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Optimizing Gene Therapy

A comprehensive guide to AAV Purification

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Foreword

We are at the forefront of a gene therapy revolution, with over 30 groundbreaking treatments for a wide range of conditions already approved by the FDA. Central to these advancements are adeno-associated virus (AAV) vectors, which have proven to be highly effective and versatile in therapeutic applications.

However, as the demand for AAV-based therapies grows, so does the need for scalable production methods. Meeting this demand requires a profound optimization of manufacturing processes to ensure efficiency and scalability, while still maintaining high quality, purity and potency.

Through a comprehensive exploration of current methodologies, technological advancements and industry best practices, this eBook aims to equip readers, whether clinician, researcher or industry professional, with a deeper understanding of the obstacles and opportunities in AAV production.

Contents

The importance of AAV purification and analytics 3

Viral vectors in gene therapy: Innovations and simplified solutions 6

AAV purification trends and techniques: Ask the experts 7

Harnessing CDMOs for optimized AAV production: ask the expert 13

Top 5 benefits of affinity chromatography for AAV purification 16

Viral clearance in a downstream AAV process: Case study using a model virual panel and noninfectious surrogate 18

Productivity optimization and process calculations for AAV affinity chromatography 25

Optimizing AAV purification for gene therapy manufacturing 34

Resources & digital tools 35

The Importance of AAV Purification and Analytics

Introduction

The rise of gene therapies marks a radical shift from traditional treatment approaches by addressing the underlying genetic causes of a disease rather than merely treating the symptoms. Initially conceived in the 1970s, gene therapy has rapidly evolved with the advent of more precise and safer delivery methods, leading to several breakthroughs in clinical applications. With the approval of over 30 gene therapies by regulatory agencies worldwide, the field has transitioned from experimental to therapeutic, offering hope for conditions once deemed untreatable.[1]

A key component of gene therapy is the efficient delivery of therapeutic genes into host cells. Viral vectors are often chosen due to their natural capability to efficiently infect human cells. These vectors are derived from viruses like adenovirus, lentivirus and adeno-associated virus (AAV) that have been engineered to be safe and effective. AAVs are one of the most promising vectors in gene therapy due to their ability to provide long-lasting gene expression, target specific cell types and exhibit relatively low immunogenicity.[2]

Large-scale production of AAV-based treatments relies on robust purification and analytical methods to ensure the consistency, potency and safety of the final product. A primary concern is the removal of impurities, such as residual host cell proteins, DNA and empty capsids, which can compromise the efficacy and safety of gene therapies. However, current methods are often difficult to scale, time-consuming and limited to specific AAV serotypes.

As the number of approved AAV-based therapies and clinical trials continues to grow, there is an increasing demand for rigorous quality control measures and improved purification techniques.[3] Continued development and refinement of purification and analytical technologies are therefore pivotal in advancing gene therapy, ensuring that these innovative treatments can safely and effectively reach the patients who need them.

This article explores the challenges associated with large-scale AAV purification and the latest advancements in purification techniques and analytical methods.

Challenges of AAV purification

The production of AAV vectors follows a complex workflow, beginning with vector design and cloning, where the therapeutic gene of interest is inserted into an AAV plasmid vector. This vector is then transfected into host cells, typically HEK293 cells.[4] Following transfection, the cells are cultured under specific conditions to produce the AAV particles. After an incubation period, the cells are harvested and lysed to release the AAV particles. The crude lysate then undergoes several purification steps to isolate and concentrate the AAV particles, followed by formulation into a final product suitable for clinical use (Figure 1).

Purifying AAVs is a critical step in the production process, ensuring that the final product is effective and safe for

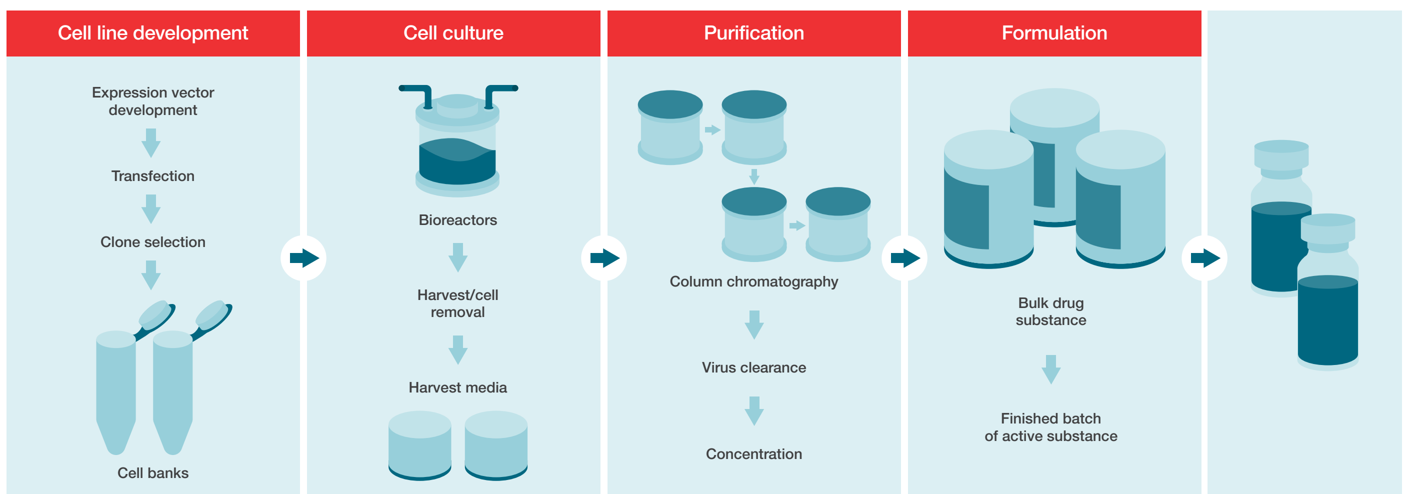

Figure 1. AAV manufacturing process for gene therapy products.

therapeutic use. The primary goal of purification is to remove impurities such as host cell proteins, DNA and other contaminants while maximizing the yield and potency of the AAV vectors. A range of techniques can be used for purification, including centrifugation and chromatography.

Downstream purification of AAV vectors presents several key challenges that significantly impact the yield and efficacy of the final product. One major issue is the increased impurity burden due to cell lysis, which releases a substantial amount of host cell proteins, adventitious viruses and other debris into the crude lysate. However, complex purification processes aimed at removing these impurities often lead to substantial loss of viral particles. Thus, improved purification techniques are key to balance efficient purification with a good recovery yield.

The large variety of AAV serotypes also complicates the purification process. AAV vectors come in many different serotypes, each with distinct capsid proteins and surface properties.[5] This diversity creates challenges in developing a standardized purification process, as different serotypes may require specific conditions or methods for optimal purification. Tailoring purification protocols to accommodate the variety of AAV serotypes can add complexity and cost to the production process.

Perhaps the most difficult challenge is the enrichment of full capsids. The production process often results in a mix of full, empty and partially filled capsids. Full capsids are crucial for delivering the therapeutic gene, while partial and empty capsids can dilute potency and increase the total viral load needed for effective treatment. Moreover, empty capsids can trigger immune responses, compromising transduction efficiency and limiting the potential for repeat dosing due to the development of neutralizing antibodies.[6] Hence, advanced separation techniques are needed to enrich the full capsids effectively.

Analytical techniques in AAV purification

Analytical techniques are vital for accurately assessing critical quality attributes (CQAs), such as the purity, potency and safety of AAV products. Several CQAs are essential for characterizing AAV products, including (i) virus titer, (ii) capsid aggregation and (iii) the ratio of full to empty viral particles (Figure 2).[7] Several analytical methods are employed to assess these attributes, each offering unique insights into the quality of AAV products.

For example, analytical ultracentrifugation (AUC) is widely regarded as the gold standard for determining the ratios of full, partial and empty capsids, in addition to providing detailed aggregation profiles. Viral titer and content ratios can be measured using a combination of quantitative PCR (qPCR) and enzyme-linked immunosorbent assay (ELISA), where qPCR measures the viral genome and ELISA quantifies capsid protein content. Additionally, transmission electron microscopy (TEM) can provide detailed visualization of AAV particles, allowing for the evaluation of capsid integrity, aggregation and morphology.

To date, manufacturing purification methods for AAV vectors typically rely on a sequence of chromatography techniques due to their ease of scalability. The process generally begins with affinity chromatography which purifies the AAV particles from crude mixtures. In this stage, specific ligands within the affinity column are engineered to selectively bind to AAV capsid proteins. As the mixture flows through the column, AAV particles are captured by these ligands, while impurities are allowed to pass through, resulting in a significantly purified AAV product. This product can then be further refined through additional chromatography techniques, such as anion-exchange high-performance liquid chromatography (HPLC). Anion-exchange chromatography (AEC) is particularly effective in enriching full capsids based on their distinct charge properties, thus enhancing the overall quality and consistency of the final AAV preparation.

However, these techniques come with various challenges.[7,8] Many of them face issues related to scalability and throughput, which are crucial for large-scale production. For instance, TEM and AUC are labor-intensive and have long turnaround times, making them less suitable for high-throughput analysis. Techniques like qPCR and ELISA, while highly sensitive, can

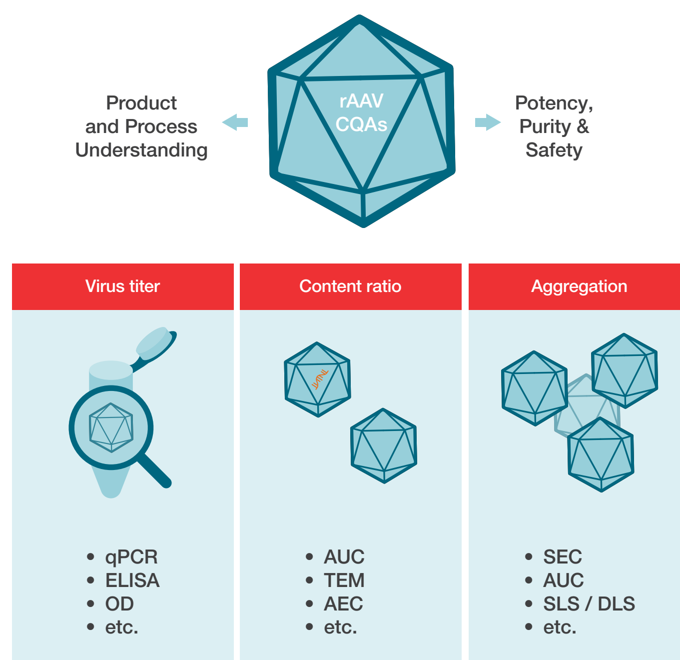

Figure 2. Several techniques can be used to measure the potency, purity and safety of AAV therapies including optical density (OD), size exclusion chromatography (SEC) and static/dynamic light scattering (SLS/DLS).

only quantify limited serotypes and often require multiple steps, increasing the complexity and time required for analysis. Anion-exchange HPLC, although effective in separating AAV particles, can be hampered by resins with small pores, limited binding capacities and long turnaround times, leading to suboptimal performance in large-scale operations.

Hence, developing more robust and streamlined analytical workflows is essential to support the growing demand for AAV-based therapies. By addressing these limitations, the gene therapy field can achieve more efficient production and quality control, ultimately leading to safer and more effective treatments for patients.

Trends and innovations

As the field of AAV-mediated gene therapy progresses, there is an increasing need for highly scalable methods for AAV purification. As a result, one-step affinity chromatography protocols have become attractive for accelerating the purification of viral vectors while meeting good manufacturing practice (GMP) requirements.[9]

HPLC-based affinity chromatography is quick and efficient, enabling the determination of both capsid titer and content ratio. With a quick run time and no need for manual sample handling or pretreatment steps, the process is ideal for rapid, high-throughput analysis in both research and production environments.[10] However, the specificity that makes affinity chromatography effective also presents a notable drawback. Most ligands are designed to capture only one or a few AAV serotypes, meaning that any change in the virus particle often necessitates a new capture ligand. This can be time-consuming and costly, hindering the flexibility needed for platform production processes.

As a result, affinity columns based on AAV-specific camelid antibodies have started to dominate the field. These resins can target a broad range of natural and synthetic AAV serotypes, significantly simplifying the purification process across various vector types.[11,12] Additionally, they have very high binding capacities, demonstrate robust viral clearance and are stable against harsh clean-in-place and regeneration methods, making them suitable for repeated use.[12,13] Similar advances in AEC are also contributing to more effective polishing steps, resulting in the enrichment of full capsids by 90% in some cases.[14]

These trends and innovations are pivotal in meeting the growing demand for AAV-based therapies, facilitating the production of safe and effective treatments on a larger scale. As the field continues to evolve, these advancements will likely play an integral role in the future of gene therapy, driving further improvements in both efficiency and product quality.

Future directions

As the number of gene therapy programs advancing to the clinical phase and commercialization continues to rise, the optimization of large-scale AAV production is essential for the future of the field. Achieving this requires analytical tools and methods capable of providing rapid and accurate assessments of sample purity at increasing scales.

Innovations in purification techniques, particularly in chromatography, are paving the way for more efficient and effective viral vector purification. Future developments will likely focus on optimizing these methods, integrating advanced analytical tools and leveraging automation to enhance consistency and scalability. Overall, continued advancements in viral vector purification will be essential to meet the growing needs of the gene therapy field and bring innovative treatments to patients worldwide.

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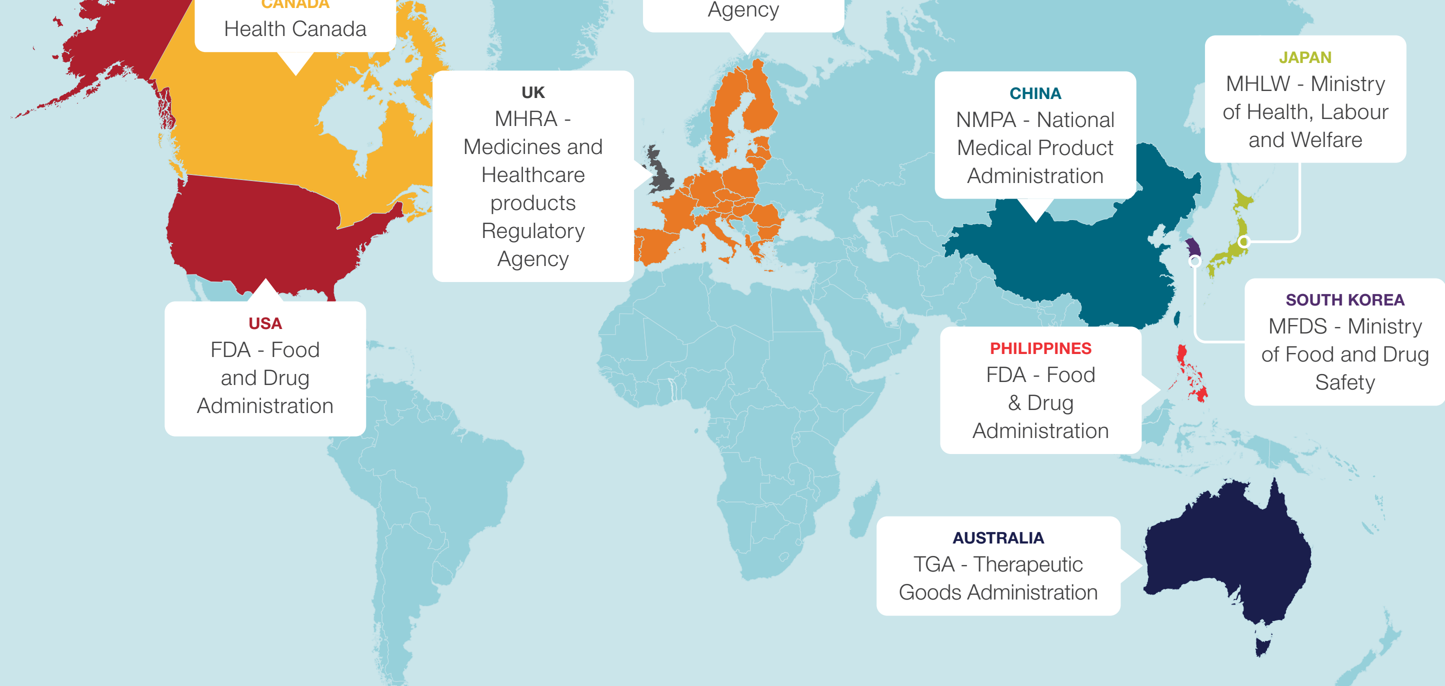
Viral Vectors in Gene Therapy: Innovations and Simplified Solutions

Gene therapy involves the introduction of specific genetic material into a patient to alter and improve cell function. Recent advancements have resulted in over 30 approved cell and gene therapies worldwide, addressing a variety of conditions ranging from congenital disorders to solid cancers.[1,2]

These breakthroughs have been possible thanks to the development of sophisticated delivery systems and the refinement of gene-editing technologies. Innovations such as viral vectors, including adeno-associated viruses (AAVs), have enabled precise delivery of genetic material to the target cells, enhancing the efficacy and safety of treatments.

This infographic explores current trends, delivery mechanisms and manufacturing challenges in gene therapy.

Key government agencies overseeing therapy approvals

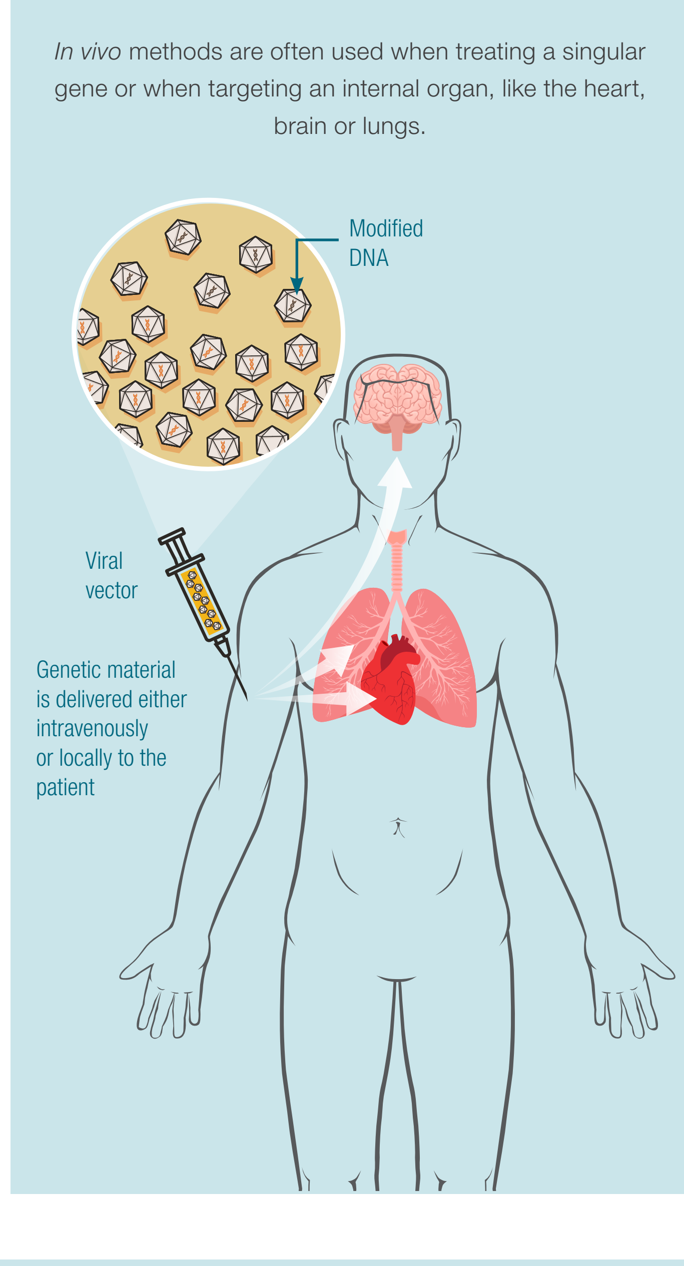


Number of gene therapies approved worldwide[3]

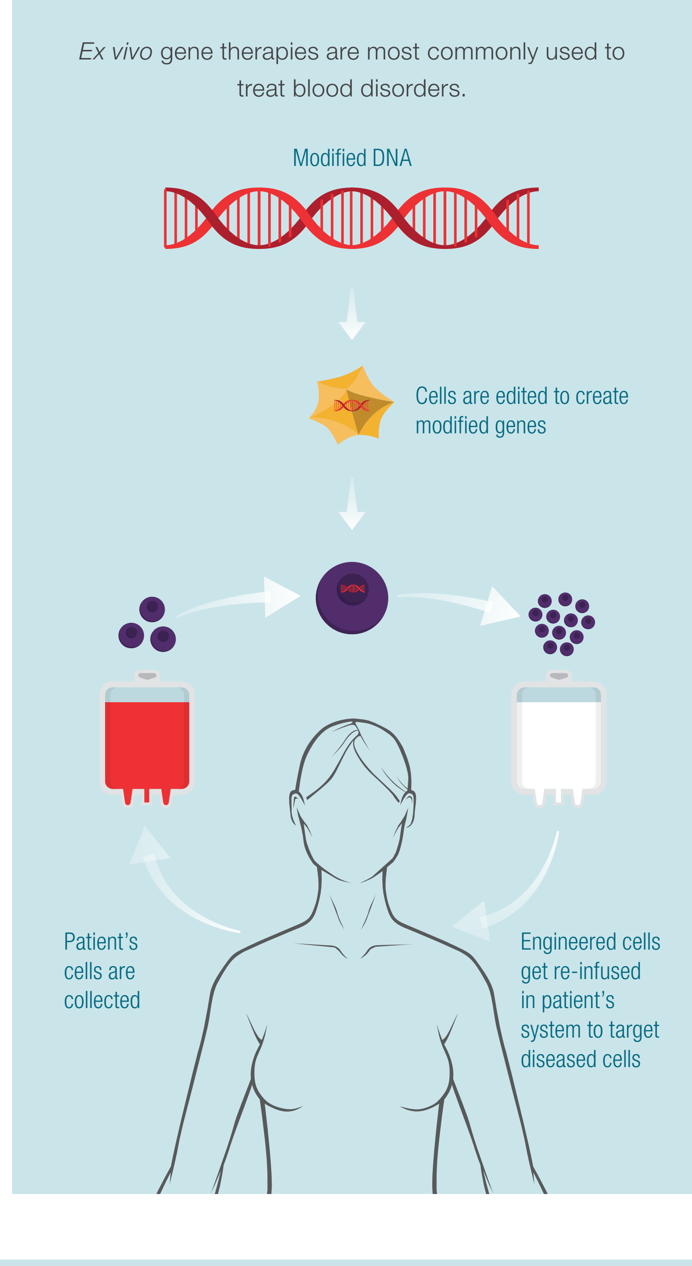
The two paths to gene therapy

There are two main ways to deliver gene therapy: *ex vivo* and *in vivo*. Each method offers its own set of benefits and considerations.

In vivo methods are often used when treating a singular gene or when targeting an internal organ, like the heart, brain or lungs.



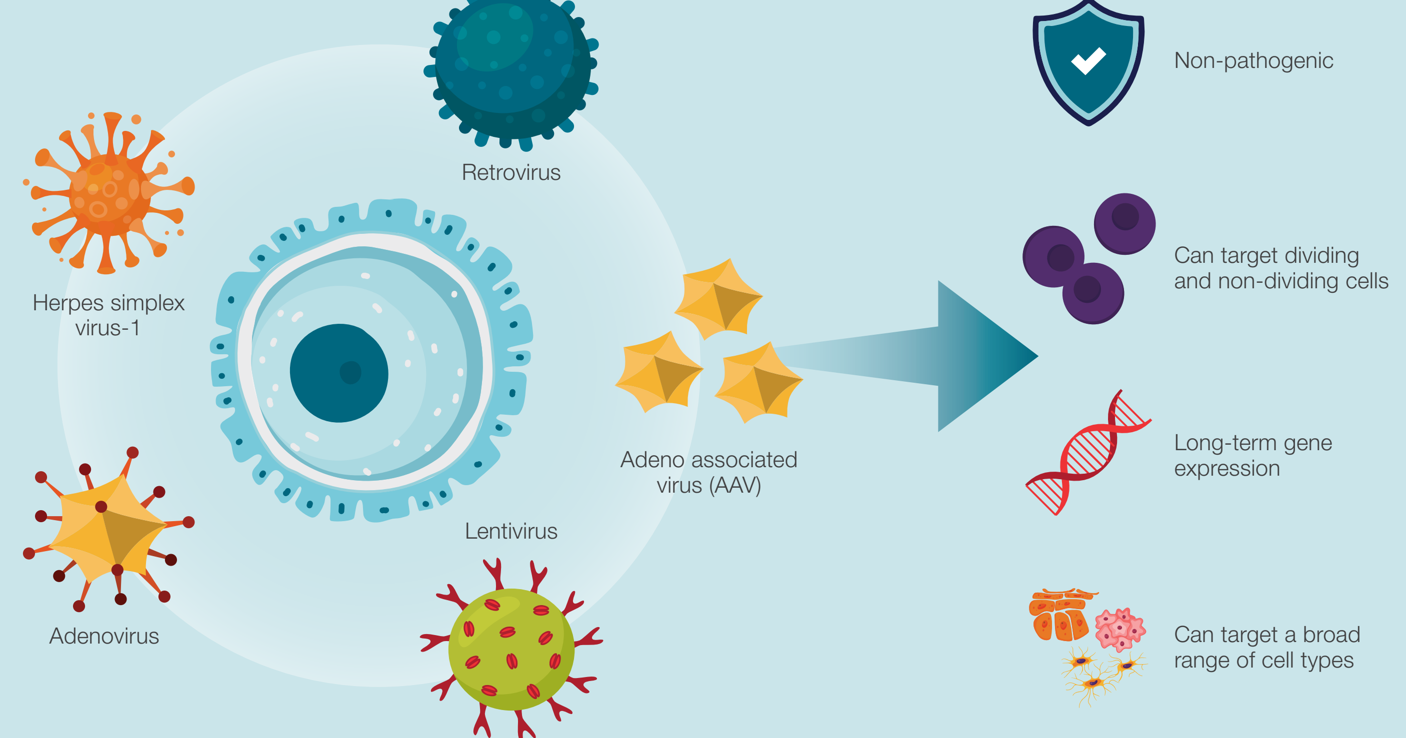
Ex vivo gene therapies are most commonly used to treat blood disorders.



Enabling efficient gene delivery

The plasma membrane acts as a barrier to large molecules, meaning that specialized methods are needed to ensure the genetic material can enter the cells effectively.[3]

The vast majority of gene therapies use viral vectors, as they are remarkably efficient at gene delivery.[4] There are different types of viral vectors available; however, AAV vectors are often chosen due to their efficiency, low risk of insertional mutagenesis and long-term gene expression.

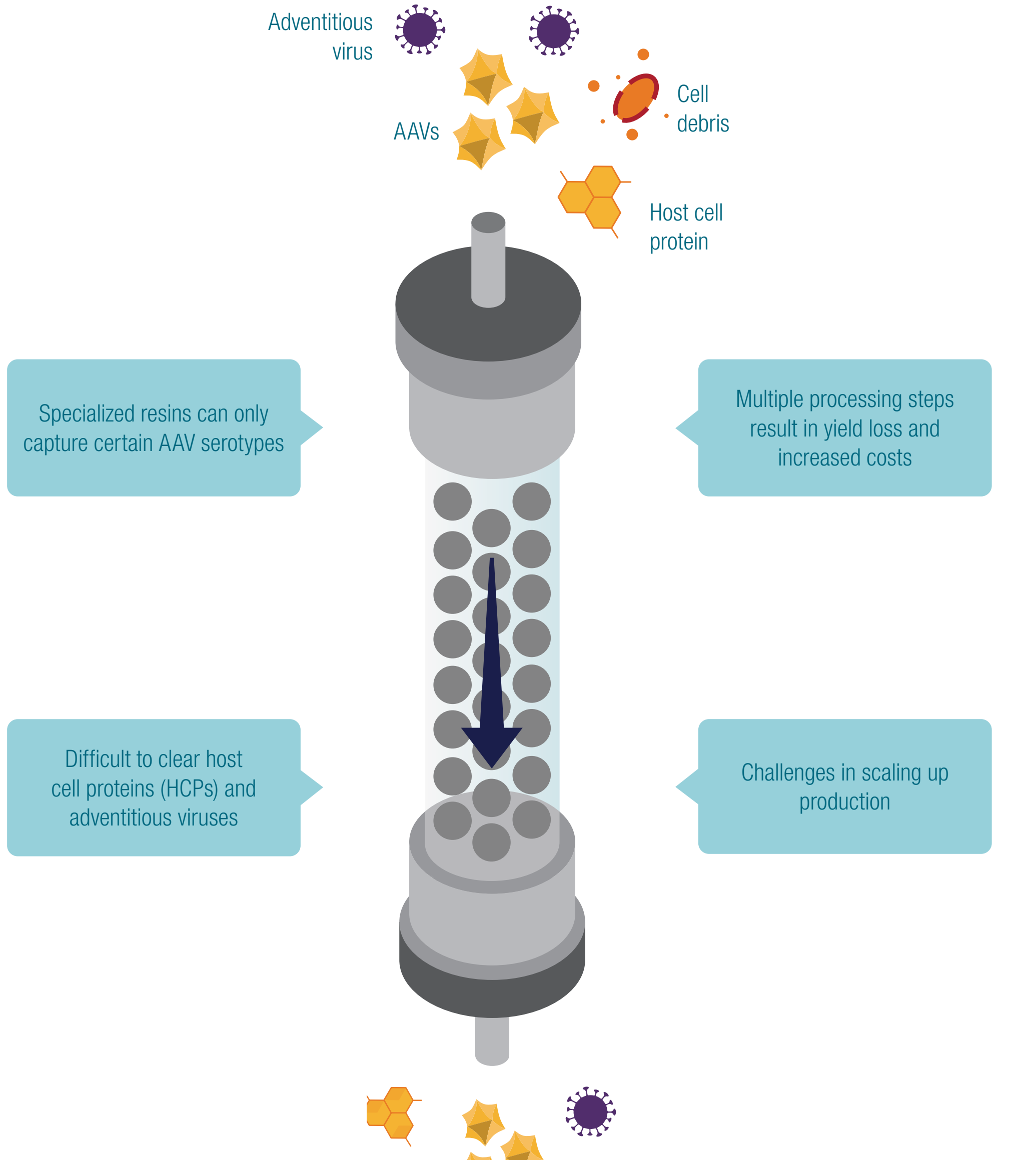


AAV purification is a critical yet challenging step

The first stage in AAV production involves the expansion of viral producer cells in culture, prior to their transfection with one or more AAV-encoding plasmids. Following transfection, the cells are broken down and the lysate is harvested for AAV purification.

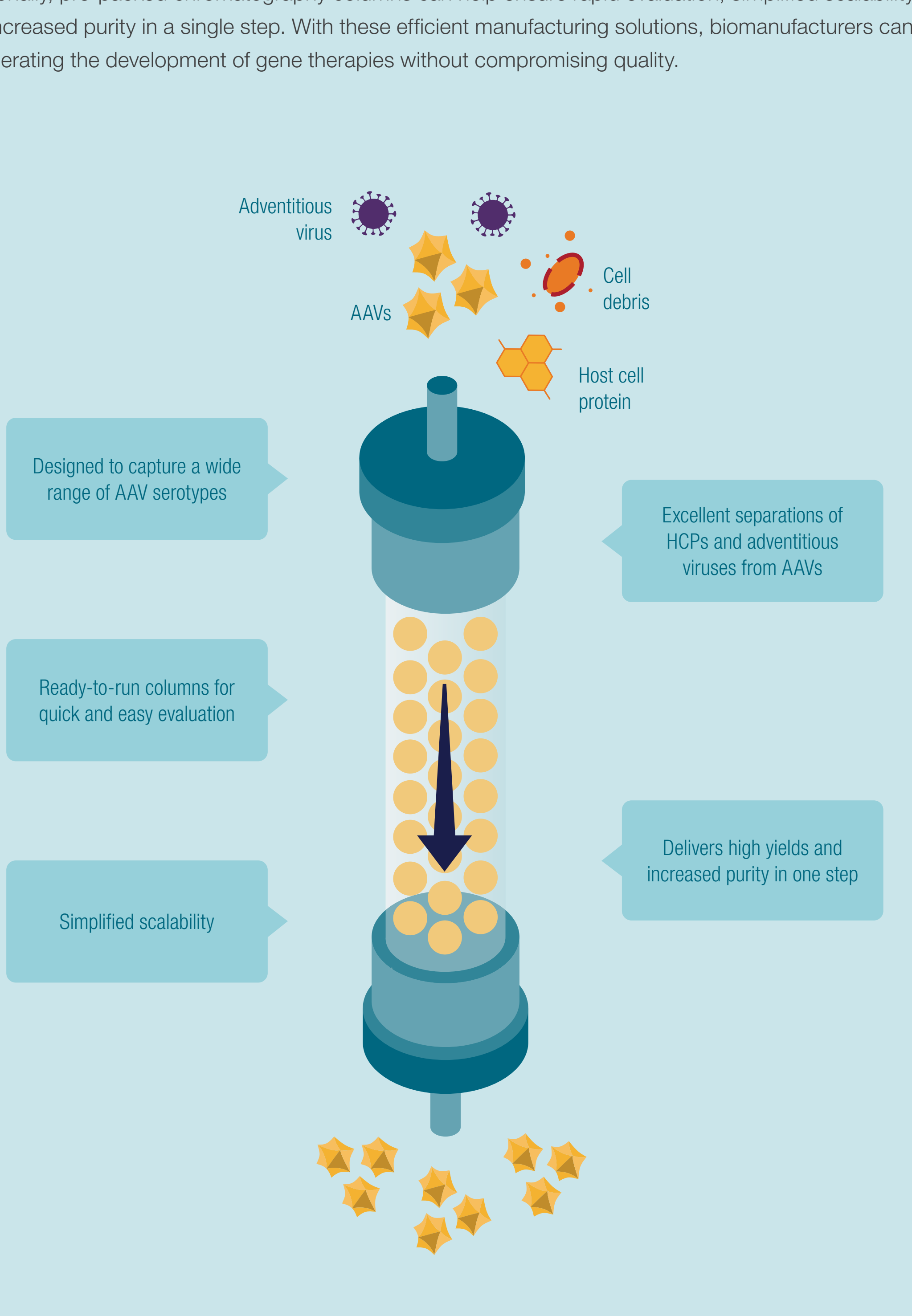
However, AAV purification is associated with several challenges:

Disadvantages of traditional affinity chromatography resins



Enhanced AAV purification with targeted solutions

Thermo Scientific™ POROS™ CaptureSelect™ resins are engineered to meet the diverse needs of AAV purification across multiple serotypes. Additionally, pre-packed chromatography columns can help ensure rapid evaluation, simplified scalability and deliver high yields with increased purity in a single step. With these efficient manufacturing solutions, biomanufacturers can streamline AAV production, accelerating the development of gene therapies without compromising quality.



The future is in our genes

Gene therapies are transforming the future of medicine. As we advance our understanding and technology, these therapies are expected to become more precise, effective and accessible. Thermo Fisher is at the forefront of this evolution, providing innovative tools and solutions that help streamline the development and production of gene therapies. By enhancing purification techniques, Thermo Fisher is working to pave the way for safer, more effective treatments that can improve patient outcomes and redefine the future of personalized medicine.

AAV-MAX Transfection Kit

Prepacked Chromatography Columns

CaptureSelect and POROS Chromatography Resins

Analytical Testing Solutions

Simplify your **AAV Purification Process**

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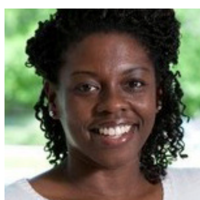
AAV Purification Trends and Techniques: Ask the Experts

Experts

Alejandro Becerra

Principal Scientist and Global Technical Lead for Purification Products, Thermo Fisher Scientific

Dr. Alejandro Becerra is a Principal Applications Scientist and Global Purification Technical Lead. Alejandro has over 15 years of experience in downstream processing and customer support having worked as Purification Team Manager and other bioprocess engineering roles prior to joining Thermo Fisher Scientific in 2018. Dr. Becerra is a subject matter expert in preparative chromatography with expertise in the development, optimization and scale-up of antibodies, recombinant proteins, viral vectors, and nucleic acid purification processes. Alejandro holds a Ph.D. in Chemical Engineering from Cornell University.



Chantelle Gaskin

Field Applications Scientist, Purification Business Unit, Thermo Fisher Scientific

Chantelle 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 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 the development of therapies for a variety of indications including ocular, CNS and systemic disease. Chantelle holds a Master's degree in Chemistry from the University of Florida and a Bachelor's in Chemistry from Smith College.

Adeno-associated viral (AAV) vectors are an increasingly popular choice for gene therapies; however, a major bottleneck in the production of AAVs is their efficient purification. A broad range of serotypes, residual contaminants and a disparity in genetic filling increases the complexity of purification processes. These factors can affect the consistency and quality of the final product, making it challenging to achieve the high purity levels required for safe and effective therapies.

We asked two in-house specialists at Thermo Fisher Scientific for their advice on overcoming AAV purification challenges and implementing innovative strategies to enhance the efficiency and reliability of AAV purification.

Q: What are the current trends in AAV purification? What challenges do process development scientists typically face in this process?

Alejandro Becerra (AB): The field of AAV purification has been advancing rapidly, particularly in the seven years since the approval of Luxturna®. Today, most of the industry is adopting a similar approach to purification with two chromatography steps after cell lysis and clarification. The primary challenge now lies in further optimizing this standardized process. Unlike other purification processes, AAV purification can be limited by difficulties in obtaining sufficient material for proper process development.

Another challenge closely related to purification is the quality of analytics. The effectiveness of any purification process depends heavily on the robustness of the associated analytical techniques, in addition to the sample type and the stage of the process. These limitations impact the ability to detect and quantify various elements accurately and develop robust unit operations.

Current efforts in clinical and commercial manufacturing are focused on eliminating product-related impurities, as these are more difficult to address. In contrast, process-related impurities are largely removed during the pre-capture and capture stages. These product-related impurities include empty particles, partially filled capsids and, occasionally, over-packaged and aggregated AAVs. The primary focus is on achieving high product purity, particularly a high percentage of full particles. As previously mentioned, the field has adopted a common downstream approach for AAV particle purification, with affinity and anion exchange chromatography serving as key unit operations.

Chantelle Gaskin (CG): There are three main trends in AAV purification that I'd like to highlight. Firstly, there's an increasing focus on engineered capsids. Many companies generate novel capsids, driven by their R&D teams, for various reasons. This includes developing viral vectors with intellectual property protection and capsids with specific tropism to target particular tissues. Additionally, there's a strong emphasis on safety and reducing immunogenic responses.

This trend towards novel and engineered capsids has escalated, and many customers are using the POROS™ CaptureSelect™ AAVX resin for this purpose. However, purifying engineered capsids requires downstream purification steps to be further optimized. Our role as field application scientists (FAS) is to support customers in optimizing their entire downstream process for these novel capsids.

Another significant trend is the enrichment of full capsids, which is generally achieved using non-affinity polish resins combined with different buffer compositions. Moreover, there is a growing focus on characterizing partially filled and overfilled capsids. Historically overlooked, this aspect is now gaining attention as companies are exploring purification processes and upstream strategies to address these issues.

Lastly, the importance of analytics in the purification process cannot be overstated; effective purification is contingent upon robust analytical methods. We're seeing ongoing trends in developing analytical assays for titer determination of various capsid species and methods for quicker titer readouts. These advancements are critical for accurately characterizing the purification process and are increasingly prevalent in the field.

Q: How does affinity chromatography contribute to achieving high purity and yield in AAV purification? Can you explain the underlying principles and mechanisms involved?

CG: Affinity chromatography has become a staple in biologics purification due to its efficiency and specificity. The technique relies on highly specific binding sites on the chromatography media, allowing only the target molecule to bind to the column while other impurities in the starting material flow through. This results in highly purified material in just one step, unlike the two or three steps often required with non-affinity chromatography.

Our CaptureSelect portfolio, for example, uses camelid antibody fragments to achieve this level of purification. These antibody fragments have high specificity for the target molecule, helping to ensure that only the desired AAV particles are retained on the column. This approach not only shortens the purification process but also increases its efficiency, delivering high purity and yield with fewer steps.

AB: Affinity chromatography offers numerous advantages for AAV purification. For example, it eliminates the need to adjust the sample before loading it onto the chromatography column. Thus, high purity can be achieved in a single affinity chromatography step. For instance, the initial load sample may contain less than 1% of the target product, with the rest being process-related impurities. After affinity chromatography, purity levels can exceed 90–95%, effectively removing these impurities. Additionally, this step concentrates the sample by several hundred-fold, depending on the specific process.

Affinity chromatography is highly specific, eliminating the need for additional unit operations, and thereby reducing the number of steps in the process. Each additional step can lead to product losses, so minimizing these steps enhances the overall AAV recovery.

Q: What are the recent advancements in affinity chromatography for AAV purification?

AB: One recent development in AAV purification using affinity chromatography involves optimization of the overall downstream process to reduce product losses. Traditionally, many processes include a concentration step using tangential flow filtration (TFF) before the affinity chromatography step. This concentration step helps to increase the amount of AAV in the sample, thereby reducing the time needed for affinity chromatography. However, it also introduces an additional unit operation, which may lead to potential product losses.

However, an innovative approach to eliminate the TFF step can be achieved by using chromatography resins with high capacity and high permeability, such as POROS CaptureSelect AAVX or AAV9. By operating at higher flow rates and using shorter bed heights, these resins can process larger volumes quickly without the need for prior concentration.

This method not only simplifies the process but also helps to minimize product losses associated with additional steps. This approach has been increasingly adopted in the field, with companies like Bristol Myers Squibb presenting related work at the American Chemical Society (ACS) meeting last year.

CG: Recent advancements in affinity chromatography for AAV purification have primarily focused on optimizing the surrounding downstream processes to enhance the efficiency of the affinity purification step. One approach involves implementing DNA removal protocols prior to affinity purification. This step helps increase the purification efficiency of the affinity chromatography process.

Additionally, there has been a focus on optimizing the cleaning and reuse of AAV resins to extend their lifecycle. This is particularly important in process development and GMP manufacturing, where the ability to reuse columns can significantly reduce costs. Historically, single-use affinity columns were preferred in GMP settings due to concerns around handling viral vector product. However, to support the development of larger-scale AAV processes, we have demonstrated that POROS CaptureSelect AAVX resin can be used for multiple cycles, enabling more sustainable and cost-effective manufacturing.

These advancements, while not the most glamorous aspects of AAV purification, are crucial for improving the efficiency and cost-effectiveness of both process development and large-scale production. We have been actively supporting customers in implementing these strategies to achieve better results in their purification workflows.

Q: The POROS CaptureSelect AAVX can capture a wide range of AAV serotypes. How does it do that?

CG: The AAVX ligand was developed through an extensive screening process of multiple ligand candidates to find the one with the highest specificity across a broad range of AAV serotypes. The key to its success lies in its ability to bind to specific sequences on the AAV capsid. These sequences are conserved across different viral vector serotypes, allowing AAVX to effectively capture a wide variety of AAV serotypes.

This binding mechanism is documented in a white paper that highlights that these conserved sequences are crucial for the ligand's broad specificity.[1] However, when customers engineer capsids and alter these proteins, the binding efficiency of AAVX can decrease. To assist with this, we provide access to the published epitopes so that customers can avoid modifying these critical binding sites during their engineering processes.

Q: What are the key factors to consider when selecting an affinity chromatography method for AAV purification? Are there any specific ligands or matrices that have demonstrated superior performance?

AB: When discussing AAV purification, it's crucial to recognize the wide range of serotypes used and the field's efforts to engineer these particles for various applications. Therefore, the first factor to consider is the specificity of the affinity chromatography resin, i.e., ensuring the affinity resin can target the specific serotypes used by an organization. The POROS CaptureSelect AAVX resin has demonstrated broad specificity, effectively binding to all natural serotypes as well as engineered capsids.

The second consideration is scalability. Chromatography has a long history and is easily scalable. However, given the typically low concentration of AAV and the relatively long processing times, it's essential to consider the binding capacity of the affinity resin, particularly at shorter residence times and higher flow rates. In this regard, chromatography resins like POROS CaptureSelect AAVX are particularly advantageous as they offer high binding capacity at high flow rates. This capability reduces overall processing time and allows smaller columns to be used, thereby lowering the overall costs.

CG: There are several key factors to consider when selecting an affinity chromatography method for AAV purification. First, you need to look at binding capacity, as this will impact the efficiency and yield of your purification process. Next, consider the material of construction, which affects flow pressure and flow characteristics. These factors can be critical when scaling up your process.

Another important aspect is the ability to clean your resins. Efficient cleaning protocols are essential for maintaining resin performance and longevity, especially in large-scale operations. Additionally, the ability to pack resins effectively is crucial. While many AAV purification processes use pre-packed columns, those who pack their own columns need a resin that is easy to pack consistently.

Q: Can you provide examples where affinity chromatography has successfully enabled one-step capture of AAV with high purity and yield?

AB: It's important to note the difference between producing recombinant AAVs for research or preclinical purposes versus clinical studies in humans. Several research groups and industry specialists have used one-step purification with affinity resins and demonstrated their effectiveness in *in vitro* or early-stage *in vivo* models.

A nice example is recent work by scientists at a biotechnology company showing that using just affinity resins can be effective for producing AAV for early stages.[2] Some of their work focused on the removal of endotoxins, which are undesirable in the context of *in vivo* studies. They were able to effectively remove those endotoxins using an intermediate wash with a detergent after binding AAV particles to POROS CaptureSelect resins. Most researchers working in research settings primarily use just one-step purification with affinity resins which provide sufficient purity for their work.

CG: One example comes from a recent study, in which they used AAVX to purify 15 divergent AAV serotypes, including AAV2, AAV9 and even the ancestral AAV serotype Anc80, known for its excellent tropism but difficult purification.[3] The

results demonstrated high levels of purification in a single step. They compared this approach to ultracentrifugation with an iodixanol gradient, a common method in early-stage research that, while effective, is difficult to scale. This comparison highlighted the advantages of AAVX, particularly in scalability, making it suitable for larger-scale applications like preparing materials for extensive animal studies.

A case study published earlier this year utilized an AAVX affinity column for analytical purposes.[4] This method is particularly beneficial for titer determination of crude samples.

Q: Are there some serotypes that prove to be more challenging when developing an AAV affinity capture step? Have these challenges been addressed?

AB: While many AAV serotypes are quite similar, which is why we can use a single affinity resin to capture all of them, they also have some key differences. One notable difference is the stability of the AAV particle itself. For example, serotypes like AAV2 are more prone to aggregation, especially under low conductivity conditions where there's not enough salt.

In affinity chromatography, we bind the particles at neutral pH and elute them at low pH. Generally, low conductivity is beneficial for good recovery. However, for serotypes prone to aggregation under these conditions, we need to address the challenge of balancing recovery and stability. We do this by including excipients like arginine to prevent aggregation while still achieving good recoveries. Additionally, after elution, we can add different salts to the neutralization buffer to prevent aggregation.

Another example is AAV5, which binds very strongly to the AAVX ligand. This means we need slightly more stringent conditions for elution, such as a lower pH – maybe half to one pH unit below what we'd use for other serotypes. We can also use excipients or modifiers to facilitate elution and maintain good recoveries.

For engineered capsids, the situation can be different. Sometimes, the binding to the resin isn't sufficient. In such cases, we can adjust the binding conditions or explore alternative custom ligands or resins to achieve the desired capture efficiency.

CG: AAV9 and AAV9-like serotypes tend to resist binding, making purification difficult. This serotype crosses the blood-brain barrier, making it particularly useful for neurological applications and diseases involving the central nervous system (CNS).

Some companies are making small modifications to the AAV9 capsid to improve its suitability as a viral vector. Despite these challenges, the AAVX resin is capable of purifying AAV9 capsids. We recommend certain considerations to optimize the purification process for AAV9, but overall, AAVX shows great binding capacity for this serotype. Additionally, we have also developed the POROS CaptureSelect AAV9 resin, which is made specifically to bind this species.

Q: In your experience, what are the main benefits of affinity chromatography compared to alternative AAV purification methods?

AB: One of the main benefits is the ability to take the sample from the previous step without needing to adjust pH or conductivity. For example, if you use cation exchange chromatography for capture, you need to lower the pH and adjust the conductivity. Some impurities may precipitate after these adjustments. This adds extra steps that require further optimization and can lead to product losses.

The second benefit is achieving very high purity levels in a single step. Affinity chromatography is scalable, and in that same step, it also concentrates the load sample. Depending on the initial concentration and specific conditions, you can achieve a concentration increase of 100- to several hundred-fold.

Compared to ultracentrifugation, the scalability of chromatography resins is also clear. Ultracentrifugation faces scalability issues, especially as the field moves toward larger doses for larger patient populations, making it challenging to produce the required amount of vector.

CG: Affinity chromatography offers significant benefits by effectively reducing the number of purification steps needed. With just one affinity chromatography step, you can achieve the same level of purification that might otherwise require two or three ion exchange steps. This translates to greater process efficiency, as you're eliminating additional chromatography steps, along with their associated costs for resins, buffers and manpower.

Q: How does affinity chromatography fit into the overall process of AAV production? Are there any considerations regarding scalability and cost-effectiveness?

AB: Affinity chromatography plays a crucial role in both research and larger-scale AAV production. Typically, it fits into the process after several initial steps and before any final processing steps. Chromatography resins have been used for decades, so the underlying physical principles remain the same, with a range of column diameters and bed heights providing flexibility compared to other adsorptive methods.

In terms of cost-effectiveness, affinity chromatography offers significant benefits by potentially eliminating the need for

additional steps. While the cost of affinity chromatography resins is a factor, it should be compared to other expensive raw materials, such as nucleases and plasmids. Importantly, these resins can be reused in both research and GMP settings. They can be cleaned and utilized multiple times, which helps reduce the overall cost of the process. We have demonstrated that these resins can maintain comparable performance over 35 cycles. Similarly, research by Florea *et al*. has shown good reproducibility over six cycles.[3]

The ability to reuse chromatography resins significantly lowers the cost per cycle, and this exponential reduction in cost with reuse makes affinity chromatography a cost-effective choice. However, it's crucial to validate the resin reuse using a qualified scale-down model and ensure the necessary analytics are in place to support this approach.

CG: Affinity chromatography streamlines the purification process. For instance, compared to ultracentrifugation – which is labor-intensive and has significant scalability issues, such as the need for precise manual band extraction from gradient tubes – affinity chromatography offers a more efficient and consistent approach.

The manual aspect of ultracentrifugation, often described as tedious or even an art form, can vary greatly between operators, further complicating scalability. In contrast, affinity chromatography using POROS AAVX resins provides excellent scalability. The resin's robust material construction supports large-scale applications and enables multiple reuse cycles, leading to significant cost savings. This allows affinity chromatography to be not only more cost-effective but also more scalable compared to traditional methods. Overall, the efficiency, consistency and reusability of affinity chromatography contribute to its advantages in AAV production.

Q: Are there any limitations associated with affinity chromatography in AAV purification? How can these be addressed or optimized?

AB: One key limitation is specificity. While the AAVX ligand has been effective for many engineered capsids, there have been instances where the resin, or even the AAV9 resin, hasn't bound to certain capsids, particularly with AAV particles similar to AAV9. Future engineered serotypes might also face similar issues.

When these challenges arise, there are a couple of options. One is to explore non-affinity approaches, such as cation exchange chromatography. However, this method involves an additional step before chromatography and requires optimization for each specific case.

The second option is to develop a custom ligand. At Thermo Fisher, we offer the capability to create tailored affinity ligands and resins for various biomolecules, including AAVs. We've successfully developed custom solutions in the past, and this could be a viable route when dealing with new engineered capsids that don't bind well with standard resins.

CG: There are some limitations and challenges with affinity chromatography in AAV purification, particularly when dealing with novel capsids. Novel capsids can present unique issues, as they may not bind as effectively or predictably to the affinity resin. This challenge extends to upstream processes, where suboptimal production conditions for the novel capsid can lead to lower viral titers, complicating downstream purification.

Our AAV-MAX system is designed to enhance upstream AAV production by optimizing culture media, additives and cell lines. Despite these advancements, issues with novel capsids can still arise, and overcoming them often requires careful troubleshooting and optimization.

FAS and purification specialists work closely with customers to navigate these difficulties, developing workarounds and refining processes to support effective purification even with novel capsids.

Q: What are the current methods to remove any additional impurities that remain after an optimized AAV capture chromatography step?

AB: The main methods used are anion exchange chromatography and ultracentrifugation. Anion exchange chromatography, especially with specific resins, is commonly employed. Each has its advantages and disadvantages depending on whether you're working in research or scaling up for GMP production.

These are the primary approaches because removing product-related impurities – similar in size and charge to the target product – is quite challenging. Fine separation is required, which is something that ion exchange chromatography and ultracentrifugation currently handle most effectively.

CG: After capturing AAV through affinity chromatography, the next step typically involves using ion exchange chromatography, with anion exchange being the most common choice. Anion exchange chromatography effectively addresses the remaining impurities, such as empty, partially filled and overfilled AAV capsids, as well as trace amounts of host cell DNA and proteins. These impurities usually account for about 3–5% of the purified material.

The focus of the anion exchange step is often on enriching the full capsid population. This step is crucial for removing empty capsids, which could potentially trigger an immunogenic

response in patients. Since most of the AAVs produced upstream are empty, it is essential to effectively separate these from the full capsids.

POROS HQ and POROS XQ are strong anion exchangers that are particularly effective in this process. Recent publications have explored advanced techniques, such as using dual salt buffer systems to create step gradients rather than linear gradients. Step gradients are more suitable for large-scale purification, enabling better separation of different capsid species and improving scalability.

Q: What particular resins are used and how does a process scientist evaluate and choose the best candidate for the process?

AB: To select the best resin, a process scientist needs to start by defining the goals of the step. This involves understanding the target enrichment of the full particles required for the process and determining the acceptable levels of other impurities, like residual ligands or DNA.

Once those targets are set, it's crucial to leverage existing knowledge and resources. For instance, since the approval of AAV therapies in the U.S. about six or seven years ago, the field has accumulated significant insights, particularly in anion exchange chromatography. Scientists should use this knowledge to guide their initial conditions and step development.

Scalability is another key factor. Anion exchange resins offer more size options compared to other adsorbents, like membranes or convective materials. Typically, this polishing step is conducted at an alkaline pH (between 8 and 9.5) because AAV particles exhibit poor binding at lower pH levels. The separation is also performed at low conductivity. Additionally, different counter ions or salts, like magnesium, have been found to positively impact the separation. While the exact mechanism might not be fully understood, it's generally considered as an additive during the process evaluation.

CG: In downstream purification, POROS HQ and POROS XQ are commonly used strong anion exchange resins. These are preferred because anion exchange chromatography effectively handles the diverse characteristics of AAV and its impurities, such as isoelectric points and binding strengths. While anion exchange is the predominant choice, there are instances where cation exchange might be used, for which POROS HS and POROS XS are available.

When selecting the best resin, process scientists evaluate several factors, including the specific binding properties and the nature of the impurities. Downstream scientists have many options here, but POROS XQ and POROS HQ are highly recommended due to their robust performance and extensive published data supporting their efficacy.

Q: Do you have any case studies that showcase the successful polishing of AAVs?

AB: Fortunately, the field is starting to share more insights and case studies on this topic. One example involves a thorough evaluation and scale-up of a polishing step using POROS HQ.[5] It's a great example of how to approach developing this step for a specific serotype, but the underlying principles are applicable to other serotypes as well.

While I've focused mainly on POROS HQ, we've found through recent customer interactions that POROS XQ might actually perform better in many cases. There aren't as many examples yet, but scientists have investigated these interactions and used POROS XQ as well.[6] These case studies highlight how our understanding and approaches are evolving.

CG: We have some notable case studies, with one of the most recent being a paper published a couple of months ago.[7] This study focused on AAV9 and demonstrated a successful full capsid enrichment step using POROS HQ. Their results were impressive, achieving over 60% full capsids, surpassing their initial target of 50%. This is just one example; there are numerous other cases, both published and unpublished, where our POROS HQ resin has been used effectively to achieve high levels of full capsid enrichment.

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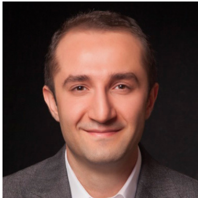
Harnessing CDMOs for Optimized AAV Production: Ask the Expert

Expert



Pouria Motevalian

Director, Viral Vector Process and Analytical Development, Thermo Fisher Scientific

As Director of Process Development, Pouria Motevalian oversees the development, scale-up and analytical characterization of novel and compliant manufacturing processes for gene therapy. He also holds a key position as a member of the Senior Leadership Team for Thermo Fisher Scientific's Plainville, MA site, our largest viral vector development and manufacturing site in North America. In this role, Pouria provides strategic guidance, shaping scientific and operational plans for implementing bioprocess technologies to meet the needs of viral vector clients. Pouria received his PhD in chemical engineering with a minor in computational science from Pennsylvania State University.

Adeno-associated viruses (AAVs) are a versatile tool in gene therapy, promising to treat a range of previously incurable genetic disorders by delivering therapeutic genes directly into patients' cells. Despite their potential, the large-scale production and purification of AAVs still faces significant challenges. Contract development and manufacturing organizations (CDMOs) play a crucial role in addressing these complexities, using their expertise to streamline and enhance production.

To gain deeper insights into how CDMOs can tackle these purification challenges and optimize AAV production, we spoke with Pouria Motevalian, Director of Viral Vector Process Development at Thermo Fisher Scientific.

Q: What have been the key advances in AAV manufacturing technology over the last few years?

A: One of the most notable advancements has been the development of scalable production platforms, particularly in the areas of triple transfection-based systems, baculovirus expression systems, and producer cell lines. These platforms have evolved significantly, enabling the establishment of scalable production processes for each approach.

Another major advancement is in capsid design and engineering, which enables the development of vectors with improved specificity, stability, and a reduced immune response. Equally notable are improvements in downstream processing, particularly in chromatography and filtration. Enhanced affinity chromatography, with improved resins for the affinity capture step, has resulted in more robust and efficient processes. Depth filtration technologies have also been significantly improved, increasing throughput and reducing process and product impurities. These advancements in recent years have propelled the field forward.

Q: What is the main bottleneck for manufacturing of viral vectors?

A: Scaling up the production process while maintaining purity, consistency and potency is a major challenge. Despite all the advancements we've seen in recent years, inconsistencies can still arise during scale-up, particularly with titer levels, impurity removal and overall product quality. These inconsistencies can

ultimately affect the final product's potency, making this a key bottleneck in viral vector manufacturing today.

Q: Are there any unique considerations or challenges that arise when purifying viral vectors compared to other types of biologics?

A: The goal is always to reduce process- and product-related impurities. Removing upstream impurities, such as host cell DNA or proteins, is standard for any biologic. However, for viral vectors, particularly AAVs, the downstream process must also remove empty and partial capsids. This is especially challenging because empty, full, and partial capsids are similar in size and have minimal differences in isoelectric points—often just 0.2 to 0.6 units—making their separation extremely difficult.

To address this challenge, we rely on high-throughput resin and mobile phase screening for optimization of anion exchange and affinity chromatography steps. Anion exchange is particularly crucial for empty/full capsid separation, and utilizing high-throughput technologies for screening is essential for developing a well-optimized process. This approach allows for substantial removal of empty and partial capsids, significantly enhancing the effectiveness of the purification process.

Q: How do CDMOs ensure the scalability and reproducibility of viral vector purification processes, particularly when dealing with large-scale production for gene therapies?

A: There are two key aspects to consider. First, it's crucial to rely on a scalable, well-developed scale-down model for each unit operation. Ensuring that the scale-down model used during process development accurately reflects larger-scale operations is essential. If the scale-down model doesn't faithfully represent large-scale production, the development process loses value as the results won't be transferable.

Second, it's important to keep the end goal of large-scale production in mind throughout the course of process development. The focus should always be on ensuring that the process designed in the lab can be successfully scaled for clinical and commercial manufacturing. This means that when developing processes and determining normal operating ranges, we must work to ensure that these parameters are feasible for large-scale production.

Q: How do gene therapy developers ensure compliance with regulatory guidelines and standards when purifying viral vectors?

A: The answer is straightforward: embrace Quality by Design (QbD) throughout the entire development process and ensure strict compliance with GMP best practices and guidelines during clinical and commercial manufacturing. By adhering to these principles—integrating QbD from the outset and following GMP guidelines—developers can ensure they meet regulatory requirements and align with industry standards.

Q: What further innovations would you like to see in viral vector manufacturing in the future?

A: First, plasmid design and optimization should be prioritized early on, because the optimized design of the plasmid (especially ITR regions) has been shown to significantly boost productivity, especially for AAV viral vectors. Second, we would like to see advancements in resins that allow for enhanced separation – not just when it comes to separating empty and full capsids, but also in removing impurities. Lastly, implementing process analytical technology (PAT) tools such as Raman, FTIR and NIR for real-time measurement of critical quality attributes is highly desired. This would help reduce bottlenecks in QC testing without compromising product quality, enabling real-time measurement and release.

Q: What are the advantages of working with a CDMO to solve purification challenges early on in the process?

A: The first major advantage is the expertise and experience that CDMOs bring. Specializing in specific areas, CDMOs possess the technical knowledge and deep understanding necessary to develop and scale processes efficiently for clinical and commercial manufacturing. Their experience working with a variety of vectors and clients, each with unique requirements, gives them a broad perspective on industry challenges. This accumulated expertise allows them to provide tailored solutions to the specific challenges each client faces.

Another key advantage is the ability to accelerate development timelines. CDMOs, equipped with advanced tools and technologies, can offer accelerated development. This is partly due to economies of scale—they handle multiple projects and pipelines simultaneously, enabling a more standardized and efficient approach. As a result, they can speed up the development process, which is particularly beneficial when time is critical.

Q: Are there any particular purification challenges for which your company has interesting and valuable solutions for AAV manufacturers?

A: One of the major challenges in AAV purification is the separation of empty and full capsids, as well as the removal of residual host cell impurities like DNA and proteins. Now,

the tricky part is that each client's process is unique, so the challenges they face are unique too. Because of that, the solutions we offer need to be tailored to each client's specific needs.

Instead of providing a one-size-fits-all purification solution, we take a more versatile approach to downstream processing, development and optimization. We rely on high-throughput technologies and techniques, which have consistently proven effective in tackling these major downstream challenges across multiple clients.

Q: Are there any specific recommendations for AAV therapeutic downstream scientists when developing a process planned to be transferred to a CDMO?

A: First, ensuring process robustness is essential, though it's a broad concept. To clarify, when transferring process parameters for a specific unit operation, it's important to provide a range of acceptable parameters rather than just a single target. This flexibility allows for a better facility fit, particularly in a GMP setting for clinical and commercial manufacturing.

Another key aspect of process robustness is the manufacturability of mobile phases used in critical chromatography steps. At times, the passing criteria for parameters like pH and conductivity are so stringent that it becomes challenging to prepare and release these buffers in a GMP environment. When developing mobile phase formulations, it's important to consider manufacturability, ensuring that the release criteria are broad enough to be practical without compromising quality.

The second point is establishing appropriate hold times for intermediates. Undefined hold times can force critical operations to be performed during less optimal shifts, increasing operational risk. Planning for these operations to occur during shifts with full manpower and expertise reduces this risk.

Lastly, it's crucial to ensure that unit operations are scalable throughout the process. For example, ultracentrifugation is often used for viral vector purification but becomes difficult to scale beyond a certain point, leading to the need to scale out rather than up, which poses operational challenges and increases capital and space requirements. Designing scalable unit operations from the start is critical for a smooth and successful tech transfer to a CDMO.

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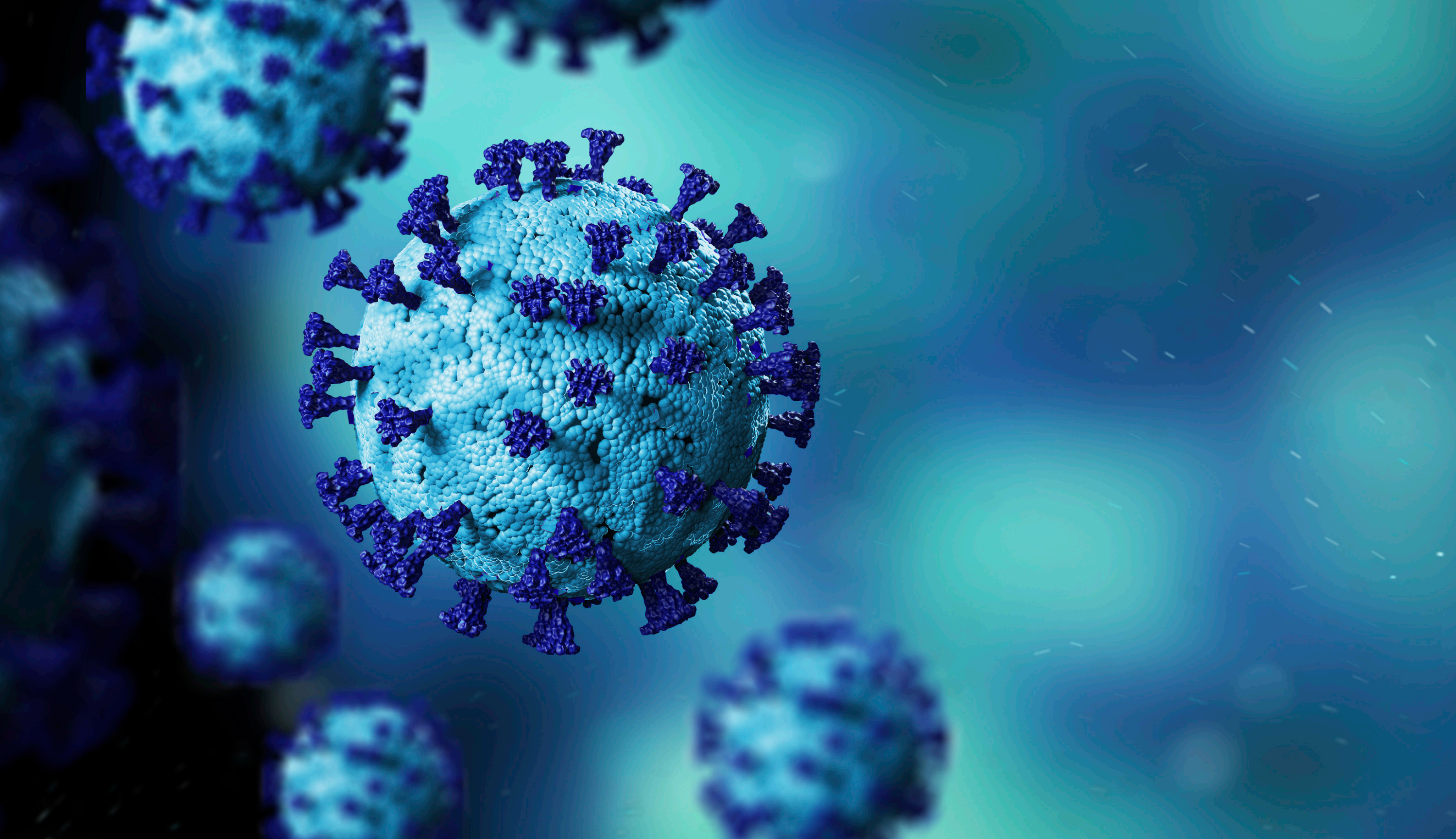


Top 5 Benefits of Affinity Chromatography for AAV Purification

Introduction

Affinity chromatography is an advanced and highly effective technique widely employed in downstream purification processes, especially for adeno-associated viruses (AAVs). By selectively binding and isolating target molecules, affinity chromatography can greatly enhance both the efficiency and yield of AAV purification. Here, we will explore the five main benefits of affinity chromatography for AAV purification and discuss various techniques and strategies to optimize your downstream purification of viral vectors.

1. High specificity and selectivity

Affinity chromatography relies on the specific interaction between a target molecule, such as a specific protein of the AAV capsid, and an immobilized ligand on the chromatography resin. This targeted binding enables the selective isolation of AAV particles from complex mixtures, yielding a highly pure sample. The high specificity and selectivity of affinity chromatography help to ensure minimal contamination and maximize the recovery of AAV particles.

2. Increased purity and yield

By specifically targeting AAVs from a complex feedstock, affinity chromatography effectively recovers AAV particles and allows unrelated impurities to pass through unretained. When optimized, this technique achieves both high purity and yield in a single step, streamlining the purification process and maximizing the recovery of AAV particles.

3. Versatility and adaptability

Affinity chromatography can employ a wide range of ligands that can be tailored to specific AAV purification needs. A key advancement in this area involves the use of VHH antibodies – camelid-derived single-domain antibodies – that can be finely tuned to bind with high specificity to a vast array of biomolecules. Through the use of advanced ligand generation platforms, researchers can design VHH antibodies that target specific properties of AAV, enabling either serotype-specific targeting or broad, pantropic AAV binding. This adaptability ensures that the purification process can be customized to address various needs and improve the effectiveness of AAV isolation.

4. Gentle purification conditions

A key advantage of affinity chromatography is its ability to operate under mild purification conditions. This is especially important for the purification of delicate viral vectors like AAV, as harsh purification methods may compromise their structural integrity and functionality. This ensures that the quality and functionality of the AAV particles are maintained throughout the process.

5. Scalability and automation

Affinity chromatography is highly scalable, making it well-suited for large-scale AAV production. The technique can be easily scaled up to meet increasing production demands while maintaining efficiency. Additionally, the purification process can be automated, enabling high-throughput purification and significantly reducing the time and labor involved. This combination of scalability and automation makes affinity chromatography a practical and efficient choice for industrial-scale AAV production.

Conclusion

Affinity chromatography presents substantial advantages for the downstream purification of AAV vectors, making it a cornerstone technique for process development scientists in this field. Its exceptional specificity, ability to achieve high purity and yield, versatility, gentle purification conditions and scalability collectively contribute to its effectiveness in AAV purification. By exploring and optimizing various techniques and strategies within affinity chromatography, scientists can further enhance the efficiency and yield of AAV purification processes, thereby advancing the development of viral vector-based therapies.

Learn more about optimizing your purification process today. Visit our webpage to find out more.

Viral clearance in a downstream AAV process

Case study using a model virus panel and a noninfectious surrogate

Michael Winkler, Mikhail Goldfarb, Shaojie Weng, Jeff Smith, Susan Wexelblat, John Li, Alejandro Becerra, Sandra Bezemer, Kevin Sleijpen, Aleš Štrancar, Sara Primec, Romina Zabar, April Schubert, Akunna Iheanacho, and David Cetlin

Over the past decade, adenoassociated virus (AAV) vectors have become established as leading gene-delivery vehicles. In 2017, the pipeline for gene therapies included 351 drugs in clinical trials and 316 in preclinical development (**1–4**). As those candidates advance, significant efforts are being made in process development and manufacturing for viral vectors, with the overall goal of reducing process impurities while maintaining the highest possible process yield.

To address that goal, industry suppliers have developed innovative AAV-specific separation technologies. Thermo Fisher Scientific's POROS CaptureSelect AAVX affinity resin provides a capture method for a number of natural and synthetic AAV serotypes irrespective of the expression system used to produce them. By leveraging a proprietary recombinant camelid antibody technology immobilized onto the highly permeable POROS backbone, the resin achieves a fine-tuned specificity for AAV recognition with an increased surface area and capacity for AAV binding. The significant impurity reduction benefits and rapid scalability of this affinity resin have led to its incorporation into several noted AAV downstream process designs.

PRODUCT FOCUS: VIRUSES

PROCESS FOCUS: DOWNSTREAM PROCESSING

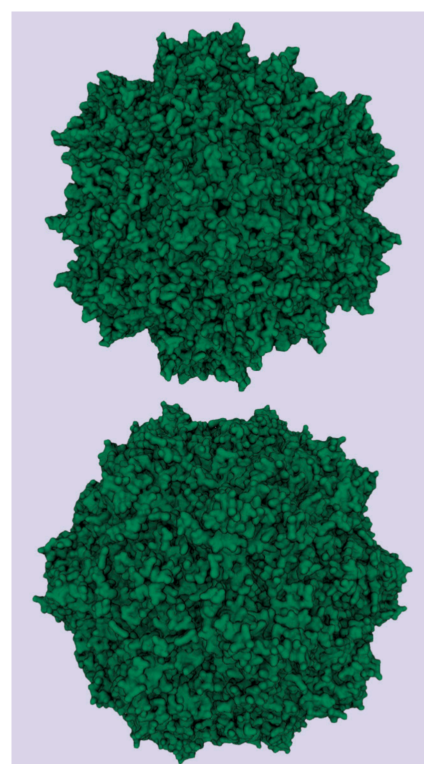
AUDIENCE: PROCESS ENGINEERS, ANALYTICAL, MANUFACTURING

KEYWORDS: MONOLITHS, AFFINITY CHROMATOGRAPHY, ADENOASSOCIATED VIRUS, $TCID_{50}$, IMMUNO-QPCR, SPR ANALYSIS

LEVEL: INTERMEDIATE

BIA Separations (now part of Sartorius AG) has developed and commercialized CIMmultus QA monoliths, which have been cited in several AAV downstream processes for their ability to separate empty and full virus particles effectively. Monolithic supports represent a unique type of stationary phase for liquid chromatography, bioconversion, and solid-phase synthesis. Aside from increased processing speed, monolithic flow-through pores (channels) also provide easy access for large molecules, which supports both purification and depletion of nanoparticles such as plasmid DNA (pDNA) molecules and AAV particles.

One elusive aspect of AAV process development is viral clearance (VC). As outlined in the ICH Q5A guidelines, VC validation is a key regulatory requirement governing all recombinant biopharmaceuticals (**5**). According to these guidelines, the risks of viral contamination should be assuaged by a three-pronged approach: prevent, test, and remove. Over the past few decades,



Similarity in structures of adenoassociated virus serotype 8 (TOP) and minute virus of mice (BOTTOM). PROTEIN DATA BANK (HTTPS://WWW.RCSB.ORG)

thermofisher.com/aav-purification

Table 1: Adenoassociated virus (AAV) process spiking runs for xenotropic murine leukemia virus (XMuLV), hepatitis A (HAV), reovirus type 3 (Reo-3), pseudorabies virus (PRV), herpes simplex virus 1 (HSV-1), minute virus of mice (MVM), and the MVM mock virus particle (MVM-MVP)

	POROS CaptureSelect AAVX						CIMmultus QA	
	Center Point							
Spiking Agent	Run 1	Run 2	POROS Alternative Ligand	POROS Base Matrix	AAV Null Load	Worst Case	Center Point	Worst Case
XMuLV	✓		✓	✓	✓	✓	✓	✓
HAV	✓					✓	✓	✓
Reo-3	✓					✓	✓	✓
PSV	✓					✓	✓	✓
HSV-1	✓					✓	✓	✓
MVM	✓		✓	✓	✓	✓	✓	✓
MVM-MVP	✓	✓	✓	✓	✓	✓	✓	✓

certain VC strategies for monoclonal antibodies (MAbs), such as low-pH inactivation and nanofiltration, have become standard for most downstream processes. However, because AAVs are viruses themselves (within the family *Parvoviridae*), it may not be possible to apply the same purification strategies to them that have served so well in MAb processes. As a result, the gene-therapy industry may depend increasingly on chromatographic modes of separation to demonstrate sufficient viral clearance.

In the study reported herein, we addressed viral removal by performing scale-down–model spiking studies and measuring VC using a clinically relevant AAV8 downstream purification process. The two-step chromatography process begins with affinity capture using POROS CaptureSelect AAVX affinity resin followed by anion-exchange polishing using a CIMmultus QA monolith. We selected as spiking agents a wide range of viruses with different sizes, molecular makeups, and physiochemical properties. For DNA viruses, we used enveloped pseudorabies virus (PRV) and nonenveloped minute virus of mice (MVM). For RNA viruses, we used enveloped xenotropic murine leukemia virus-related virus (XMuLV) and nonenveloped reovirus type 3 (Reo-3). We also included human contagions hepatitis A (HAV) and herpes simplex virus 1 (HSV-1) based on a risk assessment of possible adventitious virus contaminations with a HEK293 human-derived producer cell line used for upstream production. For the benefit of future process developers who may wish to perform similar VC testing on their own purification process steps but

Figure 1: (A) Immuno-quantitative real-time polymerase chain reaction (Immuno-qPCR) assay; (B) Immuno–qPCR standard curve

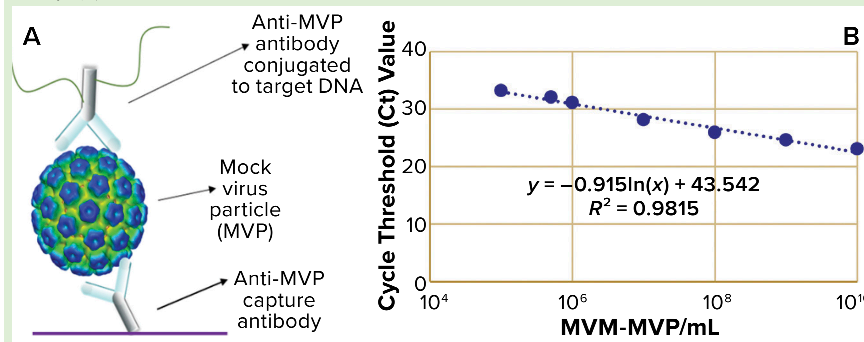
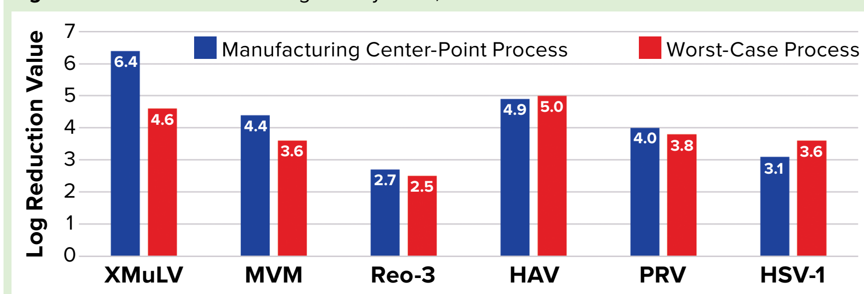


Figure 2: Viral clearance using affinity resin; see Table 1 for full virus names



lack the ability to conduct such resource-intensive studies, we assessed the MockV MVM kit from Cygnus Technologies, LLC, as an economical and rapid means to generate predictive MVM clearance data (**6**). We used the kit in parallel with MVM spiking experiments throughout our study.

Materials

All AAVs used for these viral clearance studies were produced at representative scale by a platform process at REGENXBIO Inc.

Production of Virus and Mock Virus Particles (MVPs): Viruses were propagated and purified by Texcell NA of Frederick, MD, according to standard protocols. Noninfectious MVM-MVPs were assembled after expression of the major MVM capsid protein (VP2) in a baculovirus expression-vector system (BEVS) using *Spodoptera frugiperda* 9 (*Sf*9) cells at Cygnus Technologies. Particles were purified with affinity and ion-exchange chromatography (IEC). Transmission electron microscopy (TEM) was used to verify MVP morphology, size, and concentration.

Chromatography Products: Thermo Fisher Scientific supplied prepacked, 5-mL POROS CaptureSelect AAVX affinity resin columns; BIA Separations provided scale-down CIMmultus QA monolith devices in 4-mL and 8-mL diameters. Control POROS resins were custom made by Thermo Fisher Scientific with identical base beads to

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

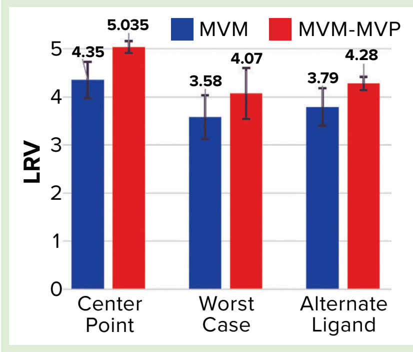


Figure 4: Characterization of nonspecific interactions based on log reduction values (LRVs) for xenomorphic murine leukemia virus (XMuLV) and minute virus of mice (MVM)

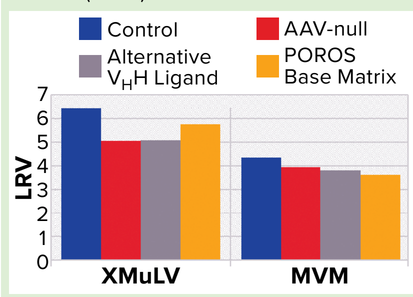


Table 2: Affinity resin data from experiments with minute virus of mice (MVM) and noninfectious MVM mock virus particles (MVM-MVPs)

		Total Particles (log_{10})			Percentage of Particles		
			MVM-MVP			MVM-MVP	
Run Type	Phase	MVM	Run 1	Run 2	MVM	Run 1	Run 2
Center Point	Load	8.1	12.3	12.2	NA	NA	NA
	FT	7.9	12.0	12.0	66.1%	52.6%	67.0%
	Wash 1	6.1	10.0	9.7	1.0%	0.5%	0.3%
	Wash 2	5.4	11.3	11.2	0.2%	10.4%	10.4%
	Wash 3	4.7	8.7	8.7	0.0%	0.0%	0.0%
	Elution	3.8	7.4	7.0	0.0%	0.0%	0.0%
	CIP	5.0	6.9	6.7	0.1%	0.0%	0.0%
Worst Case	Load	7.9	11.9	NT	NA	NA	NA
	FT	7.6	11.8		55.0%	79.1%	
	Wash 1	NT	9.9		NA	1.1%	
	Wash 2	NT	11.0		NA	14.3%	
	Wash 3	NT	9.0		NA	0.1%	
	Elution	4.3	7.8		0.0%	0.0%	
	CIP	NT	6.8		NA	0.0%	

NT = not tested; **NA** = not applicable; **FT** = flow-through fraction; **CIP** = clean-in-place solution

the affinity resin used but incorporating either an alternative V_HH ligand specificity (nonbinding for AAVs) or no V_HH functionalized ligand.

Methods

Study Design: To assess the robustness of the affinity resin and monolith polishing step within REGENXBIO's downstream process (**7**), we selected "center-point" and "worst-case" processing parameters for our viral clearance spiking experiments (Table 1). For each run, we spiked in-process AAV material with model viruses or MVM-MVPs to a target of 10.0 log_{10} MVM-MVP/mL and processed accordingly.

For affinity resin center-point and alternative-ligand runs, we loaded 150 mL of spiked material according to standard manufacturing load ratios and residence time. For worst-case conditions, we loaded 200 mL of spiked material (133% of the target) and decreased the flow rate to lengthen residence time to 170% of the center-point target. For the monolith polishing study, we applied center-point load volumes of 90 mL to 8-mL monoliths and 65 mL to 4-mL monoliths for worst case — except for the MVP- and XMuLV-spiked runs. For those, we loaded 45 mL and 65 mL onto 4-mL columns, respectively, for the center-point and worst-case conditions.

Samples were collected from each run during each step phase (flow-through, wash, and so on). We analyzed the virus samples immediately with a 50% tissue culture infectious dose ($TCID_{50}$) assay or quantitative real-time polymerase chain reaction (qPCR). MVM-MVP samples were stored at −80 °C before Immuno-qPCR analysis (described below). From those results, we determined log reduction values (LRVs) using a standard calculation (**5**).

During this study, we performed affinity-capture experiments to probe potential nonspecific binding interactions (Table 1). Both AAV-null (produced by pooling the flow-through fractions of previous AAVX runs) and AAV8-containing load materials were spiked with model virus, then affinity purified using center-point conditions and compared for viral clearance. Additionally, we evaluated interactions between viruses and base beads by performing AAV8 center-point runs using the POROS base matrix without a functionalized V_HH ligand; we evaluated virus–V_HH ligand interactions using a POROS resin with an alternative V_HH ligand specificity to the Fc portion of MAbs that cannot bind AAVs.

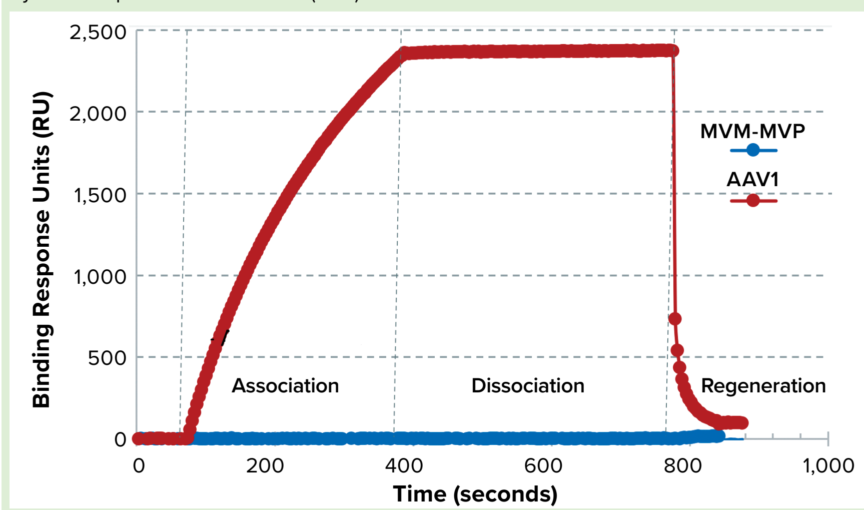
We applied an orthogonal test method — surface plasmon resonance (SPR) — to confirm the specificity of the AAVX ligand for AAV. For that, a biotinylated AAVX V_HH ligand was immobilized onto a detection surface so that binding sensograms could be generated by injection of free MVM-MVP or AAV.

Analytical Assays and LRV Determinations: Texcell scientists quantified infectious titer of XMuLV using a validated plaque-forming infectivity assay. They quantified HAV, Reo-3, HSV-1, and MVM using validated $TCID_{50}$ infectivity assays. PRV was quantified with a validated qPCR assay. From those titer determinations, we calculated LRVs by a standard method (**5**).

To analyze the concentration of noninfectious MVM-MVP within each sample, Cygnus Technologies scientists performed an Immuno-qPCR assay (Figure 1A) as described elsewhere (**8**). In brief, samples were added to microwells coated with an anti–MVM-MVP capture MAb. After incubation and washing, a DNA-conjugated anti–MVM-MVP detector MAb was added. Following another incubation and washing step, a dissociation buffer was added to each

MVM (a small, nonenveloped virus used as a model spiking agent) is a member of the same parvovirus family as AAV. Morphology and physicochemical properties (size, surface charge, and surface hydrophobicity) are **SIMILAR** in the two virus species.

Figure 5: Binding selectivity of camelid V_HH antibody-fragment affinity ligand analyzed by surface plasmon resonance (SPR)



well for five minutes. Then 5 µL of sample was transferred from each well to a qPCR plate containing TaqMan primers/probes (Thermo Fisher Scientific) directed against the conjugated DNA. To determine the quantity of particles in unknown samples, threshold cycle (Ct) values were interpolated into a standard curve generated by including a 10-fold dilution series of a known MVM-MVP standard (Figure 1B). From those concentration values, we could calculate MVM-MVP LRVs for each experiment.

Results and Discussion

Viral clearance strategies for AAV downstream processes are limited by difficulty in performing viral inactivation and filtration steps without also inactivating or removing the AAV product. Therefore, chromatographic modes of separation are paramount to achieving the desired levels of removal for viruses of concern.

To complicate matters further, MVM (a small, nonenveloped virus used internationally as a model spiking agent) is a member of the same parvovirus family as AAV. Morphology and physicochemical properties (size, surface charge, and surface hydrophobicity) are similar in the two virus species. Because of those physicochemical similarities, a step optimized to bind and elute AAV through affinity or ion interactions also might bind MVM. That would result in poor removal of such viral contaminants — or conversely, a step optimized to remove MVM could compromise AAV yield as the product is washed or eluted away along with MVM.

With that in mind, in our study we wanted to elucidate whether viruses (including MVM) could be resolved from AAV through the combination of a POROS CaptureSelect AAV column and a CIMmultus QA monolith, thereby providing effective VC.

Affinity Capture: Figure 2 summarizes the results from all VC spiking experiments using POROS CaptureSelect AAVX affinity resin. At manufacturing center-point process conditions, effective viral clearance of ≥4 LRV was demonstrated for XMuLV, MVM, HAV, and PRV. The AAVX resin also contributed to clearing ≥2.5 LRV for Reo-3 and HSV-1. During worst-case–conditions testing, similar levels of clearance were observed for all model viruses tested. Taken together, this demonstration of robust VC using POROS CaptureSelect AAVX affinity chromatography is consistent with the highly specific nature of the affinity interaction between AAVX resin and AAV vectors. Its high degree of specificity and capacity is mediated by the camelid V_HH antibody ligands functionalized to the custom-designed base beads, which in combination provide high-affinity binding to AAV vectors while minimizing nonspecific interactions.

Table 3: Monolith results for xenotropic murine leukemia virus (XMuLV)

	Total XMuLV (log_{10})		Percentage of XMuLV	
Phase	Center Point	Worst Case	Center Point	Worst Case
Load	6.7	6.9	NA	NA
FT	≤5.0	≤5.1	≤2.0%	≤1.8%
Pre-peak 1	≤4.8	NT	≤1.2%	NA
Pre-peak 2	≤4.7	NT	≤0.9%	NA
Pre-peak 3	≤3.8	NT	≤0.1%	NA
Elution	≤1.6	≤1.6	≤0.0%	≤0.0%
Post-peak 1	≤0.8	NT	≤0.0%	NA
Post-peak 2	≤1.3	NT	≤0.0%	NA
Strip	4.1	4.5	0.3%	0.4%

NT = not tested; **NA** = not applicable; **FT** = flow-through fraction

Table 4: Monolith results for hepatitis A virus (HAV)

	Total HAV (log_{10})		Percentage of HAV	
Phase	Center Point	Worst Case	Center Point	Worst Case
Load	7.5	7.4	NA	NA
FT	≤3.3	≤3.1	≤0.0%	≤0.0%
Pre-peak 1	≤3.0	NT	≤0.0%	NA
Pre-peak 2	≤3.0	NT	≤0.0%	NA
Pre-peak 3	≤2.3	NT	≤0.0%	NA
Elution	≤2.9	≤2.6	≤0.0%	≤0.0%
Post-peak 1	≤2.0	NT	≤0.0%	NA
Post-peak 2	≤2.5	NT	≤0.0%	NA
Strip	7.3	7.2	58.6%	70.3%

NT = not tested; **NA** = not applicable; **FT** = flow-through fraction

Among the panel of six model viruses tested, MVM is potentially problematic to remove based on its similarity in size to AAV and high resistance to inactivation. Being a nonenveloped DNA virus that is a member of the same *Parvoviridae* family as AAV, MVM has a similar capsid structure and therefore potentially similar viral morphology and physicochemical properties (size, surface charge, surface hydrophobicity).

Table 2 details our results for MVM and MVM-MVP (the noninfectious MVM surrogate created by Cygnus Technologies as a biosafety-level 1 safe analytical tool). Most MVM initially loaded onto the column was contained within the flow-through fractions for both center-point and worst-case runs (66.1% and 55.0%, respectively). Similarly, most MVM-MVPs also were found within the flow-through fraction (52.6–67.0% and 79.1% for center-point and worst-case runs, respectively). Affinity wash steps did little to strip the column further of MVM (0.2% for center point); moderate quantities of MVM-MVPs were removed (10.4% and 14.3% for center-point and worst-case runs, respectively). Small quantities of both MVM and MVM-MVPs were found in the elution fractions collected.

From MVM center-point and worst-case runs, 3.8 and 4.3 log_{10} total particles were detected in the elution, respectively, leading to LRV calculations of 4.35 ± 0.38 and 3.58 ± 0.46 (Figure

Table 5: Monolith results for herpes simplex virus 1 (HSV-1)

	Total HSV-1 (log_{10})		Percentage of HSV-1	
Phase	Center Point	Worst Case	Center Point	Worst Case
Load	7.2	7.1	NA	NA
FT	≤3.9	≤3.7	≤0.0%	≤0.0%
Pre-peak 1	≤3.7	NT	≤0.0%	NA
Pre-peak 2	≤3.6	NT	≤0.0%	NA
Pre-peak 3	≤2.8	NT	≤0.0%	NA
Elution	≤3.5	≤3.2	≤0.0%	≤0.0%
Post-peak 1	≤2.0	NT	≤0.0%	NA
Post-peak 2	≤2.5	NT	≤0.0%	NA
Strip	6.1	5.2	7.0%	1.3%

NT = not tested; **NA** = not applicable; **FT** = flow-through fraction

Table 6: Monolith results for pseudorabies virus (PRV)

	Total PRV (log_{10})		Percentage of PRV	
Phase	Center Point	Worst Case	Center Point	Worst Case
Load	9.9	9.7	NA	NA
FT	≤5.1	≤4.8	≤0.0%	≤0.0%
Pre-peak 1	≤4.8	NT	≤0.0%	NA
Pre-peak 2	≤4.8	NT	≤0.0%	NA
Pre-peak 3	≤3.9	NT	≤0.0%	NA
Elution	≤4.7	≤4.3	≤0.0%	≤0.0%
Post-peak 1	≤3.9	NT	≤0.0%	NA
Post-peak 2	≤4.2	NT	≤0.0%	NA
Strip	8.9	8.7	10.4%	9.6%

NT = not tested; **NA** = not applicable; **FT** = flow-through fraction

Table 7: Monolith results for reovirus 3 (Reo-3)

	Total Reo-3 (log_{10})		Percentage of Reo-3	
Phase	Center Point	Worst Case	Center Point	Worst Case
Load	8.8	8.7	NA	NA
FT	≤3.6	≤3.4	≤0.0%	≤0.0%
Pre-peak 1	≤3.4	NT	≤0.0%	NA
Pre-peak 2	≤3.3	NT	≤0.0%	NA
Pre-peak 3	≤2.4	NT	≤0.0%	NA
Elution	≤3.2	≤2.9	≤0.0%	≤0.0%
Post-peak 1	≤2.0	NT	≤0.0%	NA
Post-peak 2	≤2.5	NT	≤0.0%	NA
Strip	6.4	6.3	0.4%	0.4%

NT = not tested; **NA** = not applicable; **FT** = flow-through fraction

Table 8: Monolith results for minute virus of mice (MVM)

	Total MVM (log_{10})		Percentage of MVM	
Phase	Center Point	Worst Case	Center Point	Worst Case
Load	6.5	6.4	NA	NA
FT	≤3.6	≤3.4	≤0.1%	≤0.1%
Pre-peak 1	≤3.0	NT	≤0.0%	NA
Pre-peak 2	≤3.3	NT	≤0.1%	NA
Pre-peak 3	≤2.1	NT	≤0.0%	NA
Elution	≤1.2*	≤0.9*	≤0.0%	≤0.0%
Post-peak 1	≤2.3	NT	≤0.0%	NA
Post-peak 2	≤2.8	NT	≤0.0%	NA
Strip	7.5	7.6	941.7%	1,635.7%

NT = not tested; **NA** = not applicable; **FT** = flow-through fraction
* large-volume sampling to increase sensitivity

SIMILAR clearance results were achieved for the two particle types at each condition, and the trend in reduced clearance seen for MVM could be monitored through the use of MVM-MVPs. Our data demonstrate the utility of MVM-MVPs as a spiking/analysis tool for process development and characterization.

Table 9: Monolith results for noninfectious minute virus of mice mock virus particles (MVM-MVPs)

	Total MVM-MVP (log_{10})		% of MVM-MVP	
Phase	Center Point	Worst Case	Center Point	Worst Case
Load	11.3	11.9	NA	NA
FT	≤7.1	≤7.3	≤0.0%	≤0.0%
Pre-peak 1	≤6.5	NT	≤0.0%	NA
Pre-peak 2	≤7.0	NT	≤0.0%	NA
Pre-peak 3	≤6.1	NT	≤0.0%	NA
Elution	≤7.4	≤6.8	≤0.0%	≤0.0%
Post-peak 1	≤6.6	NT	≤0.0%	NA
Post-peak 2	≤7.1	NT	≤0.0%	NA
Strip	11.0	11.5	43.1%	45.1%

NT = not tested; **NA** = not applicable; **FT** = flow-through fraction

3). That difference in LRV probably can be attributed to the influence of process parameters (load ratio and residence time) on virus–ligand interactions. As predicted, a higher load ratio and residence time yielded nearly a 1.0 $\log_{10}$ decrease in MVM clearance. For MVM-MVP center-point and worst-case runs, 7.0–7.4 and 7.8 $\log_{10}$ total particles were determined, respectively, giving LRV calculations of 4.91–5.16 and 4.07. Thus, similar clearance results were achieved for both particle types at each condition, and the trend in reduced clearance seen for MVM could be monitored through the use of MVM-MVP. Figure 3 also shows LRV results for a center-point run using an alternative base matrix.

Overall, those results demonstrate the high selectivity of the POROS CaptureSelect AAVX affinity resin, which can differentiate between the surface epitopes of AAV and the evolutionarily similar virus MVM. Such high specificity enables the resin to partition those two particle types from a heterogenous mixture containing both of them. Our data also reveal comparable results between MVM and MVM-MVP, demonstrating the utility of MVM-MVP as a spiking/analysis tool for process development and characterization.

To probe nonspecific binding, a more detailed interaction study was performed using MVM and XMuLV, which are the two most commonly used model viruses for VC spiking studies (**9**). To probe virus–AAV interactions, we performed an AAV-null run wherein the spiked virus load was devoid of AAV8 product. As Figure 4 shows, the null run demonstrated similar performance to the manufacturing control run for both MVM and XMuLV, indicating that the presence of AAV8 had minimal effect on clearance of model viruses.

Next, to probe interactions among viruses and V_HH ligands or POROS base beads, we used two control resins designed by Thermo Fisher Scientific. The first control was an AAVX-like resin with an identical base bead but a functionalized V_HH ligand with an alternate specificity that cannot bind AAV. The second control was POROS CaptureSelect AAVX resin without a functionalized V_HH ligand. Using these control resins, we observed similar VC levels to those of the center-point run using unmodified POROS CaptureSelect AAVX affinity resin, which indicates that minimal interactions occurred between viruses and the ligand or base beads. These results strongly indicate that model viruses show no nonspecific binding to either the V_HH ligand or to the POROS base bead — and that interactions between those viruses and the AAV product are minimal.

SPR results demonstrated that the AAVX V_HH ligand bound to the injected AAV1, but not to MVM-MVP (Figure 5). For an experimental control, the binding signal was recovered when AAV was spiked back into a 0.1-µg/mL MVM-MVP background. These data suggest that the presence of virus particles (infectious or otherwise) does not interfere with the ability of AAV to bind to the specific AAVX V_HH ligand.

Monolith Polishing: Tables 3–9 show complete VC results (including MVM-MVP) from our CIMmultus QA spiking experiments. During each experiment, we captured flow-through, three pre-AAV elution peaks, two postproduct peaks, and a strip fraction along with the load and main AAV elution pool. As the data show, no virus (or MVM-MVP) was detected in any fraction other than the strip for either center-point or worst-case conditions. That indicates complete clearance for all virus types in this downstream AAV process step. Viruses also were undetectable in the flow-through, pre– and post–main-peak collections.

Virus titers within the strip fractions were significant but differed by virus. The amount of MVM detected in the strip fraction was greater than the overall challenge, which could indicate interference with the assay. In some cases, the mass-balance of total virus detected within the collected fractions did not equate to the amount of virus challenged. That may be attributable to (partial) degradation of the virus by the stripping agent and/or to using the stripping agent for too short a time to elute all the virus.

Figure 6 shows LRVs from each experiment. The monolith offered effective removal for a wide range of physicochemically distinct viruses. In addition, the LRV data demonstrate comparability between MVM and MVM-MVP clearance at both center-point and worst-case conditions.

Final Results: Table 10 lists overall process LRVs achieved after using both

Figure 6: Monolith log reduction values (LRVs); see Table 1 for full virus names

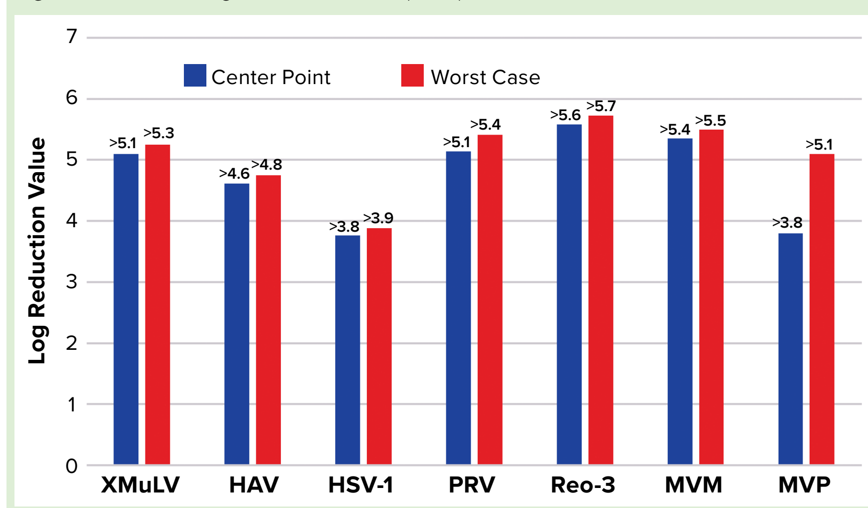


Table 10: Step-by-step and overall process log reduction values (LRVs) achieved at center-point operation

	XMuLV	HAV	HSV-1	PRV	Reo-3	MVM	MVM-MVP
POROS CaptureSelect AAVX resin	≥6.4	≥4.9	3.1	4.0	2.7	4.4	5.0
CIMmultus QA column	≥5.1	≥4.6	≥3.8	≥5.1	≥5.6	≥5.4	≥3.9
Overall	≥11.5	≥9.5	≥6.9	≥9.1	≥8.2	≥9.8	≥8.9

POROS CaptureSelect AAVX affinity resin and CIMmultus QA monolith polishing steps operated at center-point manufacturing conditions.

An Accurate and Economic Prediction Model

We sought to determine whether effective viral clearance could be achieved through chromatographic methods in an AAV purification process. Through spiking studies using a broad and inclusive panel of viruses, we determined that chromatographic modes of separation indeed can provide an effective VC strategy. Both POROS CaptureSelect AAVX affinity resin and CIMmultus QA anion-exchange monoliths demonstrated superb ability to reduce viral levels and contribute to high overall process LRVs.

During this study, we also sought to determine whether a biosafety-level 1 compliant, noninfectious mock MVM particle could serve as an accurate surrogate for predicting MVM removal. High correlation between the MVM and MVM-MVP results obtained throughout this study suggest that such an approach could provide an accurate and economic model for predicting the VC efficacy of other AAV chromatographic separation techniques.

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Productivity optimization and process calculations for AAV affinity chromatography

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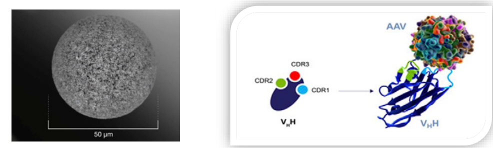
ThermoFisher SCIENTIFIC

Bioprocessing

INTRODUCTION

The use of recombinant adeno-associated virus (rAAV) as a delivery method for gene therapies continues to be successful with hundreds of ongoing clinical trials and some recent approvals. The diversity of applications for rAAV ranges from rare diseases affecting small patient populations to more prevalent inherited ailments such as hemophilia. The doses required vary widely from ~4E11 vg/eye for subretinal administration to 3.5E14 vg for intrathecal applications [1]. From a manufacturing perspective the field has moved to common approaches for production and purification of rAAV. Upstream approaches typically use transfection of HEK293 cells and titers are routinely in the 1-2E10 vg/mL although higher titers of up to 6E11 vg/mL at a 2000 L scale were recently reported [2]. These high titers will be needed for large dose and/or patient populations to meet the demand of these therapies and reduce costs. For rAAV purification the majority of the field has moved to scalable processes employing an affinity capture chromatography step [3] and commonly utilizing POROS™ CaptureSelect™ AAVX resin. In this work, dynamic binding capacity (DBC) data for multiple AAV serotypes were leveraged to estimate an optimal productivity of rAAV using the AAVX resin. An analysis of process conditions and column geometries that would fit maximum processing times and resin utilization was conducted for two case scenarios representing current titers for clinical manufacturing and high titers for commercial manufacturing scales.



POROS™ base bead technology (polystyrene divinylbenzene, left) and CaptureSelect™ ligand technology (single-domain antibody, right) are combined in the manufacturing of AAVX resin

METHODOLOGY

Dynamic binding capacity:

AAV2 breakthrough curves were generated using HEK293 clarified lysate to determine DBC at 10% breakthrough. AAV8 and rh10 DBC data were obtained from references 4 and 5, respectively.

Equation *I* was fitted to the DBC data using a linear regression numerical method.

Productivity:

Productivity curves were generated using equations *I* and *II*.

Column volumes and residence time for the non-loading steps were 25 CV and 2 min.

Column volumes and residence time for CIP steps were 10 CV and 3 min.

Scale-up and process considerations:

GMP pre-packed column pressure limitations were based on literature from multiple vendors.

Pressure drop at 3 bar was based on pressure-flow curves for POROS CaptureSelect AAVX resin (internal pressure-flow data).

Case scenarios

Processing time and resin utilization calculations were performed using Microsoft ® Excel ® assuming 20% full capsids and the results were further analyzed and plotted using MODDE® software.

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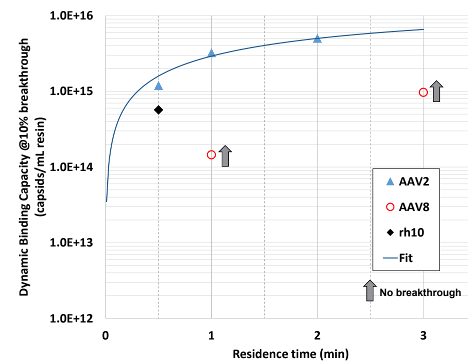
Dynamic Binding Capacity

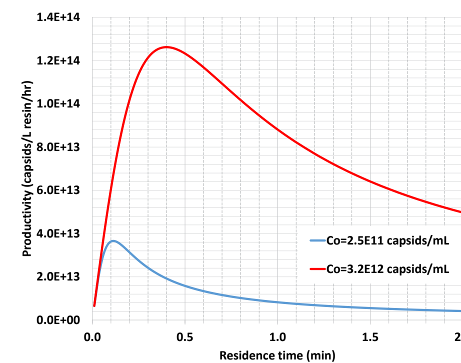


$$Q_d = \frac{Q_d^{max}\, RT}{\theta + RT} \quad (I)$$

Q_d = Dynamic Binding Capacity @10% breakthrough
Q_d^{max} = DBC at long residence times
RT = Load residence time
θ = Residence time constant

- ✓ Limited DBC data are available due to high capacity of AAVX resin, relatively low titers, and sample availability.
- ✓ DBC for AAV2 is relatively high (~1E15 capsids/mL resin) even at 30 sec residence time.
- ✓ Data fit to equation (*I*) approximates the dependence of DBC to residence time.

Productivity



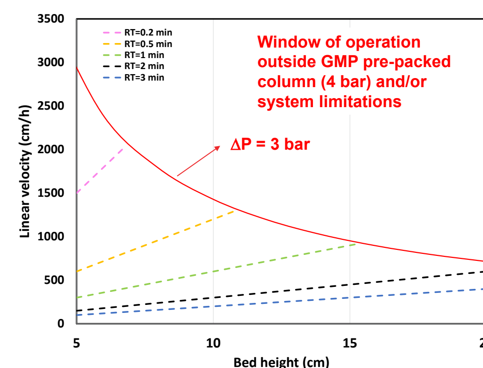
$$P = \frac{Amount\ of\ AAV\ capsids\ purified}{Unit\ resin\ volume\ \times Unit\ time}$$

$$P = \frac{\eta\, Q_d}{\frac{Q_d}{C_o} RT + CV_{non-load}\, RT_{non-load} + CV_{CIP}\, RT_{CIP}} \quad (II)$$

P = Productivity
η = Loading safety factor (% DBC)
C_0 = Load sample concentration
$CV_{non-load}$= Column volumes for non-loading steps
$RT_{non-load}$ = Residence time for non-loading steps
CV_{CIP}= Column volumes for non-loading steps
RT_{CIP} = Residence time for non-loading steps

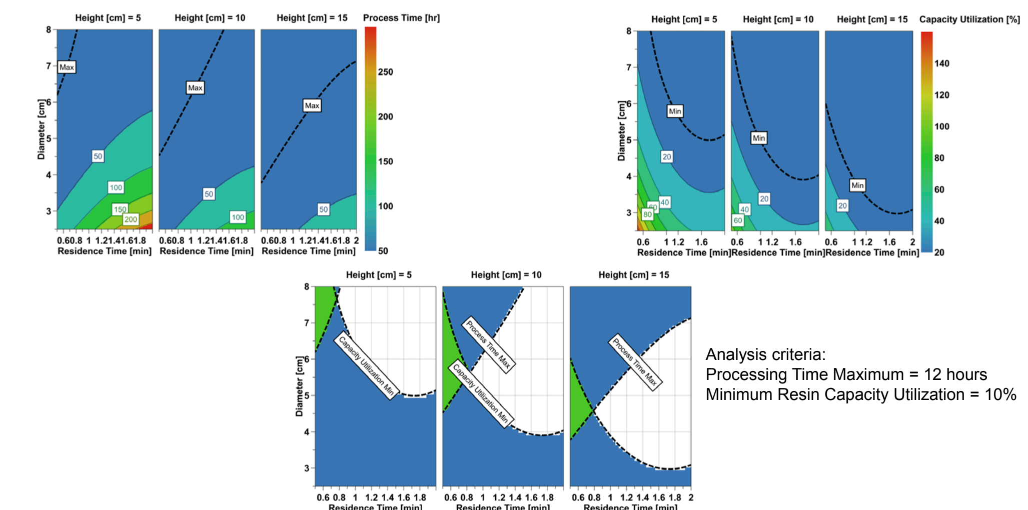
- ✓ Productivity maximum is achieved at residence times below 0.5 min.
- ✓ Productivity increases by ~3.5x with an increase in titer of ~12x.
- ✓ Increased titer shifts productivity maximum from ~7 to ~24 seconds RT for loading.

Scale-up and process considerations



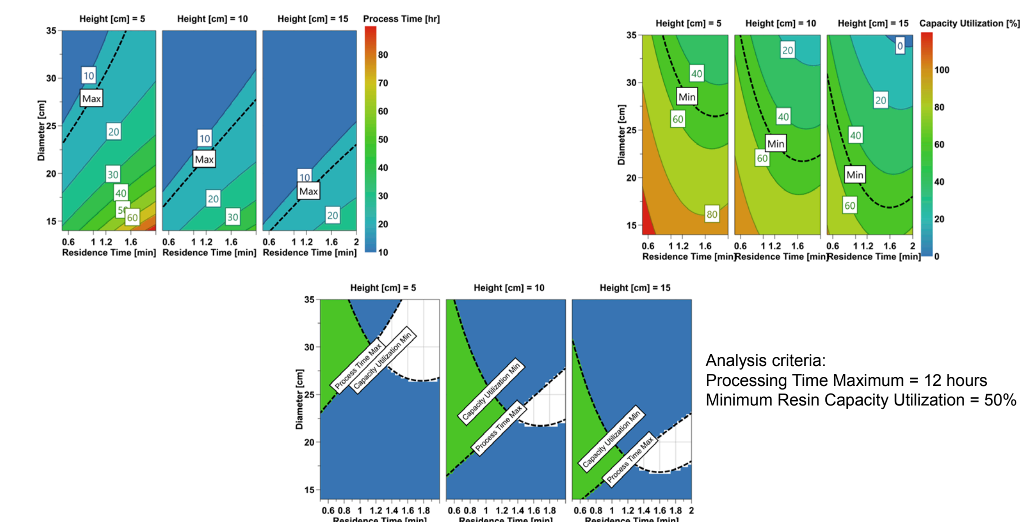
- ✓ Pre-packed columns are widely used in GMP manufacturing (4 bar limit).
- ✓ Owing to hardware limitations the optimal productivity can only be achieved with a 5 cm bed height and 30 sec residence time only with 10 cm bed heights.
- ✓ For larger columns (e.g. >25 cm i.d.) commonly used chromatography systems may limit operation to residence times >0.5 min.

Scenario 1. Clinical manufacturing, 200 L, Co=2.5E11 capsids/mL



- ✓ Residence times <0.8 min meet acceptance criteria for processing time and capacity utilization.
- ✓ Capacity utilization is low but CV are <0.4 L resin, i.e. low contribution to overall process cost.
- ✓ All column configurations in acceptable space require only 1 process cycle (data not shown).

Scenario 2. Commercial manufacturing, 2000 L, Co=3.2E12 capsids/mL



- ✓ Broader window of operation bed heights of 10 and 15 cm max res. time at 1.1-1.2 min.
- ✓ Capacity utilizations of 60-80% for column configurations meeting acceptable criteria.
- ✓ Only some configurations require 1 process cycle (data not shown). Considering potential system pump limitations optimal configurations are 20 cmD x 15cmL or 25 cmD x 10cmL.

CONCLUSIONS

- The relatively high binding capacity of POROS CaptureSelect AAVX resin was confirmed to be >1E15 capsids/mL resin at residence times >= 0.5 min for AAV2.
- Productivity is maximized at load residence times <= 0.5 min depending on titer but hardware and/or system considerations limit operation closer to 1 min.
- For clinical manufacturing the high DBC allows for a range of process conditions and requires small column volumes.
- For large bioreactor volumes and high titers the model suggests columns 20-30 cm diameter to meet typical processing limits while maximizing resin utilization.

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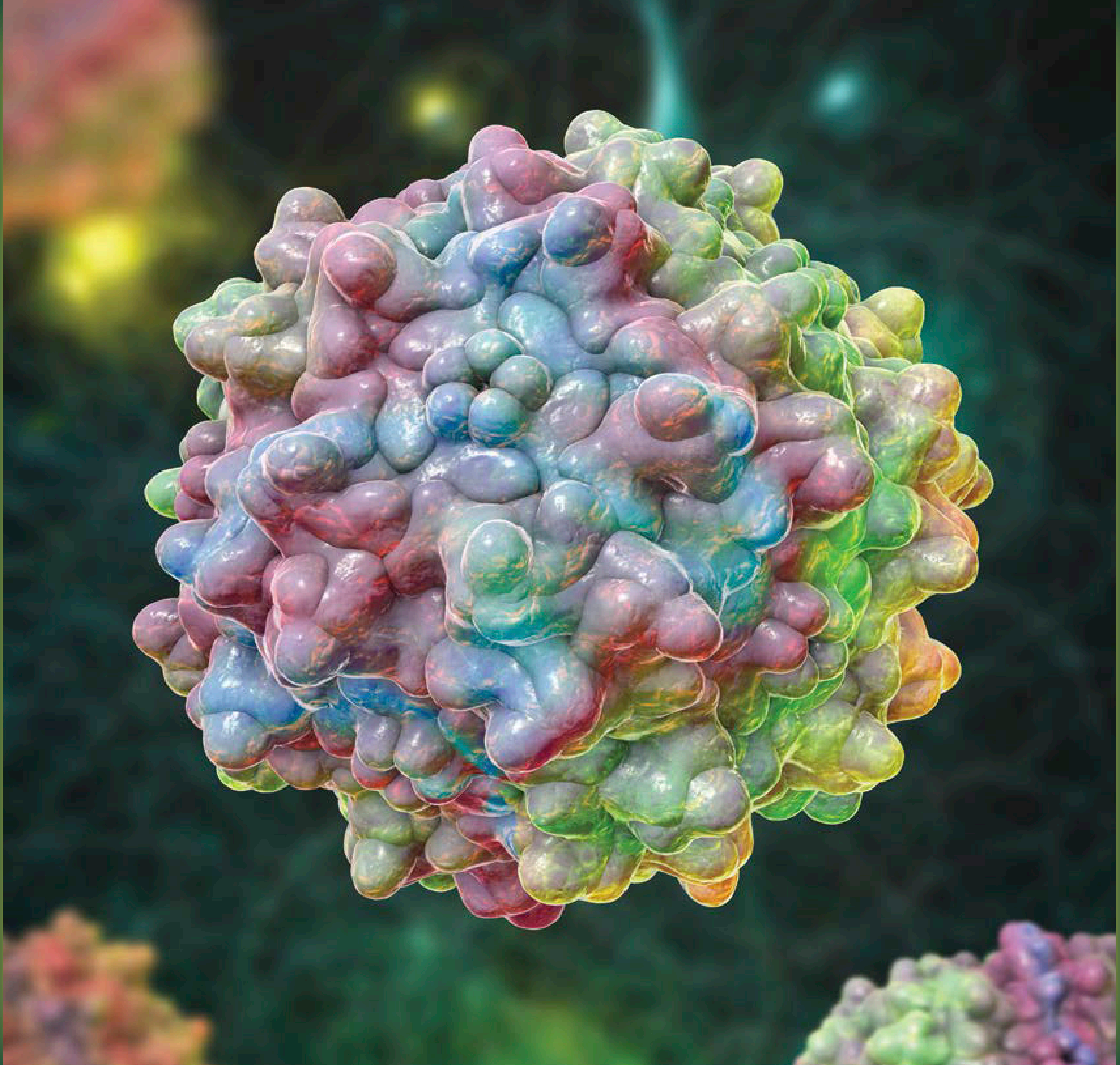
AAV Downstream Process and Product Characterization



Integrating Advanced Purification and Analytical Tools into the Workflow

BioProcess International
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AAV downstream process and product characterization

Integrating advanced purification and analytical tools into the workflow

Chantelle Gaskin, Ilaria Scarfone, and Julia Beck

Adenoassociated virus (AAV) vectors are a leading platform for gene delivery in the treatment of many human diseases. Efficient production of high-yield, high-quality AAV vectors is essential for continued advancement of the gene-therapy field, which can deliver profound and curative outcomes for patients. AAV vector mediated gene delivery has been approved for treating inherited blindness and spinal muscular atrophy, and long-term therapeutic effects have been achieved in patients with other rare diseases, including hemophilia.

As the gene-therapy field rapidly expands, regulatory guidance is evolving to help ensure the safety of such complex therapies and driving the need for efficient and effective methods of process and product characterization. To ensure proper characterization and meet regulatory expectations for product quality and safety, vector production workflows must integrate advanced purification and analytical tools. As Figure 1 shows, vector quality and purity should be monitored throughout the entire workflow using a number of methods.

Regulatory guidance recommends monitoring mycoplasma and other contaminants in upstream processes, which span a set of unit operations from plasmid development through viral vector production. For downstream processes, guidance increasingly focuses on removal of empty or incomplete capsids and clearance of adventitious viruses in addition to residual host-cell proteins and host-cell/plasmid DNA. Lot-release test expectations are comprehensive, including demonstrated clearance of process-related impurities to ensure patient safety and product quality (see box above). The final release step is governed by specifications for residual host-cell DNA and plasmid removal.

Lot-Release Test Expectations

Identity
- Capsid/serotype and transgene

Strength
- Viral genome titer
- Total viral particles

Potency
- Infectious titer
- Functional analysis

Purity
- Host-cell protein (HCP) and DNA clearance
- Residual bovine serum albumin (BSA), endonucleases, ligands, plasmids, transfection reagents, and detergents
- Genome integrity
- Protein purity
- Aggregation status
- Ratio of full to empty capsids

Compendial Assays
- Appearance
- pH
- Osmolarity

Safety
- Absence of adventitious/replication-competent viruses
- Sterility (mycoplasma, endotoxin, bioburden)

Here, we describe advanced purification strategies for AAV capture and polishing steps along with analytical tools that can be integrated seamlessly into vector production processes for simplified upstream and downstream workflows.

Mycoplasma Testing

A known contaminant of mammalian cell cultures, mycoplasma can affect the safety, quality, and efficacy of biotherapeutic products. Given the risks associated with this contaminant and the need to comply with regulatory requirements, it is critical to

Figure 1: A typical adenoassociated virus (AAV) vector production workflow showing key points in the process at which analytical methods are integrated

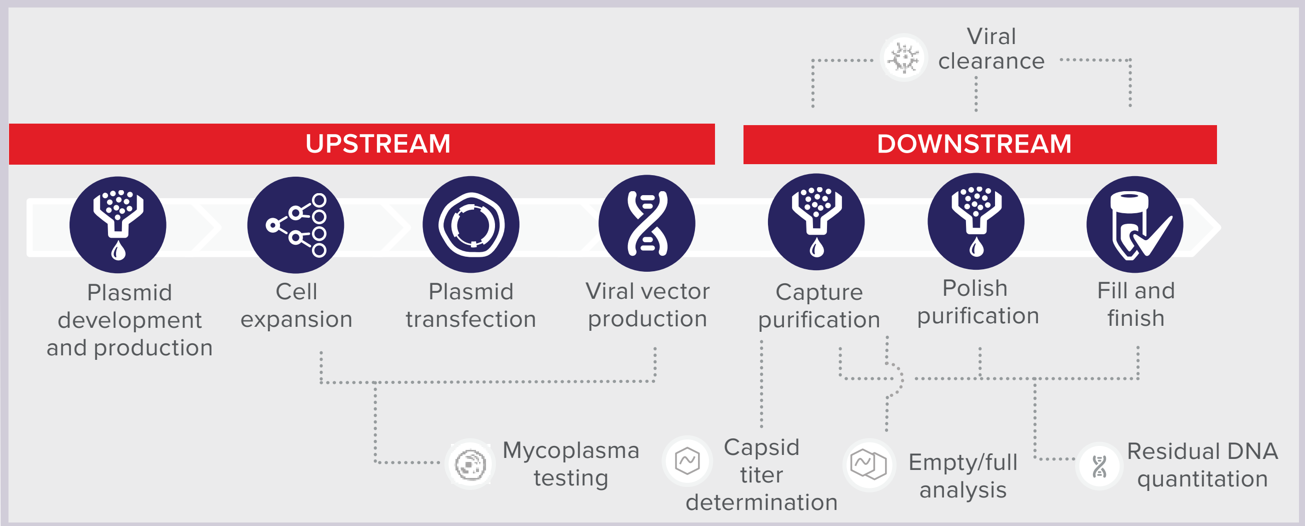
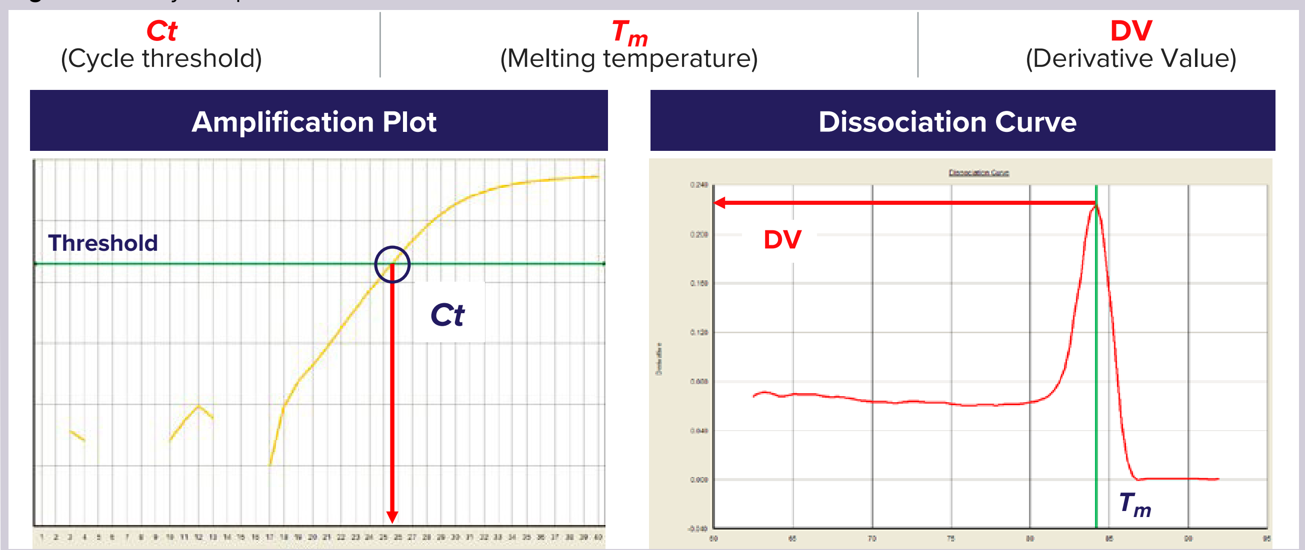


Figure 2: Analytical parameters



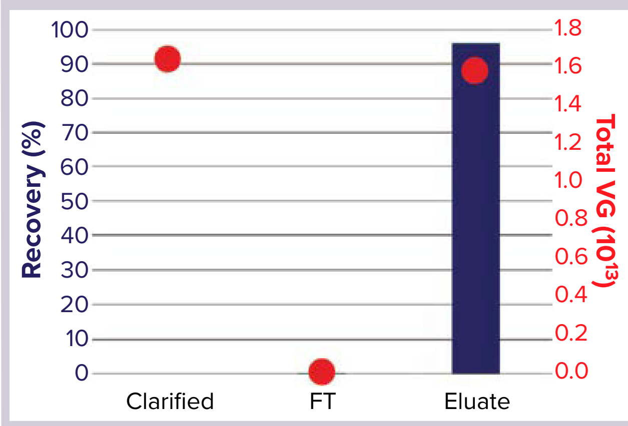
ensure that upstream cell cultures are free of mycoplasma before feeding bulk harvest material into a downstream process.

Historically, the only test method for mycoplasma accepted by regulatory agencies was based on a 28-day cell culture. Such a long testing cycle could delay lot disposition, so the industry has moved toward using nucleic-acid amplification techniques (NATs) as a faster alternative. One example with an extensive regulatory acceptance track record is the Applied Biosystems® MycoSEQ™ mycoplasma detection system, which incorporates a highly sensitive and specific real-time polymerase chain reaction (PCR) assay that delivers mycoplasma contamination results in under five hours.

Because the MycoSEQ system was designed to fulfill regulatory requirements, more than 40 marketed biologic manufacturing processes have received acceptance from regulatory agencies worldwide to use this assay for testing applications across multiple therapeutic modalities, including gene-therapy applications (following validation, regulatory filing, and review). More than 30 customers are now in the process of validation and regulatory submission using the MycoSEQ assay as a lot-release test.

The MycoSEQ system can detect more than 90 different species of mycoplasma and related species. Sensitivity has been demonstrated in both internal and external validations showing that it can detect fewer than 10 mycoplasma genome copies or colony-forming unit (CFU) equivalents. That is the required sensitivity for mycoplasma NAT detection methods according to regulatory guidelines. For a sample to be considered positive for mycoplasma, it must meet three objective analysis parameters established during validation (Figure 2):

Figure 3: Purification of a synthetic AAV serotype using the AAVX resin; recovery (blue bar) and yield are >90%.



• cycle threshold (*Ct*), a measure of the target DNA level at the start of the PCR reaction

• derivative value, a measure of specific amplicon quantity generated during the PCR reaction

• melting temperature (T_m), a measure of amplicon size and base composition that is known for mycoplasma using this assay.

A unique discriminatory positive control (DPC) significantly reduces the possibility of false-positive and false-negative results. It is used to confirm extraction and PCR performance without risk of false positives from accidental cross-contamination. The DPC facilitates amplification of a modified PCR product with a T_m well outside the range of real mycoplasma amplicons. With post-PCR melting analysis, users can determine whether samples contain mycoplasma or a positive control. Because a DPC maintains the same extraction behavior as genuine mycoplasma DNA, it can be used as a sample-extraction positive control for spiking test samples without risk of unrecognized cross-contamination. In addition, the DPC can be used as a surrogate for mycoplasma DNA during method optimization and early qualification, mitigating the requirement to use live mycoplasma and thus serving as a precaution against introduced mycoplasma contamination at early stages of method adoption. For validation, mycoplasma genomic DNA provides a suitable alternative to live mycoplasma.

The MycoSEQ system's AccuSEQ™ real-time detection software generates automated presence or absence results during data analysis. Automated calls are made based on the *Ct*, T_m, and derivative values of a test sample and inhibition control, as previously described. The software's security, audit, and electronic-signature capabilities are designed to enable 21 CFR part 11 compliance required in a good manufacturing practice (GMP) environment.

Capture Purification

The most common first purification step in an AAV downstream process is capture of capsids from cell lysate using affinity chromatography. POROS® CaptureSelect® affinity resins for AAV purification offer broad selectivity, high capacity and elution recovery (>90% recovery and 90% purity from a single step), and excellent scalability (Figure 3). The affinity ligands immobilized onto POROS beads are VHH camelid antibody fragments recombinantly expressed in yeast. Because their production process is free from animal-derived components, the resins are compatible with commercial processes. Two such ligands are serotype specific (AAV8 and AAV9), and a third ligand acts as a universal capsid affinity resin (AAVX). The AAVX resin serves as a platform solution for manufacturers developing therapies that include a range of serotypes.

The POROS backbone is a rigid, polystyrene-divinylbenzene–based solid support that allows for robust chemical stability and a linear pressure flow curve, independent of column diameter. The large pore structure reduces mass transfer resistance and results in an increased surface area, which in turn raises the binding capacity. The 50-µm bead size

Figure 4: Viral clearance capabilities of AAVX affinity resin; LRV = log reduction value

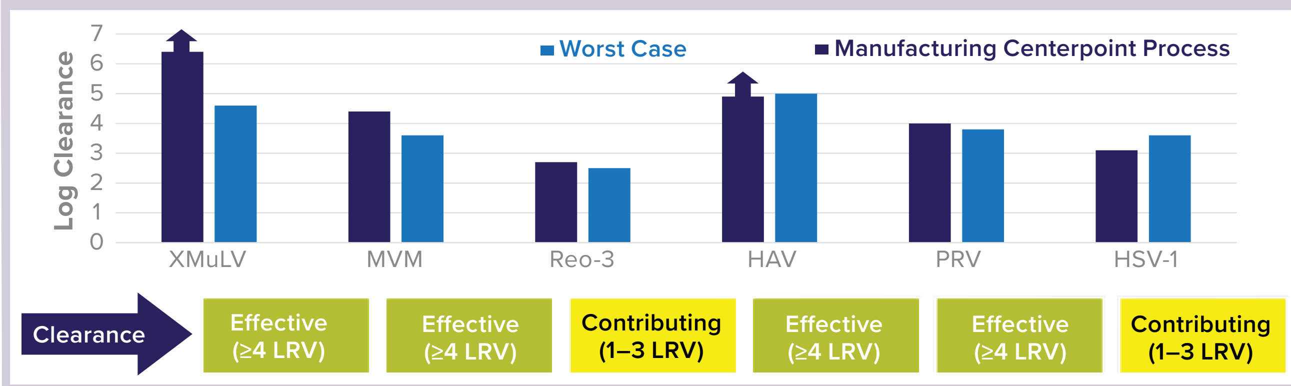
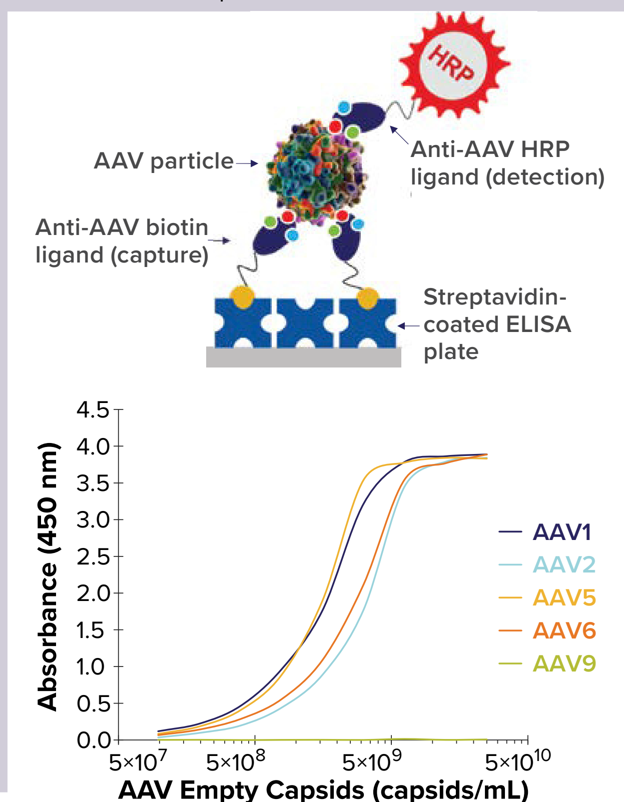


Figure 5: (TOP) Total AAV capsid enzyme-linked immunosorbent assay (ELISA) and (BOTTOM) results; HRP = horseradish peroxidase



gives increased resolution without compromising process efficiency.

Viral Clearance

In addition to host-cell and process-related impurity clearance, the AAVX resin has been demonstrated to provide effective viral clearance. Figure 4 shows robust clearance of model viruses achieved using AAVX affinity chromatography. The process was tested with a panel of six RNA- and DNA-based enveloped and nonenveloped viruses of different sizes. The AAVX resin achieved >4 log reduction of four of those and 1–3 log reduction of the remaining two viruses on the panel.

Capsid Titer Determination

CaptureSelect ligands also come in free conjugated forms. Biotinylated and horseradish-peroxidase (HRP)–labeled AAVX ligands can be used to develop enzyme-linked immunosorbent assays (ELISAs) for determination of total capsid titer. The method can be applied to both in-process and purified samples and used for monitoring the mass balance in harvest and capture unit operations.

Figure 5 illustrates use of AAVX-conjugated ligands in a highly sensitive total–capsid-titer ELISA for multiple AAV serotypes. Streptavidin-coated plates bind the biotinylated capture ligand, and the HRP-labeled ligand is used for detection. Standard curves of each serotype were prepared separately for AAV1, AAV2, AAV5, AAV6, and AAV9, then aliquoted onto the coated wells. Following a one-hour incubation, the wells were treated with a diluted preparation of the anti-AAVX HRP detection ligand and incubated for 10 minutes with a 3,3′5,5′-tetramethylbenzidine (TMB) substrate. The reaction was stopped by addition of acid. This method is intended as a starting point, and method optimization is always recommended. Under most conditions, it yields a valid assay covering the ranges of 1×10^8 and 1×10^{11} capsids/mL for most serotypes.

Table 1: Chromatography settings and mobile phases for analysis of empty and full capsids

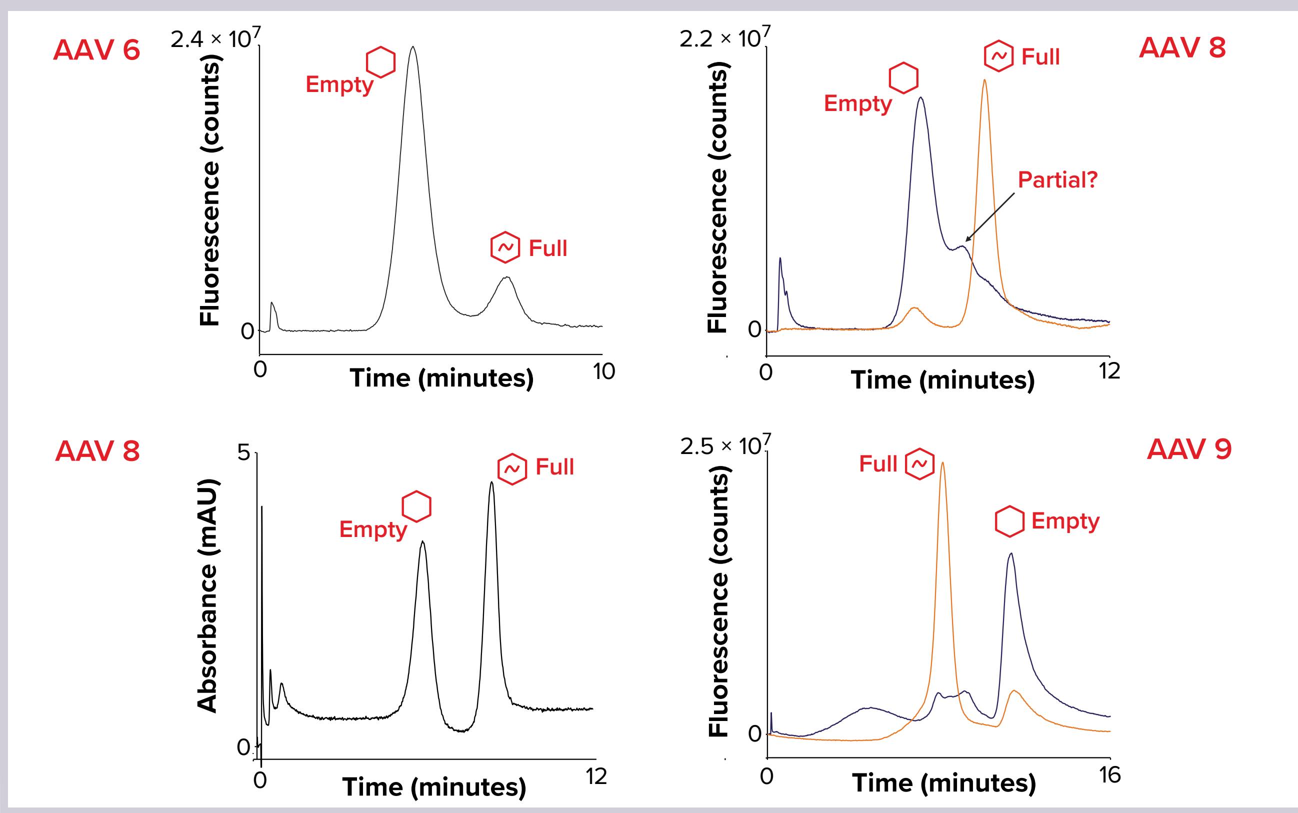
Column Format	4 × 50 mm or 2 × 50 mm
Detection	UV: Full (260 nm) and empty (280 nm) capsids **Fluorescence:** higher sensitivity, more accurate quantitative data, full capsids only
Temperature	30 °C (temperature and pressure can affect capsid structure stability)
Mobile Phase: Salt Gradient	A: 20 mM Bis-Tris propane at pH 9.5 or 8.5 (depending on serotype) B: 20 mM Bis-Tris propane at pH 9.5 or 8.5, 1 M tetramethylammonium chloride or tetraethylammonium chloride (for better resolution with a higher background signal)
Mobile Phase: pH Gradient	A: 20 mM ammonium bicarbonate and 15 mM ammonium hydroxide at pH 9.2 B: 30 mM acetic acid and 15 mM formic acid at pH 2.8

Empty/Full Capsid Analysis

Another critical step in vector-production workflows is analyzing the ratio of empty to full capsids, which can be determined using a ProPac™ SAX-10 high-performance liquid chromatography (HPLC) column. Robust separation of full and empty particles enables users to determine the ratio following both affinity purification and polishing steps. The results can be used to confirm successful removal of empty capsids and provide a baseline for further downstream purification.

ProPac SAX-10 columns are packed with polymer resin coated with a hydrophilic layer that prevents unwanted hydrophobic interactions, and the grafted polymer chains carry strong anion-exchange functional groups. Either salt or pH gradient elution can be used. With a salt gradient, protein samples bind to the stationary phase through charge interaction and elute with an increase in the salt concentration. With a pH gradient, negatively charged AAV particles become neutral as pH

Figure 6: Salt (TOP ROW) and pH (BOTTOM ROW) gradient analysis of full and empty particles



decreases, so they elute from the column. Small differences between the isoelectric points (pI) of full and empty particles allow for such separations.

Either UV or fluorescence can be used for detection. With UV detection, information on full and empty capsids is provided by the intensity of signals at 260-nm and 280-nm wavelengths, respectively. The sensitivity of UV detection is lower than that of fluorescence detection, which also provides better quantitative data because it monitors the AAV capsid signal, derived from the tryptophan residue of the capsid protein.

Table 1 summarizes conditions and mobile phases recommended for salt and pH gradients. Baseline separation of empty and full capsids from different serotypes was achieved using those conditions with tetraethylammonium chloride as the salt (Figure 6, TOP ROW). The pH gradient also provided good baseline separation for both AAV8 and AAV9 serotypes (Figure 6, BOTTOM ROW).

Polishing Purification

A range of weak and strong POROS anion-exchange resins can be used for removal of empty capsids in polishing chromatographic operations. The 50-µm POROS base bead is made of polystyrene-divinyl benzene, which provides rigidity for a stable column bed and enhanced chemical stability. As described above, these resins provide high binding capacity and linear pressure-flow curves without compromising on resolution during scale-up.

Qu et al. described empty and full capsid separation using POROS HQ resin (**3**). Capsids purified by cesium chloride gradient were applied to a POROS HQ column and then eluted using a linear sodium-acetate gradient. Empty capsids, characterized by a higher absorbing A_{280} peak, eluted sooner than the full capsids, characterized by a higher absorbing A_{260} peak. When the empty and full capsids were combined at a ratio of 16:1 and applied to the same column with the same gradient conditions, the empty capsid peak presented first followed by the full capsid peak with baseline separation, demonstrating the resin's resolving power and providing a feasible and scalable process for AAV empty capsid removal.

Residual DNA Quantitation

Purification workflows for AAV processing must remove residual host-cell DNA and plasmid DNA impurities effectively. The World Health Organization (WHO) requires documented residual DNA per therapeutic dose to be <10 ng; the US Food and Drug Administration (FDA) requests that host-cell DNA should be as low as possible and that a highly sensitive method be used to determine DNA levels. The agency also encourages companies to conduct vigorous clearance studies throughout their

Figure 7: Standard curves of (LEFT) plasmid DNA with a kanamycin-resistance (KanR) gene and (RIGHT) human embryonic kidney cell (HEK293) assays

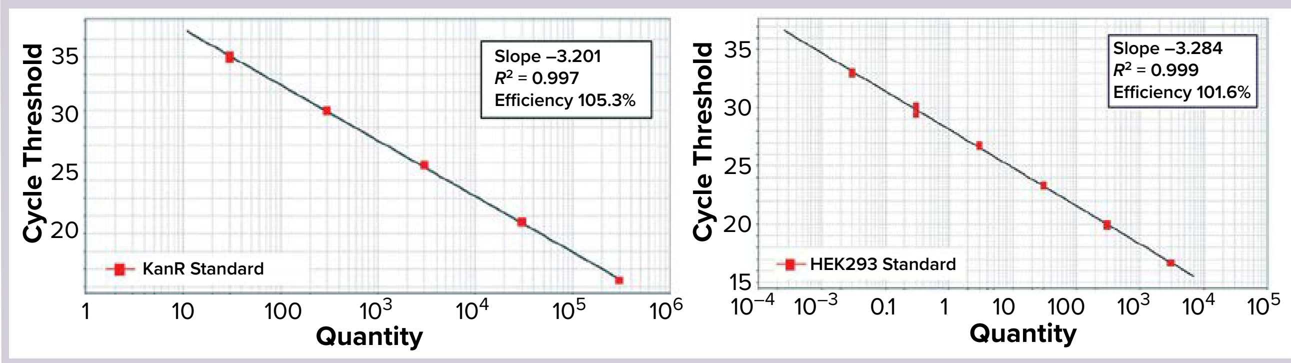


Table 2: Residual DNA kit specifications used in common AAV production platforms

Specification	Kanamycin-Resistance–Gene Plasmid DNA Kit	HEK293 DNA Kit	*Sf*9 Baculovirus DNA Kit
Linearity	R^2 > 0.99	R^2 > 0.99	R^2 > 0.99
PCR Efficiency	100% ± 10%	100% ± 10%	100% ± 10%
Precision	≤10% CV	≤10% CV	≤10% CV
LoD	15 copies	30 fg	30 fg
LoQ	30 copies	300 fg	300 fg
Range	300,000 copies to 30 copies	300 fg to 3 ng	300 fg to 3 ng

PCR = polymerase chain reaction **Sf9** = *Spodoptera frugiperda* cell line 9 **LoD** = limit of detection **LoQ** = limit of quantitation

downstream processes both to demonstrate removal of the vast majority of DNA from product streams and to monitor for process deviations.

To confirm successful removal of host-cell DNA according to regulatory requirements for lot-release testing, AAV product developers should incorporate a simple and reliable analytical kit that measures residual DNA into their workflows. To monitor DNA clearance, quantitation can be performed at different stages throughout downstream processing, from cell-culture harvest through to the final drug substance.

The Applied Biosystems resDNASEQ™ quantitative DNA system provides an effective, fully integrated, and all-inclusive approach to determining levels of residual DNA. Sample preparation, a sensitive and accurate DNA quantitation method, highly characterized DNA standards, all necessary reagents, and data analysis are included. Residual DNA testing assays are available for a number of cell lines, including two commonly used systems for AAV manufacturing: human embryonic kidney (HEK) 293 host-cell DNA quantitation and simultaneous quantitation of both *Spodoptera frugiperda* (*Sf*9) host-cell DNA and baculovirus vector DNA.

To address the need to quantitate residual vector DNA in AAV production using HEK293 cells, the resDNASEQ kit was developed for quantitative plasmid DNA with a kanamycin-resistance (KanR) gene.

The resDNASEQ assays offer ultrahigh sensitivity with a limit of quantitation (LoQ) down to 0.3 pg/reaction for HEK293 DNA, *Sf*9–baculovirus, and 30 copies for plasmid DNA with the KanR gene. A rapid, streamlined workflow with optional automated sample preparation provides results in under five hours. Table 2 summarizes the specifications of resDNASEQ quantitative HEK293, *Sf*9, baculovirus DNA, and quantitative plasmid DNA KanR gene kits. Standard curves in Figure 7 demonstrate the high sensitivity and broad dynamic range of two of those assays as examples.

The resDNASEQ quantitative plasmid DNA kanamycin-resistance–gene kit was designed to detect and quantitate the vast majority of currently known kanamycin-resistant–gene families. Careful analysis of conserved regions led to creation of a multiprimer assay to target all alleles with the same sensitivity. A number of common commercially relevant plasmids were spiked into the matrix in quantities of either 100 or 100,000 copies, then manually extracted and quantified using a resDNASEQ quantitative plasmid DNA kanamycin-resistance–gene kit. Each plasmid was recovered at >85%, and similar results have been observed for resDNASEQ quantitative HEK293 DNA kits.

To demonstrate specificity of the resDNASEQ quantitative plasmid DNA kanamycin-resistance–gene kit, a series of experiments used unrelated DNA directly spiked into the PCR reaction (Table 3) and

Table 3: Standards and reagents tested in the kanamycin-resistant plasmid kit exclusion panel (Figure 8)

Crossreactant H	3-ng spike *Escherichia coli* DNA
Crossreactant I	3-ng spike human embryonic kidney (HEK293) cell DNA
Crossreactant J	3-ng spike adenovirus 2
Crossreactant K	3-ng spike murine leukemia virus (MuLV)
Crossreactant L	3-ng spike rabbit antibody
Crossreactant M	3-ng spike bovine antibody
Crossreactant N	3-ng spike chicken antibody
Crossreactant O	3-ng spike pAV1 (AAV genome)
Crossreactant P	3-ng spike Madin–Darby Canine Kidney (MDCK) cells
Crossreactant Q	3-ng spike Chinese hamster ovary (CHO) cells
Crossreactant R	3-ng spike murine myeloma (NS0) cells
Crossreactant S	3-ng spike *Pichia pastoris*
Crossreactant T	Ampicillin (300,000 copies)
Crossreactant U	Blasticidin (300,000 copies)
Crossreactant V	Hygromycin (300,000 copies)
Crossreactant W	Puromycin (300,000 copies)
Standard	428std6 15 copies
NTC	No template control

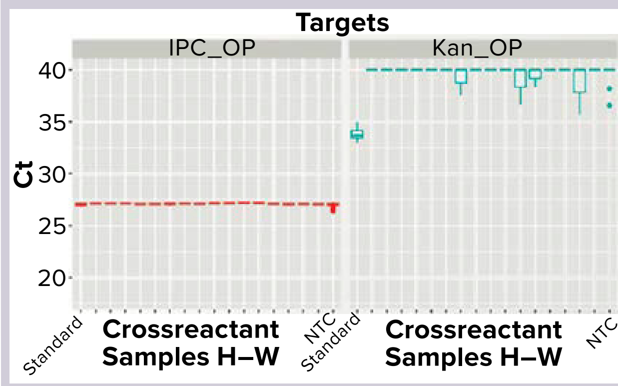
included an internal PCR control to monitor PCR inhibition (Figure 8). All reactions provided the same *Ct* value, which indicated that the PCRs performed as intended. None of the DNA in the exclusion panel was amplified by the resDNASEq kanamycin assay. The first lane in the graph shows amplification of the DNA standard included in the kit at 15 copies (*Ct* ≈ 34). Most of the other reactions provided a nonpurification curve; others provided a range of *Ct* values well above the limit of detection (LoD).

Enabling Technologies

In addition to the AAV gene therapies approved thus far, a robust pipeline of clinical candidates reinforces the potential of this modality to treat a wide range of diseases caused by single-gene defects and more complex conditions such as cancer, neurological, cardiovascular, and infectious diseases. AAV vectors are likely to remain a gene-delivery mechanism of choice for many such treatments.

Sustained growth of the AAV industry sector and the safety of gene therapies both depend on a combination of high-quality purification tools and analytical methods that are orthogonal to titer and recovery testing and are capable of meeting evolving regulatory requirements. Here, we have outlined analytical methods that work in conjunction with both capture and polish chromatography steps to

Figure 8: DNA specificity exclusion-panel test results from a kit for plasmid DNA with a kanamycin resistance gene, including standard at limit of detection (LoD) of 15 copies



create a simplified and streamlined AAV downstream process. Workflows that incorporate these advanced technologies will help ensure the quality and safety of gene therapies for their intended recipients and build confidence in this powerful therapeutic modality throughout the healthcare infrastructure.

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