How to Develop a Scalable AAV Process, from Start to Finish

Cytiva Scientists Lend Their Insights from Two Years' Work

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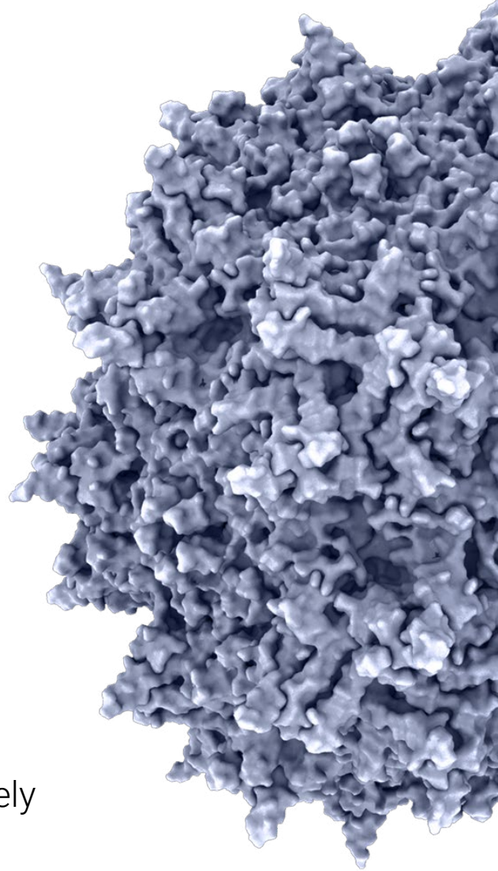

Introduction

Viral vectors are an integral delivery vehicle in modern medicine and are used in everything from oncolytic viruses to vaccines and gene therapies. With two gene therapies approved by regulatory agencies in 2021 and more in 2022, these therapies are delivering on their promise despite concerns on dosing and safety. Eyes were the first targets; there is growing interest in potential indications for targets in the central nervous system (CNS), liver, and muscles. Of the viral vectors used in gene therapy, adeno-associated virus (AAV) has emerged as the frontrunner.

In 2021, there were more than 300 gene therapies in clinical trials, about half using AAV. This virus is a versatile tool, with serotypes that target specific tissue types. This fact, combined with the prevalence of engineered variants, creates an almost boundless array of choices. On the flip side of this versatility is the challenge: How do we find ways to produce the various serotypes with high efficiency, sufficient to generate many doses of therapy?

Can we learn from monoclonal antibodies (mAbs), which use platform approaches to make grams of material? How do we achieve something similar for AAV? First off, we can't expect similar outputs for a burgeoning field. With mAbs, it took decades to get such high titers upstream that downstream became a bottleneck. And decades to achieve high recoveries following purification. But what we can do is learn more quickly than we did with mAbs to get better and better. This will take a combination of grit, perseverance, and innovation.

AAV is not a mAb. It's five times larger and is packaged into capsids, which can contain all of the necessary genetic information, partial, or no genetic information. A main challenge upstream is getting a high percentage of full capsids in the harvest. Downstream, it's effectively separating the full capsids from empties that will reduce efficacy and potentially provoke an immune response.

We don't have all the answers, but we have made progress both upstream and downstream. And we learned a lot of lessons along the way.

We believe in the promise of gene therapy, and if you're reading this you do, too. We hope you find inspiration in the articles in this eBook, so you can bring your own therapy to life.

Here we present highlights from a process that has been developed – and continues to be refined – with help from nearly two dozen Cytiva scientists. We frame these highlights around common challenges we hear from those who are developing and producing AAV.

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Meet the Team

Here are some of the Cytiva scientists who have helped to develop this start-to-finish process, with input from adeno-associated virus (AAV) process developers and manufacturers.

Ann-Christin Magnusson
Staff Research Engineer, Protein and Viral Production, Cytiva

Joining Cytiva in 2008, Ann-Christin currently leads development of cell culture solutions for virus production supporting both vaccine manufacturing and gene therapy applications. Ann-Christin holds a degree in Biomedical Engineering from Uppsala University, Sweden and has over 30 years of hands-on experience in cell culture of different cell lines.

Dr. Åsa Hagner McWhirter, PhD
Principal Scientist, Bioprocess applications R&D, Cytiva

Åsa joined Cytiva in 2003, currently leading developments in the downstream process for viral vectors. She has focused on customer collaborations to understand the specific needs for new dedicated products as well as to show use and performance of existing products in case studies for viral vector purification and analysis. Åsa holds a PhD in Medical Biochemistry from Uppsala University.

Anna Moberg
Staff Research Engineer and Project Manager, Cytiva

Anna Moberg studied analytical chemistry at Uppsala University, Sweden. Since 1998 she has worked in R&D with system, application, and consumables development for Biacore™ systems in Uppsala, Sweden. Currently she heads the Biacore™ Market Support team focusing on customer collaborations and generating marketing and training materials.

Extended Team

Pictured above: Ann Lövgren, Josefin Thelander, Åsa Hagner McWhirter, Ann-Christin Magnusson, Lena Sandberg, Sebastian Persson, Anna Mattson, Åsa Lagerlöf, Elin Monie, Eva Blanck, Viktor Björklund, Robert Magnusson

Not pictured: Albin Larsson, Camilla Estmer Nilsson, Daphne Areskoug, Anna Moberg, Marcus Kjellander, Linnea Nygren Babol, Mikaela Löfbacka, Hans Blom, Greta Hulting, Magnus Bergman, Christine Sund-Lundström, Jean-Luc Maloisel, Brigitta Németh, and Ola Lind

Challenge 1: Choose a Host Cell and Growth Conditions Suitable for GMP

Background

For research studies, adeno-associated virus (AAV) is often prepared in cells that adhere to the bottom of culture plates or flasks. Fetal bovine serum is a typical addition to spur cell growth. When it's time to increase the amount of virus for further studies, the number of containers is increased, or 'scaled out.' While this works for small quantities, there's a practical limit to the number of containers and hands-on time for larger quantities. Also, flask-based processes are prone to contamination.

Best practice for biologic production in good manufacturing practices (GMP) environments is to close and automate processing to minimize the risk of contamination and human error. Avoiding the use of serum and other animal-derived components confers a regulatory advantage. These sources can carry adventitious viruses that will be challenging to remove later since their sizes are similar to that of the desired recombinant AAV (rAAV).

So, what are the options for scalable cell growth? One possibility is to grow adherent cells in fixed-bed reactors. Another is to grow cells in suspension. We chose the latter option for our process. Furthermore, we directly adapted the cells into a serum-free medium for suspension culture that would be used for subsequent transfection, to avoid the need for a medium change.

Experimental highlights

We adapted adherent HEK293T cells to HyClone™ cell culture transfection medium, HyCell™ TransFx-H. In parallel, HEK293 cells (without the SV40 large T antigen) were also successfully adapted to the same medium using the same strategy. The illustration in Figure 1 provides an overview of the steps.

As a starting point for adaptation, cells were grown in classical **HyClone™ DMEM medium with high glucose** and 10% HyClone™ Fetal Bovine Serum (FBS). Then, they were transferred directly into the new transfection medium, which was supplemented with 4 mM of HyClone™ L-glutamine and 0.1% nonionic surfactant. The cell growth and morphology of the HEK293T suspension cells were evaluated microscopically over 10 passages. To avoid aggregates and reduce shear stress, we used 0.1% surfactant from the beginning and kept the cell density below 2.5 × 10^6 cells/mL.

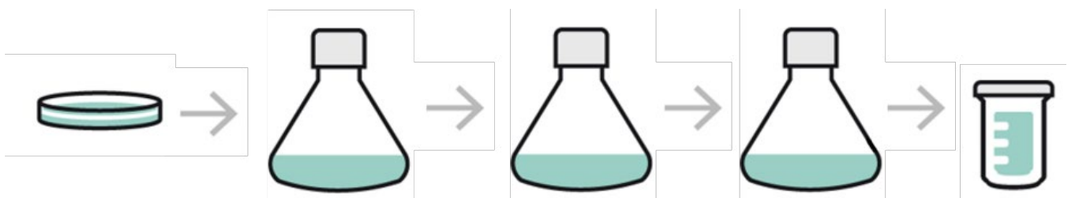


Fig 1. Illustration of cell culture adaptation: direct adaptation; detachment of adherent cells and subculture; seeding into shake-flask culture; monitoring of viable cell density, morphology, and aggregates; and creating a working cell bank.

Bottom line

Cells adapted to HyClone™ medium met our set success criteria of < 10% aggregation (small aggregates of < 10 cells) with robust cell growth.

Details

Read the full app note on AAV process development.

Next step in start-to-finish process:

Optimize the transfection conditions using a design of experiments (DoE) approach…

"Before you move into clinical studies, it's important to be using a process that is robust, reproducible, and will scale up all the way to commercialization. Otherwise, you may be forced to switch, which can cause delays trying to convince regulators that AAV from your new process is comparable to what's produced in flasks."

—Ann-Christin Magnusson

Challenge 2: Optimize Transient Transfection for Scalability

Background

Making AAV is a complex endeavor. AAV belongs to the parvovirus family and is dependent on co-infection with other viruses, mainly adenoviruses, in order to replicate in mammalian cells. The following genetic information is required for AAV production: AAV genes to make the viral capsid proteins; AAV helper Rep (Replication) genes for viral genome replication and packaging; and the gene of interest (GOI) for expression of the protein to elicit the desired genetic effect.

The main options to produce recombinant AAV include incorporating all elements into a stable producer cell or delivering the genes on multiple plasmids by transient transfection. HEK293 cell lines are typically used.

Triple-plasmid transfection is the most common method, as it's quick to implement given the pressures to be first to market with any new gene therapy. However, transient transfection is challenging and costly to scale.

Because it's the most common and accessible method, we chose to perform triple-plasmid transfection using polyethyleneimine (PEI), as shown in Figure 2.

Experimental Highlights

A series of DoE studies were performed to evaluate the optimal conditions for the transfection. Five studies were performed with four being applied to rAAV2. During our process development, the goal was to achieve the following criteria to meet the requirements for the downstream process: 10^{14} virus particles (VP)/L, 10^{13} viral genomes (VG)/L, and 10% or more full capsids in harvested material.

We started to investigate the effects of plasmid DNA concentration, different cell densities, PEI:DNA ratio, transfection volume, and incubation time. We continued with fixed parameters from the previous DoE and added new parameters to the next DoE. Evaluation of temperature, as well as DNA plasmid ratio, were investigated and evaluated. DoE studies 1 through 4 showed that we could not achieve all these requirements for rAAV2, and consequently, the developed transfection protocol was tested for rAAV5. DoE5 was therefore designed as a verification study that confirmed the optimal DNA concentration and DNA-PEI complex incubation time for rAAV5 production.

Transfection efficiency and transduction assays detecting GFP-expressing cells were evaluated by flow cytometry. Viral genomes (VG, full capsids) were determined by qPCR (VG/L), and the total viral particle titer (VP/L) was determined by ELISA. The ratio of qPCR:ELISA was used to estimate the percentage of full capsids.

In our DoE experiments, we found that a high concentration of DNA improved VG titer and transfection incubation time did not significantly affect the VG titer. However, when we looked at the percentage of full capsids, we saw the opposite effect; by reducing the DNA

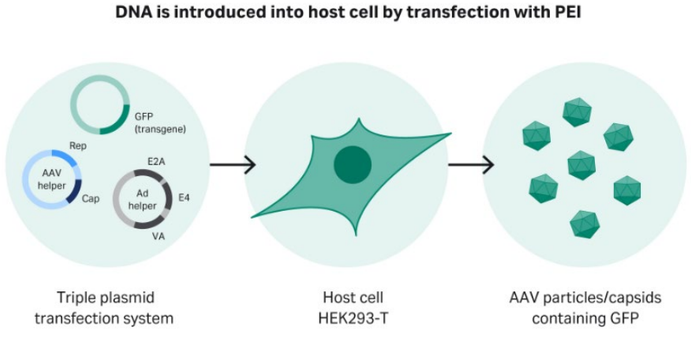


Fig 2. Transient transfection of adapted HEK293T cells using a triple plasmid transfection approach. PEI forms a complex with DNA, which is then introduced into the host cells.

"Of course you want to have good productivity with a high viral titer. But at the same time, you want a high percentage of full capsids in the harvest material. It's important to find the balance in different parameters to create a robust production process."

—Ann-Christin Magnusson

concentration and having a short incubation time, the percentage of full capsids increased.

Bottom Line

The verification for rAAV5 production in the fifth DoE study showed that a reduced transfection incubation time positively affected the percentage of full capsids. From the responses in the DoE experiments, we conclude that during transfection, a DNA concentration of 0.75 µg/mL and an incubation time of 15 min is likely optimal under the conditions tested.

We could summarize our DoE studies by doing a design space optimization based on a more realistic scenario, calculating the percentage of full capsids we were aiming for. Figure 3 shows the green area, which is the operating space if we were aiming for 15% full capsids. The red x marks the conditions selected.

Details

Read the full app note on AAV process development.

Next steps in start-to-finish process:

Use the optimized process to produce virus in bioreactors…

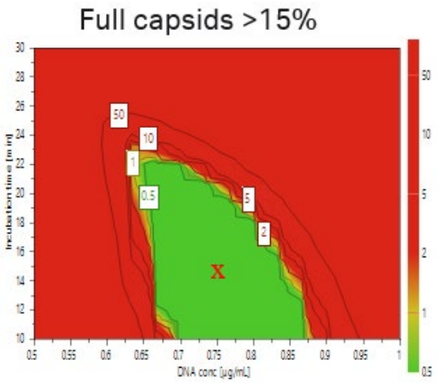


Fig 3. Results from DoE5 to determine the operating space to achieve > 15% full rAAV5 capsids. The red x marks the conditions that were selected (DNA concentration and incubation time).

Challenge 3: Produce Virus in Single-Use Bioreactors

Background

It's one thing to make AAV in shake flasks; it's another to make it in vessels suitable for a good manufacturing practices (GMP) environment. Bioreactors are the preferred systems for clinical and commercial production of biologics. With the right bioreactor platform, a thoughtfully developed process can be scaled up in a straightforward manner as larger volumes are required. Single-use bioreactors are preferred for biologic production, including viral vectors, for several reasons. The risks for cross-contamination are minimized, and it's possible to increase the production capacity with shorter changeover procedures between batches compared with stainless steel. Also, single-use bioreactors provide a flexible option for multi-product manufacturing.

Transient transfection is known to be challenging to scale up; however, it remains the predominant method of introducing the viral genes, helper gene, and GOI into cells. During our process development work, we optimized the conditions to balance the resulting titer and percentage of full capsids with the goal of developing a robust protocol that would scale up well.

Experimental highlights

Using the protocol we optimized during process development, we expanded HEK293 or HEK293T suspension cells from small-volume cell culture (20 mL) in shake flasks up to a 10 L culture in

Xcellerex™ XDR-10 bioreactor
- rAAV5 production: 10 L
- ELISA: 1.1 × 10^{14} VP/L
- qPCR: 1.8 × 10^{13} VG/L
- Full capsids, 16%

ReadyToProcess WAVE™ 25 bioreactor
- rAAV5 production: 10 L
- ELISA: 2.2 × 10^{14} VP/L
- qPCR: 5.4 × 10^{13} VG/L
- Full capsids, 24%

Fig 4. Verification batches for rAAV5 at 10 L scale using Xcellerex™ XDR-10 (left) and WAVE™ 25 bioreactor (right) confirmed that criteria for rAAV5 production could be achieved with the developed transfection protocol.

a benchtop **Xcellerex™ XDR-10 single-use bioreactor system**. Also, we produced rAAV5 in **ReadyToProcess WAVE™ 25 bioreactor system**, up to 25 L. Our goal with the study was to achieve 10^{10} viral genomes (VG)/mL (10^{13}/L), and 10^{11} of viral particles (VP)/mL (10^{14}/L) in crude harvest material. These criteria are based on levels described in the literature, which of course can be improved by further process development.

We aimed for at least 10% full capsids in the harvest material to allow efficient AAV full and empty capsid separation during the purification process. The percentage of full capsids was calculated as a ratio between VG/mL and VP/mL.

We performed verification batches to confirm that these criteria could be achieved, which they were (Fig 4). Then, we performed three production runs in each bioreactor type.

To confirm transfection efficiency in the XDR-10 bioreactor, we performed brightfield and fluorescent microscopic evaluation of cells in addition to flow cytometry analysis. Figure 5 shows GFP-positive fluorescing cells in a diluted sample of rAAV5 at harvest.

Bottom line

We confirmed that both the ReadyToProcess WAVE™ 25 and Xcellerex™ XDR-10 bioreactors are suitable for AAV production with similar productivity. While good results were observed for both bioreactors, the XDR process offers scalability up to 2000 L with the Xcellerex™ XDR family. Three production batches in XDR-10 bioreactor gave a similar viral titer with approximately 10^{14} VP/L. All three production batches successfully yielded > 15% full capsids, as shown in Figure 6. The consistency between the three batches indicates that the process design is robust and suitable for manufacturing purposes.

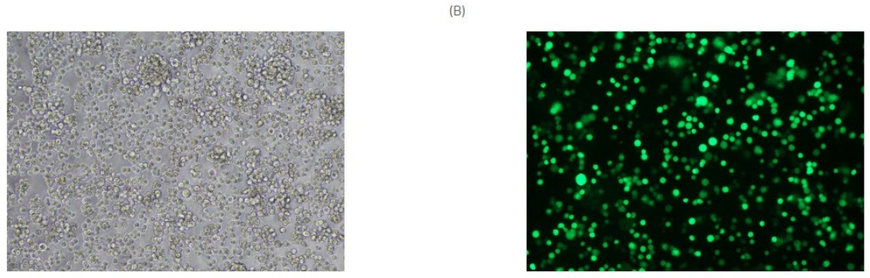


Fig 5. (A) Bright field and (B) fluorescent microscope images from diluted sample of rAAV5 at harvest from the XDR-10 bioreactor.

Details

Read the full app note on AAV production in single-use bioreactors.

Next step in start-to-finish process

Determine whether the developed process works for other AAV serotypes…

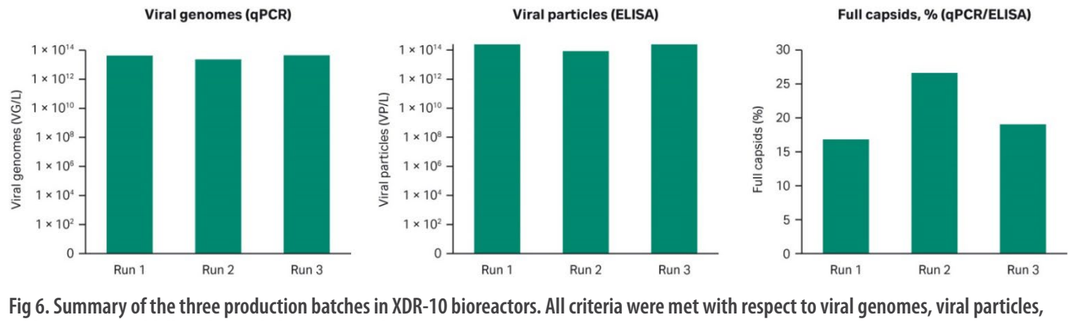


Fig 6. Summary of the three production batches in XDR-10 bioreactors. All criteria were met with respect to viral genomes, viral particles, and percentage of full viral capsids for rAAV5 production.

"Our mindset is to create a 'simple' process which will improve reproducibility, robustness, and flexibility in our AAV production process." —Ann-Christin Magnusson

Challenge 4: Produce Different Serotypes

Background

Research and development in the AAV field is highly active. Efforts are aimed at improving efficacy and tissue specificity as well as reducing some of the potential safety concerns. Numerous natural and synthetic or engineered serotypes are in various stages of development. It would be useful to have a scalable process that can produce a variety of serotypes.

Experimental highlights

In addition to rAAV5, we used our optimized protocol to produce rAAV2, rAAV8, and rAAV9. The same method was applied using shake flasks and the XDR-10 and WAVE™ 25 bioreactors. We achieved similar titers as for rAAV5.

Bottom Line

Figure 7 shows the average productivity from shake flask (20 mL to 2 L) and bioreactor (3 L to 25 L) demonstrating good scalability and productivity from shake flask to bioreactor for all four serotypes.

We conclude from these findings that the transfection protocol we describe for bioreactor scale-up should allow production of different AAV serotypes at large scale.

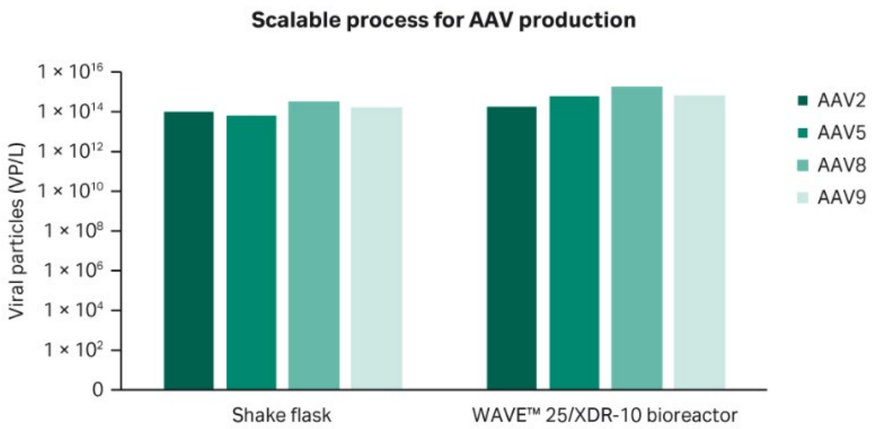


Fig 7. Average of the titer for viral particles shows scalability of the transfection protocol for varioust AAV serotypes.

Next steps in start-to-finish process

Harvest, clarify, concentrate, and capture the viruses…

Details

Read the full app note on **AAV production in single-use bioreactors**.

"We have heard from many people who want the flexibility to use different serotypes in their programs. When developing a scalable process, consider the serotypes you're using now – as well as the ones you may use in the future." —Ann-Christin Magnusson

Challenge 5: Capture Virus Efficiently

Background

Affinity capture is an efficient way to specifically bind and concentrate the target. At the same time, this step removes a large portion of impurities. However, affinity ligands don't discriminate between the full rAAV capsid (containing the VG and GOI) and empty rAAV capsids (product-related impurity lacking viral genomes).

Experimental highlights

The starting materials for all experiments were obtained from rAAV production by triple plasmid transfection in HEK-293T cells, followed by harvest using detergent lysis (Tween 20), clarification, and concentration and buffer exchange by tangential flow filtration using hollow fibers. We optimized the affinity capture step using Capto™ AVB resin.

Capto™ AVB is an affinity chromatography resin designed for the purification of rAAV. The ligand of Capto™ AVB resin is a single domain antibody fragment that specifically binds rAAV 1, 2, 3, and 5 serotypes. The ligand also binds genetically engineered recombinant variants depending on the presence of the AVB binding epitope region.

We applied concentrated and buffer-exchanged rAAV2 and rAAV5 samples to Capto™ AVB and eluted in 50 mM citrate at pH 3.0 with gradient elution from 500 to 0 mM NaCl. rAAV2 elutes completely at 500 mM NaCl. The rAAV5 eluted to some degree at high salt, but the majority of the rAAV5 was eluted at the end of the gradient when the salt concentration was close to zero. These results indicate that rAAV5 binds more strongly to the Capto™ AVB. We also applied rAAV8 samples to Capto™ AVB, but binding was very low.

We further optimized the conditions for both rAAV2 and rAAV5. The final protocol for rAAV2 uses elution buffer with 50 mM citrate pH 3.5, 500 mM NaCl, and 500 mM arginine. The final protocol for rAAV5 uses elution buffer of 50 mM glycine pH 2.7. The capacity was 2 to 5×10^{14} VP/mL resin and typically, the yields were in the range of 80% to 100% VP for rAAV2 and rAAV5. Removing the salt from elution buffer did not cause aggregation of rAAV5 (analytical size exclusion, data not shown).

Bottom line

The high purity of the affinity capture eluates for both rAAV2 and rAAV5 can be seen in Figure 8.

Details

Read the full app note on **AAV capture**.

Next step in start-to-finish process

AAV full and empty capsid separation…

"Affinity capture is very efficient and hard to beat for target specificity, concentration, and impurity removal." —Åsa Hagner McWhirter

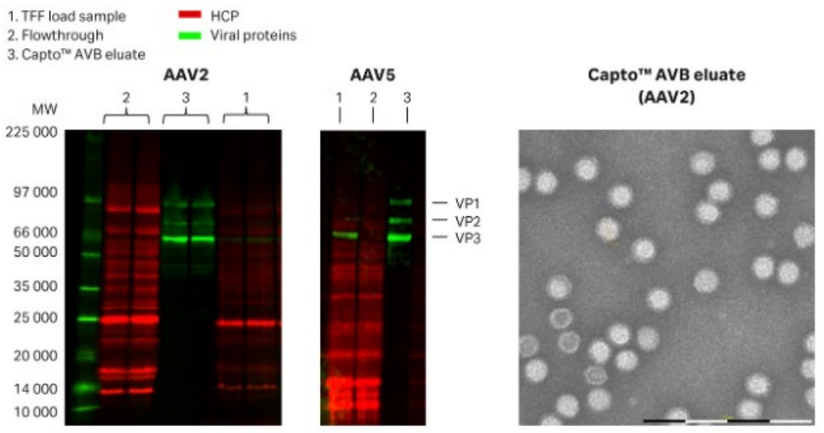


Fig 8. Purity analysis of Capto™ AVB in (left) rAAV2, and (middle) in rAAV5 eluates using multiplex fluorescence Western blot and (right) TEM image (MiniTEM from ViroNova) with negative staining. Host cell protein (HCP) was detected by Cy™5 prelabeling, and rAAV proteins were targeted by primary anti-AAV2 or AAV5 antibody and Cy™3 labeled secondary antibody.

Challenge 6: Perform AAV Full and Empty Capsid Separation

Background

Even with an optimized upstream process, the proportion of full capsid product in the harvested material is usually well below 50%. High levels of empty capsids are problematic – they decrease efficacy and contaminate the product. For these reasons, they must be minimized. In addition to empty capsids, partially filled capsids containing truncated genomes or process-related impurities can be packed into the capsid during upstream production. Traditionally, CsCl gradient ultracentrifugation – which separates based on density differences – has been used to purify full rAAV capsids in small scale. But this method doesn't scale up well. Scalable methods, such as those based on chromatography, are more suitable for manufacturing environments.

Full and empty capsids have slightly different isoelectric points (pI) – 5.9 vs 6.3 on average, respectively. The charge difference can be used to separate them by ion exchange chromatography using salt or pH elution. When developing a process, it's important to keep in mind that other capsid properties might affect the binding behavior depending on serotype. Cation or anion exchange could be used. However, anion exchange is the most common, and we have successfully developed a protocol for several serotypes.

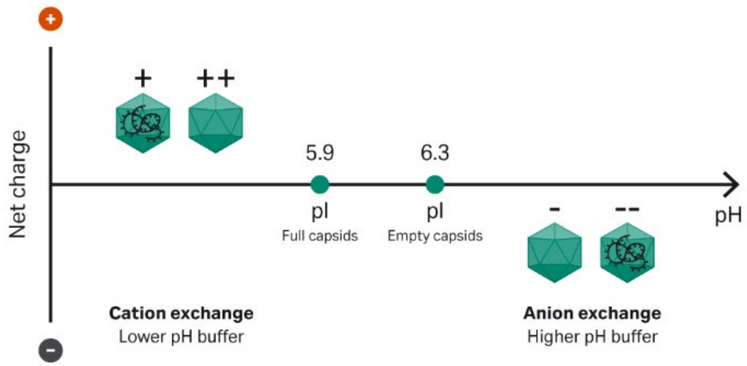


Fig 9. Principles of AAV full and empty capsid separation using ion exchange.

With anion exchange, the capsid net charge is negative with a buffer pH higher than the average capsid pI. In this case, the empty capsids elute first in a salt gradient since they are less negatively charged compared to the full capsids (Fig 9). Additives such as detergents, carbohydrates, and metal ions can enhance the separation. Dextran extenders, step elution rather than gradient elution, as well as salt type, enhances the separation.

Accurate analysis of full and empty rAAV capsids is critical for optimizing the polishing step. In current chromatography polishing steps, peaks often overlap. Incomplete separation can enrich full capsids with some VG losses, depending on the pooling strategy. Therefore, it's critical to optimize the polishing step. An additional challenge is that the polishing step typically needs to be optimized to maximize separation for each serotype.

Experimental highlights

We have now optimized the affinity capture step using CaptoTM AVB resin. After neutralization, the highly purified eluate contains both full and empty capsids in similar ratio as in the harvested material. How efficiently the empty capsids will be removed, depend on the separation performance in the anion exchange polishing step.

For the polishing step, we screened several resins for AAV full and empty capsid separation. The anion exchange resins **Capto™ Q ImpRes** and **Capto™ Q** were the most promising candidates. We further optimized the separation by screening conditions - such as pH, salt type, additives, and mode of elution.

Using Capto™ Q ImpRes, we developed a protocol using a high constant $MgCl_2$ (18 mM) to elute the empty capsids before a linear shallow gradient of NaCl was applied to elute the full capsids (Fig 10). The mechanism of how $MgCl_2$ enhances the separation is unclear, but it may be due to the differential binding of Mg^{2+} ions between full and empty capsids, which in turn affects binding to the anion exchange ligand. Depending on how the pooling was made with

"We developed a simple two-step elution protocol with anion exchange that works for AAV2, AAV5, AAV8, and AAV9 with excellent separation and viral genome recovery." —Åsa Hagner McWhirter

a trade-off between viral genome recovery and % full capsids, we obtained 60% to 70% viral genome recovery with 40% to 65% full capsids.

We then applied similar conditions but switched to Capto™ Q resin with dextran surface extenders. Capto™ Q resin clearly enhances the separation compared to the Capto™ Q ImpRes resin, which lacks the dextran surface extenders. We used high constant $MgCl_2$ (18 mM) and a 2-step elution with NaCl. Instead of linear gradient elution we used step elution, and we achieved excellent AAV full and empty capsid separation (Fig 11).

For small scale optimizations, it is important to use a 10 mm path length UV 260:280 detector for higher sensitivity and bypass the mixer on the ÄKTA chromatography system to sharpen the conductivity steps (reduce dead volumes).

The capacity was similar for both Capto™ Q impRes and Capto™ Q resins – at least 1-3 × 10^{13} VP/mL resin.

We continued to explore the separation of full and empty capsids for more AAV serotypes using Capto™ Q. We developed a protocol that could separate AAV2, AAV5, AAV8 and AAV9 using 20mM bis-Tris propane (BTP), pH 9.0 containing constant 2 mM $MgCl_2$ without additives. We also replaced NaCl as an elution salt with sodium acetate, which is a milder, more kosmotropic (order-inducing) salt. The high $MgCl_2$ concentration used in the above alternative protocols gave too high load conductivity (both full and empty capsids end up in flowthrough) for AAV9 that binds much less strongly than the other serotypes. It is critical to ensure that the sample load has a low conductivity, < 3 mS/cm and <2 mS/cm for AAV9. This can be achieved by dilution in buffer A or buffer exchange. The elution conditions required to elute the empty capsids were identified in a prescreening procedure using incremental 5% elution buffer (buffer B) steps of 3 CV each. The % B at which empty capsid elution was initiated without leaking of full capsids (judged by the UV 260:280 ratios in each peak) was selected, and the step 1 elution was prolonged to 20 CV to maximize the empty capsid removal. This was followed by a second shorter (5 CV) step for elution of full capsids using 100% B or lower depending on the

Step	Column volume (CV)	Buffer/material
Equilibration	3	20 mM Tris pH 9.0 + additives[1]
Load	~ 5	Capto™ AVB eluate diluted 10-fold in equilibration buffer (~ 2 ×10^{13} VP/mL resin)
Wash 1	4	20 mM Tris pH 9.0 + additives[1]
Elution hold at 0% buffer B	5	Buffer A: 20 mM Tris pH 9.0 + 18 mM $MgCl_2$ + additives[1] Buffer B: Buffer A, +1 M NaCl
Elution	15	Linear gradient, 0% to 20% buffer B
Elution hold at 20% buffer B	5	

[1] 1% sucrose + 0.1% poloxamer-188

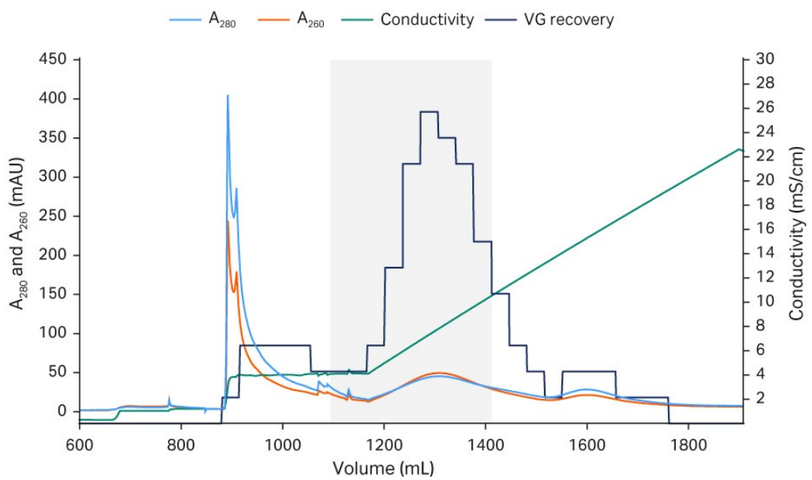


Fig 10. Separation of rAAV5 full and empty capsids using Capto™ Q ImpRes anion exchange chromatography resin. The optimized protocol included an 18 mM $MgCl_2$ wash to elute the empty capsids followed by a NaCl linear gradient to elute the full capsids. The gray shaded area represents the pooled fractions containing the highest percentage of full capsids and sufficient viral genome recovery.

Columns: Capto™ Q ImpRes or Capto™ Q resin packed in Tricorn™ 5/100 columns, 2 mL
Sample: ~ 1 × 10^{12} virus particles (VP) of rAAV8 (35% to 40% full capsids as determined by qPCR:ELISA)
Buffer A: 20 mM BTP pH 9.5, 2 mM $MgCl_2$ + 1% sucrose and 0.1% poloxamer 188 additives
Buffer B: Buffer A + 400 mM NaCl
Gradient: Linear gradient elution with buffer B, 0 to 200 min
Flow rate: 1 mL/min

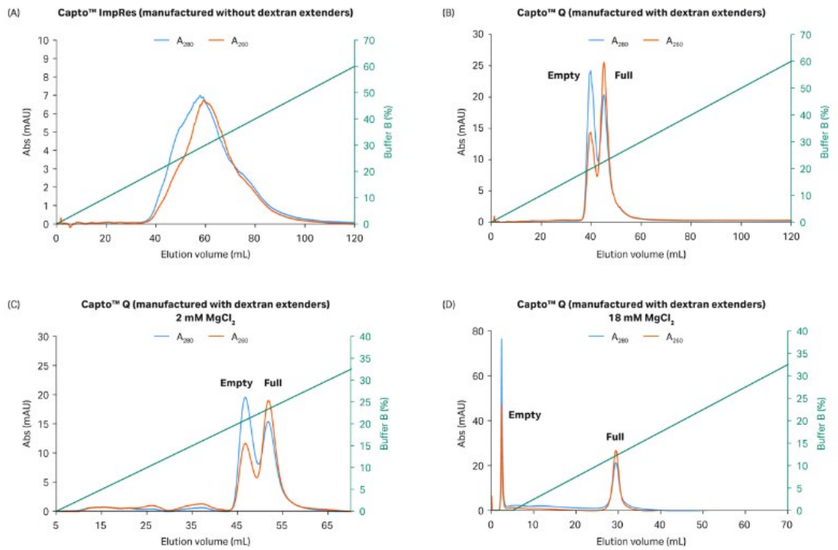


Fig 11. Separation of rAAV8 capsids on (A) Capto™ Q ImpRes (manufactured without dextran surface extenders) vs (B) Capto™ Q (manufactured with surface extenders) in the presence of 2 mM $MgCl_2$. Separation performance of (C) Capto™ Q with 2 mM $MgCl_2$ and (D) 18 mM $MgCl_2$ is also shown. Elution was performed with an increasing NaCl linear gradient up to 400 mM.

elution concentration for the full capsids in the prescreening run (Fig 12). The peak UV 260:280 ratios (~ 0.6-0.7 for empy capsids and ~ 1.1-1.25 for full capsids) were confirmed by qPCR and ELISA analysis. The VG recovery and % full capsids in peak 2 were above 80%. (Fig 12 Table).

Bottom line

Affinity capture can require optimization depending on serotype to maximize recovery. Higher viral genome recovery and % full capsids can be obtained in the polishing step by using Capto™ Q with surface extenders, which significantly enhances the separation. We also learned that step elution and $MgCl_2$ enhance the separation. To be able to achieve a protocol with good performance for AAV9, we reduced the conc. $MgCl_2$ and changed to sodium acetate as elution salt. Our new protocol is compatible with AAV2, AAV5, AAV8, and AAV9. The developed process is suitable for large-scale clinical-grade production.

Details

Read the full app note on **separation of empty and full rAAV capsids** and the app note on **capture and polishing**.

Serotype	Start sample	Peak 1 (empty capsids)			Peak 2 (full capsids)		
	qPCR:ELISA (% full capsids)	UV 260:280 (peak area)	VG recovery (%)	qPCR:ELISA (% full capsids)	UV 260:280 (peak area)	VG recovery (%)	qPCR:ELISA (% full capsids)
AAV2	7-10%	0.75	NA	NA	1.14	NA	NA
AAV5	47%	0.65	7	5	1.20	80	100
AAV8	11-35%	0.60	3	1	1.24	80	95
AAV9	40%	0.63	0.3	1	1.25	91	100

Mass balance based on total UV signal 70%-100%

Capto™ Q - Baseline Separation in 2 Steps

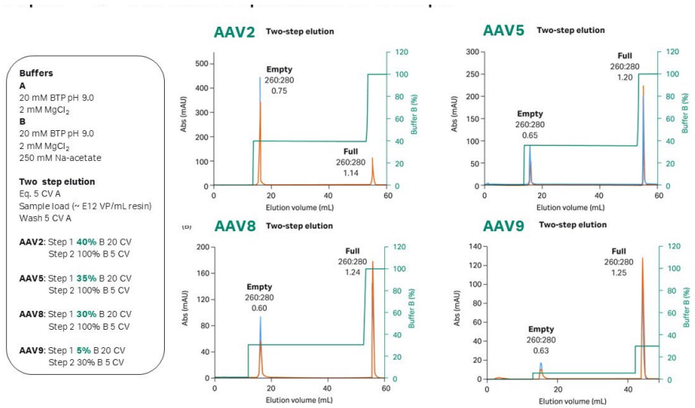


Fig 12. Separation of rAAV2, rAAV5, rAAV8, and rAAV9 full and empty capsids using Capto™ Q with dextran surface extenders. We used 20 mM BTP, pH 9.0 containing 2 mM $MgCl_2$ without additives. Elution was performed with sodium acetate (250 mM as 100% buffer B). The % buffer B in step 1 and 2 was selected based on prescreening using 5% buffer B incremental steps. Step 1 was extended to 20 CV eluting the empty capsids, and step 2 was 5 CV, which led to elution of the full capsids. The mass balance based on total UV signal was 70% to 100%.

Underpinning all this work: **Analytics…**

Challenge 7: Determine Titer Accurately

Background

During process development, final production, and purification, we monitor a range of critical quality attributes including infectious titer, viral genome titer, viral total particle, host cell protein (HCP), and DNA levels. Many of the techniques used for this are labor-intensive with low precision and a low degree of automation. In addition, the analyses are often time-consuming and costly. Simplified, high-precision analytics like surface plasmon resonance (SPR) can enhance today's workflows with a high degree of automation.

Standard titer analysis uses ELISA, an assay that requires a lot of manual interaction and can come with challenges in precision and reproducibility.

Experimental highlights

We developed SPR assays for both rAAV2 and rAAV5. For rAAV2 we amine coupled an anti-AAV2 in one flow cell on Sensor Chip CM5 in **Biacore™ T200**. We also used Biacore™ T200 for analysis of AAV5 virus titer. The latter assay is run using capture to **Sensor Chip Protein A** and doesn't require separate immobilization. We used a capture format

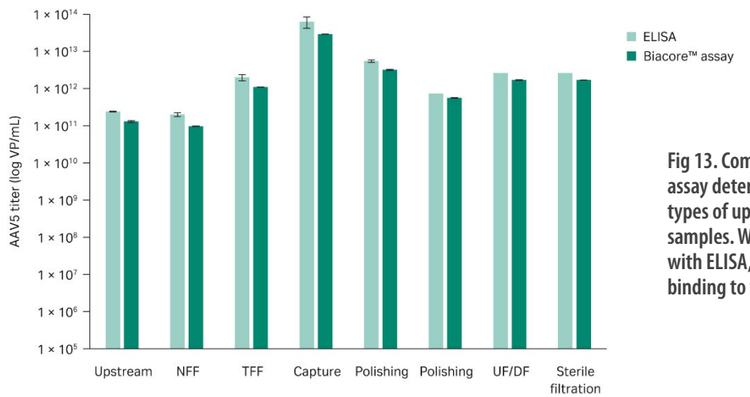


Fig 13. Comparison between ELISA and Biacore™ assay determination of virus titer in different types of upstream and downstream AAV5 samples. We obtained slightly higher titers with ELISA, which might be due to nonspecific binding to the ELISA plate.

instead of covalent coupling because of the binding antibody for this assay.

Also, we set up the rAAV2 assay on a **Biacore™ 8K system**, which significantly increases sample throughput. For a full 96-well plate of samples, the system reduces run time to less than 3 h, compared to 19 h on a single-needle system.

We performed a Biacore™ analysis of a set of upstream and downstream AAV5 process samples and compared the results with ELISA (Progen AAV5 Titration ELISA, art no. PRAAV5). Our results correlate well (Fig 13, on the previous page).

For this sample set, the Biacore™ assay determined the average intra assay precision to be 2%, while the ELISA determined it to be 15%.

Bottom line

The developed assays for both rAAV2 and rAAV5 provide repeatable results and correlate well with established total AAV capsid titer assays using ELISA. Compared to ELISA, Biacore™ assays have a higher degree of automation and precision and are compatible with process samples from harvest to final purified bulk. Their performance and ease-of-use make them very suitable as process development tools.

Details

Read the full app note on **titer analysis using SPR**.

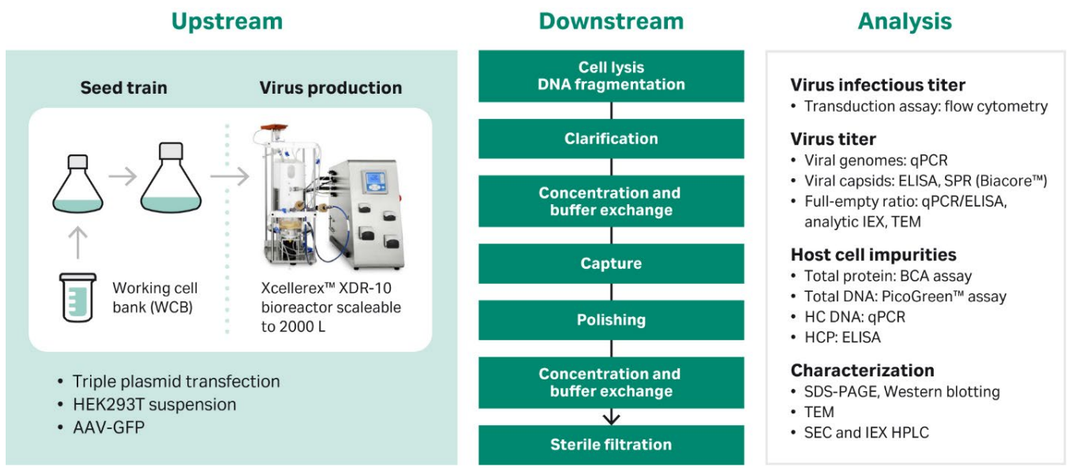


Fig 14. Summary of our start-to-finish process for rAAV, showing upstream, downstream, and analysis.

What we learned

We learned that:

- It's crucial to obtain harvest material with high levels of full capsids. Therefore, it's worthwhile to put in the time to develop an upstream process that will deliver > 10% full capsids.
- Cell lysis gives challenging feeds.
- Conditions for affinity capture may differ by serotype.
- We have developed a step-elution protocol for full and empty capsids separation using Capto™ Q with high performance for AAV2, AAV5, AAV8, and AAV9.
- Analytics are critical, especially for process development of the polishing step. In addition to qPCR and SPR/ELISA, use at least one orthogonal method to confirm the full and empty capsids ratio. Confirm performance for compatibility with different buffers, and include controls and references.

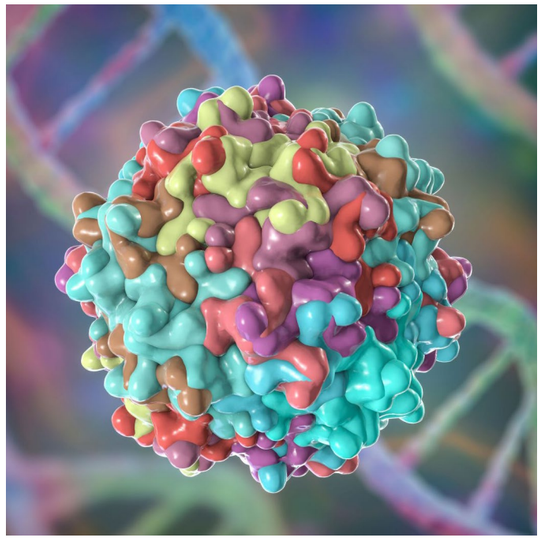
The start-to-finish process we developed for recombinant AAV5 (rAAV5) is presented in Figure 14.

For details on the work presented here, please visit our website to read the full application notes:

- **Cell culture process development for AAV vector production in suspension cells**
- **Adeno-associated virus production in suspension HEK293 cells with single-use bioreactors**
- **Optimizing capture and polishing steps in an rAAV purification process**
- **Biacore™ surface plasmon resonance for titer analysis of adeno-associated virus**
- **Recombinant adeno-associated virus serotype 5 production process**
- **Effective separation of full and empty rAAV capsids by anion exchange**

"During production and purification it is crucial to monitor critical quality attributes such as viral genome titer, HCP, and DNA levels. This is often done with labor-intensive, low precision techniques. By using Biacore™ SPR systems for titer analysis, we get better precision compared to ELISA. We also save time since the assays are very easy to run and more highly automated." —Anna Moberg

A Report on the Present Science of Gene Editing



AAV Vectors Advance the Frontiers of Gene Therapy

Technological developments and therapeutic applications of third-generation AAV vectors.

By Anis H. Khimani, PhD, Christian Thirion, PhD, and Arun Srivastava, PhD

Gene delivery vehicles have helped realize the concept of treating human diseases by introducing normal alleles of genes into appropriate target cells. These gene delivery vehicles include recombinant and nonrecombinant lentiviral vectors and adeno-associated virus (AAV) vectors.

Recombinant retroviral vectors have been used in clinical trials for nearly three decades. Initial results with retroviral vectors were encouraging,[1,2] but the use of these vectors in nonhuman primate studies was reported to lead to T-cell lymphoma.[3] These vectors were also implicated in the development of T-cell leukemia in several children who received gene therapy for X-linked severe combined immunodeficiency in clinical trials.[4,5]

Adenoviral vectors in gene therapy of cystic fibrosis were reported to lack efficacy.[6] Also, an adenoviral vector was suspected of playing a role in the death of a patient in a trial for gene therapy of ornithine transcarbamylase deficiency.[7]

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Senior Strategy Leader, PerkinElmer Life Sciences

Additionally, early-generation lentiviral vectors have shown clinical efficacy in more than 300 patients who received gene therapy for a number of diseases[8–10]; however, a recent gene therapy trial to treat cerebral adrenoleukodystrophy was halted after a participant in the study developed myelodysplastic syndrome, a bone marrow disorder that can lead to leukemia.[11]

Christian Thirion, PhD
CEO, Sirion Biotech

Meanwhile, recombinant AAV vectors, based on a nonpathogenic parvovirus, have been used or are currently in use in 264 Phase I/II/III clinical trials for diseases such as cystic fibrosis, Batten's disease, α1-antitrypsin deficiency, Parkinson's disease, Pompe's disease, and Duchenne's muscular dystrophy.[12] In some cases, such as Leber's congenital amaurosis (LCA),[13] hemophilia B,14 lipoprotein lipase deficiency,[15] aromatic L-amino acid decarboxylase deficiency,[16] choroideremia,[17] Leber hereditary optic neuropathy,[18] hemophilia A,[19] and spinal muscular atrophy (SMA),[20] unexpected, remarkable clinical efficacy has also been achieved.

Thus far, two AAV "drugs"—Luxturna for LCA and Zolgensma for SMA—have been approved by the FDA.21 However, in some cases, relatively large vector doses are needed to achieve clinical efficacy. The use of high doses has been shown to provoke host immune responses culminating in serious adverse events[22] including the deaths of four patients.[23]

Arun Srivastava, PhD
Professor of Genetics, University of Florida College of Medicine

Although gene therapy with AAV vectors continues to be a promising treatment modality, it has also become increasingly clear that none of the first generation of AAV vectors currently in use is ideal for the following reasons:

1. The use of AAV vectors composed of naturally occurring capsids induces immune responses, especially at high doses, because the host immune system cannot distinguish between AAV as a virus and AAV as a vector.[24]
2. Like the naturally occurring AAV, most of the recombinant vectors contain single-stranded DNA, which is known to be transcriptionally inactive. Viral second-strand DNA synthesis is known to be a rate-limiting step during AAV-vector-mediated transgene expression.[25]
3. Most of the AAV serotype vectors currently in use lack selective tropism for human cells and organs.

Next-generation AAVs come into focus

These limitations have been addressed in various ways. First, scientists have developed capsid-modified next-generation (NextGen) AAV serotype vectors. (These vectors are up to 80-fold more efficacious at reduced doses[26,27]; they are also less immunogenic.[28]) Second, scientists developed genome-modified generation X (GenX) AAV vectors. (These vectors mediate up

to eightfold enhanced transgene expression.[29]) Third, scientists have combined both strategies to produce optimized AAV serotype vectors. (These vectors are about 20–30-fold more efficient at further reduced doses.[30]) Fourth, scientists have identified AAV3 and AAV6, serotypes that possess remarkable tropisms for human liver and primary human hematopoietic stem cells, respectively.[31–36]

In vivo evolution of large AAV libraries in combination with massive parallel screening of barcoded AAV vectors are now being actively employed to identify AAV vectors with further enhanced transduction properties and improved specificity.[37,38]

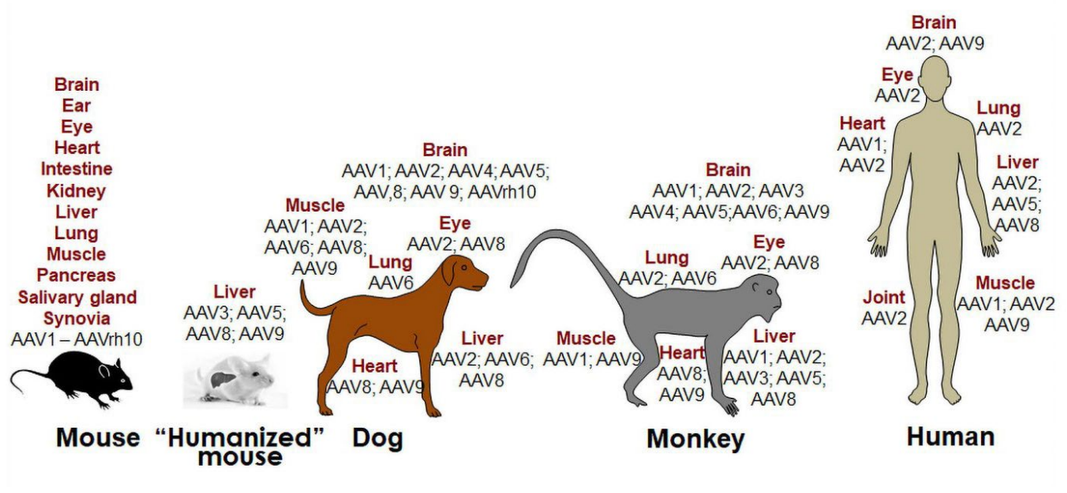
AAV Serotype Considerations

Multiple AAV serotypes have been isolated from tissue culture stocks from humans and nonhuman primates.[39] To date, 13 distinct AAV serotype vectors (AAV1–AAV13) have been described, and this number is likely to grow. The precise mechanism of tissue tropism of AAV serotype vectors in vivo remains unknown, but it is clear that attachment to putative cell surface receptors is the initial step for successful transduction. A wealth of information has been obtained from studies in mice, where different AAV serotype vectors have been shown to exhibit distinct tropism for various tissues and organs. The efficacy of some of the AAV serotype vectors has also been evaluated in other animals, small and large, such as rodents, canines, and nonhuman primates.[40]

Innovative Tools to Advance Discovery

For therapeutic vector design, optimization of the size of the packaged therapeutic expression cassette can be achieved. Expression cassettes that are oversized (>5 kb) can result in the packaging of truncated genomes,41 whereas packaging of expression cassettes that are undersized can result in increased cross-packaging of plasmid-derived prokaryotic sequences that incorporate antibiotic resistance genes.42 Inclusion of large non-immunogenic inverted terminal repeat (ITR)-flanking spacer sequences significantly reduces unwanted

Viral vector characterization is also an important application in gene therapy. It facilitates the evaluation of critical quality attributes (CQAs) such as identity, potency, purity, and stability.



In this schematic illustration, the recombinant AAV serotype vectors that have been used in various animal models and Phase I/II/III trials in humans are indicated. The animal models shown are the ones most commonly used to evaluate vector safety and efficacy. Also indicated are the organs that were targeted by the different vectors.

packaging of plasmid-derived prokaryotic sequences.

The design, development, and production workflow for gene therapy vectors such as AAV is similar to the workflow for classical large-molecule biotherapeutics. Living producer systems that support an efficient, sustainable, and scalable growth environment for the vectors are desirable. Such systems can drive the development of the surrounding infrastructure for workflow management, automation, and regulatory compliance.

A commonly used cell system for AAV transient transfection and production is HEK293. For other producer biological model systems, either mammalian or insect cell lines have been used to scale up AAV sequences containing the transgene. For stable mammalian cell lines, either BHK cells or HeLa cells are used. With the insect system, Sf6 cells infected with recombinant baculovirus have been used as well to scale up recombinant AAV. With either of the above cell systems, production levels of 104 to 105 genome copies/L are obtained.

Viral vector characterization is also an important application in gene therapy. It facilitates the evaluation of critical quality attributes (CQAs) such as identity, potency, purity, and stability. To evaluate CQAs and comply with regulatory guidelines, companies engaged in gene therapy need to adopt reproducible and robust methods of development and validation.

A number of analytical techniques enable chemical and physical characterization. Viral structure and particle integrity and aggregation can be assessed with transmission electron microscopy (TEM), contributing to the quality control of viral vectors. TEM can also be used in

combination with molecular techniques such as droplet digital PCR (ddPCR) or quantitative real time PCR (qPCR) to determine AAV identity and purity.

Recent attention has been focused on analytical ultracentrifugation and mass spectrometry as well. However, some of these technologies have limited throughput. Higher throughput microfluidic capillary electrophoresis platforms, such as the LabChip GXII Touch with the ProteinEXact assay, can enable AAV capsid protein (VP1, VP2, VP3) analysis.

Some of the above technologies are critical to the orthogonal validation of conventional or well-established methods such as electrophoresis, spectroscopy, and chromatography.

Artificial intelligence and machine learning, along with analytical data management solutions, are significantly supporting analytical techniques to achieve automation, predictability, and decision making along the vector development, characterization, scale up, and QA/QC workflows.

Advanced tools, methods, applications, and services can facilitate the design, manufacture, and characterization of gene therapy vectors. End-to-end solutions can incorporate platforms for liquid handling and microfluidics; platforms for the analysis of macromolecules; platforms for cellular analysis and in vivo imaging; as well as platforms for bioinformatics applications. In addition, enhanced security software tools that enable 21 CFR Part 11 compliance to meet the regulatory requirements within the GMP lot release environment are critical.

Therapeutic gene and cell therapy research and development can progress more readily when advanced technologies and services for viral vector design and manufacturing are adopted. Such technologies, in combination with technologies for CRISPR-based gene editing, RNA interference, base editing, and prime editing, can move innovative therapeutics forward.

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