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# Upstream and downstream solutions for AAV manufacturing

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With the advancement of gene delivery vectors and gene editing technologies, cell and gene therapies are a very real solution to many previously untreatable or difficult to treat diseases. With this heightened interest in cell and gene therapies, the need for powerful, cost-effective, and scalable methods to deliver these therapies has intensified. Whilst here are a number of non-viral methods for delivery of gene therapies already being utilized, viral delivery remains the most commonly employed method. This article discusses the current AAV manufacuring workflows and identifies opportunities, both upstream and downstream, for process optimisation to support the scalable manufacture of viral vectors to support the increasing demand.

**Submitted for Peer Review**: 24 Jun 2019 ▶ **Published**: 8 Aug 2019

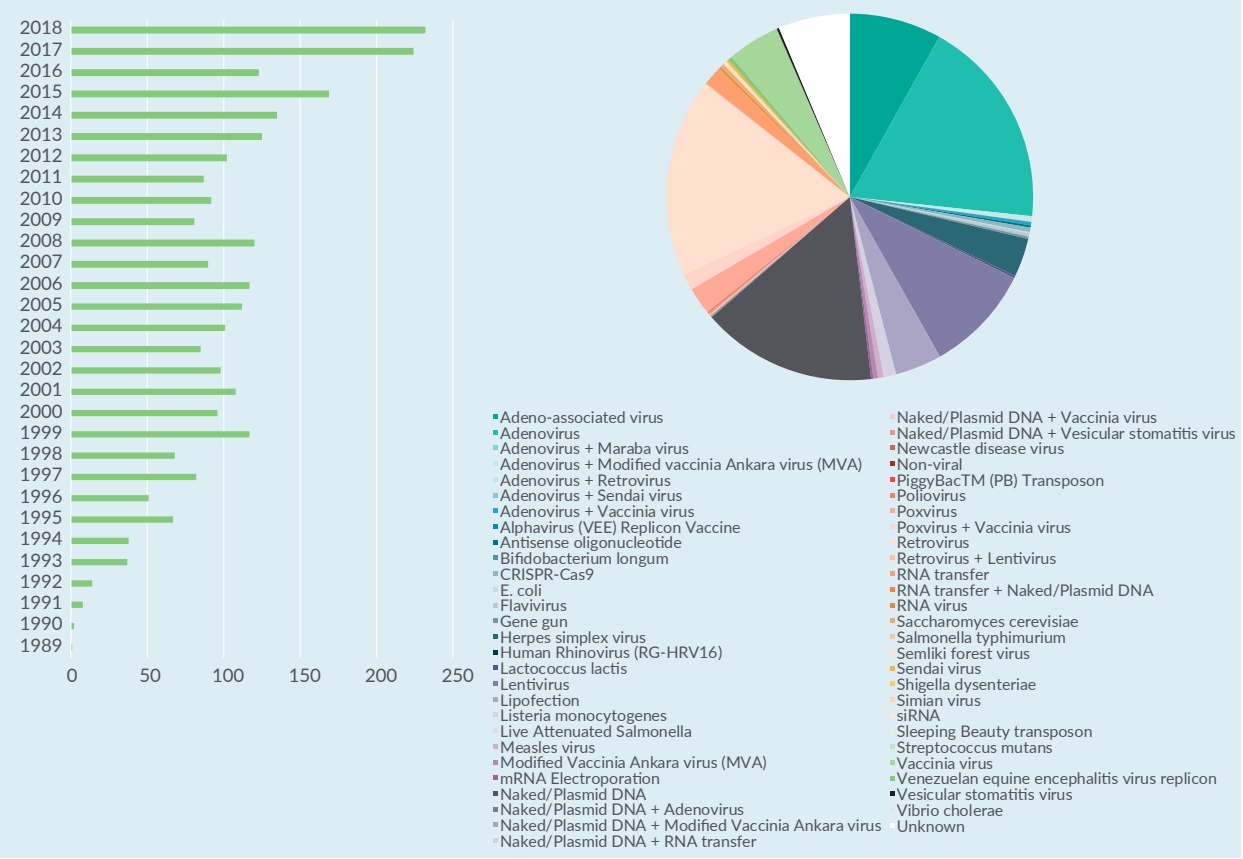
*Cell & Gene Therapy Insights* 2019; 5(Suppl. 5), 1017–1029

DOI: 10.18609/cgti.2019.110

With the advancement of gene delivery vectors and gene editing technologies, cell and gene therapies are a very real solution to many previously untreatable or difficult to treat diseases. It is now possible to add, silence, replace, and edit genes to treat diseases like neuromuscular disorders, immunodeficiencies, and ocular and liver diseases as well as develop enhanced cells for the treatment of diseases like cancer. The interest in gene therapies has continued to rise with the number of gene therapy clinical trials steadily increasing internationally since the first approved trial in 1989 (Figure 1A) [1,2]. The FDA alone is

## FIGURE 1

**Gene therapy clinical trials by year and delivery mechanism from 1989 through December of 2018.**




**(A)** The number of gene therapy clinical trials approved internationally by year. **(B)** Breakdown of the different delivery mechanisms used for gene therapy. ©Journal of Gene Medicine a Wiley Publication.

expecting to receive over 200 INDs per year by 2020 with the approval of 10–20 cell and gene therapies per year by 2025 [3].

The heightened interest in cell and gene therapies has increased the need for powerful, cost-effective, and scalable methods to deliver these therapies. There exists many non-viral methods for delivery of gene therapies, including electroporation and chemical-based systems; however, viral delivery is the most commonly employed method (e.g., adenovirus, retrovirus, lentivirus, and adeno-associated virus [AAV]) (Figure 1B) [1]. AAV stands out for its ability to infect dividing and non-dividing cells, its ability to maintain long-term gene expression, and increased safety. In fact, AAV vectors are particularly safe because infection is not pathogenic, AAV cannot replicate on its own, and the vector is maintained as an episome instead of directly integrating into a host genome. Furthermore, AAV can target different tissue or cell types depending on the composition of its capsid proteins, making it an attractive candidate for delivery of gene therapies *in vivo* [4]. By contrast, lentivirus, retrovirus, and electroporation are often used for *ex vivo* gene therapies [4]. AAV has already demonstrated efficacy as a method


DOI: 10.18609/cgti.2019.110

of gene therapy delivery in approved gene therapy products, and at least 238 AAV gene therapy clinical trials were approved internationally by December of 2018 [1,4,5].

With the increased interest in gene therapy over the past several decades, companies need to consider the cost of bringing a gene therapy to market. For example, Glybera – an AAV based therapy treating patients with familial lipoprotein lipase deficiency (LPLD) and the first gene therapy approved in Europe – had a $1 million price tag per treatment [6]. Virus production alone can make up 40% of the cost of goods, so development of a robust and cost-effective virus production process is very important not only during R&D but also for the clinical development and commercial use of a gene therapy product [7]. Some of the major challenges with virus production are the development of technologies and acquisition of knowledge to create a workflow that is facile, reproducible, and scalable while maintaining a high viral titer. Many technological advances in viral manufacturing now exist to help address these challenges, thereby reducing cost. Additionally, the expansion of contract development and manufacturing organizations (CDMOs) like Brammer Bio help scientists successfully scale up their viral production for gene therapies.

## AAV MANUFACTURING WORKFLOW

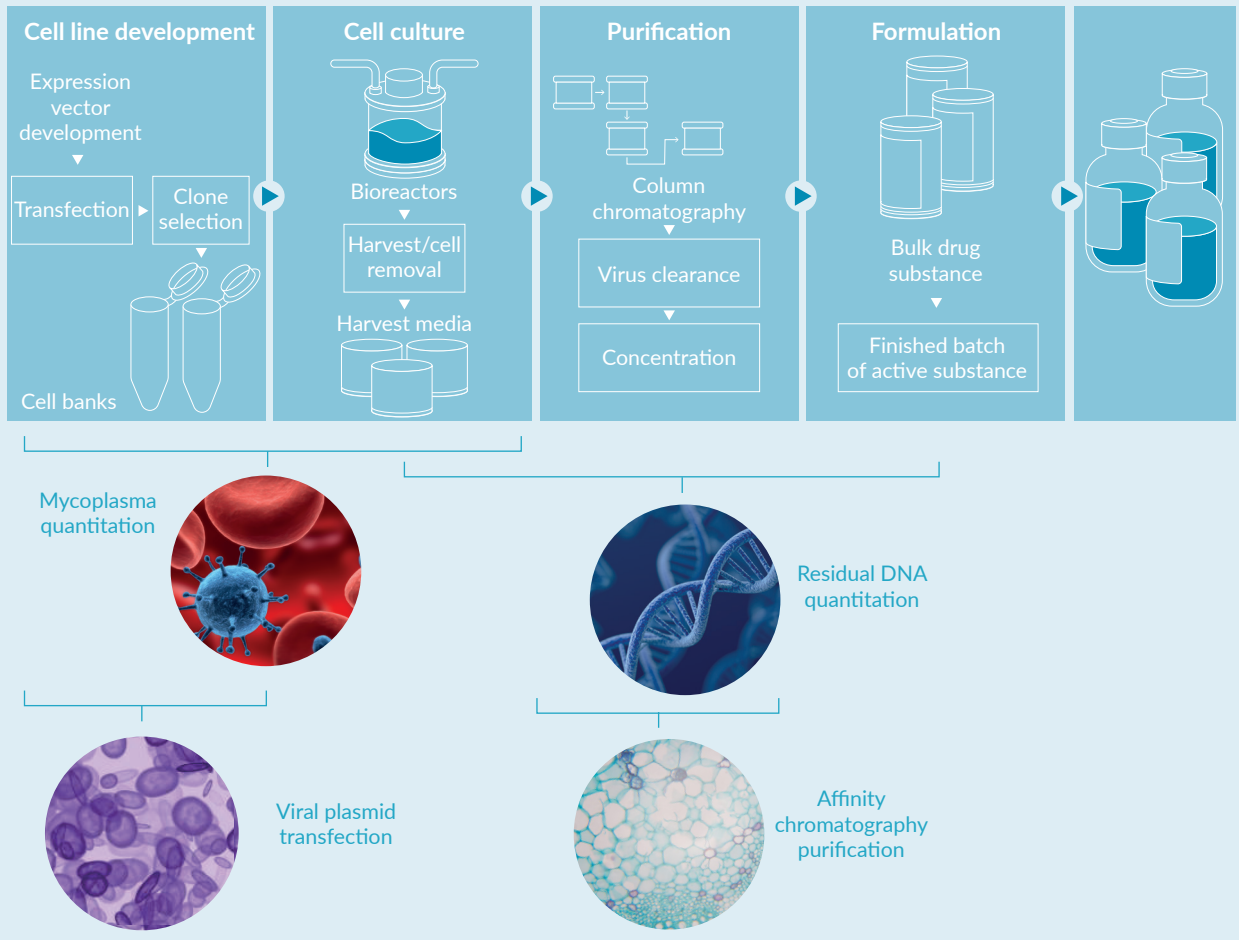
Viral manufacturing workflows can be broken down into upstream and downstream processes, with all steps requiring cell culture constituting the upstream processes and viral purification, batch formulation, and filling constituting the downstream processes (Figure 2). A successful viral manufacturing pipeline must deliver a consistent, pure, and high-titer product that exhibits good safety and efficacy to meet regulatory expectations.

Upstream viral production begins by generating cells that express the components necessary to create functional viral particles. There are many different ways of accomplishing this, but the most common method for viruses like lentivirus and AAV involves transiently transfecting HEK293T or HEK293 cells with multiple expression plasmids. AAV production commonly makes use of three separate plasmids: a *cis*-plasmid that encodes the AAV inverted terminal repeats ITRs along with the gene therapy of interest, a *trans*-plasmid that encodes the AAV *rep* and *cap* genes, and a helper plasmid – commonly encoding Adenovirus helper genes – which AAV is dependent on for viral production. The transiently transfected cells are then allowed to produce virus for several days before virus is harvested by chemical or mechanical lysis of the producer cells and treatment with a nuclease to remove any free DNA [4,8]. The cell lysate is then moved to downstream processes for viral purification.

Each virus has its own unique challenges for process development. AAV is no exception, requiring high viral titers after separation of the virus from process and product related impurities, host cell material left over from cell lysis and empty AAV capsids respectively, making purification challenging. Just as every virus has its own production hurdles, each step in the manufacturing process poses its own difficulties. Broadly, the biggest challenges consist of saving time and

## FIGURE 2

**Upstream and downstream processes of the AAV workflow.**




The upstream processes include cell line development, cell culture, and viral harvest. The downstream processes include purification, formulation, and filling.

money, having confidence in every step through QA/QC protocols, and generating a pure, high-titer product. Luckily, advances in the technologies used throughout the AAV manufacturing process have greatly improved viral production. Furthermore, CDMOs can help companies develop an effective viral production workflow for IND filings. For example, Brammer Bio has extensive experience as a viral vector manufacturer for gene therapies and provides such services as process and QC analytical validation, cGMP manufacturing at both small and large scale, product release and stability testing, analytical qualification, process qualification, and commercial supply. Brammer Bio is therefore able to support companies with both their upstream and downstream AAV manufacturing workflow for pre-clinical through to commercial-scale production. With the development of new technologies for viral production and purification, it is becoming easier and more cost-effective to produce virus for gene therapies whether developing viral production in-house or contracting out to a CDMO.


DOI: 10.18609/cgti.2019.110

## SOLUTIONS TO UPSTREAM AAV MANUFACTURING CHALLENGES

Production of high-titer virus is important during R&D all the way through commercial manufacturing and requires: 1) effective production methods that 2) mitigate the risk of adventitious agents in the final product and 3) are capable of scaling up through product development. A large volume of cell culture is necessary to produce gene and cell therapy products that rely on high-titer dosages. While transient transfection is popular in the development space, it tends to be more difficult to scale up. Additionally, the scale up of this process increases demand for cGMP plasmids used during transfection, quickly outpacing current commercial supply. In order to make this process commercially feasible, scientists must painstakingly optimize their process to maximize productivities, minimizing further scale up and relative plasmid consumption. To address this challenge, scientists can transition to other systems such as helper virus–mediated transfection or the insect cell platform. Furthermore, the development of stable producing cell lines is an ideal solution, however, there are no commonly used AAV products in the market today.

The HEK293-based transient transfection process is the simplest for most scientists, despite its increased cost, difficulty scaling up and automating, and use of serum [4,7,9,10]. The increased surface area required for adherent cells as production increases can make viral production unwieldy quickly. Additionally, the use of animal-based products such as serum can be a source for adventitious agents and cell lines that express large T-antigen can raise safety concerns because of the large T-antigen oncogenicity. However, during development, transient transfection provides flexibility to easily change the AAV construct or the transgene. One solution is the use of suspension HEK293 cells that do not express large-T antigen, grow to high density in serum-free chemically defined medium, and can easily be used during R&D or scaled up for use in large bioreactors. Industry is looking to help scientists bring gene and cell therapies to market, and several products are in development at Gibco™ to help support this platform, including chemically defined medium for serum-free cell culture.

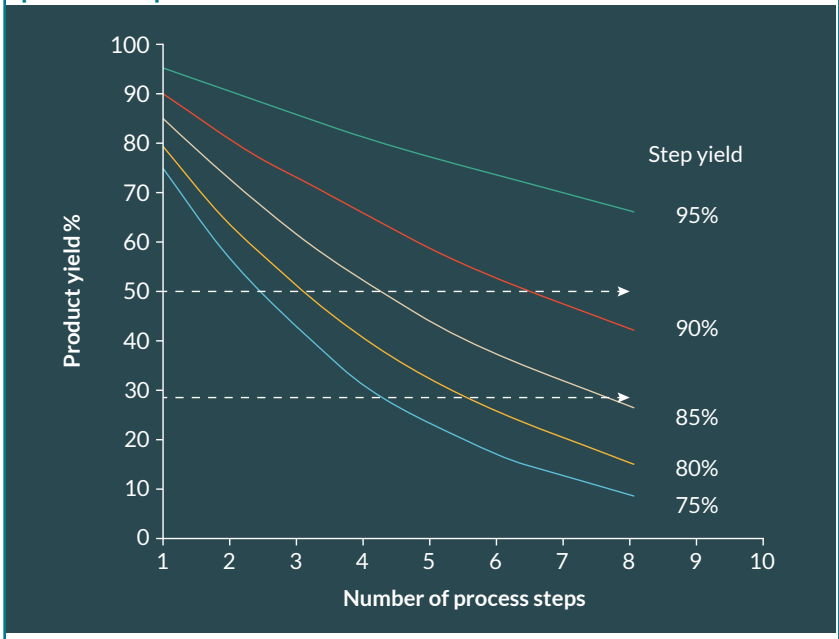
## SOLUTIONS TO DOWNSTREAM AAV MANUFACTURING CHALLENGES

The second half of the virus production workflow requires the purification of virus particles from process- and product-related impurities, including host cell material, plasmid DNA, and empty capsids. These downstream processing steps can make up a large part of the total cost of virus production, so effective methods of generating high purity virus are important.

During viral harvest, AAV is released from host cells through physical or chemical cell lysis, meaning the virus must be separated from host cell proteins (HCPs) as well as host and residual plasmid DNA. Viral preparations will often be treated with a nuclease to remove residual DNA. Removal

## FIGURE 3

**Final percentage product yield expected with increasing number of process steps.**




Even steps with a high percent yield, shown in green (95% yield/step), result in a significant decrease in product the more steps that are required.

of HCPs is a more involved process and often requires many long, labor-intensive steps that lead to loss of viral yield as well as possible delays in process development timelines. Traditional downstream workflows require the use of many different processing steps, including cesium chloride or iodixanol gradients as well as multiple chromatography steps. This is not ideal, as even process steps with high yields result in a significant reduction in the final yield when a large number of steps are required (Figure 3). To address this challenge, recent advances in affinity chromatography have reduced the number of steps required to purify AAV, boosting yield and reducing process lead times. Furthermore, iodixanol and cesium chloride gradient purification are non-scalable purification steps to separate empty from full capsids, which can be replaced with high-resolution ion exchange chromatography.

## TABLE 1

**Percentage binding of different AAV serotypes using the AAVX resin in a static binding assay.**

| POROS™ CaptureSelect™ AAVX resin. | |
|---|---|
| AAV serotype | Binding % (in eluate) |
| AAV1 | 99.63 |
| AAV2 | 97.8 |
| AAV2_HSPG | 98.33 |
| AAV4 | 98.05 |
| AAV5 | 97.88 |
| AAV6 | 97.45 |
| AAV6.2 | 98.93 |
| AAV7 | 98.37 |
| AAV8 | 97.76 |
| AAV9 | 98.43 |
| AAVrh10 | 96.28 |
| AAVrh32.33 | 99.29 |
| AAV9PHPB | 98.51 |
| AAV7m8 | 98.39 |

Clarified lysates containing AAV was mixed with AAVX resin for ten minutes. AAV was then eluted using acidic elution buffer at pH 2 (0.1M citric acid). Percentage binding was determined by qPCR. Data kindly provided by Genethon.

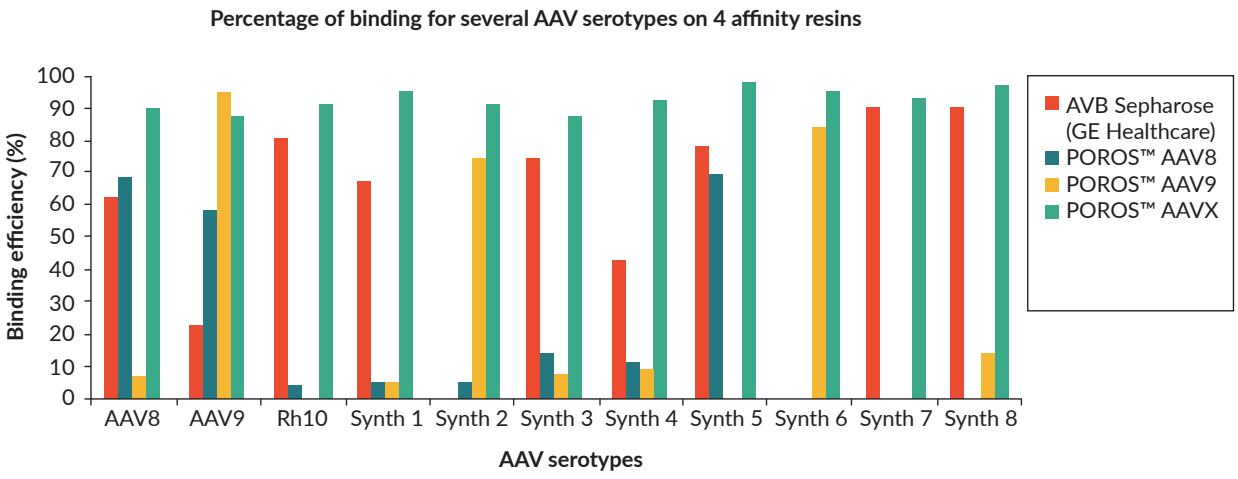

DOI: 10.18609/cgti.2019.110

A major hurdle in the current market is the lack of an industrialized platform to effectively and consistently purify AAV from large volumes of cell lysate. Scientists have started turning to affinity chromatography to fill this need. Initially affinity resins were developed against individual or a subset of AAV serotypes, so a true pan-AAV affinity chromatography platform did not exist until the development of the Thermo Scientific™ POROS™ CaptureSelect™ AAVX Affinity Resin (Table 1 & Figure 4). For example, the AVB Sepharose™ High Performance (GE Healthcare™) resin performs well for a variety of AAV capsids, but performs poorly when attempting to purify AAV9 and certain synthetic capsids (Figure 4). The CaptureSelect AAVX affinity resin consists of the single-domain heavy chain ($V_HH$) fragment of the heavy chain–only antibodies normally produced by Camelids. This $V_HH$ fragment is both highly specific and compact, allowing it to perform well in the strenuous conditions encountered during column chromatography. Furthermore, these $V_HH$ domains are produced by *Saccharomyces cerevisiae*, rendering them animal-origin free. These $V_HH$ domains are tethered to 50 µm POROS polystyrenedivinylbenzene beads that allow for large volumes of cell lysates to be purified quickly because of their large through pore size [11–13]. The POROS CaptureSelect AAV resins can be used at different flow rates without compromising capacity, resolution, and purity. This results in a high degree of flexibility in designing a process (Figure 5) [13]. With all these features, the POROS CaptureSelect AAV affinity resin is a simplified and robust platform for the generation of high-purity virus.

While affinity chromatography is capable of generating high yields of purified AAV, it cannot discriminate between empty and full viral capsids. It can be important to separate out empty capsids

## FIGURE 4

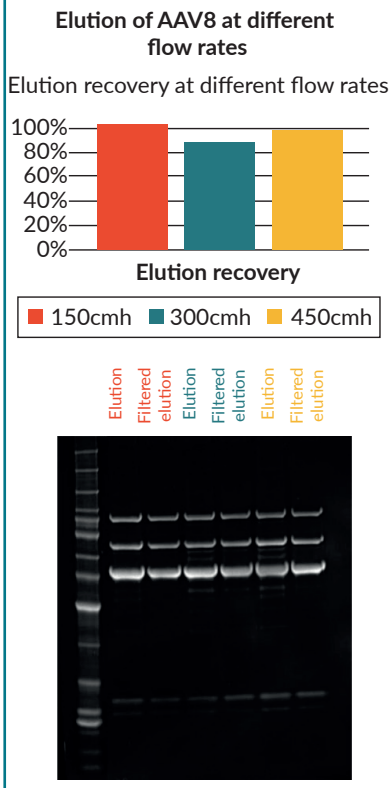
**Comparison of the AVB Sepharose High Performance resin with the POROS CaptureSelect AAV8, AAV9, and AAVX resins.**




These data were generated from purification of AAV8, 9, 10, and 8 synthetic capsids using 25 µL of resin in a 96-well plate format. Data kindly provided by Genethon

**FIGURE 5**

**Elution recovery and purity of the viral capsids using the POROS Capture Select AAV resins is not influenced by flow rate.**




**(A)** Percentage elution recovery for each of the different flow rates. **(B)** SDS-PAGE gel indicating AAV purity. The three strong bands indicate each of the three viral capsid proteins. (cmh = cm/hr)

as they contribute no benefit to the therapy and may even increase the immunogenetic potential [14]. The separation of empty and full capsids can be accomplished by a separate polishing step following affinity chromatography. Since empty and full viral capsids have only a slight difference in isoelectric points (pIs), only high-resolution anion exchange resins can effectively separate empty and full capsids (e.g., Thermo Scientific™ POROS™ HQ (Figure 6) [11,15–17]. Together the POROS CaptureSelect AAVX Affinity Resin and POROS ion exchange resins are able to separate AAV from both process- and product-related impurities. These resins can be easily incorporated into large-scale production platforms, thereby reducing the number of processing steps required for AAV purification to save time and increase product yield.

## VIRAL PRODUCTION ANALYTICS

Once a group is ready to scale up to clinical manufacturing, many QA/QC protocols must be implemented to help ensure the regulatory approval of the cell or gene therapy. Two such regulations, important for the viral production process are the absence of *Mycoplasma* in the cell culture and the absence of residual DNA following virus harvest and purification. New analytics to ensure regulatory compliance were required with the advent of gene therapies. For example, traditionally, *Mycoplasma* detection required a 28-day culture-based test; however, faster detection methods are needed because of the quick turnaround times required for cell and gene therapies. To fill this need, rapid, qPCR-based tests were developed by a number of different companies. Therefore, to identify the best kit on the market, Duguid et al. reviewed 21 different commercial kits and ranked them based on critical risk attributes. They used their ranked list to select three kits to test for specificity, sensitivity, and ease of use and identified the Applied Biosystems™ MycoSEQ™ Mycoplasma Detection Kit as the best kit according to all three metrics (Table 2) [18].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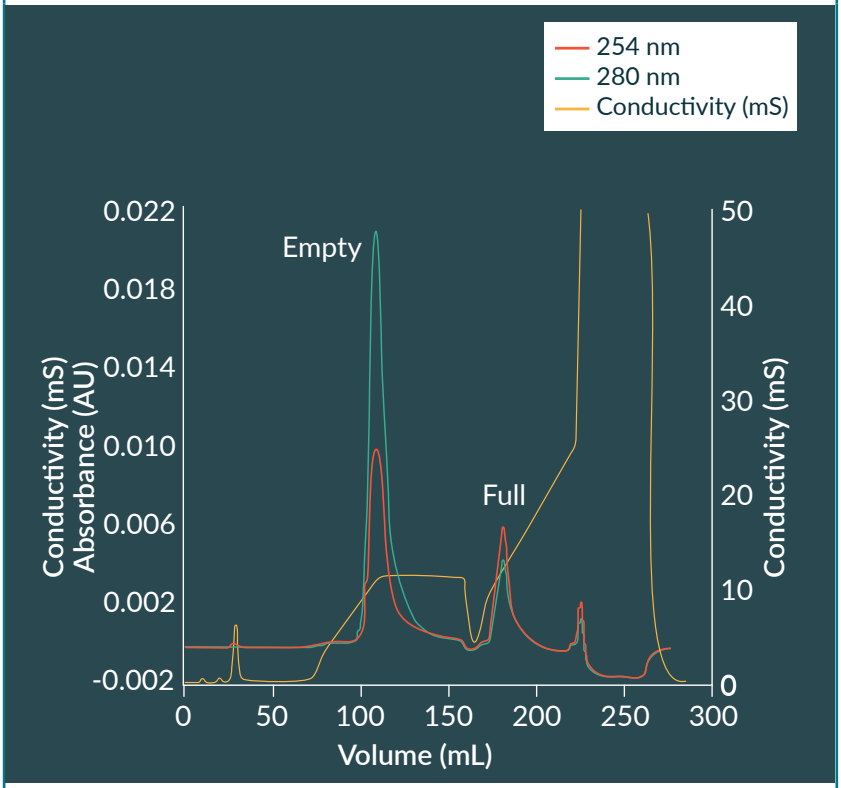
The MycoSEQ Mycoplasma Detection Kit is a straightforward method to specifically recognize *Mycoplasma* in accordance with regulatory compendia. The kit uses 31 *Mycoplasma*-specific primers to identify >90 different species, including those recommended for testing and validation. This allows for the sensitive detection of *Mycoplasma* in a sample – complying with regulations requiring a limit of detection of at least 10 CFU/mL – with no cross-reactivity (Table 2). This kit is easy to use with such features as a positive control with a modified melting temperature compared to the naturally occurring *Mycoplasma* amplicons to help reduce the risk of false positives (Figure 7 & Table 2). Furthermore, The MycoSEQ kit is paired with the Applied Biosystems™ PrepSEQ™ 1-2-3 Mycoplasma Nucleic Acid Extraction Kit to quickly isolate genomic DNA (gDNA) from cell culture samples. The PrepSEQ kit allows for high recovery of gDNA using both automated and manual methods, generating flexibility in the workflow. These kits make *Mycoplasma* detection for R&D or lot release possible in hours instead of 28 days. A variety of companies have now successfully used the MycoSEQ kit for compendial testing of their gene and cell therapy products for regulatory approval by both the FDA and EMA.

Gene therapy products must also be tested for residual DNA for lot release, with the FDA requiring <10 ng of residual DNA per dose [10]. Residual DNA testing requires methods that are able to reliably recover DNA and specifically asses the amount of residual DNA with high sensitivity. The Applied Biosystems™ resDNASEQ™ Human Residual DNA Quantitation Kit was created to accurately quantify residual DNA in a sample in as few as five hours. The resDNASEQ system includes the Applied Biosystems™ PrepSEQ™ Residual DNA Sample Preparation Kit which can be used either manually or with automation to consistently recover DNA from challenging matrices. This means that residual DNA can be assessed during the early stages after viral harvest through to final lot release (Figure 2). When used together, the resDNASEQ and PrepSEQ kits are able to reliably detect residual DNA across a broad dynamic range with a limit of quantitation of 1.5 pg/mL and a low coefficient of variation

**FIGURE 6**

**Chromatogram of an AAV mixture of empty and full particles.**




Empty capsids were separated by CsCl gradient ultrafiltration and mixed together with vector in a ratio of 16:1. The AAV mixture was applied onto a POROS HQ50 column and demonstrates excellent separation of the empty from the full AAV particles. The larger peak had an $A_{254/A280}$ ratio <1, while the smaller peak had an $A_{254/A280}$ ratio >1, indicating that the larger peak was enriched for empty capsids and the smaller peak was not. Further validation of the smaller peak was performed by ELISA and qPCR, and the ratio of empty capsid to vector was 0.9 [15].

## TABLE 2

**Results of a side-by-side study of three different qPCR kits for the detection of Mycoplasma.**

| Criteria | | qPCR Kit 1 | qPCR Kit 2 | qPCR Kit 3 |
|---|---|---|---|---|
| Specificity | Un-spiked samples | Negative | Negative | Negative |
| | PC-spiked samples | Positive | **Mixed** | **Mixed** |
| | Organisms detected | Wide range | Wide range | **8** |
| | Cross-reactivity | None detected | ***S. pyogenes*** | N/A |
| Sensitivity | Minimum detected | 4 copies (0.004 µL PC) | 0.016 µL PC | 50 copies (0.25 µL PC) |
| | Minimum reproduced | 8 copies (0.008 µL PC) | 0.03 µL PC | 50 copies (0.25 µL PC) |
| Other | Sample preparation | Optimized | **None** | **<5% recovery** |
| | Kit Stability | >1 month | >1 month | **<1 month** |
| | Complexity | Least | **Most** | N/A |
| | Optimized for various instruments | Yes | Yes | **No** |
| | Delivery | TBD | **2 wk – 2+ mo** | 1 day |
| | Logistics | None | **Difficult** | None |

Issues are in bold.

(Figure 8 & Table 3). The resDNASEQ kit can therefore be used to qualify products for lot release.

## CONCLUSIONS

With the increased interest in gene and cell therapies, it is apparent that efficient and cost-effective strategies for viral production are necessary to meet market needs. Recent technological advances have significantly improved the AAV production workflow. This includes technologies like serum-free HEK293 cell lines capable of growing to high confluency

## TABLE 3

**DNA quantitation by the resDNASEQ Human DNA Residual DNA Quantitation Kit from samples prepared using the PrepSEQ Residual DNA Sample Preparation.**

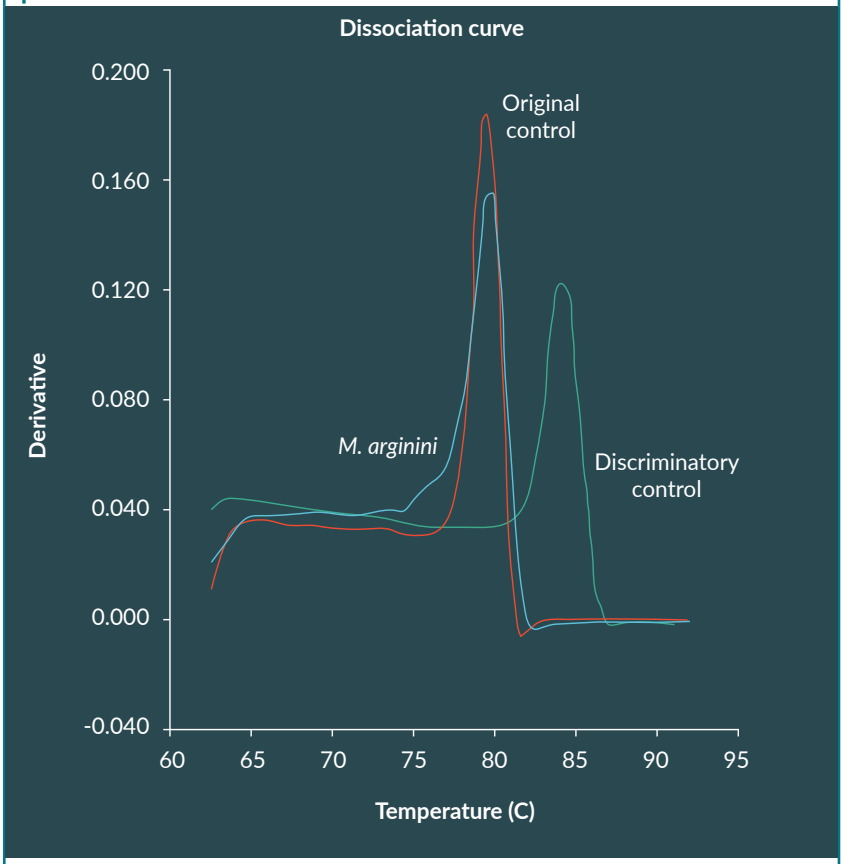
| Test sample | 100 pg Spike | | 10 pg Spike | | 1 pg Spike | |
|---|---|---|---|---|---|---|
| Buffer | pg DNA recovered | % CV | pg DNA recovered | % CV | pg DNA recovered | % CV |
| Assay 1 | 75.2 | 11% | 7 | 7% | 0.65 | 5% |
| Assay 2 | 65.4 | | 6.2 | | 0.69 | |
| Assay 3 | 61.9 | | 6.1 | | 0.63 | |
| Assay 4 | 58.3 | | 6.3 | | 0.63 | |
| Assay 5 | 55 | | 5.9 | | 0.66 | |
| Assay 6 | 60 | | 7.1 | | 0.72 | |
| Average | 62.63 | | 6.43 | | 0.66 | |

Six samples were spiked with 100 pg, 10 pg, and 1 pg of human standard DNA respectively. The average and percent coefficient of variation (%) were calculated for each DNA amount tested.


DOI: 10.18609/cgti.2019.110

## FIGURE 7

**Melting curves of qPCR amplicons using Mycoplasma specific primers.**

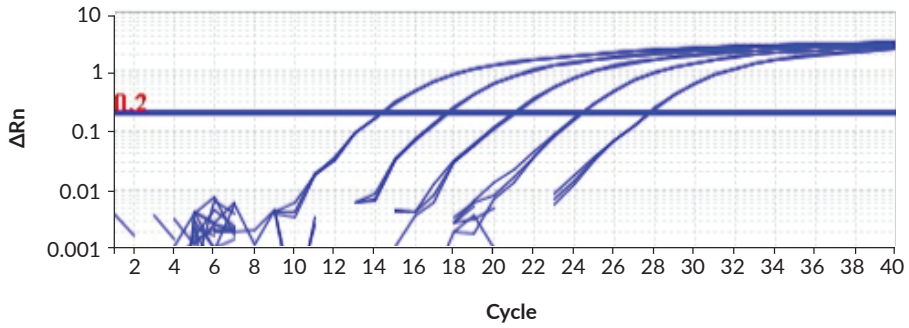



Melting curves for Mycoplasma arginini and an unaltered positive control are shown in blue and red respectively. The melting curve for the modified positive control is shown in green.

in optimized medium to improve safety, help with downstream processing, and easily scale up manufacturing. Additionally, the affinity chromatography-based pan-AAV CaptureSelect AAVX affinity resin allows for the development of a robust platform for downstream AAV purification with increased yields and less processing time. Furthermore, qPCR-based assays like the MycoSEQ and resDNASEQ kits meet regulatory requirements for specificity and sensitivity and can be performed in under five hours to accommodate the short shelf life of gene and cell therapies. These kits are also automatable, reducing labor time. Last, CDMOs, such as Brammer Bio, can help throughout manufacturing with process optimization and analytics to help ensure successful IND filings. Significant improvements to the traditional AAV manufacturing process now exist, so high-purity, high-titer virus is possible with lower costs and shorter lead times.

## FIGURE 8

**Amplification plot of a 10-fold serial dilution of human DNA using the resDNASEQ Human DNA Residual DNA Quantitation Kit.**




| | DNA per rxn |
|---|---|
| 1 | 3 ng |
| 2 | 300 pg |
| 3 | 30 pg |
| 4 | 3 pg |
| 5 | 300 fg |


### AUTHORSHIP & CONFLICT OF INTEREST

***Contributions:*** *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

***Acknowledgements:*** *The authors wish to thank Genethon for the provision of data contained within this article.*

***Disclosure and potential conflicts of interest:*** *The authors are employees of Thermo Fisher Scientific. The authors declare that they have no conflicts of interest.*

***Funding declaration:*** *The authors received no financial support for the research, authorship and/or publication of this article.*





***Article source:*** *Invited; externally peer reviewed.*

***Submitted for peer review:*** *24 June 2019*; *Revised manuscript received: 26 July 2019*; *Publication date: 7 August 2019*.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DOI: 10.18609/cgti.2019.110

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