust, scalable tool that provides high capacity, high specificity, and high throughput to obtain product at high yield and purity.

By immobilizing a proprietary recombinant V_HH fragment, derived from a single-domain antibody, onto the highly

permeable POROS™ backbone, the resin achieves a fine-tuned specificity for AAV recognition with an increased surface area and high capacity for binding.[2]

"The physical properties of the resin result in a fully linear pressure flow curve making scaleup predictable in terms of back pressure," said Laroudie. "Different flow rates have no significant impact on binding capacity or elution, enabling process flexibility and a reduction in processing times." Greater than 80% vector recovery has been reported on scales from 10-2000L.

The robustness of POROS CaptureSelect AAVX resin was demonstrated by purifying AAV6 from two production systems, HEK293 and Sf9 cells. The resin showed high dynamic binding capacity for both feed streams resulting in high yields independent of the production system. Similar to other chromatography resins, the affinity resin can be used multiple times for the same serotypes with negligible AAV carryover.

Because of its high specificity and its affinity towards the broadest range of serotypes, the use of POROS CaptureSelect AAVX resin for platform manufacturing simplifies the downstream process. The resin binds greater than 10^{13} vg/mL of resin allowing the use of smaller columns and reducing the manufacturing footprint.

"Optimization produces the most robust process possible," said Laroudie. "Specialists are available to assist in optimizing parameters to achieve easy, predictable scaleup."

Ensuring a Safe Product

One elusive aspect of AAV process development is viral clearance. According to the ICH Q5A guidance all biotechnology products derived from cell lines of human or animal origin must demonstrate that the products are safe by implementing appropriate testing and demonstrating that the manufacturing process is capable of clearing any endogenous or adventitious viruses.

"Manufacturers need to demonstrate that they are providing a safe product through a robust process," said Alejandro Becerra, PhD, Principal Applications Scientist, Global Purification Technical Lead, Thermo Fisher Scientific.

Prevent, detect, and remove are the three tenets of viral safety programs. Virus-free cell lines, control of raw materials, and the use of closed-process steps and single-use systems minimize the entry of adventitious viruses into the process. As it is impossible to guarantee prevention of adventitious viruses from entering the process, detection tests monitor critical points of the production process in cell banks, harvest, and bulk drug substances. However, assays have detection limits, so it is crucial that the process is capable of removing residual viruses.

"In the AAV field there is limited information in the public domain on the subject of viral clearance specifically focusing on the purification processes," said Becerra. "This was an opportunity for us to contribute knowledge."

Four companies, REGENXBIO, Texcell NA, MockV Solutions, and Thermo Fisher Scientific, joined forces to assess the adventitious virus removal capability of a two-step chromatography process that began with affinity capture using POROS CaptureSelect AAVX followed by anion-exchange polishing step.[3,4]

Designing the Viral Clearance Study

The viral clearance study employed REGENXBIO's production process for AAV8 using HEK293 cells adapted for suspension culture and a triple transfection process without helper viruses. The purification work was performed at the Texcell NA specialized facility.

As stated earlier, AAV capsids are less than 0.1% of the proteins generated by the cells. The main product impurities are the host cell proteins, DNA/RNA, plasmid DNA, cell culture medium components and supplements. POROS CaptureSelect AAVX resin was used to remove these impurities.

Viral spiking studies evaluate removal of viruses by

downstream steps such as chromatography. For these studies, concentrated stock virus solutions are spiked into the load, and the purification step is executed under scaled-down conditions representative of the manufacturing scale.

Both the load and the step product are evaluated for viral titers to determine the LRV (log reduction value)–Log 10 of the ratio of viral concentration in the load compared to the viral concentration in the product. Due to assay variability, LRV <1 is considered negligible and cannot be counted towards the overall viral clearance. LRVs 1-3 are considered contributing and LRVs ≥4 reflect a robust viral clearance step.

Spiking viruses were propagated and purified by Texcell NA according to standard protocols. For DNA viruses, the study used enveloped pseudorabies virus (PRV) and non-enveloped minute virus of mice (MVM); for RNA viruses the enveloped xenotropic murine leukemia virus-related virus (XMuLV) and non-enveloped reovirus type 3 (Reo-3) were used. Since a HEK293 human-derived producer cell line was used for upstream production, two human viruses, hepatitis A (HAV) and herpes simplex virus 1 (HSV-1), were added based on the risk assessment of possible operator sources of contamination.[3]

To determine viral clearance across the affinity purification step, viral concentration in the load and elution were measured by infectivity for non-enveloped viruses or by qPCR for enveloped viruses. AAV8 elutes at a low pH, and low pH elution inactivates the enveloped viruses; therefore, an infectivity assay is not suitable for demonstrating that the chromatography step removes enveloped viruses.

The chromatography step evaluated performance at the target manufacturing parameters as well as worst case conditions. The worst-case conditions were selected as high load ratio, challenging the column with a higher viral load, and high load and elution residence time to allow viruses more time to bind to the resin during load or to dissociate from the resin into the product fractions during elution. Worst case conditions were evaluated separately and in combination.

Although, theoretically, the spiked viruses should not bind to the affinity ligand and would flow through the column, mass balance testing of all fractions determined where the viruses were going during the chromatography step.

Another set of experiments evaluated product-virus interactions by using an AAV null load that was generated by collecting the non-bound fraction of the affinity column run, basically the same matrix and impurity composition as an affinity load but without product. The AAV null load was then spiked with viruses and purified, and the LRV compared to the control run with AAV capsids.

To assess nonspecific interactions between the viruses and camelid V_HH ligand or POROS™ beads, control POROS™ resins were custom made by Thermo Fisher with identical base beads to the affinity resin but incorporating either an alternative V_HH ligand specificity (nonbinding for AAVs) or no V_HH functionalized ligand. For this experiment, due to capacity constraints, only the most commonly-used viruses in viral clearance studies, XMuLV and MVM, were used. XMuLV represents the large enveloped retro viruses and MVM the small, hardy, non-enveloped Parvo viruses.

Viral Clearance Results

"The study achieved a clearance of greater than 3 LRVs for most of the viruses tested, particularly for the center-point condition," said Becerra. "To put this into context, in the mAb field using Protein A affinity chromatography resins the viral clearances are usually lower than what we observed, typically 1–3 logs. The POROS CaptureSelect AAVX resin had a viral clearance higher than the most commonly used affinity resin in bioproduction under the process conditions tested."

The POROS™ CaptureSelect™ AAVX columns showed robust clearance at target manufacturing parameters.

Reo-3 and HSV-1 were the only viruses with observed clearances below 4 LRV. However, they still showed significant viral reduction by the affinity resin (*Table 1*).

For the worst case conditions the level of viral clearance remained the same when the flow rate was increased. When 33% more was loaded onto the column, the clearances were reduced slightly but even at conditions of combined higher load and reduced flow rate the observed viral clearances were essentially the same, within 1 LRV except for XMuLV which was 1.8 LRV, of the clearances achieved under the control run (*Table 1*).

The AAV null run containing the spiked virus showed similar clearances compared to the control run indicating minimal, if any, interactions between the capsid and the viruses. The non-significant effect of the presence of AAV capsids on the LRVs suggests the possibility of bridging viral clearance results generated with one AAV8-based product to other AAV8-based projects (*Figure 1*).

The experiments testing the nonspecific interactions to the V_HH ligand and the POROS bead showed clearances similar to the control run. No nonspecific interactions were found. The mass balance results also were in line with expectations. The majority of the viruses were in the flow through, some were detected in the first wash, and the amount of the virus actually binding and remaining on the resin was less than 1%.

"The findings of the viral clearance study suggest that the POROS CaptureSelect AAVX resin contributes to and can be used for the overall viral clearance in a process," said Becerra. The study demonstrated that robust clearance of XMuLV, MVM, HAV and PRV viruses could be achieved at REGENXBIO's target manufacturing conditions for AAV8.

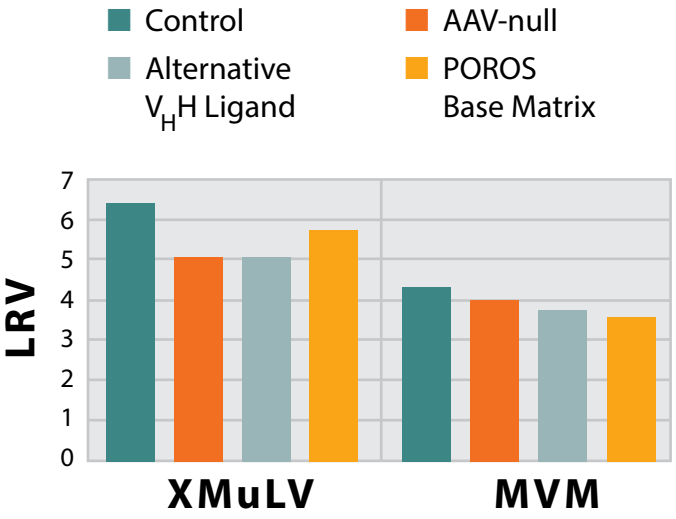

Figure 1. Characterization of nonspecific interactions based on log reduction values (LRVs) for xenomorphic murine leukemia virus (XMuLV) and minute virus of mice (MVM).[3]

Virus name	xMuLV	MVM	Reo-3	HAV	PRV	HSV-1
Virus type	RNA env	DNA non env	RNA non-enveloped		DNA enveloped	
Centerpoint process conditions	>6.4	4.4	2.7	>4.9	4.0	3.1
Worst case conditions (high load ratio and residence time)	4.6	3.6	2.5	5.0	3.8	3.6
Clearance level	Effective	Effective	Contributing	Effective	Effective	Contributing

Table 1. Viral Clearance Results using six various model viruses. Clearance was tested at standard manufacturing conditions and under worst case conditions.

This is especially important for MVM because chromatography is one of the few options to clear this virus. MVM belongs to the same virus family as AAV. It is approximately the same size, and it is highly resistant to inactivation steps such as incubation, detergent, or low pH conditions.

The MockV MVM Kit

"Most companies do not have the capability to work with viruses since they require special containment laboratories, such as those at Texcell NA," said Becerra. The MockV™ MVM kit uses a non-infectious MVM surrogate (MVM-MVP) that mimics MVM's physiochemical properties and can be used in any BSL-1 laboratory. The LRVs are quantitated using immuno-qPCR.

The kit is a useful tool to make an assessment of the viral clearance in a process before a CRO performs the enabling studies. "Using it for an AAV process was novel but the mock particles have demonstrated similar correlations with multiple types of unit operations in mAb purification processes," said Becerra.

The study showed comparable LRVs using MVM and MVM-MVP under center-point conditions, worst-case run conditions and alternate ligand runs (*Figure 2*). This demonstrated the utility of MVM-MVP as an analysis tool for AAV process development and characterization.

Conclusion

The results of the viral clearance study demonstrate the contributions of the POROS CaptureSelect AAVX resin to the clearance of adventitious viruses during AAV downstream purification and its applicability as a platform purification product. The universal capsid affinity resin showed robust viral clearance at target manufacturing parameters, particularly for the center-point condition, and no interactions between the viruses and the AAV capsids or nonspecific resin interactions.

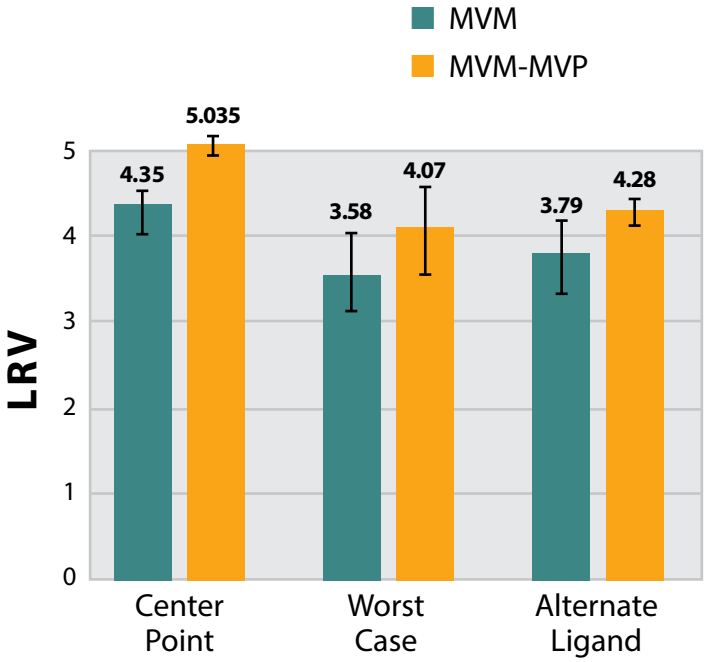

Figure 2. Log reduction value (LRV) determinations for affinity resin runs spiked with minute virus of mice (MVM) and noninfectious MVM mock virus particles (MVM-MVPs).[3]

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INNOVATOR INSIGHT

Platform optimization for efficient AAV purification: insights from a CDMO

Vincent Ravault & Nicolas Laroudie

Over the last decade, the number of clinical trials involving recombinant adeno-associated viral (AAV) vectors has dramatically increased, the diversity of serotypes has expanded, and the demand for larger quantities of highly purified material manufactured to cGMP standards has rocketed. For contract development and manufacturing organizations (CDMOs) like Yposkesi, the manufacturing challenges are centered around flexibility, robustness, and productivity, especially with regards to purification. Universal tools able to address any serotype with minimal process adjustments are critical. In this article, we describe how POROS™ CaptureSelect™ AAVX resin can be used as a pan-affinity tool for the universal capture of AAV vectors, and how Yposkesi optimized the operational parameters to make the resin an efficient, robust, and productive purification platform that fits within the constraints faced by CDMOs.

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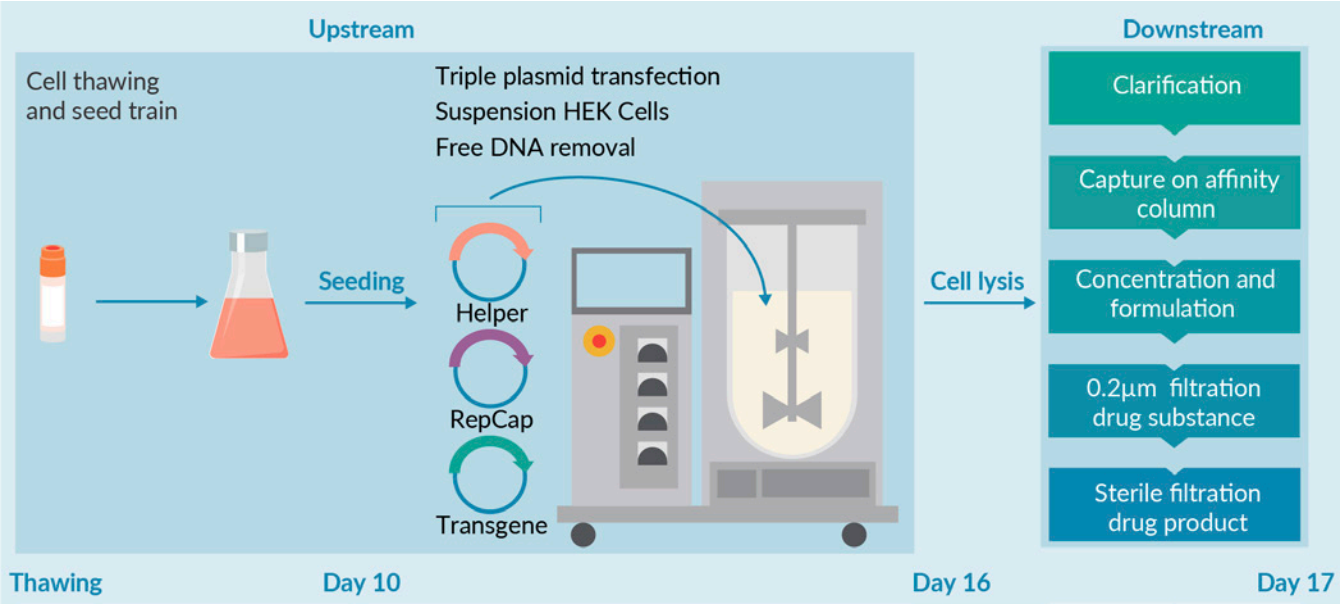
As a full-service CDMO for innovative gene therapy products, Yposkesi supports customers from early-stage development, including process and analytical development, through to large-scale production and commercial supply of gene therapy products.

Yposkesi produces recombinant adeno-associated virus (rAAV) and recombinant lentivirus (rLV) vectors using adherent- and suspension-adapted cell platforms. The manufacturing platform at Yposkesi currently includes four independent production suites equipped with 200 L single-use bioreactors, which will evolve to include a 1000 L single-use bioreactor from 2023. Yposkesi is currently building an additional 5,000 m^2 clinical/commercial

FIGURE 1

AAV manufacturing process at Yposkesi.



manufacturing plant to support the growing demand for viral vector supply.

This article describes how Yposkesi developed an AAV purification platform for a range of serotypes based on Thermo Fisher Scientific's POROS CaptureSelect AAVX Affinity Resin.

YPOSKESI'S AAV MANUFACTURING PROCESS

The established AAV manufacturing process at Yposkesi is shown in Figure 1. AAV vectors are produced by triple plasmid transfection in human embryonic kidney (HEK) cells. The lysate is clarified and then directly loaded onto an affinity column. The eluted vectors are concentrated and formulated, before being sterile filtered. The full process lasts 17 days, from cell thawing to drug product filling.

The current AAV purification process involves the use of different affinity sorbents according to the AAV serotype to be produced. The POROS CaptureSelect AAVX Affinity Resin leans on the use of a ligand derived from a heavy-chain antibody that can bind AAV serotypes 1–9 and synthetic or recombinant AAV vectors, offering a great opportunity to develop the next AAV purification platform at Yposkesi (Figure 2) [1].

EVALUATION OF DYNAMIC BINDING CAPACITY

As a first step to evaluate the AAVX resin as a platform purification solution, the dynamic binding capacity was evaluated using an AAV8 serotype. The binding capacity was assessed using 1 mL-prepacked columns, packed with either POROS CaptureSelect AAV8 or POROS CaptureSelect AAVX. The binding capacity was assessed at 1 and 3 mins residence time on two different feedstocks, each with different initial virus titers.

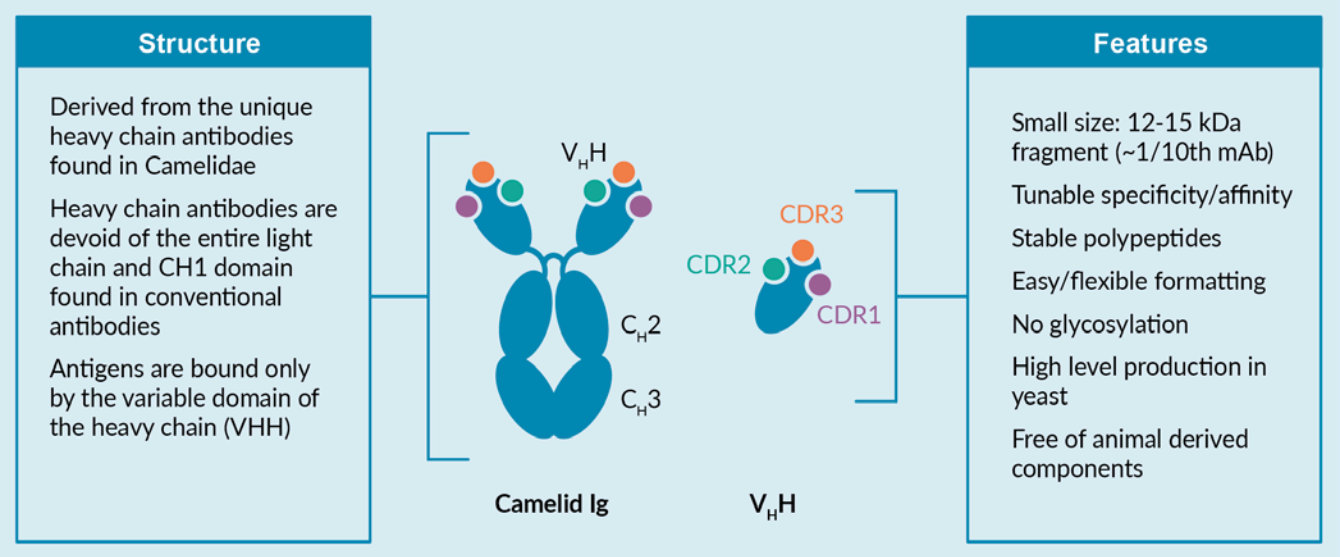
Clarified supernatant containing AAV8 vectors was directly loaded on the affinity columns until a 10% breakthrough in AAV8 was observed in the flowthrough.

Multiple fractions (column volumes [CV]) were collected at the outlet of the column during the loading phase, and the quantity of capsids was determined by ELISA assay in each collected fraction. The results for the 3 mins residence time are presented in Figure 3.

No breakthrough was observed on the AAVX resin at loading volume of up to 1,500

FIGURE 2

Key features of camelid-derived, recombinant expressed ligands used in CaptureSelect™ Affinity Resins.

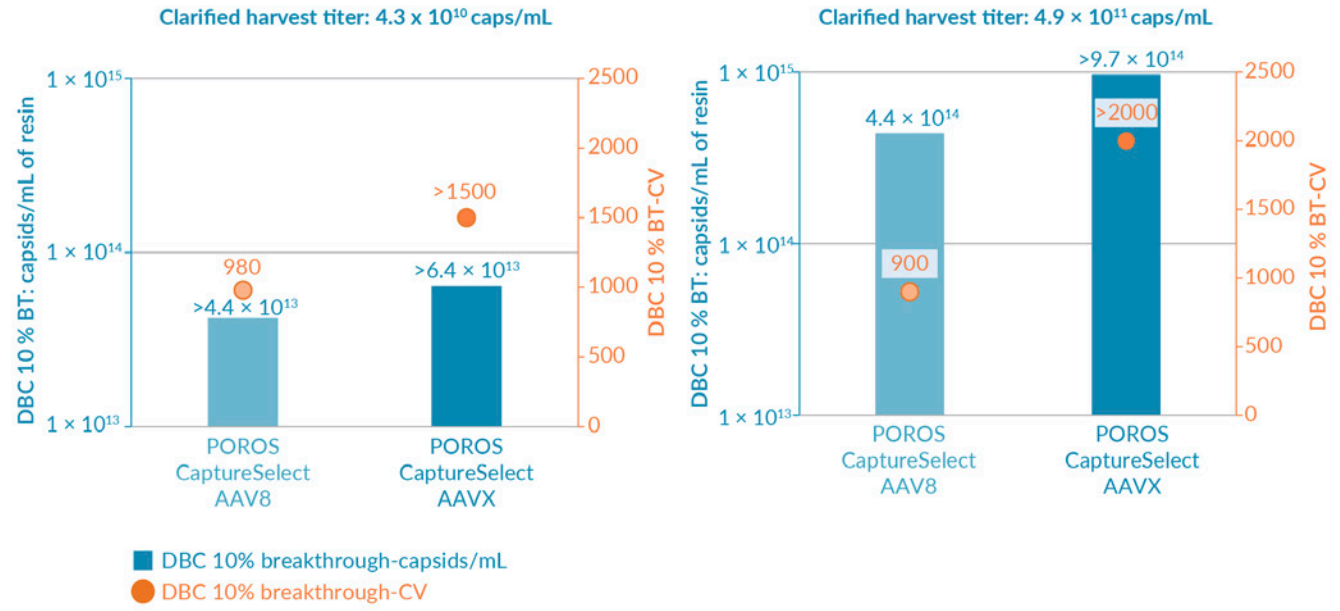


or 2,000 column volumes for the low viral titer and higher titer feedstock, respectively. Both resins showed higher binding capacity when feeds contained a higher vector titer, but overall, the AAVX resin showed a higher binding capacity for AAV8 than the Poros CaptureSelect AAV8 resin.

Figure 4 shows the binding capacity of AAV8 vectors measured at 1 min residence time on both resins, showing similar binding

FIGURE 3

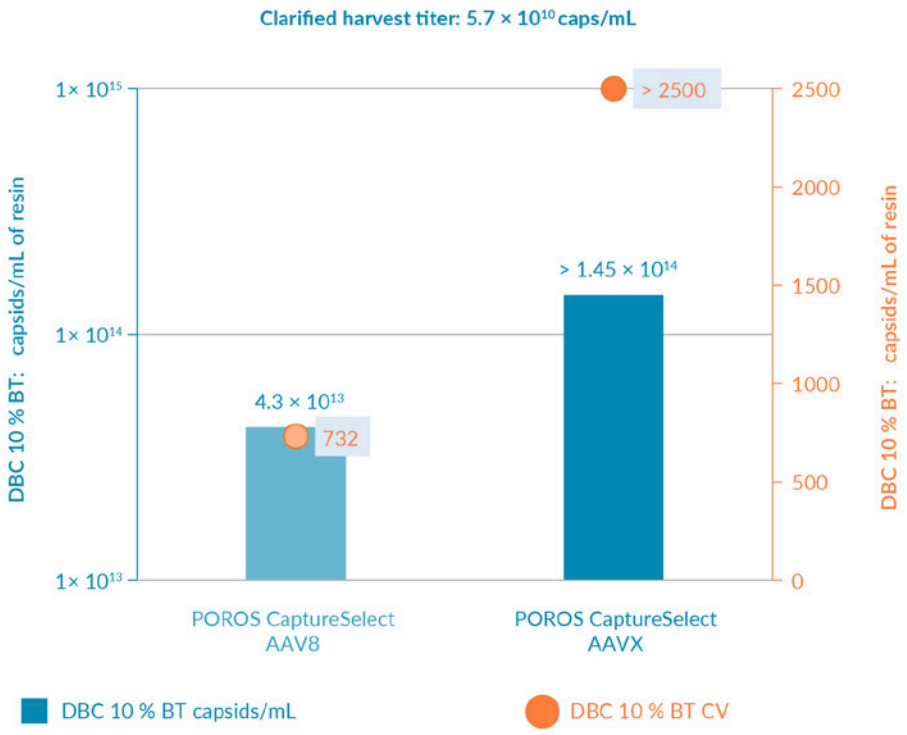
Binding capacity at 3 mins residence time and 10% breakthrough for POROS CaptureSelect AAV8 and POROS CaptureSelect AAVX at AAV titers of 4.3×10^{10} caps/mL (left) and 4.9×10^{11} caps/mL (right).



The blue bars represent the binding capacity in terms of capsids per mL of resin at 10% breakthrough. The orange dots represent the column volumes that lead to 10% breakthrough.

FIGURE 4

Binding capacity at 1 min residence time and 10% breakthrough for POROS CaptureSelect AAV8 and POROS CaptureSelect AAVX at an AAV titer of 5.7×10^{10} caps/mL.



The blue bars represent the binding capacity in terms of capsids per mL of resin at 10% breakthrough. The orange dots represent the column volumes that lead to 10% breakthrough.

capacities compared with the 3 mins residence time. At both residence times, there was no breakthrough on AAVX, with loading volumes up to 2,500 column volumes (CV).

The results from these binding capacity studies led to three main conclusions:

1. The AAVX resin has a better AAV8 binding capacity than the AAV8 resin
2. Binding capacity increases with harvest titer
3. Residence time has no significant effect on the binding capacity

DEFINING OPERATING CONDITIONS FOR PURIFICATION OF AAV8 & AAV2

The operating conditions for the capture of the AAV8 serotype were defined according to the DBC data obtained previously. Screening of capture conditions was performed on 1 mL-pre-packed columns with AAV8 or AAVX resin (Figure 5).

The material loaded onto the columns was a clarified supernatant containing AAV8 vectors. To align with our AAV manufacturing operating conditions, the maximum loading time selected was 18 hours – (overnight loading). Two residence times were evaluated: 3 mins and 1 min.

The loading volumes selected were 356 CV (for AAV8 and AAVX) with a 3 mins residence time, and 600 CV (AAV8) and 1080 CV (AAVX) with a 1 min residence time. These CVs are all below the resin binding capacities at 10% breakthrough defined earlier (Figures 3 & 4).

After loading and washing, purified product was recovered during the elution step at low pH and was immediately neutralized.

The clarified harvest and eluent were tested for viral genome (VG) titer.

Similar quantities of AAV vectors were loaded on the AAV8 and AAVX resins at 3 mins residence time. As shown in Figure 6, the quantity of AAV8 vector recovered after elution and the AAV8 yield was very similar for both resins. The resins showed no significant difference in performance when loading at 3 mins residence time or at 18 hours loading time.

Results at 1 min residence time are shown in Figure 7. As a consequence of the different binding capacities at 1 min residence time, the loading times were different for the two sorbents – 10 hours for the AAV8 resin and 18 hours for the AAVX resin. Thus, the total quantity of AAV8 capsid loaded on the resins was around 1.8 times higher for the AAVX resin compared with the AAV8 resin. As a result, the quantity of purified recovered product for AAVX was approximately 1.7 times higher. The step yields for both resins were also very similar and close to 90% which is higher than the yield of around 70% obtained with a residence time of 3 mins.

These results indicate that it will be possible to switch from POROS CaptureSelect AAV8 to AAVX for the purification of AAV8 serotype.

Based on these results with POROS CaptureSelect AAV8, the AAVX resin was also evaluated for the capture of another serotype of AAV: AAV2 (Figure 8). The aim was to compare POROS CaptureSelect AAVX with an affinity resin from another supplier, which is currently used at Yposkesi for AAV2 processes.

The screening of the capture conditions was performed on 1 mL prepacked columns. Two residence times were applied for the AAVX resin: 3 mins and 1 min. The residence time applied to the other affinity resin was 8 mins, according to supplier's recommendation.

Three purification conditions were screened for the capture of the AAV2 vector. At 3 mins residence time, the volume of clarified harvest loaded on the column was 455 CV, whereas at 1 min residence time the volume loaded on the column was 1,440 CV. The same starting material was used for all trials.

For the other resin, only 340 CV were loaded since the residence time applied was higher. After column washing, the product was eluted at low pH, and the loading and elution fractions were tested for VG titer.

Using the AAVX resin and decreasing the residence time from 3 min to 1 min resulted in an increase in VG yield from 57% to 89% (Figure 9). Using the affinity resin from another supplier with higher residence time

FIGURE 5

Experimental plan for the definition of operating conditions for purification of AAV8.

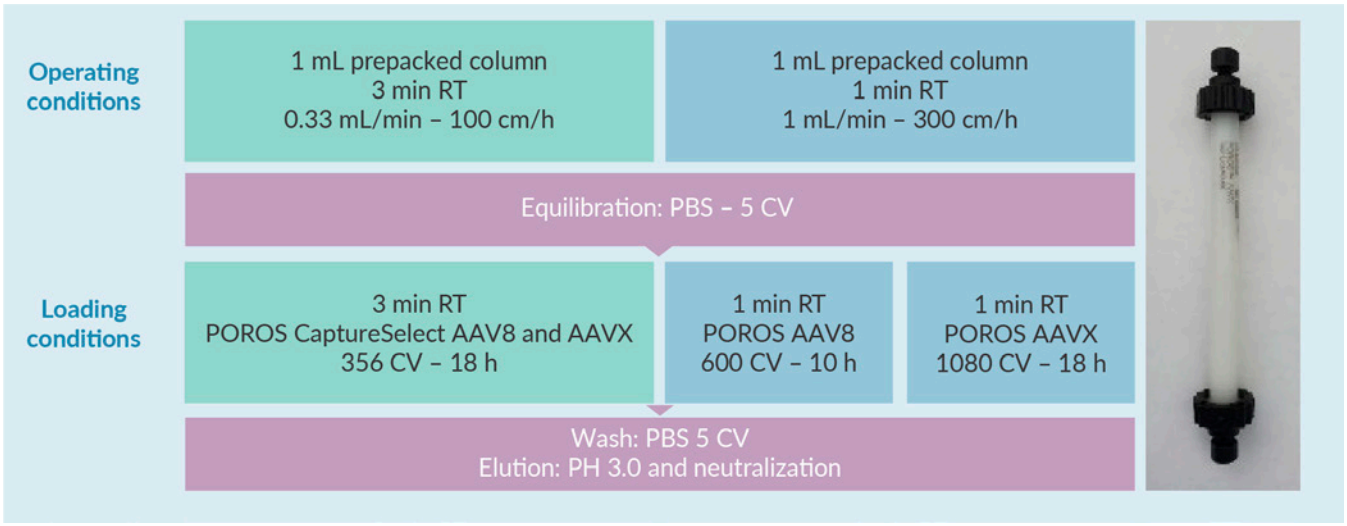
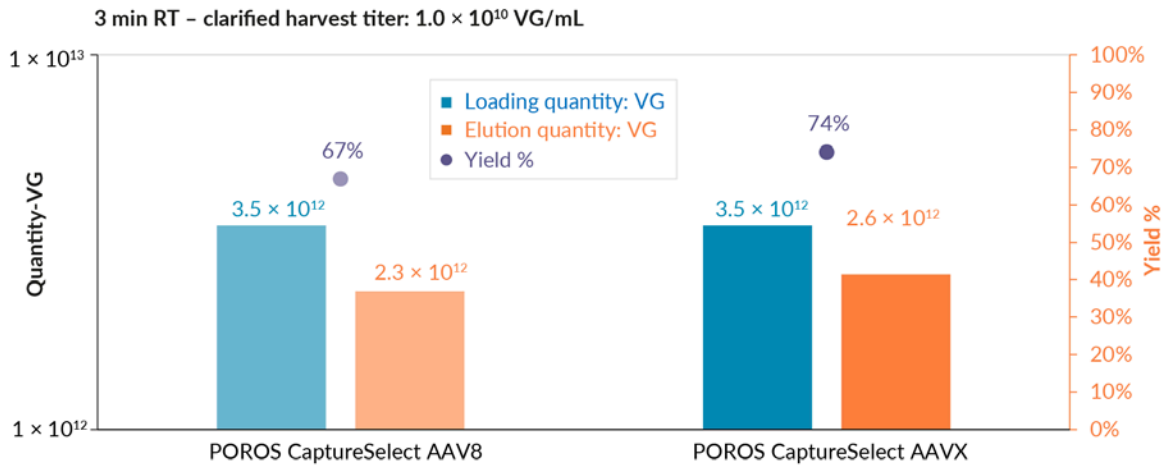


FIGURE 6

AAV8 capture conditions – results for 3 mins residence time.



The blue bars represent the product quantity loaded on each column. The orange bars represent the quantity of purified product recovered during the elution. The red dots represent yield.

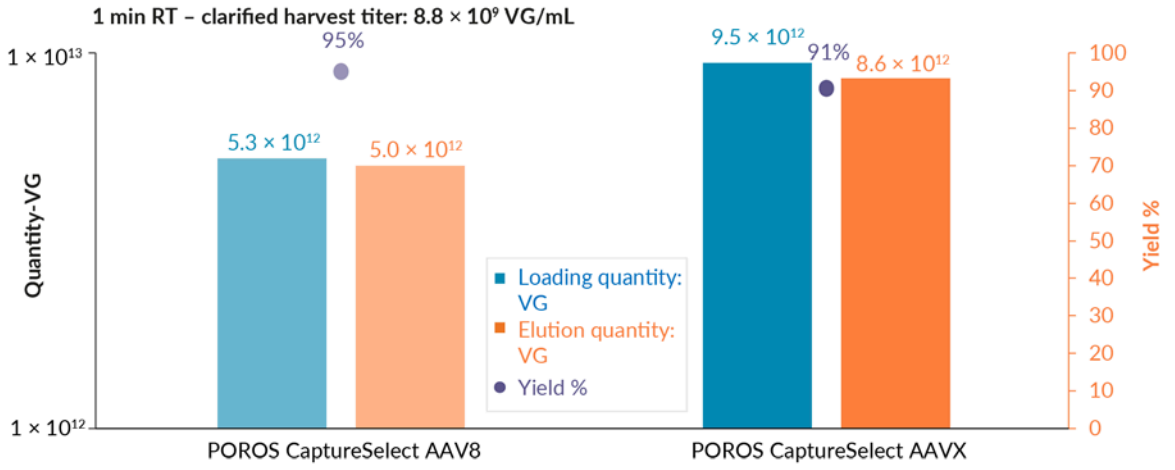
(8 mins, imposed because of the compressibility of the media, and as recommended by the supplier), resulted in a low volume loaded on this column. The AAV2 yield is significantly lower than the yield obtained with AAVX: 48% yield, versus 70–90% yield obtained with AAVX.

This part of the study demonstrated that using a lower residence time results in higher AAV binding capacities for both Thermo Fisher Scientific resins, and that the AAVX resin shows better results for the capture of AAV8 and AAV2 vectors. The volumes of clarified harvest that can be loaded on AAVX without any AAV breakthrough in the flowthrough are 1080 CV for AAV8, and 1440 CV for AAV2.

The promising results obtained with POROS CaptureSelect AAVX led us to select this resin for the next part of the study and to work with a residence time as close as possible to 1 min.

FIGURE 7

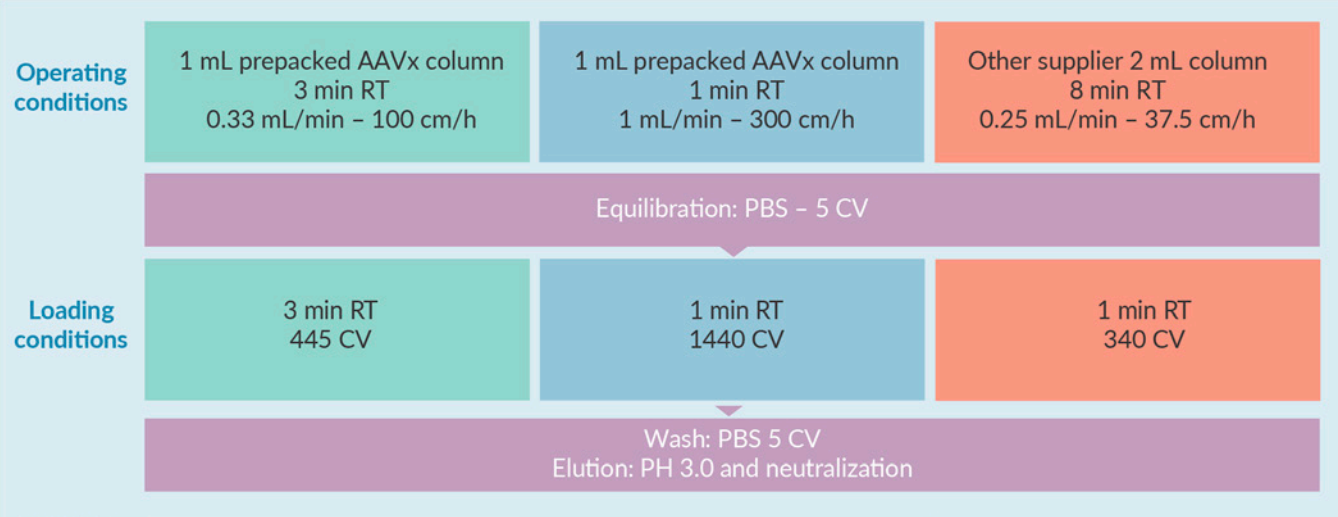
AAV8 capture conditions – results for 1 min residence time.



The blue bars represent the product quantity loaded on each column. The orange bars represent the quantity of purified product recovered during the elution. The red dots represent yield.

FIGURE 8

Experimental plan for the definition of operating conditions for purification of AAV2.



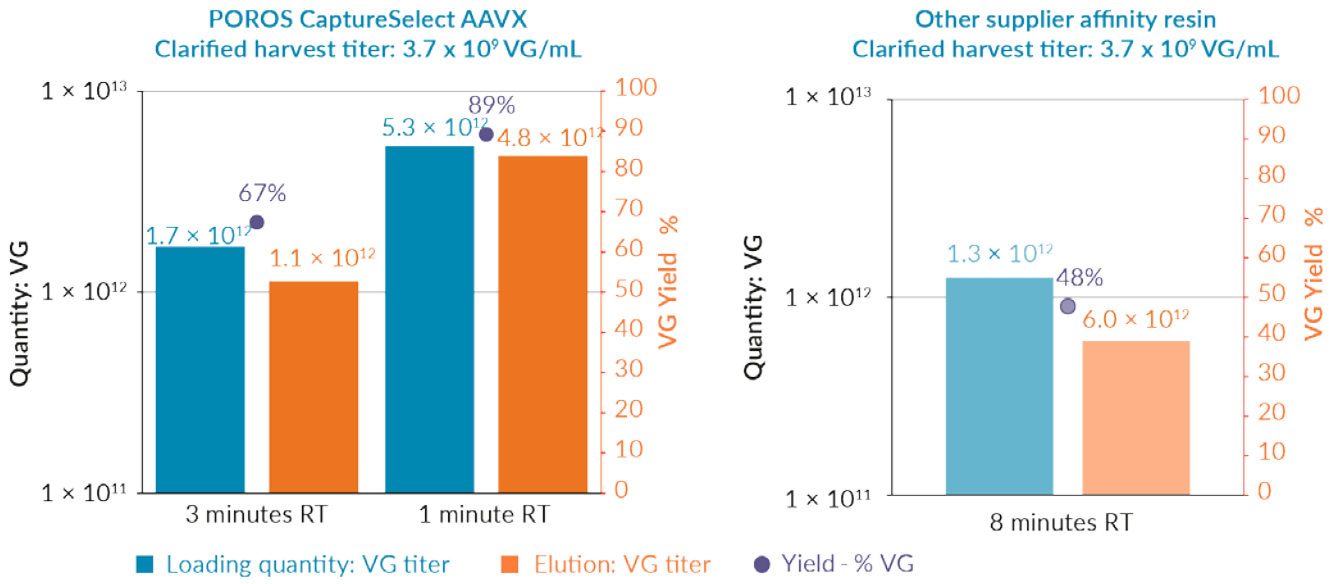
SCALE-UP OF THE CHROMATOGRAPHY STEP

The experimental conditions determined using AAVX for the capture of AAV2 and AAV8 vectors were adapted for the purification of clarified harvest from a 10-liter bioreactor (Figure 10).

The volume of resin necessary to purify a 10 L clarified harvest was calculated by applying the column loading capacity in terms of CV determined previously during the screening for AAV8 and AAV2 processes. This AAVX resin volume was found to be 14.3 mL for AAV8 capture and 11.3 mL for AAV2 purification process.

FIGURE 9

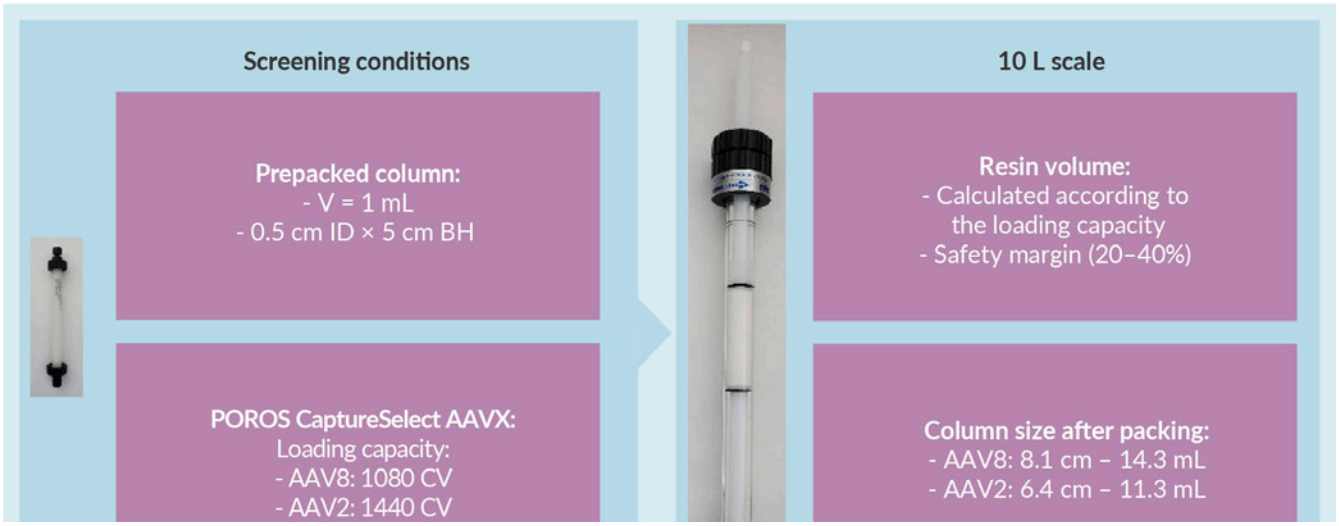
Definition of the operating conditions for the purification of AAV2 using POROS CaptureSelect AAVX (left) or alternative supplier's affinity resin (right).



The blue bars represent the product quantity loaded on each column. The orange bars represent the quantity of purified product recovered during the elution. The red dots represent yield.

FIGURE 10

POROS CaptureSelect AAVX chromatography scale up.



Conditions to be tested are listed for small-scale (left) and 10-liter scale (right).

AAVX resin was packed in a 15 mm internal diameter glass column, which allowed for a resin bed height that would be easily transferrable to GMP scale. The column bed height was 8.1 cm for AAV8 purification and 6.4 cm for AAV2 purification.

In order to obtain the starting material for resin evaluation, two 10 L bioreactors were used to produce AAV2 and AAV8 vectors from HEK cells.

After AAV production, cells were lysed, and the lysate was clarified and filtered using a 0.22 µm filter. After lysate filtration, the pool titer was 1.10 x 10^{11} VG/mL for AAV8 vectors and 3.70 x 10^{9} VG/mL for AAV2 vectors.

The selected operating conditions for the AAVX resin to purify AAV8 and AAV2 from a 10 L clarified harvest are shown in Figure 11.

FIGURE 11

Selected chromatography operating conditions at larger scale.

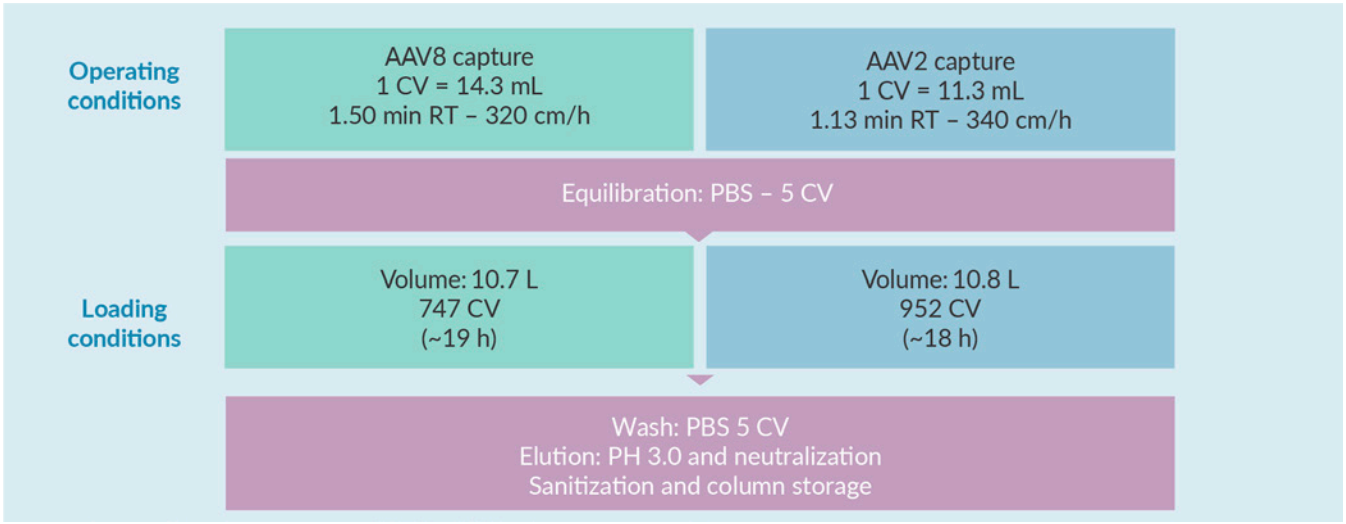
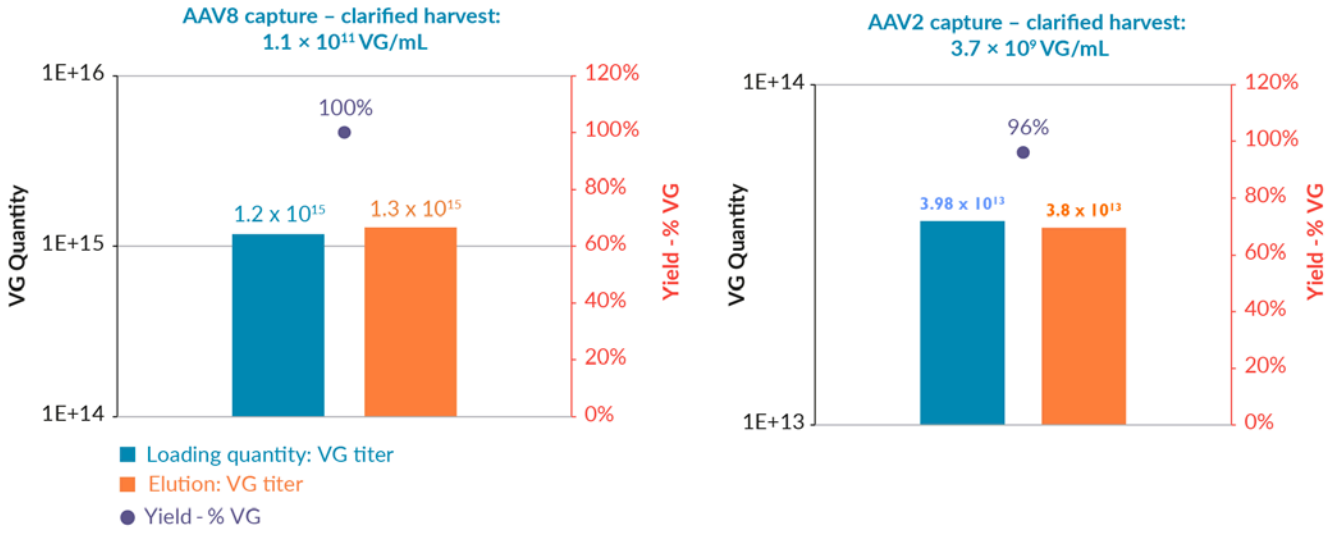


FIGURE 12

AAV capture on POROS CaptureSelect AAVX – yields for AAV8 (left) and AAV2 (right).



The blue bars represent the product quantity loaded on each column. The orange bars represent the quantity of purified product recovered during the elution. The red dots represent yield.

The residence time for both AAV processes was close to 1 min. 747 CV of clarified harvest were loaded on to the AAVX resin for AAV8, and 952 CV for AAV2, while the loading times were in the same range. The purified products were recovered during elution at low pH and then neutralized. VG titers, total protein content, and residual DNA levels were assayed in the clarified harvests (starting materials) and in the elution fractions.

The pressure was monitored at the inlet of the column during the loading step for the AAV8 and AAV2 capture process. The pressure slightly increased during the loading stage but stayed within an acceptable range. The pressure was around 1.5 bars at the end of the loading step, which helps to provide good conditions for a transfer to GMP scale.

Even though VG titers in the starting material were very different for the AAV2 and AAV8 serotypes, the final yields of the capture step are close to 100% for both serotypes and there was good scalability from lab scale development to the 10 L scale (Figure 12).

Additional experiments revealed that the purity of AAV vectors captured with AAVX resins appears to be very high. There was an impurity reduction of over 99% in the purified product after capture on AAVX for each serotype. This clearance rate could be even further optimized by adding an intermediate washing step or implementing a polishing column after the AAV capture step.

CONCLUSIONS

This long-term study with POROS CaptureSelect AAVX resin has highlighted several advantages of AAV capture using this resin compared to other affinity resins commercially available:

- Flexibility in terms of serotypes: capture of AAV1 to AAV9 serotypes and synthetic and recombinant serotypes
- Possibility to standardize a purification platform for several AAV serotypes with only a few adjustments
- Cost reduction due to shorter residence times and very high loading volumes.

- Low level of impurities captured on the resin. This could be further optimized for each serotype if needed (wash conditions screening or addition of a polishing step)
- Good scalability of the downstream platform. It is compliant for a large-scale GMP AAV manufacturing process

Overall, Yposkesi concluded that the PO-ROS CaptureSelect AAVX resin appears to be a great tool to improve purification processes in terms of quality, cost, and standardization. Yposkesi plan to implement this resin for the purification of other AAV serotypes.

ASK THE EXPERTS

Nicolas Laroudie (Thermo Fisher Scientific) joins **Vincent Ravault** (Yposkesi) to answer readers' questions on implementing POROS CaptureSelect technology into viral vector production.

Q Can the POROS CaptureSelect resin be cleaned and re-used?

NL: Yes, the resin can be cleaned and reused. Many customers use the resin once, particularly CDMOs that deal with multiple serotypes and multiple transgenes and want to avoid spending a lot of time validating cleaning. But the resin can absolutely be cleaned and reused, and many customers are doing that.

Notably, the resin is not very caustic stable, and so for cleaning, we do recommend using acidic solutions such as phosphoric or citric acid. In case of very dirty resin, we advise additional cleaning with chaotropic agents, such as guanidine hydrochloride or urea.

I would encourage people who want to clean and re-use the resin to reach out to their local application specialist, who can help them develop a process for this.

Q Apart from AAV2 and AAV8, do you have experience with other AAV serotypes and POROS CaptureSelect AAVX?

VR: The goal for Yposkesi now is to expand this platform to a broad range of AAV serotypes. With our experience of AAV2 and AAV8 serotypes, we know that the AAVX resin is a good solution to use as a purification platform.

Currently, we are working with AAV5, 6, and 9, and the results so far are promising. We also know that we can work with modified capsids.

Q Is the resin available in a pre-packed format?

NL: Yes, we do have pre-packed formats. We have 1 and 5 mL pre-packed formats available that are compliant with standard benchtop chromatographic systems. We also have robocolumns available, at 200 µL and 600 µL, for high-throughput screening.

Of course, the resin can be purchased as bulk material and our local Field Application Specialists are happy to support customers in packing the resins in their own columns, whatever the scale.

Q Which additional washing conditions would be suitable for host cell protein and host cell DNA reduction?

VR: Several washing conditions are interesting to assess. For example, you can add an extra washing step using high salt concentration. You can also wash your column with a low pH buffer in order to remove impurities from the column before recovery of AAV in the eluate.

If you decide to implement the second washing step you have to be careful that your washing condition won't affect the integrity of your capsid. Moreover, if the washing step pH is too close to the pH of the elution buffer, a significant quantity of capsids could be eluted during your washing step, and as a result, lower your AAV yield during the elution.

Q Is the resin GMP compliant?

NL: The resin is used in GMP manufacturing by many of our customers. While not manufactured in a cGMP process, the resin is produced under an ISO 13485 environment.

When you purchase the resin, you can request the regulatory support package, including documents regarding quality, stability, production, control method, and so on. Those documents are useful when you make a product and submit a dossier to a regulatory agency.

For each of our commercially available CaptureSelect resins, we developed an ELISA assay to monitor the level of ligand leakage over the purification process.

Q Vincent, why did you use two different analytical methods during this study – ELISA for dynamic binding capacity and viral genome titers at termination during your screening?

VR: During our DBC study, a lot of fractions were collected in the flowthrough at the outlet of the column in order to calculate 10% breakthrough for AAV vectors, so we needed to use a high throughput assay for the analysis of the first full fractions. The ELISA assay allowed us to test several samples in parallel and to get the results quickly, in around half a day.

In the screening study, the number of samples was much lower – only two samples for each set of conditions screened were produced – so here we used an internal assay for the quantification of the viral genome titer in the product. The viral genome titer was determined by qPCR for each serotype.

Q Which resin can be implemented for a polishing step?

VR: Several different resins can be implemented for this step. Commonly, an anion exchanger is implemented in order to reduce host cell protein and host cell DNA. Anion exchange also has the capability to separate empty and full AAV capsids, and some suppliers have developed resins specifically for the polishing step. For more information, you can contact chromatography resin suppliers.

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BIOGRAPHIES

Vincent Ravault

DSP Expert, Process Development and Industrialization Department, Yposkesi

Vincent Ravault is a technician within the Process Development and Industrialization department at YposKesi since 2018. He focuses on upstream and downstream process development and optimization for AAVs and Lentiviruses vectors to support and advance their large-scale production for gene therapies. He is also involved in the technical transfer of processes for viral vectors from pilot scale to manufacturing scale. Graduated of a Biochemistry degree, Vincent Ravault has over 15 years of experience at Pall Life Sciences where he was in charge of the technical support for chromatography resins. His main role at Pall was to provide purification solutions and strategies to customers. He was also involved in the promotion and evaluation of new products for downstream.

Nicolas Laroudie

Staff Scientist, Field Applications, Thermo Fisher Scientific

Biochemist by education, Nicolas Laroudie used to work for Généthon, France, between 2001 and 2011 as Head of Downstream Development. He was leading a team in charge of developing and scaling-up purification processes for AAV, retroviral and lentiviral vectors

used in gene therapy treatments. He then joined Merck Millipore as a BioManufacturing Engineer where he used to technically support European customers for all DSP technologies - from clarification to sterile filtration, including TFF and systems - with a strong focus on chromatography. In particular, he took an active role in the establishment of a fully continuous, large-scale disposable DSP process for the purification of a monoclonal antibody, within the framework of a large multi-company European consortium. He eventually joined ThermoFisher Scientific in 2019 as Field Application Specialist for purification, technically supporting the implementation of POROS and CaptureSelect chromatography products for south-western European customers.

AUTHORSHIP & CONFLICT OF INTEREST

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INNOVATOR INSIGHT

Current technological trends & advancements in vector purification

Elisa Manzotti speaks to Ying Cai, Nathalie Clement, Chantelle Gaskin, Matt Roach & Ashish Saksule

YING CAI is the Sr. Director of Process Development at Ultragenyx Pharmaceutical. She heads AAV downstream process development and formulation development functions, also a CMC lead of AAV clinical programs. Prior to joining Ultragenyx, Ying worked at Sanofi, Biogen, Merck, and a few CDMOs. Ying has over 20 years' experience in the development, validation, manufacturing and commercialization of different modalities including AAV, plasmid DNA, oligonucleotides, antibodies, antibody conjugates, and fusion proteins. Ying holds a Ph.D. in Chemical Engineering from the University of Arkansas at Fayetteville and a B.S. in Biochemical Engineering from Zhejiang University in China.

NATHALIE CLEMENT has more than 25 years of experience in the field of Gene Therapy, with a strong expertise in viral vectors, specifically adeno-associated vectors, in the academic and industry settings. Her research focus has strongly been focused on optimizing processes to support large-scale production of high quality rAAV stocks and their implementation into the GMP settings. During her thesis work at the University Libre of Brussels, Belgium, she developed new recombinant viruses derived from the parvovirus Minute Virus of Mice (MVM) for cancer-selective gene therapy treatments. She then joined Dr. Michael Linden's laboratory at Mount Sinai School of Medicine, New York, where she developed novel recombinant AAV vectors and directed the AAV Vector Core. She next joined the Powell Gene Therapy Center in 2008 as the Associate Director to supervise AAV production and

testing at research, preclinical and clinical grades. She led the Process and Development Group and the Quality Control group responsible for the production and release of all AAV pre-clinical and clinical lots. During her time at UF she oversaw manufacturing, release and stability campaigns of more than 7 AAV INDs from start to finish, including CMC preparations and interactions with FDA. More recently, she spent several months at Resilience, Alachua, Florida, as the Director of Process and Development of the Viral Vaccines and Gene Therapy franchises. IN that role she oversaw viral vaccine and AAV production scales up to 200L in suspension format and in the icellis 500 platform for adherent platforms of a variety of viruses and AAV vectors. Currently Nathalie is taking a break before starting a new adventure in 2022.

CHANTELLE GASKIN is a Field Applications Scientist, specializing in protein and viral vector purification and downstream process development. She held leadership positions at Applied Genetic Technology Corporation and Brammer Bio, prior to joining the Thermo Fisher Scientific Bioproduction Division in 2020. With over 10 years of experience in gene therapy, Chantelle has accumulated comprehensive knowledge of standard industry practices and regulatory standards, applying this knowledge to advance development of therapies for a variety of indications including ocular, CNS and systemic disease.Chantelle holds a Master's degree in Chemistry from University of Florida and a Bachelor's in Chemistry from Smith College.

MATT ROACH leads the AAV Process Development group at Precision BioSciences, which is focused on designing and implementing new strategies for the production and purification of adeno-associated virus. Matt completed his Bachelor's degree in Biological Sciences at North Carolina State University and his Master's degree in Microbiology and Cell Science at the University of Florida. Prior to Precision, Matt spent time at Pfizer working on the purification of AAV and the Biomanufacturing Training and Education Center training industry professionals on downstream bioprocessing operations.

ASHISH SAKSULE is the Cell and Gene Therapy process development lead and technical expert on bioprocessing platforms for viral vectors (Lentivirus and Adeno-associated virus vector) and non-viral vectors with more than 7 years of experience. Ashish has graduate degree in Chemical Engineering from Michigan Tech University, and Biotechnology graduate degree from Harvard University. His experience spans research & drug development, clinical stage and CRO/CMO settings. Ashish is currently working at Takeda within Global Gene Therapy and have previously worked at MilliporeSigma and Miltenyi Biotec.

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Q Can you sum up the key current technological trends and advancements in AAV vector downstream processing?

YC: There are three key trends regarding AAV gene therapy. First, we want the enrichment for full AAV particles to be as high as possible. This is not only done by removing empty capsids, but also partially filled AAV, which is quite challenging. Secondly, there is a rising regulatory bar for the control of adventitious agents including viral clearance and inactivation. The third trend is manufacturing cost reduction from the clinical phase to commercial. Manufacturing cost consideration is becoming more important. We have seen high cost per dose, especially for AAV and cell therapies. Moving forward, we not only need to improve product quality, but we also need to reduce manufacturing cost per dose. Our ultimate goal is to make these drugs affordable to all patients.

MR: Somewhat unsurprisingly, we are all still working towards improved recovery and purity. There have been innovative revelations on the separation of empty and full capsids that have added to this potential solution. There has been a move to continue reducing the number of purification steps whilst also maintaining sufficient purity, especially around the harvest and capture purification steps.

NC: One substantial advancement over the past five years is the CaptureSelect™ column, or affinity capture column, specifically for AAV8 and 9. Having worked on AAV9 for more than 10 years, this was a huge change in the field. We have seen it widely implemented in downstream processes in the industry.

Another new trend is the enrichment in full capsids. There has also been an effort to develop new reagents to better remove DNA and RNA residuals. Instead of or in addition to benzonase, there are current efforts to remove some DNA species that may be more resistant.

AS: A newer trend I have seen is regarding novel variants and new serotypes. Generation and screening of libraries for AAV variants has emerged as a powerful method for identifying novel capsids. Novel capsids are emerging with numerous advancements in the construct design, and we have multiple synthetic capsid variants that can outperform their natural counterparts. These include new liver-tropic serotypes such as AAV-DJ or AAV-DJ/8, muscle-tropic AAV9MYO, or even the newer AAV7m8.

For downstream processing of this novel capsid, we are still using traditional methods, which were developed for the proteins and monoclonal antibody (mAB) space. There is a key technological need to focus on the newer novel serotypes. There are tools that are being developed specifically for AAV such as CaptureSelect™ AAVX. There are also new key players emerging who can provide custom AAV serotype-specific affinity ligands, as well as newer formats of chromatography media such as monolith or membrane adsorbers formats, which can deliver higher performance as compared to traditional resin formats.

The separation of empty and full creates a mandate for chromatography suppliers to explore new surface chemistries and methods with the goal of achieving adequate separation for all the serotypes. Until then, many of us are still relying on traditional methods such as ultracentrifugation.

Lastly, the application of fast and high-resolution analytical tools is important. Confirmation of all the results with the techniques that we work with for weeks to months is not a problem. Relying on them for day-to-day guidance, especially within process development where decisions need to be made on the spot, is a burden. That is where high-resolution and quick analytical technologies will be necessary.

CG: From the vendor side of things, I personally am looking at the column-free systems on the horizon. One example is essentially a liquid-liquid phase separation approach, based on a hydrophobic affinity reagent binding to the target molecule in the crude harvest phase. This is combined with tangential flow filtration to produce purified material. Another example is a single-use flow-path system using a chromatography resin in a recirculation flow path. The different process buffers are connected and are allowed to circulate in the flow-path along with the crude material. If they are applied at the correct time, then the purified material is eluted in a separate vessel.

Q How are current solutions helping to address the challenge of empty/full capsid separation?

MR: This is an exciting topic that has made significant progress in the last few years. Companies are moving towards designing platforms for AAV. It has become more apparent just how different various AAV capsids are from each other. Additionally, you need to account for the differences in production systems, heterogeneity of viral proteins, and heterogeneity of packaging, which can be a challenge.

The good news is that many groups are tackling this. We have seen an increase in the number of resin and column manufacturers providing specific solutions to empty/full capsid separation. Four years ago, vendors had no specific solutions, only general recommendations and examples of model proteins, like BSA being separated with an anion-exchange resin. We have seen a large increase in the number of vendors approaching us personally with initial methods that have been tested for AAV. It is pretty promising.

There is still a large space to be explored regarding additives. We are seeing that start to develop, and it is promising that people are willing to share that information.

CG: From a regulatory perspective, people are finding they want to get ahead of the bar being raised, as there are not many regulatory guidelines yet. Like Matt said, it is very interesting how people are willing to share methods. A handful of papers and posters published in 2021 have tackled the subject. I have seen the use of divalent salts and other additives to modulate the retention times between the two species, so that you could get baseline separation and even proceed to step gradients in some cases. We have made some advancements, but it is still challenging.

NC: I would like to emphasize the challenge of separating full and empty capsids. The innate nature of these capsids is that the isoelectric point (pI) is so close and requires a specific method. This is why chromatography methods have been slow in becoming efficient,

though we have seen progress here. Successful separation may end up being very product and platform specific. We will be able to develop standard protocols, but we need to keep in mind that each product will be different. The percentage of empty in a harvest is affected by the AAV construct itself: the length of the genome and the sequence of the genome. It is also affected by the type of helper being used, such as a *rep/cap* helper or adenovirus.

AS: We still need more technological advancements in platforms that can be applied for multiple serotypes. Right now, it is time-consuming, and we need to develop a process individually for each serotype. If we are working with 10 different targets, a lot of hours and resources are spent developing a process for the individual serotypes.

There are technical difficulties and challenges existing specifically around elution. For example, the close similarity of elution conditions leaves the separation vulnerable. The variation in temperature, buffer formulation or lot-to-lot differences among the buffers, the chromatography media, or the AAV feed material itself, can add a lot of challenges. Even small variations can compromise the separation and recovery of AAV.

Empty capsids are reported to have some beneficial effects, under certain conditions, based on their immunological similarity. Empty capsids can act as an effective decoy to reduce the neutralization of AAV vectors by pre-existing antibodies, thus increasing the target tissue transduction following systemic administration. We need to find out exactly how much percent empty and full AAV are beneficial, and whether we should focus on removing all the empty particles. We must balance both sides of the separation, and this will be useful for systemic administration.

YC: Empty/full separation is based on small differences in the pI. Recently, in the October issue of *Cell and Gene Therapy Insights*, my team published a paper using capillary isoelectric focusing to explore this. We demonstrate that measured pH is different from calculated pI; there is also a heterogenous species of different charge profile ranking between 6.2 to 7.0, which is very different from the theoretical pI being reported: 5.9 to 6.3.

This is caused by several factors including the capsid post translational modifications (PTM), not just the length and sequence of the genome. Certain PTMs can shift the charge profile drastically. This is also highlighted during this forced degradation study, where the shifting of the charge profile is visible. The heterogeneity of charge profiles are observed in different products, as well as the same product of different origin (clone, serotype, or different upstream conditions).

Right now, it is more of an art than a science, as we do not understand all the root causes contributing to this charge profile heterogeneity. Mixed mode chromatography will become interesting to apply to this field. We are also exploring gradient separation, and how can we apply it to the industry, but the challenge from the GMP environment is whether it is possible for it to become single use.

Q If you were starting a new process development initiative today, would you recommend utilizing ultracentrifugation or would you bank on chromatography techniques or other new technologies to enrich full capsids?

MR: We have been through this at Precision, and our choice was to go with chromatography. We have devoted effort on the process development side, which has been no small feat. As you get to the later stages of a clinical trial, you think more about comparability. When you are transitioning from phase I/II to phase III, you are going to modify your initial production process. If this is a chromatography method, which it more than likely is for commercial production, it is better to start early. You are likely going to get a worst-case scenario for your percent full at the beginning, but it is ideal to build it early and then improve upon it. That being said, you must hit certain metrics. I would not recommend doing that if you are 10 to 20% full, for example. As you approach higher than 40% full, you are probably in a good state to switch over to chromatography.

Q What are the chief implications of residual testing, for example in terms of cost and time, and what are the keys to optimizing this aspect?

NC: Measuring residuals, whether DNA, protein, or product or process derived, has become a very hot topic over the past few years. In my opinion, there are two reasons. The technology has advanced tremendously, so all the testing has become more sensitive and more accurate, for example ddPCR, next generation sequencing, and RNA sequencing. In parallel to the technology improvements in the assay itself, the clinical doses have dramatically increased, mostly because of the type of indication treated. With higher doses in the clinic, there comes a higher burden of residuals, and therefore a need to better determine the amount and the type of residuals. We have seen toxicity in humans during the course of several trials, furthering the importance of measuring residuals.

One of the keys to success in residual testing is to determine the type of residuals you are going to face in your own platform with your own product. Separate the ones that are very common to every single product and platform like host cell DNA and host cell protein. CMOs have invested in the field, and they are going to be able to offer assays that have been, to some extent at least, validated and standardized.

Spend more time looking at what is going to be specific to your product. If you are using a specific helper virus, for example baculovirus or HSV, you are going to require different types of assays. Keep in mind that developing specific assays for your product could be more time consuming and therefore more expensive, so take this task on early. You also need a well-defined clinical dosing regimen early on. It is sometimes difficult to think about the clinic if you are just about to start screening candidates. However, the importance of your residuals will be impacted by the dose you are going to choose, the route of administration, and also the localization of your administration. Being in the eye, the liver, or being systemic will present very different impacts.

There is still a significant need for improving the technologies, mostly for accuracy and consistency across various products. It is becoming more important to know that the techniques developed are validated across multiple Investigational New Drugs (INDs), so you can have a

basis for comparability between a product that may show some toxicity or immune reaction in patients and another product that would not. Moving the field towards sharing and standardizing more is critical for everyone, especially in terms of residuals.

AS: There are a lot of guidelines available for residual testing. For example, with cell-substrate DNA, we want to have less than 10 nanograms per dosage with a median DNA size of 200 bp or lower. From a process development point of view, these guidelines need to be addressed by establishing process optimization strategies when the residual host cell DNA is present as a nuclease-sensitive process-related impurity. One of the challenges is that there is residual nuclease-resistant host-cell DNA that has been packaged within the AAV capsid.

For process optimization, based on the close similarity with the desired vector product, it is difficult to eliminate the AAV package host cell DNA impurities by regular vector purification methods. The separation of AAV particles based on density or by gradient ultracentrifugation can remove the AAV package nucleic acid impurity, as they can differ significantly in length from the vector genome based on different densities of the respective particles. In addition to chromatography, gradient ultracentrifugation has been shown to improve this to four to five-fold. This again can represent a scalability challenge when we move into large clinical programs.

CG: One of the more critical aspects of assay development is getting hold of representative reference standards, which the downstream process development team is usually responsible for. It can pose a bit of a problem because before a process is finalized, you must start assay development; it must be developed concurrently with the process development. This means the process that you use to make any reference standards for assay development might not be your final process.

It can be helpful for a sponsor company to establish your formulation buffer early on during the process development. This allows you to use a standard platform column chromatography, or even an affinity chromatography step, then buffer exchange your material into your final formulation buffer, to serve as a surrogate for your reference standard in the interim.

YC: We should always push for improving methods and the manufacturing process. We are always being asked for the residual specifications from a safety perspective as early as possible, but we are reluctant to set the specifications based on very early data. It is equally important to demonstrate impurity clearance as early as possible, by designing scale-down studies to analyze impurities that could introduce certain safety concerns with higher doses. Overall, the keys are demonstrating testing, improving methods, and also demonstrating the process capability for downstream operations as early as possible.

MR: To echo what other panelists have said, an interesting way to approach this is to prioritize testing for final material studies, supply and animal studies, initial animal studies, then compare to confirmation runs as the process improves as a check. These tests, especially if they are outsourced, can get quite expensive. Designing specific Design of Experiments (DOEs) carefully around key steps like the harvest process and in various buffer conditions for possible chromatography steps is important.

Q Is there any trend of companies being more open with sharing critical quality attributes (CQAs) and residuals information, to better understand how products are affecting patients?

CG: In industry, there is a sense of keeping CQAs and other material information close to the chest. However, there is a very slow-moving trend towards being more open with data on reduction of host cell protein and other types of residuals.

NC: Being able to see data, especially on residuals, would be critical to the field. I do agree with Chantelle that there is a trend there, but we are far from being where we should be. I hope that the FDA will push towards sharing this information, because this is exactly how we are going to understand the role of residuals and their toxicity, if any, in a human body.

Turning to adventitious agent inactivation, removal, and viral clearance – what is the current state of the art?

YC: Currently, we inactivate and remove adventitious agents through more traditional approaches. For example, inactivation is typically done through heat, detergent, or lower pH. The removal process typically uses different chromatography modes, including affinity-based modes to find the protein, and different anion exchange steps during separation.

With AAV, we need to be careful when selecting viral filters. Viral clearance is dependent on the manufacturing platform being used. In the early clinical stages, it is possible to get away with not executing viral clearance, especially if you have a low-risk manufacturing process. This is a small part of the control strategy, and you can still test your raw materials, cell bank, starting material, or seed bank. If we have a high-risk process using adenovirus or helper virus, then removal needs to be demonstrated with a viral clearance study, as well as inactivation. If you do not have a key inactivation step, then it can be difficult to add during a later clinical phase.

Due to the rising regulatory bar, I recommend thinking about what the risks of your process are. Also, justify the choice of your model virus. For example, AAV is relatively small, so consider the smallest model virus you want to use. We are not currently using very small viruses; we are not trying to use MNV yet, although we have tried SV40, which has been quite successful.

MR: Having worked on later stage projects, this should be dealt with earlier rather than later. If you do not have something like detergent or an inactivation step built in, it can be quite disruptive to the process to add later. Otherwise, the general steps that people are going through – affinity purification and anion exchange – will help in providing the appropriate log removal values. There may be slight modifications, like low pH holds, that can be added to achieve sufficient viral clearance. It is important to rely on the quality organization within companies as well and have robust raw and starting material qualification.

NC: Focusing on testing your raw material and your cell banks early on is critical. Viral clearance, as Ying said, is not required for phase I or II, so it may not prevent you moving to the clinic, but it is still something to consider. An issue I faced myself is when you are using a virus as a helper, like HSV in my case, testing the raw material, your HSV stock, is a challenge

in itself because you may get false positives. This makes the development of your adventitious assay a little more complicated.

CG: I have seen the introduction of older technologies like viral reduction filters specifically designed for the removal of larger viruses. This has been adopted in some processes and has worked really well with high recovery.

Q Chantelle, regarding a previous answer: did you observe high aggregation levels in your process intermediates, and what did you include to reduce or remove aggregation? Could you find a good purification solution?

CG: There are certainly some serotypes that have more of a tendency to aggregate, such as AAV2. Some other novel capsids might have some aggregation problems depending on the buffer background. I have worked with a few processes where aggregation was alleviated by adding different excipients throughout the process. Intermediates can suffer aggregation because sometimes you need to include longer hold times between unit operations.

If you have an unstable intermediate product, I recommend looking into either non-ionic detergents or potentially different amino acids in a small-scale screening study. Including a stability study early on during process development allows you to get an idea of what your stability really is.

Q What issues can a lack of serotype-specific technologies present to process development, and what solutions are available?

AS: Unfortunately, due to current lack of serotype specific technology, the approaches that we are using are still traditional methods such as cesium chloride gradient or iodixanol gradient ultracentrifugation combined with filtration technologies. One benefit of this is that we can distinguish our serotypes based on the physical characteristics versus chemical characteristics.

It is easy to develop a process based on the physical characteristics of AAV, because regardless of the serotypes and the capsid differences, we still see similar physical characteristics. Due to this, we can utilize many filtration-based technologies, making process development easier. We can adapt the process based on the physical characteristics of the viruses. On the other hand, if we consider chemical properties, we see multiple differences. As an industry, we need to continuously work on bringing new technologies that can address multiple serotypes and novel variants.

YC: For AAV we have some choices for accommodating different serotypes. We need more publications regarding the fundamental mechanism, regarding which part of the serotype the peptides are binding to associated with the resin. There is continual work to do around developing the technology and working closely with resin manufacturers and vendors.

This includes possible work on the isolation of certain peptides or antibodies which have more specificity to the serotype the company is using.

NC: If you are lucky enough that your serotype or capsid variant works well on any of the current tools, specifically the affinity resins that are available, such as AVB or CaptureSelect™ resins, there is no problem with using the same method for each serotype you have. The exception is the possibility for cross contamination that needs to be assessed once you are in the clinical environment.

MR: We have taken the approach where we have a whole platform, then we test a given serotype as it comes through. We may have to modify a portion of the platform, but the rest will ideally stay intact. However, something like empty/full separation may have to be modified significantly. The various serotypes we have tested to date fall in a few buckets depending on their homology. They may need slightly different buffer conditions, or slightly different load conditions. These options for manufacturing make things easier when novel serotypes come through.

CG: Putting the time in to design a high-throughput screening experiment usually gets overlooked. Often, people want to brute force through small-scale experiments using 1 or 5 ml columns. Static mode small-scale screening tools can be useful in this case, to give good data early on in your process run. I would encourage people not to shy away from doing something like that.

AFFILIATIONS

Ying Cai, PhD
Senior Director of Process Development, Ultragenyx Pharmaceutical

Nathalie Clement
Unicorn Consultations, Formerly Resilience

Chantelle Gaskin
Field Applications Scientist, Thermo Fisher Scientific Bioproduction Division

Matt Roach
AAV Process Development Team Leader, Precision BioSciences

Ashish Saksule
Cell and Gene Therapy Process Development Lead, Takeda

AUTHORSHIP & CONFLICT OF INTEREST

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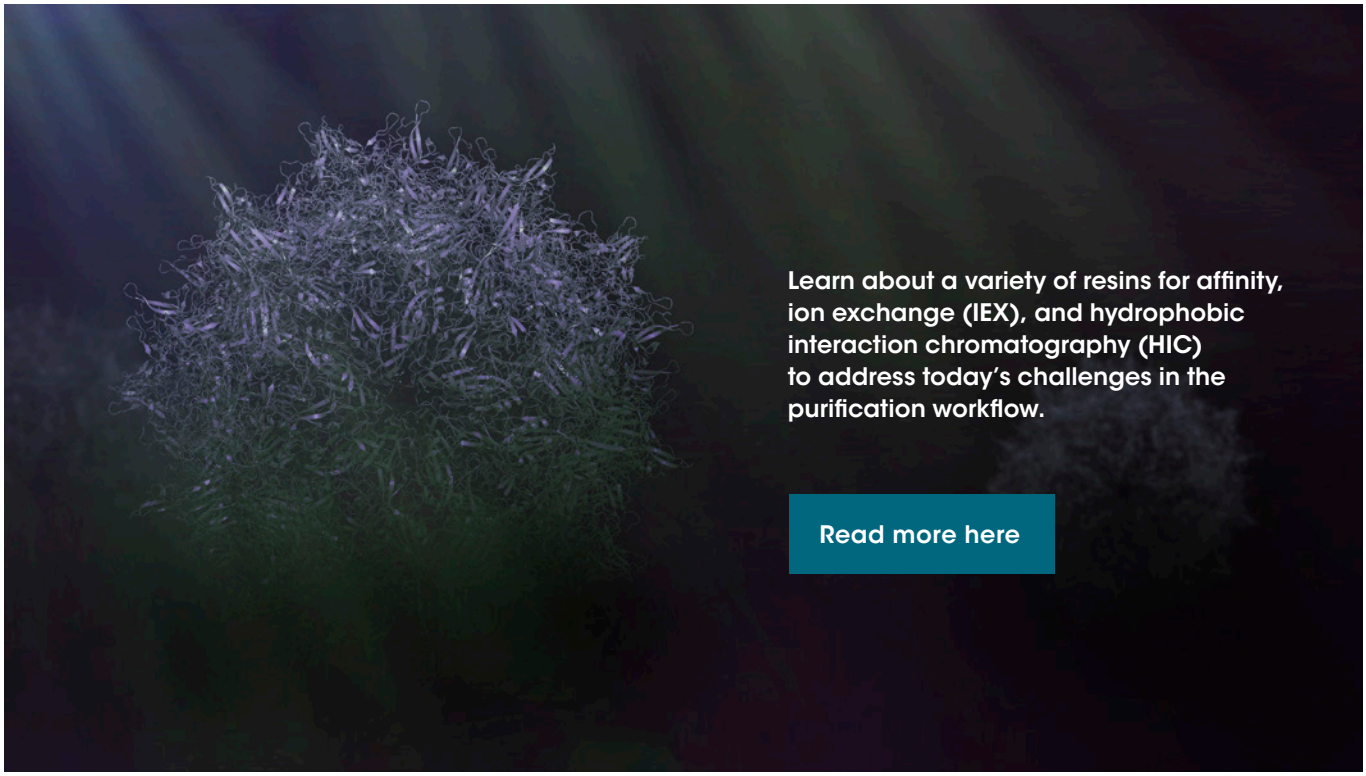

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