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CELL & GENE THERAPY INSIGHTS

SPOTLIGHT ON:

Vector bioprocessing

CELL & GENE THERAPY INSIGHTS Volume 8, Issue 3

CONTENTS

SPOTLIGHT: Vector bioprocessing

LATEST ARTICLES

SUPPLY CHAIN CHANNEL EDITION: Best practices for ensuring cell and gene therapy supply chain scalability

REPORTS: Regulatory Insights

Spotlight

Vector bioprocessing

FOREWORD
Vector bioprocessing spotlight
Franz Gerner
451–453

EXPERT INSIGHT
Adeno-associated virus process development: optimization & development of a scalable elution for polishing chromatography
Daniel Martin
421–429

RESEARCH ARTICLE
Clarification of recombinant adeno-associated virus (rAAV) & lentivirus from adherent culture
Rajeshwar Chinnawar & Nicholas Marchand
483–493

EXPERT ROUNDTABLE
Thinking big: the need for innovation in the production of lentivirus-based cell and gene therapies
John Moscariello, Will Junker, Lee Davies, Hanna Leinonen, Scott Cross
341

EXPERT INSIGHT
Adeno-associated viral vector process development challenges in early research & preclinical study
Ashish Saksule
411–420

INNOVATOR INSIGHT
Flexible & scalable solutions for AAV viral vector production using a templated platform
Eva Fong
499–512

FAST FACTS
Detecting residual host cell DNA with Droplet Digital PCR technology
Mark White
443

Volume 8, Issue 3

INTERVIEW
Exploring commercial upstreamprocessing of AAV
Hugo Rojas
371–376

LIVE 30
Manufacturing of RCA-free adenoviral vectors
Nico Scheer
351

INNOVATOR INSIGHT
Accelerating cell & gene therapy workflows with next-generation analytical tools
Chris Heger
463–473

INTERVIEW
Considerations for demonstrating comparability of AAV processes
Laura Griersch
475–479

VIEWPOINT
Future trends in viral vector process & product development
Ramji Krishnan
447–450

INNOVATOR INSIGHT
Process development innovations to improve gene therapy manufacturing
Jessica Hillmoe & Michael Shen
541–548

Latest articles

FAST FACTS
Reducing processing risks & operational challenges for low volume fluid transfer
Troy Ostreng & Jayanthi Grebin
353

FAST FACTS
Laying the foundations for success with scale-down models
Rohit Saklecha & Andres Castillo
431

INNOVATOR INSIGHT
The digital revolution: technological innovations to enable automation in cell therapy manufacturing
Sean Chang, Bruce Greenwald & Krish Roy
355–369

WEBINAR DIGEST
Rapid translation of a cellular therapeutic from research to clinic
Ashish Patel & Joseph Candiello
445

LIVE30
Supply chain efficiency and sourcing for scaling your gene therapy operation
Celine Martin & Don Young
523

FAST FACTS
POROS CaptureSelect AAVX Wash Optimization Study for Optimized Recovery and Purity of AAV6 Capsids
Jenny England
621

INNOVATOR INSIGHT
From cells to purified capsids: How to develop a scalable rAAV process
Åsa Hagner McWhirter
611–620

Supply Chain Channel

Best practices for ensuring cell and gene therapy supply chain scalability

PODCAST INTERVIEW
Precisely for CGT: automating aseptic filling for lowest volumes
Barbara Fischer
403–408

FAST FACTS
Key factors in cell cryopreservation and impact on manufacturability of cell and gene therapies
Robert Newman
433

INTERVIEW
A collaborative approach to cell & gene therapy supply chain management
Anthony Johnson
435–441

Report

Regulatory Insights

REGULATORY PERSPECTIVE
Current global regulatory landscape for biodistribution & shedding assessment of rAAV gene therapies & recommendations of the IMI ARDAT consortium on future directions
Natalie Anne Schmidt, Jon Giblin, Timothy K MacLachlan, Shera Dandapat, Gabor Veres, Tatiana Anna Reimer, Martin Schulz, Eva Hatzmann, Andeleeb Dahy, Gregory LaRosa, Mimoun Azzouz, & Christopher J Mann
377–394

EDITORIAL
Unproven stem cellbased interventions & harm to existing & future patient
Flora Naylor & Kirstin RW Matthews
397–401

FOREWORD

Vector bioprocessing spotlight

FRANZ GERNER is Chief Technology Officer at Excision BioTherapeutics, a clinical-stage CRISPR company using AAV as transfer vehicle to treat persistent infectious diseases, where he is responsible for CMC and related activities internally and at external contract organizations. Dr Gerner has over 25 years' experience working in the gene therapy field, in process and analytical development, manufacturing, Quality Control, Quality Assurance and vector development. Prior to joining Excision, he was Vice President of Technical Operations at Axovant/Sio Gene Therapies where he oversaw the activities of CMC activities at contract organizations. Previously, he was one of the early members of Regenxbio, responsible for Process Development, including the establishment and subsequent expansion of in-house capabilities. In addition, he was responsible for new innovative approaches to produce recombinant AAVs. Prior to Regenxbio, he has held increasing positions in the biotech industry. He holds a MS in Chemistry and a PhD in Chemistry with focus on Gene Therapy by the Ludwig-Maximilians-Universität München.

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Since the early days of gene therapy in the 1970s, significant efforts have been made to improve the manufacturing of viral vectors, starting with the availability of efficient plasmids for generating recombinant adeno-associated viral (AAV) vectors by Samulski *et al.* in the early 1980s. Despite the challenges in the gene therapy field over the last decades, including the death of Jessie Gelsinger in 1999, and the pullback of investments during the great recession at the start of the 21st century, several groups continued to advance gene therapy programs, leading to the first commercially available products.

To support such advancements from a CMC perspective, continued improvements of gene therapy viral vector production were performed by implementation of methods to enhance the scalability of the viral vector manufacturing. This includes improved plasmid constructs for adenoviral, AAV, and lentiviral-based vectors, alternate manufacturing

methods using viruses to produce viral vectors, scalable manufacturing methods leading to larger scale production, and significant improvements to analyze the produced viral vectors. In particular, the use of bioreactors for adherent and suspension cell cultures and replacing centrifugation for chromatographic purification led to increased interest in the use of gene therapy due to the improvements of yield and quality of the manufactured vectors. In addition, the advancements in the analytical characterization of viral vectors gave significant insights into the quality of vectors produced, and feedback for manufacturing improvements.

Optimizations for increasing the yield and the purity of viral vectors are often primarily focusing on the bioreactor conditions and transfection, as well as the chromatographic purification. Rajeshwar Chinnawar and Nicholas Marchand (Pall) discuss the important step between these upstream and downstream steps, with a focus on clarification of the harvest material. By optimizing this step, the clarified material used for subsequent purification steps can be significantly improved, leading to an advanced performance of the downstream process and therefore, enhanced viral vector product. The use of depth filters and membrane filters for use in AAV and lentiviral production for efficient separation of particulates and soluble impurities while preventing fouling are reviewed.

A critical step for improving the purity of the viral vector is the separation of product-related impurities, such as empty and partially filled capsids from full capsids during AAV manufacturing. Since the goal of the downstream process is to be scalable, chromatographic purification methods have been employed, but this makes the separation of the product-related impurities significantly more challenging compared to ultracentrifugation-based methods. Daniel Martin (Precision Biosciences) shares an approach of optimizing the downstream process to reduce process-related impurities. Martin demonstrates that by investigating different columns and the run conditions, the purification process can be improved, reducing the amount of empty and partially filled capsids in the product.

For better targeting of viral vectors, several AAV serotype or capsid mutants are often evaluated for specific organ or cell type targeting. With the changes in the capsid, the performance of the viral vector during purification on chromatographic columns can change, leading to a suboptimal yield and/or quality of the viral vector. Ashish Saksule (Takeda) is discussing the challenges of process development supporting the evaluation of modified serotypes in pre-clinical settings in order to use the most suitable vector in clinical applications.

Developing efficient manufacturing processes is a suitable goal through the clinical stages all the way to commercial manufacturing. This requires the upstream and downstream unit operations to not only be fit for purpose to enable production of the projected amount of material, but also controllable and amenable to automation for improved performance and robustness of the manufacturing process. Hugo Rojas (uniQure) explores requirements and approaches for establishing a commercially viable manufacturing process.

Despite recent advances, significant challenges remain to be resolved for the future of gene therapy, including the need to further improve the yield to reduce cost as well as enabling high-dose gene therapy approaches, optimization of the purification of gene therapy vectors to further reduce impurities, and new analytical methods to enhance the understanding of the vector to support the knowledge of, and potentially improve the consistency of, the manufacturing of viral vectors. In his Viewpoint article, Ramji Krishnasamy (Rocket Pharmaceuticals) discusses some of the approaches to address these challenges, including the importance of comparability due to process changes while going through the different stages of development.

Most, if not all companies have started with processes that were changed over the course of the life of a program. This can be caused by the scale not being sufficient to

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include a broader patient population in late-phase or commercial stages, the improvement of the purity profile of the product, and more advanced analytics giving feedback to process development and manufacturing. Laura Giersch (Lysogene) discusses the considerations that should occur prior to developing a new process, including the cost of goods, regulatory implications, and the feasibility of the anticipated new process. In addition, comparability between the new and previous processes needs to be planned, including the availability of relevant assays. Giersch points out that one of the most important considerations for comparability, which was also discussed during a recent 2-day conference on comparability in Boston, is the retention of enough samples for being able to perform comparability.

I hope you find these articles about advances and challenges in the bioproduction of gene therapy vectors as inspiring as I did, and I would like to thank the experts who contributed to this edition for their valuable discussion points and insights.

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AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

VECTOR BIOPROCESSING

SPOTLIGHT

EXPERT INSIGHT

Adeno-associated virus process development: optimization & development of a scalable elution for polishing chromatography

Daniel Martin

In vivo gene therapy utilizing adeno-associated virus (AAV) is a rapidly developing and highly focused area of the biopharmaceutical industry. As the regulatory landscape is shaped there is an increased expectation for companies to develop manufacturing processes generating safe and efficacious AAV. A highly discussed and difficult aspect of AAV manufacturing is the separation of empty capsids from full capsids. Undesirable capsids have unknown impact on the patient's immune system; consequently, it is desirable to remove them from the final product altogether and de-risk the potential impact to patient safety. From a process development perspective removal of undesirable capsids is challenging due to the similar quality attributes that are shared with full capsids. Anion exchange chromatography is often used as the final polishing step and is primarily used to separate empty capsids from full capsids. In order to achieve this separation a shallow increasing salt gradient with fractionation is used and these two modes of operation are not ideal for large scale manufacturing. Empty-full separation chromatography for a promising AAV8 construct underwent initial development and optimization for technology transfer to large-scale manufacturing at Precision BioSciences. This was accomplished by modifying the platform downstream process and screening both different elution conditions and resin modalities for improved resolution. The eluting peak order for this AAV8 construct is the empty capsid peak first followed by the full capsid peak and is then followed by a final impurity peak. Screening to improve resolution between the peaks created an opportunity to include an additional wash step that removes the empty capsid peak before elution occurs. This was accomplished without negative impact to product recovery and empty-full content. With the removal of the empty capsid peak the elution profile can be further optimized to not rely on a gradient fractionation at the manufacturing scale.

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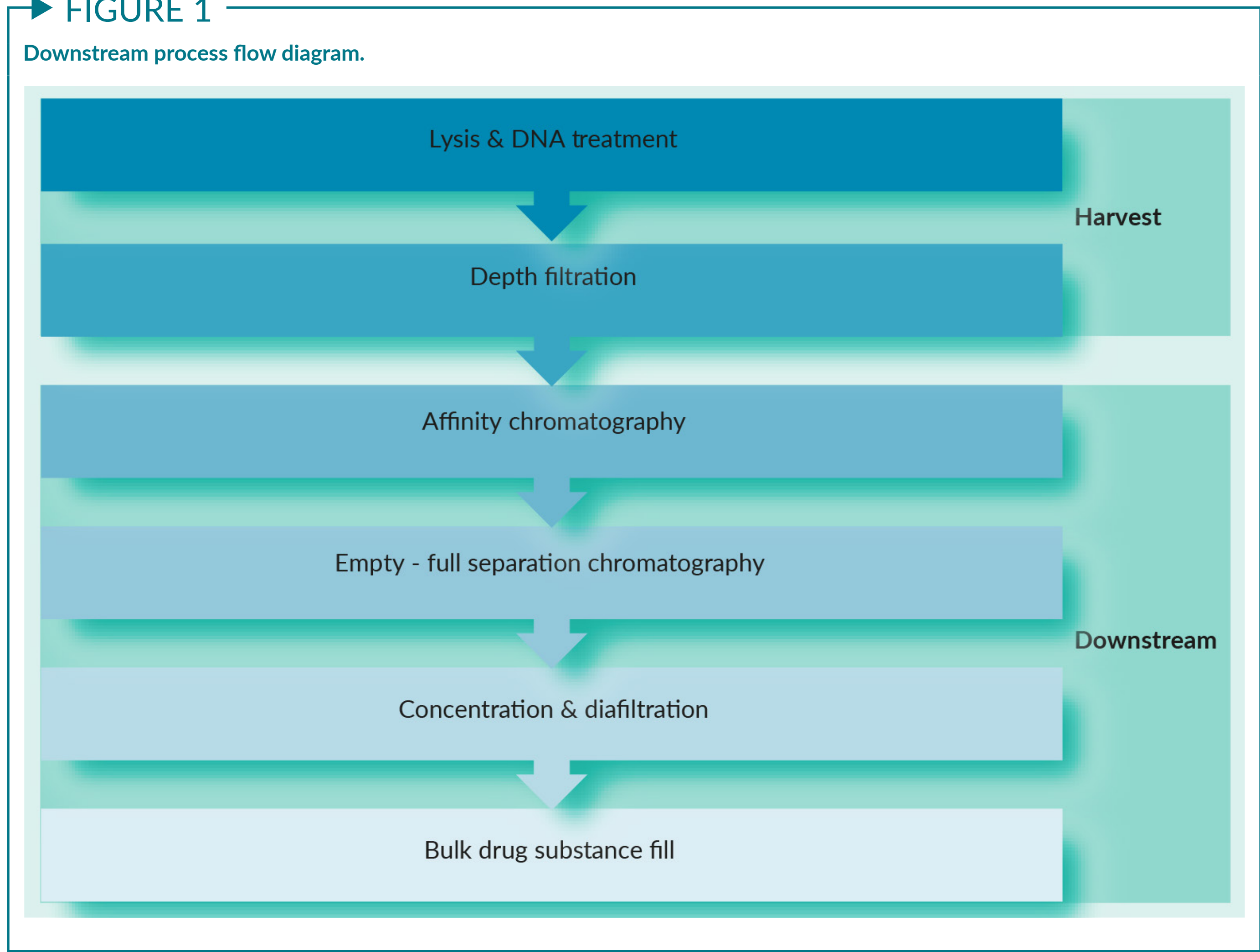
Adeno-Associated Virus (AAV) is a non-pathogenic, replication-deficient virus that can deliver DNA to different tissues in the body. We can utilize our ARCUS™ technology to use different AAV serotypes to deliver many potential transgenes via *in-vivo* and *ex-vivo* treatments. AAV Process Development (PD) at Precision BioSciences have developed a platform purification process to evaluate different AAV serotypes combined with different transgenes called constructs (Figure 1). For promising candidates, the platform process is modified specifically for each construct. In the current AAV landscape empty-full separation remains a difficult polishing step to optimize without the use of gradients and fractionation. Both gradients and fractionation can present challenges with scaling to a large-scale manufacturing process. Gradients are utilized often and can be accommodated, but large-scale fractionation is highly undesirable. The AAV downstream process development team conducted experiments that investigated developing a robust isocratic elution operation. To do so different salts were screened using Anion exchange chromatography (AEX) chromatography and the resolution between different capsids were measured. Utilizing the salts with the highest resolution, an isocratic elution was developed by first washing off undesired capsids preceding the full capsids. Additionally, the team found that when utilizing a mixed mode chromatography system that exploits differences in hydrogen bonding combined with traditional AEX that separation now depends on differences in pH as opposed to conductivity. Experiments with mixed mode chromatography showed improved resolution with the possibility to develop a robust isocratic elution

FIGURE 1

Downstream process flow diagram.



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operation scalable to manufacturing. This article will provide insight into AAV PD activities for a AAV8 construct that underwent initial empty-full separation optimization.

MATERIALS & METHODS

Samples & sample preparation

Sample and sample preparation were produced in-house at our AAV PD laboratory. Lysate of Expi293F cells producing AAV8 were harvested using depth filtration and is then further 0.2µm filtered. Harvest filtrate is loaded directly onto the affinity capture chromatography column.

The affinity chromatography capture step utilizes POROS™ CaptureSelect™ AAVX affinity resin from ThermoFisher Scientific. AAVX resin utilizes cross-linked polystyrene-divinylbenzene POROS beads with a CaptureSelect AAVX affinity ligand that have an average particle size of 50µm. AAVX loading falls into the range of 5.6×10^{12} to 9.1×10^{13}vg/mL resin. The buffering system can be seen in Table 1. A 5 CV step elution is utilized, and the eluate is neutralized with 500 mM BTP, 200mM NaCl, pH 9.0. Typical neutralized AAVX eluate concentration for this AAV8 construct is 5×10^{12} vg/mL.

The AEX chromatography step utilizes a CIMultus™ (CIM) QA monolithic column from BIA Separations. The CIM QA columns utilize a Polyglycidyl methacrylate-co-ethylene dimethacrylate support matrix with a strong quaternary amine for separating full capsids from empty capsids in the neutralized AAVX eluate. Neutralized affinity eluate is prepared by performing a 1:10 total parts dilution with the equilibration buffer. On a 1mL CIM QA column, 10mL of the prepared load is used per run and for this construct this equates to ~5×10^{11} vg/mL resin. Constructs are evaluated and purified using the platform AEX process using the system parameters shown in Table 2. A 65 CV increasing conductivity gradient elution is utilized for this unit operation and is fractionated into 1 mL fractions. Fraction pooling is done on a case-by-case basis using the resulting chromatogram. This platform process serves as the foundation for further development and optimization for scale up operations. Both chromatography steps are preformed using an ÄKTA Avant 150 from Cytiva.

TABLE 1.

Affinity chromatography system parameters.

Phase	Buffer	CV	Residence Time (min)
Equilibration	10mM BTP, 700mM NaCl, pH 6.8	5	2
Load	0.2µm Harvest Filtrate		
Wash 1	10mM BTP, 700mM NaCl, pH 6.8	5	
Wash 2	10mM BTP, 200mM NaCl, pH 6.8	5	
Elution	20mM NaCitrate, 200mM NaCl, pH 2.5	5	

CIM QA salt screening

When promising constructs are identified the CIM QA B buffer is then modified with commonly used salts in place of 400mM NaCl and then screened against the platform process using the same elution strategy. In the case of this AAV8 construct, development first started with screening different salts to compare against the platform process. The B buffers used can be seen in Table 3.

Improved CIM QA elution development

Based on the resulting chromatograms from the different salt screening development runs, the best candidates that show improved resolution compared the platform process are then further developed improve the elution profile. In the case of this AAV8 construct the peak order that is generally observed is a predominately empty capsid peak (A260/280 < 1) followed by a predominately full capsid (A260/280 > 1) that is then followed by a final peak that contains additional impurities (shown in Figure 2). For this construct a

TABLE 2

AEX chromatography system parameters.

Phase	Buffer	CV	Residence time (min)
Equilibration	10mM BTP, pH 9.1	10	0.25
Load	1:10 Diluted Neutralized Affinity Eluate, pH 8.7		
Wash 1	10mM BTP, pH 9.1	10	
Elution	A: 10mM BTP, pH 9.1 B: 20mM BTP, 400mM NaCl, pH 9.1	65 Gradient: 0-100% B	

secondary wash step is developed to remove the initial empty peak and further isolate the full peak during elution. To do so there are three conductivity conditions that are chosen to evaluate. These conditions consist of the conductivity observed at the beginning of the empty peak (11.00 mS/cm), the conductivity observed at the peak max of the empty peak (12.25 mS/cm) and the last condition is chosen at the end of the empty peak (12.85 mS/cm) (shown in Figure 3). For this construct sodium chloride, tetramethylammonium and sodium acetate were chosen to develop a secondary wash step.

CIM PrimaS platform implementation

As will be discussed later in the results section of this article it was observed for this AAV8 construct the conductivity ranges established for the secondary wash steps using CIM QA were too tight to robustly scale up to manufacturing. Consequently, CIM PrimaS was also screened for this AAV8 construct.

CIM PrimaS utilizes a multimodal ligand that introduces hydrogen bonding with anion exchange to enhance selectivity. It uses the same support matrix but with a weak anion exchange ligand. CIM PrimaS load is prepared in the same manner as CIM QA, it is diluted 1:10 total parts with equilibration buffer. The elution strategy changes from an increasing conductivity gradient to an increasing pH gradient. The platform buffering system used can be seen in Table 4. and was implemented as per recommended by BIA Separations with minor modification. Results from platform implementation were screened against platform CIM QA and salt screening CIM QA experiments.

TABLE 3

CIM QA elution (B) screening buffer composition.

Elution buffer composition
20mM BTP, 1M Sodium Chloride (NaCl), pH 9.1
20mM BTP, 1M Tetramethylammonium Chloride (TMAC), pH 9.1
20mM BTP, 0.5M Magnesium Chloride ($MgCl_2$), pH 9.1
20mM BTP, 1M Sodium Acetate (NaAc), pH 9.1

Improved CIM PrimaS elution development

Similar to CIM QA the same peak order was observed for CIM PrimaS. The same improved elution strategy was used to wash the preceding empty capsid peak off with a secondary wash. Only the change to the buffer system was to pH as opposed to conductivity. The elution or B buffer was titrated to target pH values chosen from the platform CIM PrimaS runs.

RESULTS & DISCUSSION

Analytical evaluation

Product fractions were pooled based on resulting chromatograms and then submitted for analytics. Digital Drop Polymerase Chain Reaction (ddPCR) was used to quantify viral genome content of the product pools and viral genome content for the load. Recoveries are calculated using results from ddPCR. A260/A280 ratios were calculated by using Unicorn™ v7.6 software to find the peak max absorbance of both 280 nm and 260 nm wavelengths for the full capsid peaks and

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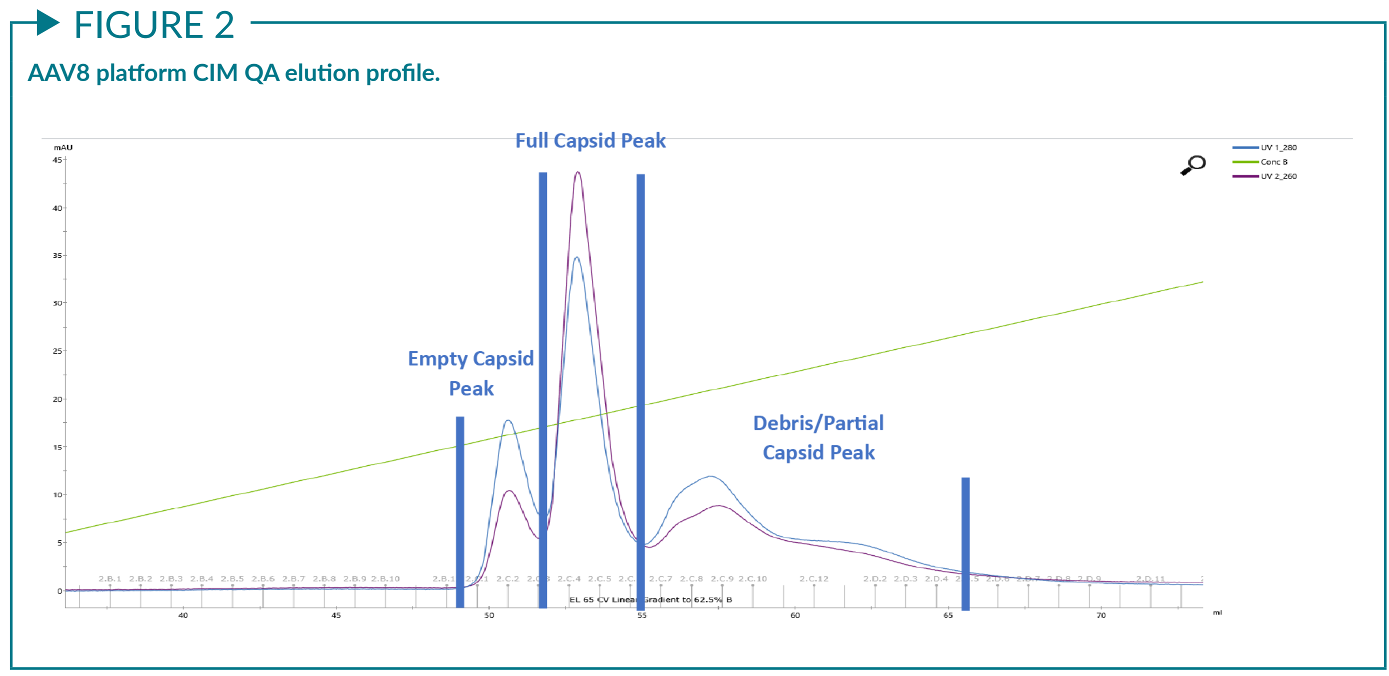
FIGURE 2

AAV8 platform CIM QA elution profile.



then dividing these numbers to determine the ratio. This ratio is used as a rough guideline of empty-full ratio with A260/280 < 1 being considered a empty capsid peak and A260/280 > 1 being considered a full capsid peak. Additionally, resolution between peaks is calculated using Unicorn™ v7.6 software and the equation used can be seen in Figure 4. Lastly, a Stunner™ by Unchained Labs was used to analyze total viral capsid content. Utilizing the viral genome content determined by ddPCR and total viral capsid content determined by Stunner analysis was used to characterize empty-full capsid content. It is important to note that this method of determining empty-full capsid content

FIGURE 3

AAV8 NaAc wash conductivity selection.

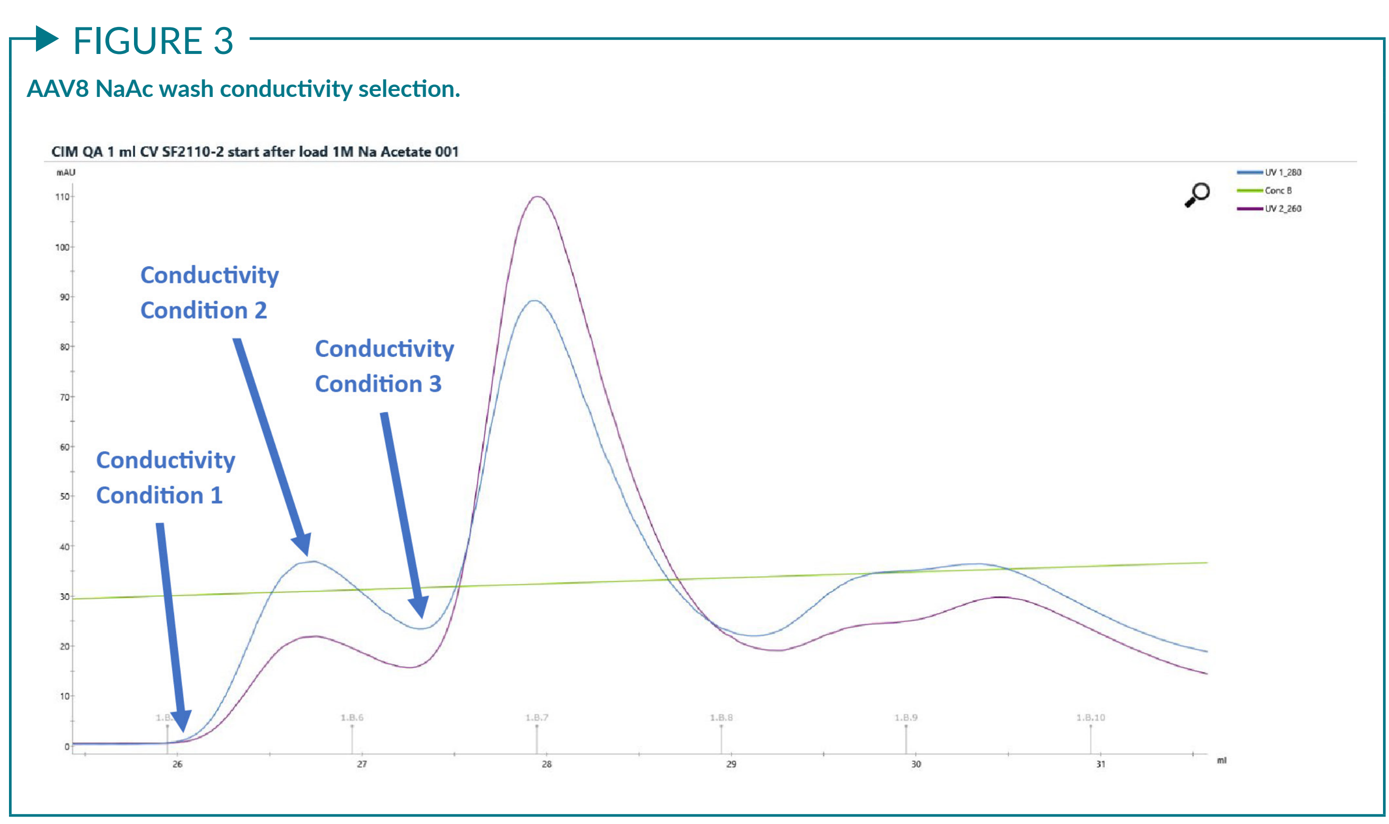


TABLE 4

CIM PrimaS chromatography system parameter.

Phase	Buffer	CV	Residence Time (min)
Equilibration	10mM Tris, 10mM BTP, 2mM $MgCl_2$, pH 8.0	10	0.25
Load	1:10 Diluted neutralized affinity eluate, pH 8.7		
Wash 1	10mM Tris, 10mM BTP, 2mM $MgCl_2$, pH 8.0	10	
Elution	A: 10mM Tris, 10mM BTP, 2mM $MgCl_2$, pH 8.0 B: 10mM Tris, 10mM BTP, 2mM $MgCl_2$, 13mM NaAc, pH 10.0		

is utilized only for preliminary development since samples were close to the bottom LOQ of the Stunner. Subsequent development utilizes ddPCR and ELISA analysis to determine empty-full content.

Empty-full chromatography peak resolution

Results from the salt screening study on CIM QA can be seen in Table 5. Compared to the platform process, only Tetramethylammonium Chloride and Sodium Acetate improved resolution between full capsid and empty capsid peaks for this AAV8 construct. Recoveries (mass balance utilizing ddPCR) were seen to improve with the use of Tetramethylammonium Chloride and recovery was slightly reduced with Sodium Acetate. As a result, these two salts and Sodium Chloride were picked to further develop a secondary wash step. It is interesting to note that the presence of Magnesium Chloride impacted the binding characteristics of the empty and full capsids. Magnesium is thought to have interactions with glutamic acid residues on capsid protein borders which might explain why there is an impact on binding. Consequently, the full capsid peak eluted first followed by empty capsid peak and lastly the final impurity peak. The results from CIM QA were compared to the platform CIM PrimaS process and significantly more resolution is observed between all the capsid species when utilizing CIM PrimaS. In addition to much improved resolution there is also significant improvement in the empty-full ratio while maintaining similar recovery. Since empty-full was calculated using ddPCR viral genome quantification with Stunner, total viral quantification requires further investigation using ddPCR and ELISA analysis is necessary.

Empty-full chromatography improved elution development

Results from the CIM QA secondary wash results can be seen in Table 6. below. Three conductivity conditions were chosen using the methodology previously mentioned for Tetramethylammonium Chloride (1M), Sodium Acetate (1M) and Sodium Chloride (1M). It is important to note that during this study one sample was compromised for Sodium Acetate and was not analyzed. Additionally, empty-full ratios via Stunner analysis were not performed on these samples due to unavailability of the equipment at the time of this study. Results from these conditions show some negative impact to recoveries when compared to the CIM QA and

FIGURE 4

Resolution algorithm.

$$\text{Resolution} = \frac{\left[\dfrac{(2^{nd}\text{ peak retention} - 1^{st}\text{ peak retention})}{(2 * (2^{nd}\text{ peak width at half height} + 1^{st}\text{ peak width at half height}))}\right]}{2.354}$$

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TABLE 5

Screening study result.

Condition	Resolution (full and empty)	Resolution (full and partial)	Full Peak A260/280 ratio	Empty-full (%)	Viral genome recovery (%)
CIM QA Platform	0.97	1.01	1.26	33	50
CIM QA 1M NaCl	0.91	0.95	1.24	29	43
CIM QA 1M TMAC	1.33	0.84	1.16	28	57
CIM QA 0.5M $MgCl_2$	0.34	1.37*	1.13	19	30
CIM QA 1M NaAc	1.01	0.88	1.24	15	40
CIM PrimaS Platform	1.89	1.98	1.27	62	50

The presence of Magnesium changed the order of the peaks to full capsids followed by empty capsids and then partial capsids or debris. This value is the resolution between empty and partial peaks.

CIM PrimaS processes. However, improved A260/280 ratios were observed with Sodium Acetate and Sodium Chloride conditions and lower recoveries, so this suggests there is most likely improved empty-full ratios. Results from these experiments show that washing off the empty peak with a second wash step is a feasible approach. But when analyzing the conductivity ranges established by these wash conditions it presents challenges to scalability to a manufacturing environment due to a tight operating range. Such a tight operating range does not allow for a robust normal operating range (NOR) with an acceptable operating range (AOR) the allows for adequate empty-full ratio and acceptable recoveries.

It is because of the tight conductivity ranges found when implementing a second wash step that CIM PrimaS was evaluated for this AAV8 construct. With greatly improved resolution along with improved empty-full ratios while maintaining equivalent recoveries, CIM PrimaS is a more favorable candidate to implement a secondary wash step (seen in Table 7). A wide range of pH conditions were evaluated as secondary wash steps. Most conditions resulted in similar A260/280 ratios when compared to platform processes. Additionally, empty-full ratios remain improved over CIM QA while maintaining more comparable recoveries to the platform process. Based on the results of the experiments conditions 8.84, 8.78 and 8.73 show a robust range where A260/280, empty-full, and recovery is optimal. This provides a range of 0.11 pH units between the high and low condition. While this is still a tight range it is feasible to control the pH in manufacturing within this range. Thus, there exists more scalability when using CIM PrimaS with this AAV8 construct to develop a secondary

TABLE 6

CIM QA secondary wash results.

Wash salt	Conductivity (mS/cm)	Full peak A260/280 ratio	Viral genome recovery (%)
CIM QA Platform		1.26	50
CIM PrimaS		1.27	50
TMAC	10.53	1.26	41
TMAC	10.92	1.21	38
TMAC	11.27	1.19	45
NaAc	9.98	1.33	49
NaAc	10.49	1.32	39
NaCl	9.93	1.30	40
NaCl	10.52	1.30	35
NaCl	10.86	1.28	33

TABLE 7

CIM PrimaS secondary wash results.

Condition	Full Peak A260/280 Ratio	Empty-Full (%)	Full Peak Recovery (%)
CIM QA Platform	1.26	33	50
CIM PrimaS platform	1.27	62	50
pH 8.68 Wash	1.25	23	50
pH 8.73 Wash	1.26	53	55
pH 8.78 Wash	1.23	101	47
pH 8.84 Wash	1.32	58	49
pH 8.88 Wash	1.26	76	24
pH 9.07 Wash	1.17	75	12

wash step to help isolate the product peak in elution.

TRANSLATION INSIGHT

Further optimization with CIM PrimaS has not been performed at this time. These experiments demonstrate a strong proof of concept and potential to utilize this CIM PrimaS with this AAV8 construct to develop a scalable secondary wash step and further elution optimization. The advantage of utilizing a secondary wash step to remove the empty capsid peak before elution allows for further development of the elution step. A step elution can be explored to further improve elution robustness. Regardless the elution pH gradient now has a large product peak with improved resolution from the partial capsid peak (shown in Figure 5). This can allow for use of UV gates to capture the full peak and eliminate the need to fractionate at scale.

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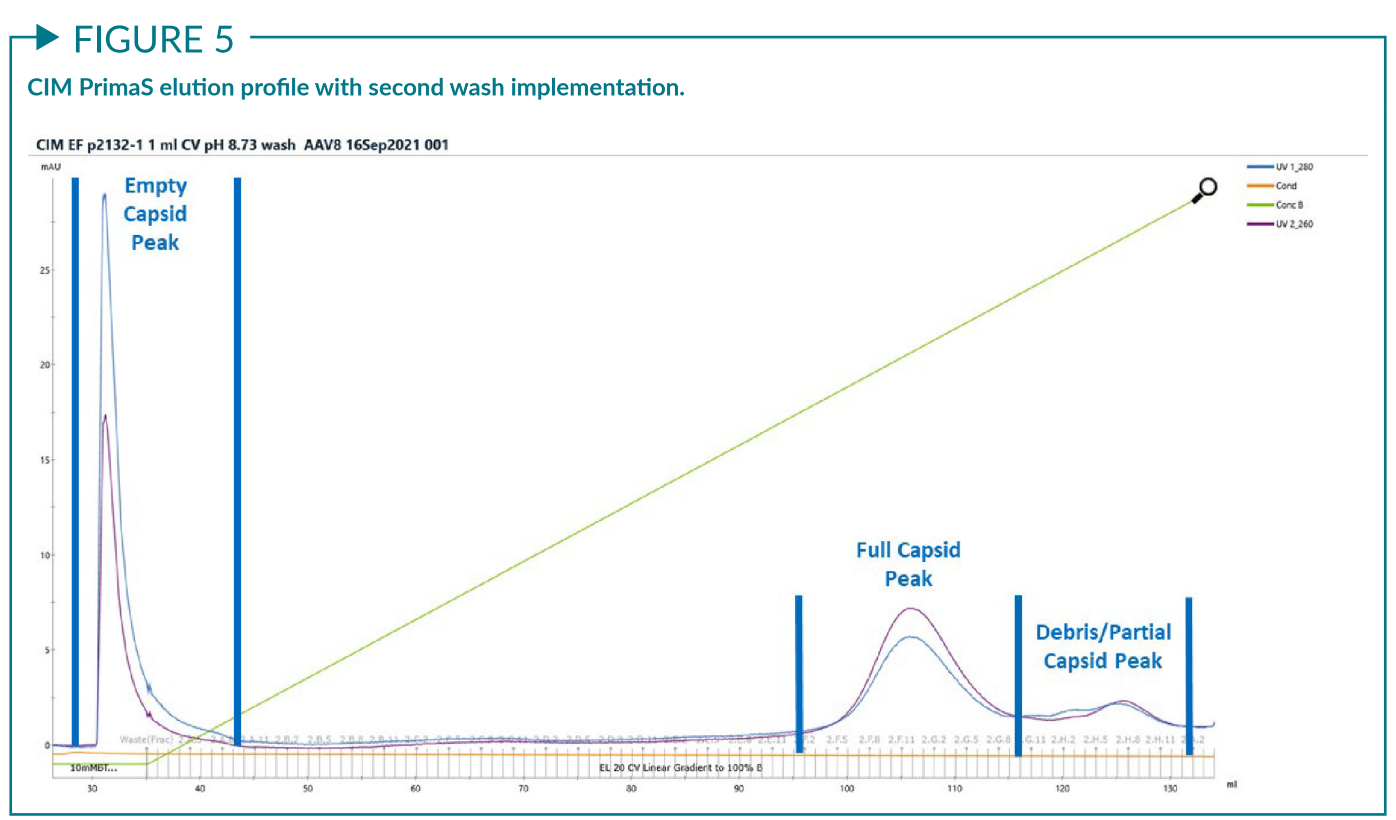
FIGURE 5

CIM PrimaS elution profile with second wash implementation.



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CELL & GENE THERAPY INSIGHTS

VECTOR BIOPROCESSING

SPOTLIGHT

RESEARCH ARTICLE

Clarification of recombinant adeno-associated virus (rAAV) & lentivirus from adherent culture

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In recent years the cell and gene therapy industries have been rapidly expanding, with two of the most utilized viral vector classes being adeno-associated virus (AAV) and lentivirus. With clinical success comes the need to develop and scale-up efficient manufacturing processes. As both of these vectors are produced in cells, the first step in their purification is to clarify them from the cell culture. There are many technologies traditionally used for cell culture clarification but given the projected manufacturing scales and need for single-use consumables a combination of depth and membrane filtration is a logical fit for batch processing of viral vectors. This work focuses on developing filtration-based clarification processes for both AAV and lentivirus. The data shows robust turbidity reduction and step yields across batches, scales, and AAV serotypes. We discuss how capacity can be impacted by feedstream characteristics and how capacities translate to manufacturing footprints. Finally, we discuss some process considerations that are unique to viral vector processing and critical to successful vector harvest.

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In recent years, development in the gene therapy industry has grown rapidly [1,2]. As of 2022 there are over 20 gene and gene-modified cell therapies approved by regulatory bodies across the world with hundreds more in clinical trials. The two largest classes of viral vectors in development today are recombinant adeno-associated virus (AAV) and lentivirus [3–5]. AAV is a non-enveloped virus ~20 nm in diameter. The recombinant vector can package ~4.7 kilobases of DNA and shows relatively low immune response

compared to retroviruses and adenoviruses [6]. Furthermore, it is relatively stable under standard bioprocessing conditions [7,8]. Lentiviral vectors are enveloped and are ~120 nm in diameter. They deliver an RNA payload and are used in both gene therapy as well as many chimeric antigen receptor (CAR) T cell-based cell therapies [9].

Both vectors are expressed from host cells which are often grown on substrates in adherent cell culture bioreactors. In typical rAAV production the product can be found both intra- and extracellularly, and many processes therefore include a cell lysis step to maximize product recovery. In contrast, the majority of lentiviral vectors are secreted from the host cells and can therefore be harvested from the bioreactor supernatant without cell detachment or lysis. Similar to recombinant protein processing, once the crude harvest is collected from the bioreactor the next step is to clarify the product from the complex mixture of insoluble impurities which can include cell debris and any precipitated host-cell protein and DNA.

A typical filtration-based clarification step will include a membrane filter with a thin structure and tight pore rating down to 0.2 µm or in some cases 0.45 µm. This filter is responsible for bioburden removal and some additional particulate removal to protect subsequent purification technologies from fouling. In many cases the membrane filter is preceded by a prefilter with a thicker structure and wider pore range. These can dramatically improve capacity on the membrane filter translating to improved overall process economics and footprint. Depth filters made of a mixture of cellulose, inorganic filter aids, and resins are commonly used prefilters and in addition to the particulate removal can provide some soluble impurity removal through adsorption. Both depth and membrane filters have a long history of use in the biopharmaceutical industry and offer a robust, cost-effective solution for clarification over a wide range of scales [10–12]. However, there are many filter options across the industry ranging in materials of construction, structure, pore size, and available formats which must be considered against the feedstream and product characteristics. AAV and lentivirus have been shown to carry a negative surface charge [8,13], but there can be slight differences based on serotype [14,15]. Depth filters carry a mix of both positive and negatively charged surfaces [16,17], and of course any charge interaction will be dependent on the ionic strength of the spent media and process buffers. There is some evidence showing lentivirus will bind to diatomaceous earth, a filter aid used in many depth filters [18]. Finding the optimal set of filters for clarification remains a challenge for these emerging fields.

In this work we evaluate filtration-based clarification options for recombinant AAV and lentivirus coming from adherent cell culture. The data demonstrates that depth filtration combined with membrane filter clarification can be an effective solution for viral vector manufacturing and provides some guidance in filter selection and screening. Finally, we discuss some subtle differences in filter options to consider during process development to improve the chances of successful scale-up, tech transfer, and production.

MATERIALS & METHODS

Crude harvest supply

All recombinant AAV5 used in this work was supplied through transient transfection of HEK293T cells using a PEIpro® transfection reagent (Polyplus-transfection). For transfection, plasmids pCDAAV-Helper, pCDAAV-CMV-eGFP, and pCDAAV5-R/C (Creative Biolabs) were used in a 1:1:1 ratio. A DNA:PEIpro ratio of 1:1 was used for transfection. Adherent cultures were either produced with Corning® CellSTACK® chambers or in Pall's iCELLis® Nano bioreactors. Following transfection the cultures were grown for 5 days at which point the culture supernatant was removed, the cells were lysed using a detergent buffer (10 mM Tris (pH 8.0), 160 mM NaCl, 2 mM $MgCl_2$, 1%

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Tween 20), and the lysate collected from the bioreactor. The supernatant and lysate were treated with an endonuclease (25 U/mL) and the total NaCl concentration was increased to 500 mM prior to clarification. rAAV5 concentration in the crude harvest averaged 7.4×10^9 with a 95% confidence interval of $\pm 2.0 \times 10^9$ gene copies per milliliter (gc/mL). HEK293 cells producing AAV8 and AAV9 vectors encapsulating a green fluorescent protein (GFP) reporter gene were procured from Vector BioLabs. The cells were grown in adherent CellSTACK chambers and contained the expressed vectors intracellularly upon arrival. Treatment of the cells was designed to mimic that of the AAV5 harvest from the iCELLis Nano bioreactors. Cells were lysed with the same detergent buffer as described above, diluted into culture medium, and endonuclease-treated prior to clarification. Concentrations for AAV8 and AAV9 crude harvests were 1.4×10^8 and 1.7×10^8 gc/mL, respectively.

All lentivirus pools used in this study were produced by transfection of adherent HEK293T cells grown in CellSTACK chambers or iCELLis Nano bioreactors. The Lentivirus produced was HIV-1 derived with a VSV-G pseudotype carrying a gene for GFP. Lentivirus plasmids were purchased from Aldevron and used in a ratio of 2 pALD-VSV-G-A:2.5 pALD-GagPol-A:1 pALD-Rev-A:2.5 pALD-LentiEGFP-A. A DNA:PEIpro ratio of 1:2.75 was used for transfection. Supernatants were collected from the bioreactor 48–72 h after transfection, 2 mM $MgCl_2$ was added, and the pool was endonuclease treated (25 U/mL). Lentivirus concentration in the crude harvest averaged 7.1×10^7 with a 95% confidence interval of $\pm 4.9 \times 10^7$ infectious particle per milliliter (IP/mL).

Filtration

A description of the prefilters and membrane filters used in this work is summarized in Table 1. Pall's PreFlow™ UB media is made of resin-bonded glass fiber and provides a gamma-stable option for protecting membrane filters in bioprocessing. In this work 47 mm discs were tested using stainless steel holders (effective filter area (EFA) = 11.1 cm²). Seitz™ P-series depth filter sheets are made up of a combination of cellulose, inorganic filter aids, and a binding resin. The V100P is a sheet designed specifically for processing viral vectors that is low-charge and free of diatomaceous earth. The PDK11 filter is a dual-layer filter made up of the same V100P sheet on the bottom and a K900P sheet on top. The K900P media is a standard grade in Pall's Seitz P-series depth filter line made of cellulose, filter aids, and resin with a retention rating of 8–20 µm. For screening, the V100P and PDK11 filters were tested in Supracap™ 50 capsules (EFA = 22 cm²). For larger scale work PDK11 filters were also evaluated in Supracap 100 capsule format (EFA = 0.025 m² for 127 mm and 0.05 m² for 254 mm).

In select studies filtrate pools from a single prefilter was run over Pall's Supor® EKV sterilizing grade or Supor EAV bioburden reduction filters. These were tested in Mini Kleenpak™ syringe filters (EFA = 2.8 cm²) and Mini Kleenpak 20 capsule (EFA = 20 cm²) formats. For larger scale work the Supor EKV filters were also evaluated in Mini Kleenpak capsule format (EFA = 220 cm²).

TABLE 1

Description of prefilters and membrane filters used in this work.

Role	Filter media	Materials of construction	Layers	Retention rating
Prefiltration	PreFlow UB	Resin-bonded glass fiber	1	0.45 µm
	Seitz V100P	Cellulose fibers, perlite, and resin	1	2–4 µm
	Seitz PDK11	Cellulose fibers, filter aids, and resin	2	8–20 µm/2–4 µm
Bioburden reduction	Supor EAV	Single-layer polyethersulfone (PES)	1	0.2 µm
Sterilizing grade	Supor EKV	Dual-layer PES	2	0.2 µm

All filtration work described here was run at constant flux on PendoTECH Filter Screening System (NFF) control systems with peristaltic pumps on the feed lines. Pressures and filtrate volumes were recorded over time. In all trials filters were equilibrated using a 1× phosphate buffered saline (PBS; pH 7.4) solution at ≥50 L/m^2. Prefilter capsules were drained prior to loading process fluid. Experiments used to determine filter capacity were run at constant flux to a terminal pressure of 0.7 bar (10 psi). AAV capacity trials were run at 200 liters/m^2/hour (LMH) on the prefilters and 1000 LMH on the membrane filters. Lentivirus capacity trials were run at 200 LMH on the prefilters and 500 LMH on the membrane filters. A post-use buffer chase of 1.5× hold-up volumes was also employed to maximize virus recovery. This flush was pooled with the product filtrate and sampled for virus titer to determine yields.

Analytics

Pool turbidities were measured offline on a Hach® 2100Q portable turbidimeter. AAV concentrations were measured by a digital droplet polymerase chain reaction (ddPCR) method using the BIORADQX200 AutoDG Droplet Digital PCR System. Non-encapsidated DNA was digested at 37°C for 1 h using an RNAse Free DNase I kit (Qiagen). Once digested, the samples were diluted 1:100 in 1× TE solution (Integrated DNA Technologies), supplemented with Pluronic PF-68 to 0.01% and ddPCR was performed using primers targeting an amplicon in the gene of interest. Lentivirus concentrations were quantified using a flow-cytometry based transduction unit (TU) assay. HEK293 cells were seeded into 24-well plates at 1 × 10^5 cells/well and incubated overnight at 37°C in 5.0% CO_2. Serial two-fold dilutions of samples were performed prior to addition to cells. A spinoculation was then performed for 2 h at 1000 xg at 25°C. Post-spinoculation additional media was added to the wells and the plates were incubated for 48 h at 37°C in 5.0% CO_2. The wells were aspirated of media, washed with 1× PBS, aspirated again, and then TrypLE (ThermoFisher) was added to detach the cells for fluorescent cytometric analysis on a CytoFLEX (Beckman Coulter). Step yields were calculated using Equation 1 below where V_f and V_p refer to feed and filtrate pool volumes and C_f and C_p refer to feed and filtrate pool concentrations respectively.

$$\text{Equation 1: Yield } \% = \frac{(V_p * C_p)}{(V_f * C_f)} * 100$$

RESULTS

AAV screening

Initial AAV screening work was conducted on the prefilter to identify an appropriate filter train for clarification. The V100P was selected as a single-layer depth filter option as it was specifically designed for the processing of viruses. This filter media contains no diatomaceous earth and a relatively low overall charge. A dual-layer PDK11 depth filter was also evaluated which contains the same V100P sheet with a more open K900P sheet on top. Both filters were evaluated with an adherent AAV5 crude harvest pool measured at 36 Nephelometric Turbidity Unit (NTU) and 9.0 × 10^9 gc/mL. Capacity was defined through constant flux (P_{MAX}) studies run at 200 LMH to a terminal pressure of 0.7 bar (10 psi). As shown in **Figure 1**, both V100P and PDK11 filters demonstrated high yields (≥95%) and strong turbidity reduction (<3 NTU in the filtered pool). However, with this feedstream there was a significant capacity benefit from the dual-layer PDK11 **(Figure 1a)**, reaching >500 L/m^2 at 0.7 bar (10 psi). Both depth-filtered pools were then taken offline and used to measure capacity on Supor EKV sterilizing-grade filters with both showing capacities of >1700 L/m^2 and AAV5 yields of >99% (data not shown).

AAV process robustness & scalability

Turbidity of the crude harvest is often used as a rough measurement to encompass key

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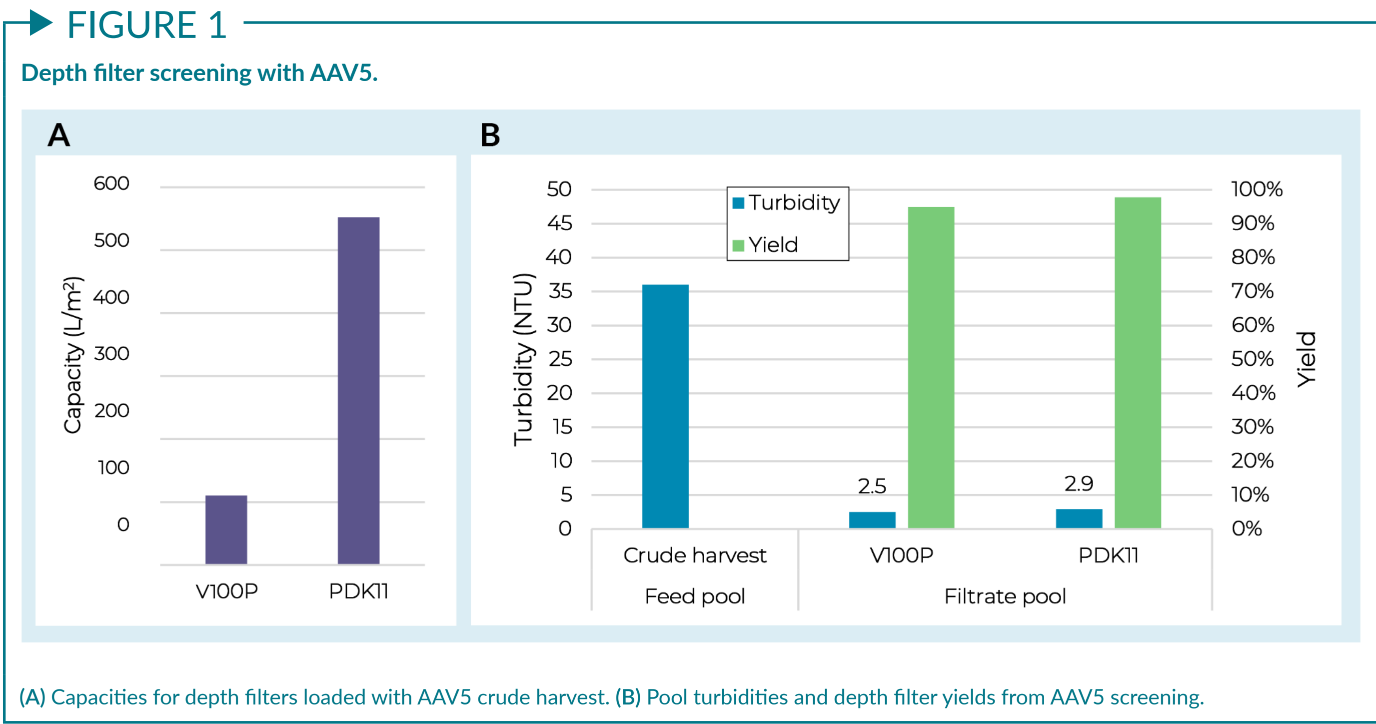
FIGURE 1

Depth filter screening with AAV5.



(A) Capacities for depth filters loaded with AAV5 crude harvest. **(B)** Pool turbidities and depth filter yields from AAV5 screening.

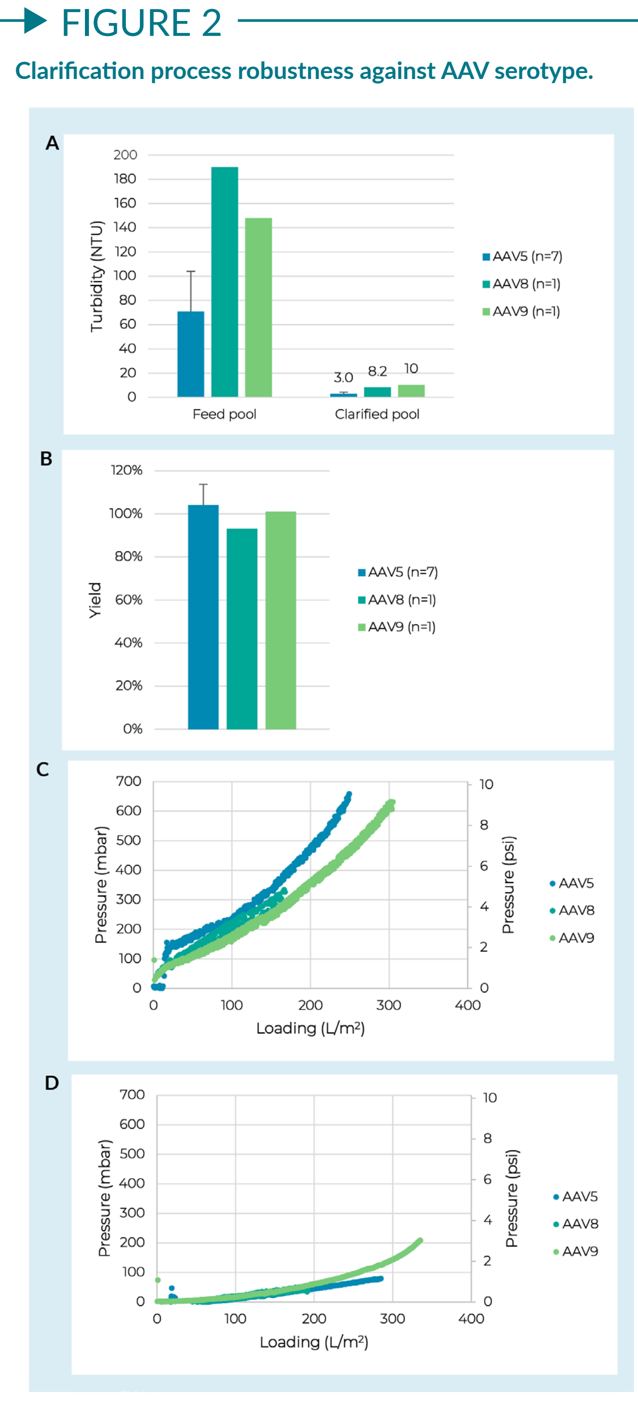
feedstock characteristics including culture cell density and viability prior to harvest, particle concentration, and particle size distribution. Using AAV5 crude harvest pools produced in iCELLis Nano bioreactors we ran seven replicate trials with feedstocks ranging from 29–133 NTU. As expected, we did generally see higher feed turbidites translate to lower prefilter capacities (consistent with the lentiviral data shown below). However, there was not a consistent trend and we saw one example of a highly turbid feed leading to low fouling on the prefilter and high fouling on the membrane filter. We hypothesize this was due to a difference in particle size distribution and highlights that while turbidity is a useful tool, it is not a comprehensive measure of crude harvest characteristics. Regardless of the crude harvest turbidity, over the seven batches tested capacities were all >250 L/m^2 on a PDK11 depth filter and >400 L/m^2 on a subsequent Supor EKV filter. The key finding was that across the range of feed turbidities we observed strong robustness for turbidity reduction and yield with pool turbidities at 3.0 ± 1.3 NTU **(Figure 2a)** and yields at 104% ± 9.6% **(Figure 2b)**.

Next, we evaluated two additional AAV serotypes (AAV8 and AAV9) produced in adherent culture grown in CellSTACK chambers. Here we saw no significant difference in pressure curves on the PDK11 **(Figure 2c)** or Supor EKV filter **(Figure 2d)** when run with an AAV8 or AAV9 feed compared to an AAV5 feed with a similar turbidity. The clarification train showed strong robustness to serotype for turbidity reduction and yield with all clarified pools at or below 10 NTU **(Figure 2a)** and yields >93% **(Figure 2b)**.

Assessing scalability is another critical step in the development of a clarification process. Using the adherent AAV5 material, performance of the PDK11 + Supor EKV filter train was evaluated across process development and pilot-scale capsules. Throughputs ranged from 180 to 550 L/m^2 on the depth filters and 300 to 1900 L/m^2 on the sterile filters. Note that in most cases the entire batch was processed before reaching capacity on either filter and that the different scales were tested with independent feedstocks making it difficult to comment on scalability of filter capacity. Pool turbidities and AAV yields are shown in **Figure 3** between the development-scale PDK11 in Supracap 50 capsules + Supor EKV membrane in Mini Kleenpak syringe filters or Mini Kleenpak 20 capsules and the pilot-scale PDK11 in Supracap 100

FIGURE 2

Clarification process robustness against AAV serotype.



Turbidity reduction (A) and step yield (B) for clarification of three different AAV serotypes using a combination of PDK11 and Supor EKV filtration. Where multiple trials were run error bars represent a 95% confidence interval. Differential pressure vs. loading curves from PDK11 (C) and Supor EKV filters (D) run with crude harvests of AAV8, AAV9, and a representative batch of AAV5 which had a crude harvest turbidity closest to the other two serotypes (133 NTU).

capsules + Supor EKV filters in Mini Kleenpak capsules. Critically, there was no statistically significant difference observed between scales for pool turbidity or AAV yield ($p > 0.05$ from two-sample T-tests).

Lentivirus screening

Lentiviral work started with prefilter screening using an adherent crude harvest pool taken from an iCELLis Nano bioreactor with a turbidity of 38.5 NTU and lentivirus concentration of 3.7×10^7 IP/mL. Previous data has demonstrated successful clarification of lentivirus from adherent cultures using various combinations of glass fiber prefilters and PES or PVDF membrane filters [19,20]. Using crude harvest pools with turbidities of approximately 10 NTU these synthetic filter options provided capacities of >1500 L/m^2 and infectious particle recoveries of >75%. The PreFlow UB resin-bonded glass fiber filter was therefore included in this screening. Due to a significantly higher crude harvest turbidity in this work the V100P and PDK11 depth filters were also included. Lentivirus crude harvest was loaded onto all prefilters at a constant flux of 200 LMH to a terminal pressure of 0.7 bar (10 psi). The data revealed similar capacities for the PreFlow UB and V100P prefilters at approximately 250 L/m^2 whereas the PDK11 provided an approximately four-fold higher capacity, achieving 1000 L/m^2 (Figure 4a).

While all three prefilters reduced the turbidity below 5 NTU, the cellulose-based filters did show slightly lower turbidity levels than the PreFlow UB prefilter (Figure 4b). This turbidity difference correlated to capacity differences on the downstream membrane filters. Pools from each prefilter were run over Supor EKV and Supor EAV filters in parallel. The capacities for those loaded with PreFlow UB filtrate were 34 and 32 L/m^2 respectively. In contrast, the cellulose depth filtered pools led to membrane filter capacities between 390 and 480 L/m^2.

The cellulose depth filter + membrane filter combinations were evaluated for lentiviral step yields (Figure 4C). The V100P combinations appeared to have slightly higher yields than the PDK11 combinations. This could be expected as the dual-layer PDK11 does contain some diatomaceous earth

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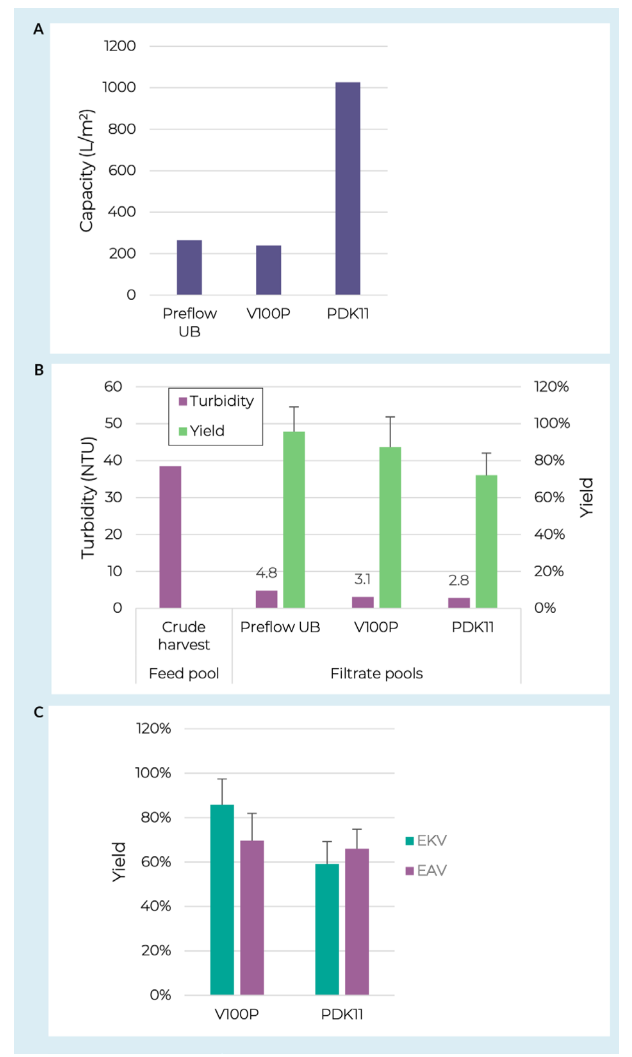
FIGURE 3

AAV clarification scalability.

Turbidity reduction (A) and yields (B) from AAV5 crude harvest clarified over a range of depth and sterile filter scales. SC50 indicates PDK11 Supracap 50 depth filters run over Supor EKV media in Mini Kleenpak syringe filters or Mini Kleenpak 20 filter capsules (n = 3). SC100 indicates PDK11 Supracap 100 depth filters run over Supor EKV media in Mini Kleenpak filter capsules (n = 3 for 127 mm; n = 2 for 254 mm). Error bars represent a 95% confidence interval.

FIGURE 4

Depth filter and sterile filter screening with lentivirus.



A & B. Filter capacity, pool turbidity, and step yields for lentiviral crude harvests processed over three different prefilters. C. Lentiviral step yields over the full clarification (prefilter + membrane filter) for four different filter combinations. Error bars represent 95% confidence intervals based off TU assay technical replicates.

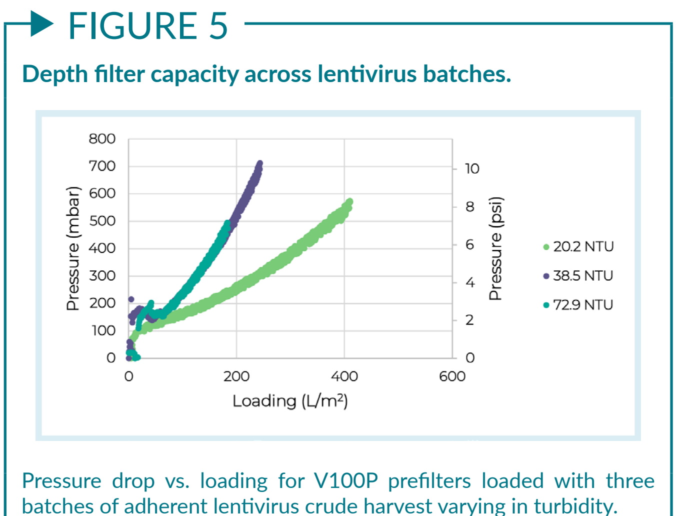
which has been previously shown to reduce filtrate lentivirus levels [18], however more replicates will need to be run to determine if the difference is real and reproducible. Regarding the membrane filters, we did not observe a clear benefit for capacity or yield between the two tested here.

Lentivirus process robustness

Based on the balance between yield and capacity the V100P prefilter was selected for additional testing. The Supor EKV filter was selected as the membrane filter as it provides a sterile filtrate stream and showed no drop-off in capacity or yield. Two additional batches of adherent lentivirus crude harvest from CellSTACK chambers were processed over the V100P + Supor EKV filters. Over the three runs feed turbidity ranged from 20.2–72.9 NTU, feed concentration ranged from 3.7×10^7–1.2×10^8 IP/mL, and the step yield over the depth and membrane filters averaged 74%. Note we did not observe any clear trend between feed turbidity and yield. Prefilter pressure vs. loading curves are presented in Figure 5 and reveal how feed turbidity can impact depth filter capacity. However, despite the range in crude harvest turbidities, the clarified pools showed consistently low turbidity averaging 2.7 ± 0.6 NTU.

FIGURE 5

Depth filter capacity across lentivirus batches.



Pressure drop vs. loading for V100P prefilters loaded with three batches of adherent lentivirus crude harvest varying in turbidity.

DISCUSSION

The work described here provides a case study for clarification process development of adherent lentivirus and AAV cultures. For both viral vector classes we found filter combinations that could provide consistently low filtrate turbidities (≤10 NTU) despite relatively large variance in feedstream turbidities. The feedstream turbidity range observed here is likely an extreme case as the cell culture process was being developed in parallel with this clarification work. However, even in tightly controlled processes there is some variability in crude harvest characteristics known to impact clarification such as cell density, viability, particle concentration, and particle size distribution. Therefore, the crude harvest variability tested here provided a nice challenge for assessing process robustness. Step yields also showed strong consistency, particularly for AAV which averaged 103 ± 7.7% across all batches, serotypes, and scales. This consistency could make a depth + membrane filter harvest process fit into a platform to be used across an AAV product portfolio.

While the data shared here should provide some guidance for process development, the optimal filter train will depend on many factors including product quality, process economics, and facility footprint constraints. The lentivirus data presented previously [19] and here provides a nice case study of the trade-offs. Take for example, an iCELLis 500+ bioreactor with a 10 cm bed that produces ~570 L of crude harvest. If the feed turbidity is <15 NTU it could be possible to get >1000 L/ m² through a Preflow UB + Fluorodyne® II DBL filters. This would translate to a single 254 mm (10 in.) PreFlow UB filter capsule and a single 254mm (10 in.) Fluorodyne II DBL filter capsule with the important benefit of both being available in closed and presterilized assemblies. However, with feed turbidites tested in this work, a similar filter train would need to run six 762 mm (30 in.) membrane filters in parallel which would be logistically challenging. In this case the cellulose depth filter options may be needed to simplify the process and reduce the footprint down to a single Stax™ capsule and one 508 mm (20 in.) membrane capsule. Note that these estimates do not include a safety factor which should be included [21], but nonetheless illustrate how capsule formats and manufacturing-scale can help define the optimal process.

Another interesting example of process trade-offs was seen in the lentiviral membrane filter comparison. While we did not observe a significant difference between the Supor EKV and Supor EAV filters in terms of capacity, pool turbidity, or yield, they each carry unique process benefits. The Supor EKV filter is a sterilizing-grade filter which may allow for more flexibility in pool hold time. However, because the Supor EAV bioburden-reduction filter incorporates a single-layer membrane it can hold more filter area per capsule, and therefore can have some footprint benefit over the Supor EKV filter in some situations, whilst still providing a high level of bioburden reduction assurance.

The long history of success for these depth and membrane filters in the biopharmaceutical industry combined with the data presented here makes them a low risk for successful implementation in viral vector manufacturing. Future work could include additional development of lentivirus clarification to further improve yield. Work could include fractionating the filtrate from various filter chemistries and pore sizes to investigate how yield loss may be split between adsorption and size

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exclusion. Previously work [19] has demonstrated similar clarification development of AAV from suspension cultures, but clarification from suspension lentivirus cultures remains a target for future work. Data demonstrating full scalability from development scale to manufacturing scale would also be of value. In this work we observed some trending between feedstock turbidity and filter capacity, but there were some notable deviations suggesting that additional feedstock characterization would be needed to predict impact on filtration performance. Particle concentration and size distribution in crude harvests and their relation to filter performance could be an interesting follow up. Finally, we highlight the need for new technologies. This may include new filter media to improve viral yields. Furthermore, as gene therapy manufacturing has limited options for adventitious virus and endotoxin clearance there is a strong desire for closed, aseptic processing, highlighting the need for depth filter options that fit these requirements.

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AUTHORSHIP & CONFLICT OF INTEREST

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Thinking big: the need for innovation in the production of lentivirus-based cell and gene therapies

In a recent Expert Roundtable discussion, we spoke to five experts about strategies for scale up of lentivirus-based cell and gene therapies. Here, we sum up some of their key thoughts.

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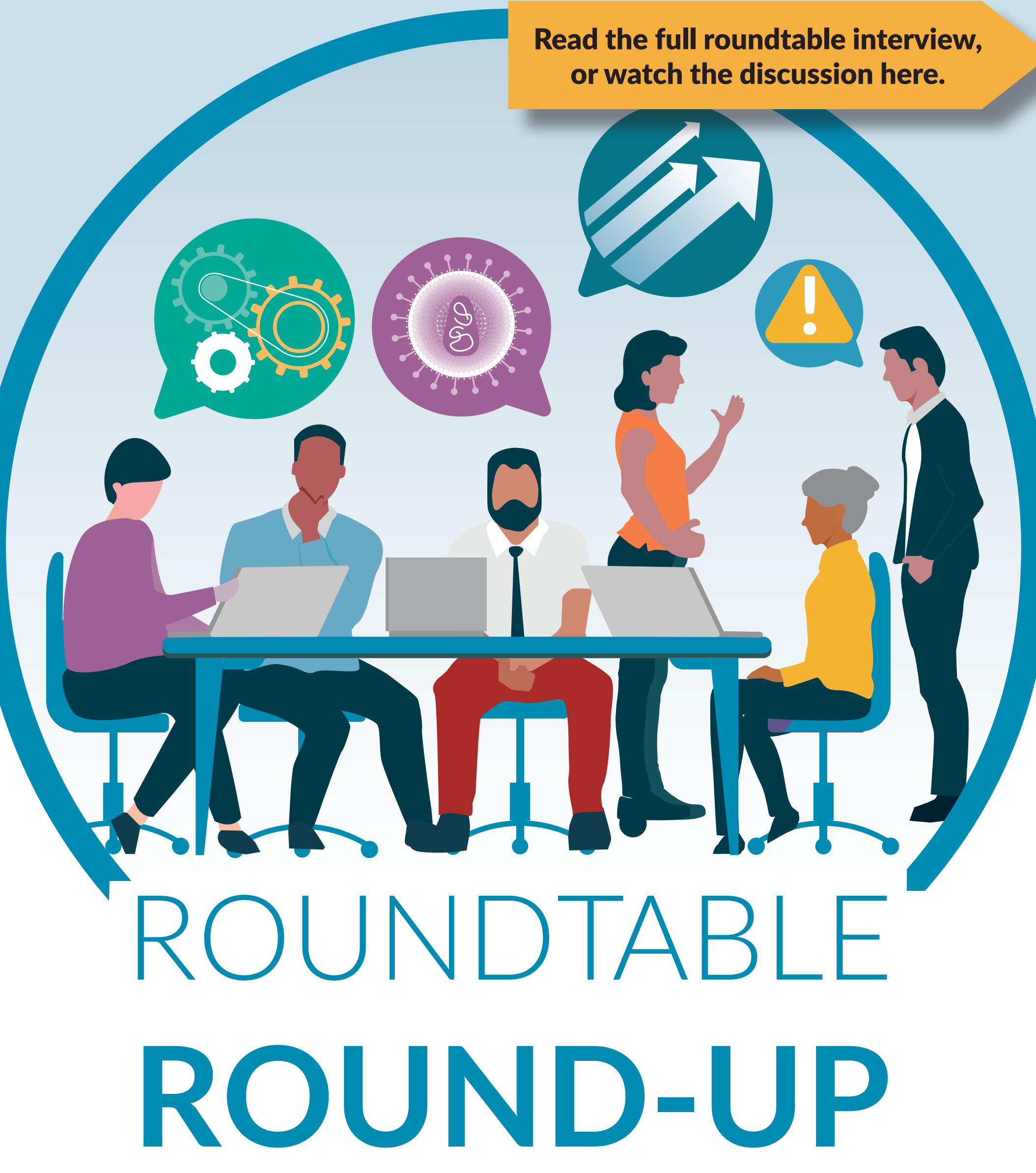
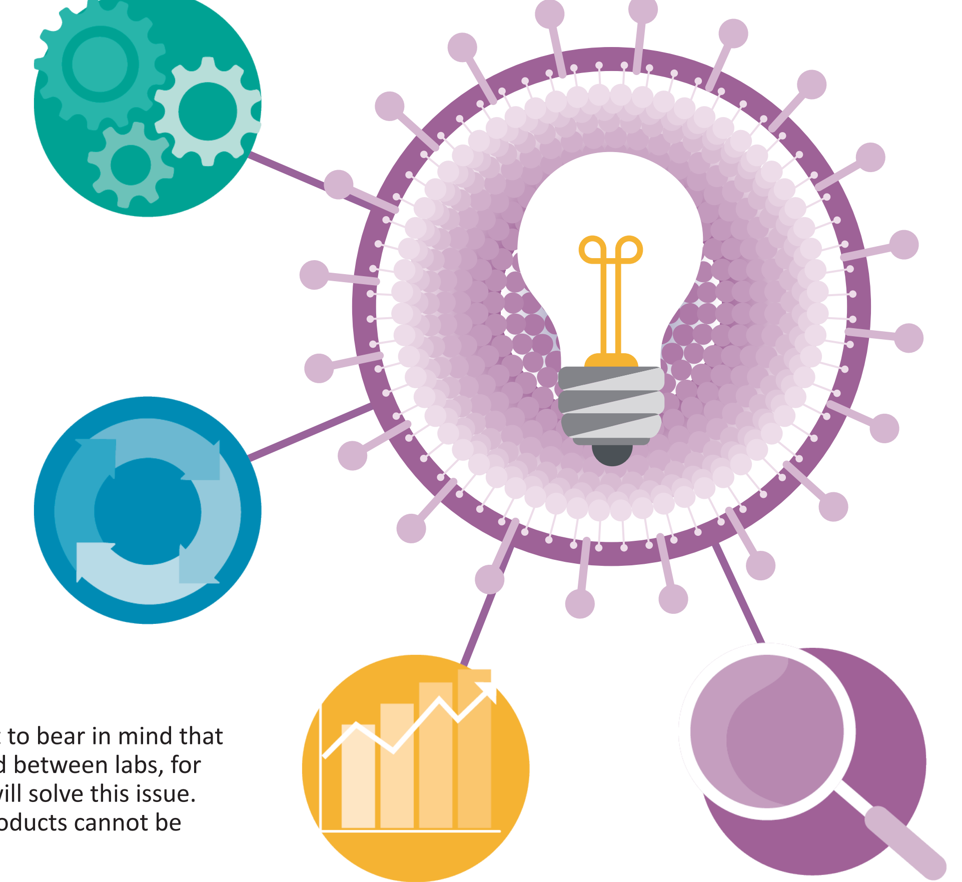


EVOLUTION OF LV-BASED CELL AND GENE THERAPY MANUFACTURING:

"The field has made good progress in the last few years, moving from an idea of clinical demand to things that will enable commercial demand. There has been a lot of focus on what technologies are scalable, and how to make sure we can meet the demand of a commercial product. CAR T programs and *ex vivo* uses of lentiviral vectors (LVs) to make cellular therapies have come to the forefront, and with this great progress people are now excited about the commercial possibilities of these products."

John Moscariello, *Bristol Myers Squibb*



PROCESS DEVELOPMENT CONSIDERATIONS FOR ENSURING A COMPARABLE TITER AND DRUG PRODUCT:

"Looking at process development, the most important aspect is quality by design – quite early during process development ranges for different process elements such as pH, cell density and so on should be statistically defined. With biological processes there is always some variation in productivity and impurity levels. It's important to bear in mind that LV titer is dependent on cell line, and titers cannot be directly compared between labs, for example. Reference standards should be available quite soon, and we will solve this issue. But without reliable analytical tools, comparability of titers and drug products cannot be ensured."

Hanna Leinonen, *Kuopio Center for Gene and Cell Therapy*

"We are in a relatively new part of the biotech industry, and robust, established and even compendial analytical methods are still a long way away. Measurement is a problem. Looking at titer and genome copy is important. The dialogue we've had with agencies in our jurisdictional scope are always about patient safety – what is it that makes the vectors efficacious? Reproducible and consistent transduction results in T cells. This is the biggest analytical space that we're dealing with. Out of every lot of vector generated, how is it comparing to the ones that came before, and how are things changing? In the end, what is going to sell everything you do is demonstrating safety."

Will Junker, *Kite Pharma*

THE BEST TIME TO BEGIN BUILDING YOUR ANALYTICAL ASSAYS:

"Start developing your analytical strategy as soon as possible. You will need the analytical titer and infectious titer during your development – this will help you ensure the constructs you are making are producing enough titer. You also need to make sure the titer is functional on your target cells – we always recommend doing this before you get too far into the process. You also need assays for residuals: your host cell DNA, plasmid DNA, host cell protein, everything you want to clear through your purification process. Overall, this will help you characterize and understand your manufacturing as you move through the process."

Scott Cross, *Dark Horse Consulting*

WHERE INNOVATION IS STILL NEEDED TO IMPROVE THE COST EFFECTIVENESS AND SCALABILITY:

"When considering cost, primarily what we're looking to do is increase the yield of the process, both at large and smaller scale. It fundamentally comes down to cost per dose for the patient. I don't see one huge improvement to the current transient transfection process that will solve our problems. It will be lots of small increases across the whole process that add up to make a significant difference. One area that hasn't received a lot of attention in the past is the vector constructs themselves. We're largely still using vector plasmids and genome constructs that are 10-15 years old and haven't changed much. Something we're doing is taking a deeper dive into these sequences to see if we can improve the overall efficiency of the process."

Lee Davies, *Oxford Biomedica*

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VECTOR BIOPROCESSING

SPOTLIGHT

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Adeno-associated viral vector process development challenges in early research & preclinical study

Ashish Saksule

Significant advances have been made in gene therapy using recombinant adeno-associated virus (rAAV) in recent years. Given the interest in and success of rAAV as a novel therapeutic modality, there has been an increase in preclinical proof of concept studies and clinical trials. However, with the momentum and competition in gene therapy, many research groups are coming up with novel AAV variants, new combinations of rAAV constructs, combined with targeted genes of interest to outperform traditional and competitor therapies. Also, screening and generation of rAAV libraries have helped identify novel rAAV serotypes and variants. The preclinical research studies require the production of highly pure rAAV particles with multiple serotypes and different gene of interest, thus creating a major challenge for the production and purification of rAAV. It is worth mentioning that the preclinical phase often overlooks the process development aspect and manufacturability of targeted rAAV and thus undermines the challenges that may arise as the rAAV-based gene therapy program progresses. This article outlines some of the major challenges, quality and quantity requirements, and scalability considerations during process development in the research preclinical phase. The process development and research scale production can be challenging considering volume, serotypes, and ever-changing GOI whilst maintaining the high-speed delivery of vectors. Thus, there is an urgent need to create a platform for the production and purification systems that can generate a high quantity of pure rAAV.

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Process development for high yield rAAV vectors is focused on building robust and reproducible processes that can meet manufacturing design considerations. Typically process development involves scale-up and scale-out in the mid to late-stage of development to meet quality, quantity, and regulatory aspects of manufacturing. But it is critical to start thinking about process development in the early stages while working on the discovery and preclinical phase.

Typically, pre-IND studies include multiple preclinical studies including safety, efficacy, bio-distribution assessment. For early preclinical studies, the rAAV vector quantity requirements are lower so typically we would need to set up multiple, smaller rAAV production batches. But as the study progresses to multiple animals and large animals (non-human primates), the dosage requirements are higher often reaching the 1×10^{15} to 1×10^{16} vector genome range. While still operating in the research phase the timelines and flexibility are demanding and we cannot necessarily perform process development on each of the targeted vectors with a combination of GOI & rAAV serotypes or variants.

Currently, there are more than 100 rAAV serotypes, hybrid constructs, or variants that are reported in the literature [1,2,3]. Every year we see the literature and publications on the discovery of new rAAV serotypes or variants. Each rAAV type can be different from the other based on capsid components, the combination of *Rep, Cap* with complimentary ITR from another serotype. Preclinical research studies often start with multiple rAAV serotypes based on close similarities of some serotypes for cellular tropism, targeted interactions and biodistribution, and transduction efficiency. Each rAAV serotype comes with its unique advantages, disadvantages, and challenges in upstream production and downstream purification. Illustrative Figure 1 shows examples of the most commonly used rAAV serotypes that are used for research and preclinical as well as clinical studies. Based on the number of serotypes used for early screening, can increase considerations for early production and process development [1]. The production platforms for rAAV particles are often complex and challenging due to the lack of platform technologies and the lack of understanding of different rAAV serotypes.

This review article provides an overview and challenges of small-scale and large-scale production of rAAV for preclinical studies. It is noteworthy that preclinical studies are often focused on the efficacy and safety of rAAV and do not provide full details on the production and purification methods used for rAAV. The rAAV upstream production is dependent on the cell lines used, the transfection methods, and plasmid systems. Upstream cell culture can be used in adherent and suspension systems, while many studies start with the adherent system due to ease of operating and the short time required for establishing a process. For preclinical studies, considering the quantity requirements, an adherent system can be a good start for proof of concept [4]. On the other hand, the suspension system can take a longer development time while it is truly scalable from lab-scale to large-scale stirred tank bioreactors [5]. The suspension system provides ease of collection of supernatants and cells & simple in-line sample collection cell culture analysis. There are currently 3 major types of production platforms commonly used for rAAV production which are listed in Table 1. Each platform comes with its unique advantages and disadvantages and can provide different performance for vector quality, quantity, and scalability.

In the long run, the choice of upstream production platform and downstream processing used in preclinical applications should be used for clinical and commercial considerations for an easier transition. Henceforth, appropriate investment in process development and pre-capital investment is vital for optimization and validation of process ahead of time for timely FDA compliance with current good manufacturing practices (cGMP).

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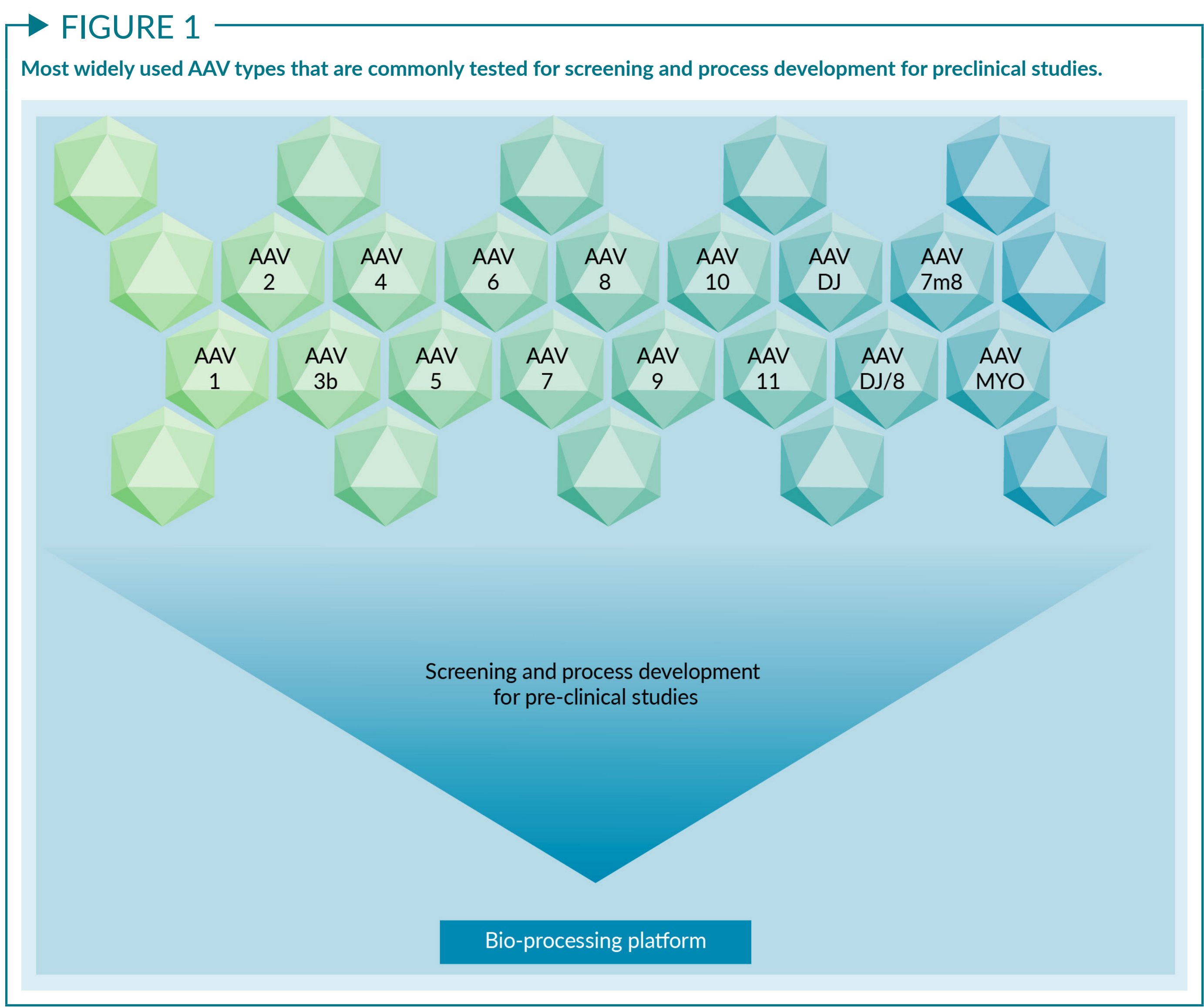
FIGURE 1

Most widely used AAV types that are commonly tested for screening and process development for preclinical studies.



SOME OF THE MAJOR CHALLENGES IN THE PROCESS DEVELOPMENT IN THE EARLY RESEARCH, PRECLINICAL PHASE

Scale-up is not linear & simple

The logical interpretation of scaling up a process is critical when developing a process at a small scale and transferring it to a large scale. But viral vector-based processes are often susceptible to batch-to-batch variation and scalability variations. In early research process development, individual methods are developed for each rAAV type product, creating a large portfolio of methods. Small-scale processes for research utilize non-scalable, benchtop processes such as adherent flasks for cell culture, centrifugation for clarification, syringe filter-based sterile filtration, and ultracentrifugation for separation of empty and full rAAV particles. While the large-scale production process often looks different utilizing more scalable unit operations including clarification by depth filtration, multiple chromatography unit operations, and concentration using tangential flow filtration [5,6,10]. Figure 2 showcases the outline for small-scale production and purification processes compared to large-scale processes. As we can see the number of unit operation at each scale are different that can contribute towards balancing

TABLE 1

Overview of the common upstream production platform for rAAV.

Production platform	Preclinical considerations (Reference)
Transient transfection using HEK293 or similar cells	Transfection complex scalability and high manufacturing cost [6].
Baculovirus infection/ rHSV Infection	Precursor baculovirus or rHSV is required and can add extra development time [7, 8].
Producer cell lines (PCL)	PCL additional development time & stability of PCL cells [9].

purity over yield (higher number of unit operation can result in increased purity but the rAAV yield can be lower).

Often scalability is not considered while creating individual methods, e.g., separation of empty capsids is performed using cumbersome density ultracentrifugation methods using cesium chloride or iodixanol, the formulation buffer exchange is performed using bench-top dialysis methods [10,11].

Table 2 shows an overview of common downstream unit operations and linear scalability challenges. Linear scalability can be defined differently for each unit operation, but a process without any scalable challenges and easy transfer & application of a small-scale study into a large-scale study can be considered a linear scalable process.

Thus, downstream processes developed in early research for rAAV preclinical studies are not always linearly scalable. Scientists often conduct optimization at a smaller volume and consider that it will be proportionally linear when scaling up, but due to differences in scale-down and scale-up devices processes often are not linearly scalable.

FIGURE 2

Outline and comparison of downstream purification of rAAV for small scale and large-scale production.

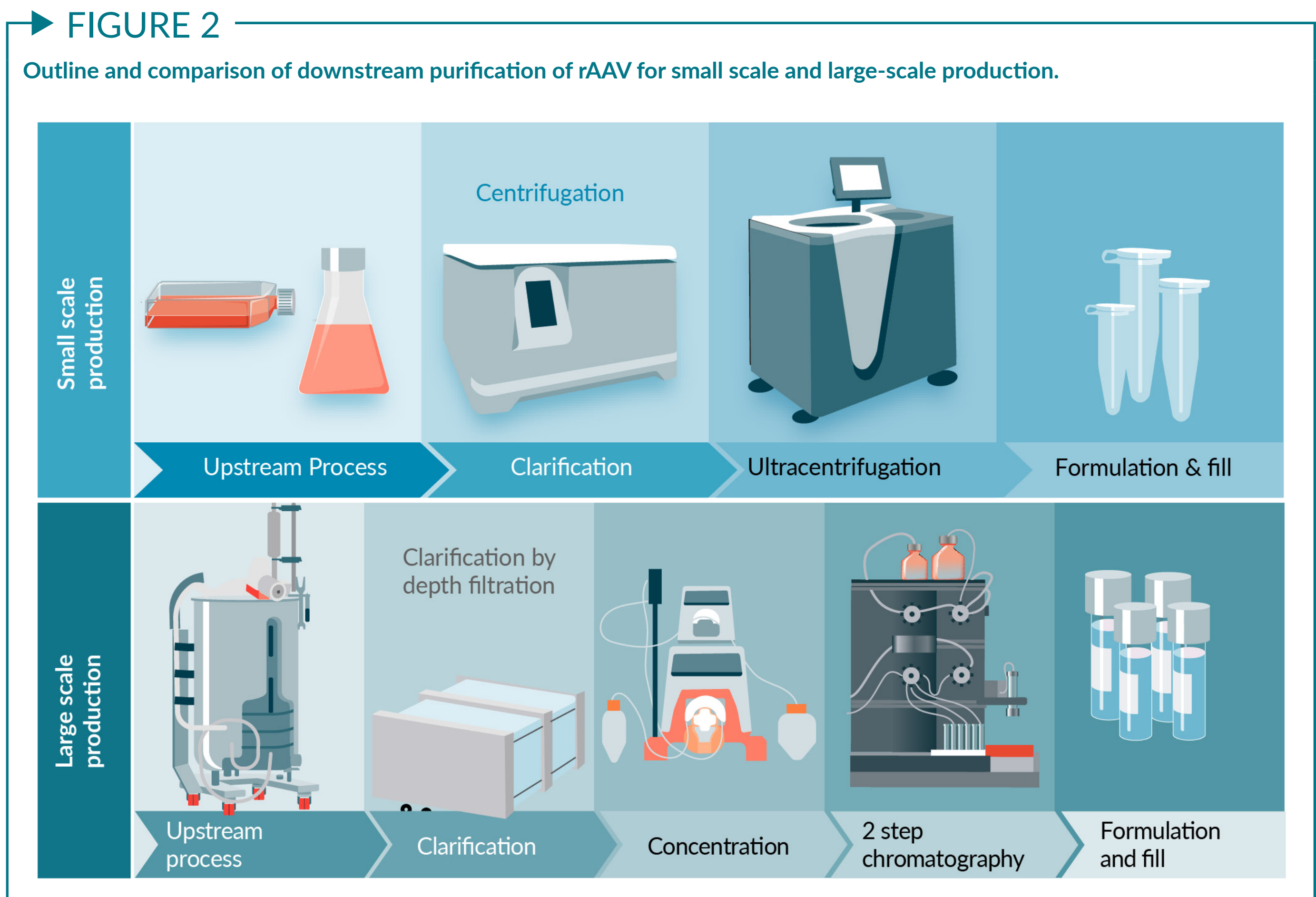


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TABLE 2

Outline for downstream unit operations and linear scalability for commonly used processes in rAAV purification.

Unit operation	Linear scalability	Linear scalability challenges
Centrifugation (primary clarification)	No*	Limited scalability due to processing volume, often conducted in batch mode * Continuous centrifugation devices available
Clarification by depth filtration (primary and secondary clarification)	No	Scale-down models are not equivalent/ comparable with large-scale models Linear scale-up can be challenging
Tangential flow filtration (concentration and diafiltration)	Yes*	Scale-down and scale-up models are available and linearly scalable *Based on TFF device format (Flat sheet cassette TFF – limited scale-down models are available, good for very large-scale models. Hollow fiber TFF – small and medium scale models are available and linear, lacks large-scale models)
Dialysis (buffer exchange)	No	Benchtop dialysis is not scalable
Ultracentrifugation (separation of empty and full rAAV)	No	Discontinuous CsCl or iodixanol ultracentrifugation is not scalable beyond small scales
Chromatography (purification)	Yes	Linearly scalable Challenging for newer formats such as membrane adsorbers, monolith-based chromatography columns due to the lack of each scale model availability

Quality & quantity for preclinical studies

Dosage requirements for preclinical studies with *in-vitro*, *in-vivo* animal models with mouse and non-human primate (NHP) studies vary hugely in terms of total vector genome (VG) per study. Dose levels that are safe, non-toxic, and biologically plausible are key considerations while designing preclinical studies. Typically, we need less than 1×10^{10} to 1×10^{11} Total VG (considering small animals and µl dosage allowed) of rAAV for *in-vitro*, small animal studies. This rAAV vector material is typically produced at small scales (100 to 1000 ml) with traditional purification methods. While the dosage requirements for the NHP study are very high and often required large-scale production (>50–200 L scales) based on the initial titer for specific rAAV types [12,13].

Often NHP studies include multiple NHPs and can require total vector requirements higher than 1×10^{15} or 1×10^{16} VG. Manufacturing such higher amounts of rAAV is often difficult with the same specifications as small scale. In such cases, many research groups need to outsource manufacturing externally to CDMO, which can introduce multiple variations from previous preclinical studies thus increasing complexity in comparability and study conclusions (Figure 3).

The purification method for large-scale production is different than small-scale production. Thus, the quality and purity of vectors change significantly with changes in small animal study vs NHP and can affect study comparison. Examples of some of the critical quality attributes (CQA) and quality control (QC) test to support preclinical testing are listed in Table 3 [14,15]. (Note: release testing or GMP specifications are not always considered during preclinical assessments). Lack of standardized characterization (due to differences and compatibility of various vector capsid variants) can also be a major challenge in controlling process development for multiple serotypes. Thus, quality control and purity comparison are critical when transitioning from small to large production scale and purification methods.

While FDA typically requires CQA considerations for clinical manufacturing, it is advised to start defining CQA for the overall process as early as possible during the preclinical studies to avoid profile changes as

FIGURE 3

rAAV vector dosage and production scale comparison between early *in-vitro*, *in-vivo* studies

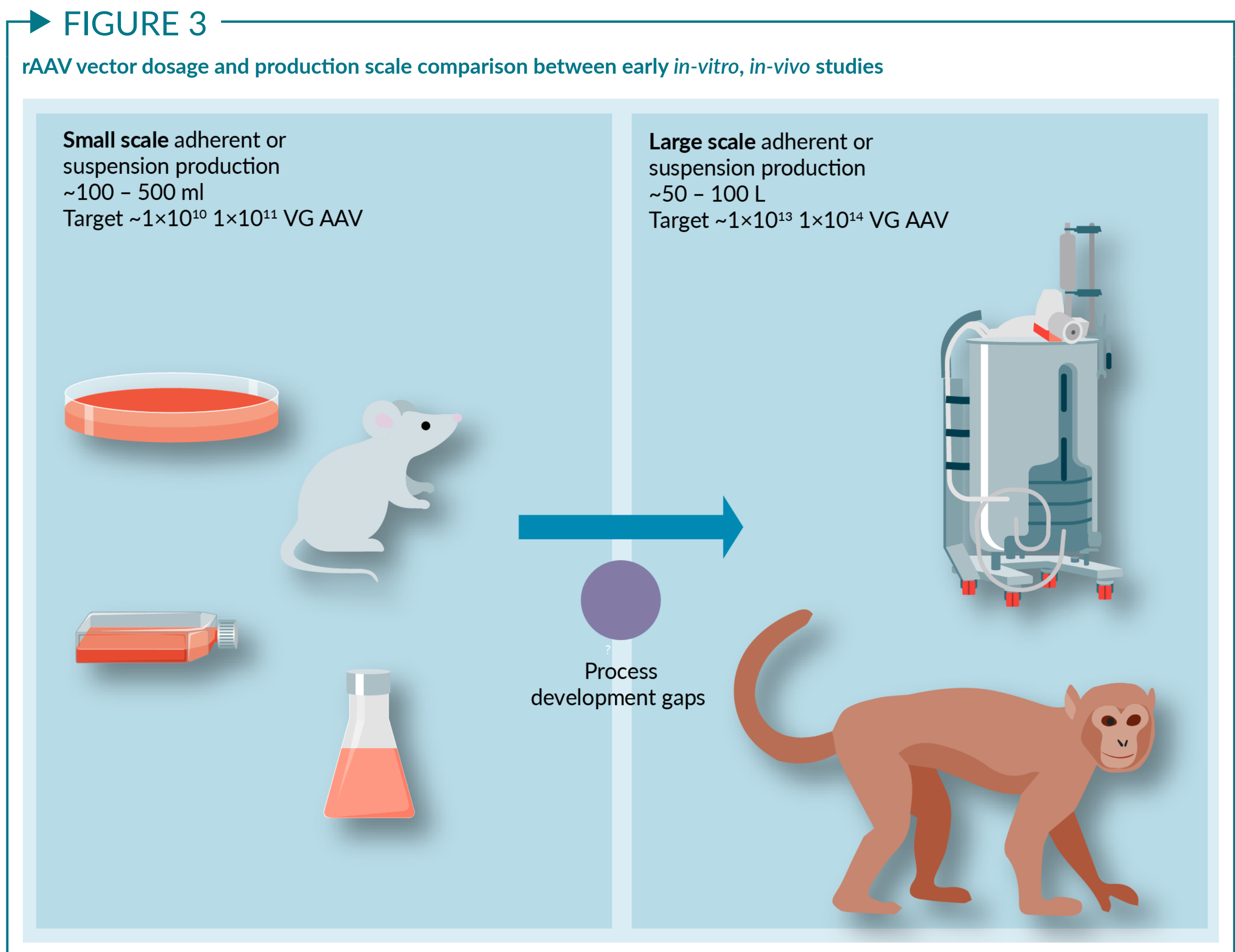

TABLE 3

Examples of CQA and QC test with expected variability in characterization due to difference in operating scale.

QC Test	Test/purpose	Expected variability between small-scale vs large-scale
Identity: vector capsid	Capsid ELISA: total vector copies based on capsid specific ELISA	
Identity: vector genome	PCR: genome containing particles/ sequencing-based	
Purity: aggregation	SEC/ DLS: size exclusion chromatography or light scattering to identify particle size distribution and aggregation	X
Purity: residual DNA/ host cell proteins/ benzonase/ other impurities	ELISA/ PCR	X
Purity: Empty capsids	AUC/ CryoTEM: analytical ultracentrifugation or transmission electron microscopy for determining % full and % empty rAAV	X
Potency: VG concentration	PCR (qPCR/ ddPCR)	
Potency: infectivity/expression activity	*In-vitro* potency assay	X
Safety: standard (sterility/ mycoplasma, bioburden, etc.)	Standard 21CFR safety tests	

CQA: critical quality attributes, QC: quality control.

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we transition from small-scale to large scale. Investing time and resources early in defining CQA can be helpful for a faster and smooth transition into the late phase and commercialization.

Following are the non-exhaustive and few examples to find regulatory guidelines documents related to CMC, analytical development of Gene Therapy products.

FDA: https://www.fda.gov/vaccines-blood-biologics/biologics-guidances/cellular-gene-therapy-guidances

- Human Gene Therapy Products Incorporating Human Genome Editing 3/2022
- Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs); Guidance for Industry 1/2020
- Design and Analysis of Shedding Studies for Virus or Bacteria-Based Gene Therapy and Oncolytic Products; Guidance for Industry 8/2015
- Considerations for the Design of Early-Phase Clinical Trials of Cellular and Gene Therapy Products; Guidance for Industry 6/2015
- Guidance for Industry: Preclinical Assessment of Investigational Cellular and Gene Therapy Products

EMA: https://www.ema.europa.eu/en/human-regulatory/research-development/scientific-guidelines/multidisciplinary/multidisciplinary-gene-therapy

- Guideline on quality, non-clinical and clinical requirements for investigational advanced therapy medicinal products in clinical trials
- Quality, preclinical and clinical aspects of gene therapy medicinal products
- Quality, non-clinical and clinical aspects of medicinal products containing genetically modified cells
- Non-clinical studies are required before the first clinical use of gene therapy medicinal products
- Non-clinical testing for inadvertent germline transmission of gene transfer vectors
- Reflection paper on quality, preclinical, and clinical issues related to the development of recombinant adeno-associated viral vectors

Proof of concept & research-grade production using scale-down models

Scale-up and scale-down modeling to design any production process are critical for process development. While working with multiple target rAAV serotypes and GOI, scale-down models are often required for initial proof of concept which can be used with less volume and low vector concentrations. But the real challenge can arise when we need to produce preclinical research-grade material in smaller quantities for several different constructs. Scale-down models are often too small to produce and purify enough viral vector quantity, while the regular size and scale-up models can be too large. Chromatography tools are relatively easy for scale-down modeling with loose resin, 96- well plate formats. Also, there is an option for packing custom-sized columns at a small scale. While filtration operations such as depth filtration, tangential flow filtration, sterile filtration are more challenging due to the lack of single-use scale-down models. The product gap between the smallest format filter and the next available size filter is a challenge for research-grade production. While working with multiple rAAV serotypes, downstream purifications have some challenges:

- Platform technology doesn't exist or limited scale-down models available to cover a wide range of rAAV types, so each purification step must be tailored to a specific rAAV capsid variant

- Batch-to-batch variability in rAAV titer and impurity levels can cause overloading or underloading on scale-down models and create challenges to scale-up
- Variations in packaging efficiency of each rAAV type changes the production of empty capsids and full rAAV particles; thus, the same separation-purification process is not applicable every time with the changing ratio of empty capsids to full rAAV particles.

Therefore, equal consideration towards development of scalable and miniature models for proof-of-concept studies and small-scale bioprocessing is critical for faster preclinical studies and smooth transition into clinical phase.

Resources: generalist vs specialist

While we have seen impressive investment and growth in the cell and gene therapy space, there is a significant shortage of skilled and experienced personnel for handling viral vector production and process development. This is a critical topic that has been brought out at multiple forums and we will need to work together to develop and train more skilled scientists to thrive in the competitive CGT landscape [16].

In disciplines of small molecules and biologics, where manufacturing is a well-known commodity highly skilled personnel are easily available.

We often have to hire new talent from small molecules and biologics industries and provide essential training required to handle and process viral vectors. A recent survey report from the UK Cell and Gene Therapy Catapult shows that about 63% of the candidates being hired in the CGT space do not have CGT relevant experience (31% from new graduates/postgraduates, 23% from outside the CGT industry and 9% apprentice) (Figure 4).

The big challenge we see while finding and allocating resources for research scale process development is whether we seek a generalist or specialist. A small team of cross-functional generalist scientists (example: vector designing, upstream processing, downstream processing, and analysis) is often required to meet the demand of constantly producing research-grade vectors. At the same time, constant process improvement is required which may need a specialist for process optimization. The batch sizes are often so small that sometimes it doesn't make sense to bring highly specialized scientists who can handle one specific aspect of the production.

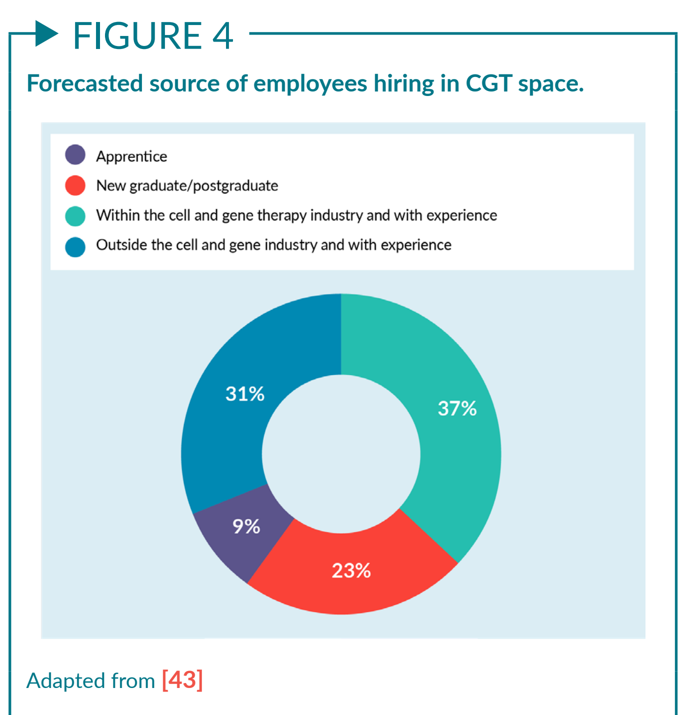
While specialists are critical for PD, at a research scale PD, generalists who can handle all aspects of the end-to-end process can be more valuable. Thus there is a significant need for cross-functional training for lab scientists and associates specifically focused on preclinical production labs and Vector core labs.

CONCLUSION

Key challenges and limitations for the production and purification of multiple rAAV serotypes, combined with the different gene of interest have been highlighted in the

FIGURE 4

Forecasted source of employees hiring in CGT space.



Adapted from [43]

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article. Considering the growth and early phase in gene therapy using rAAV as novel modalities, we must take efforts into building robust, scalable production technologies that can help with increasing demand in the future. Early investment and early process development towards a platform technology are critical and they can help with a smooth transition into late-phase clinical trials and ultimately successful commercialization.

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AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

VECTOR BIOPROCESSING

SPOTLIGHT

INNOVATOR INSIGHT

Flexible & scalable solutions for AAV viral vector production using a templated platform

Eva Fong

Adeno-associated virus (AAV) has become an increasingly popular viral vector for gene therapy. Currently, there are two approved AAV-based gene therapies, and the number of clinical trials is steadily increasing. As with most viral vector-based therapies, production is labor-intensive and expensive due to the use of adherent cell culture production processes. Consequently, for process intensification, the industry has begun to utilize bioreactors. This article explores the development of a suspension-based AAV upstream process to provide a more efficient and cost-effective bioprocessing solution for large-scale production. The use of template tools for upstream process development will be examined, as will development of the clarification and tangential flow filtration unit operations immediately following harvest.

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AAV UPSTREAM PROCESS DEVELOPMENT USING TEMPLATE TOOLS

The VirusExpress® 293 AAV Production Platform consists of a HEK293 host cell line and a chemically defined media that was developed to be compatible with polycation polyethylenimine (PEI)-based transfection methods. The cell line is a HEK293 derivative that was selected for enhanced virus production and suspension cell growth. Both master cell bank and the working cell bank have been manufactured and characterized according to cGMP regulations.

The objective was to develop a suspension-based production process at bench-scale using the Mobius® 3-L Bioreactor. A model

virus for multiple AAV serotypes was used, all of which packaged the gene for green fluorescent protein (GFP). A shake flask production process for AAV2 was developed to benchmark the bioreactor and scale-up process development. A triple plasmid transfection protocol using PEI as a transfection reagent was followed. Figure 1 summarizes the AAV production process flow. The target seeding density in the shake flask at day zero was 1.1 million viable cells/mL. Transfection was performed 24 h post-seeding. At 72 h post-transfection (on day four of the process) harvest activities were performed, which entailed detergent cell lysis and a concurrent DNA digestion. Following the specified lysis time, a salt spike was added to the cell lysate. Analytical samples were clarified via centrifugation or filtration.

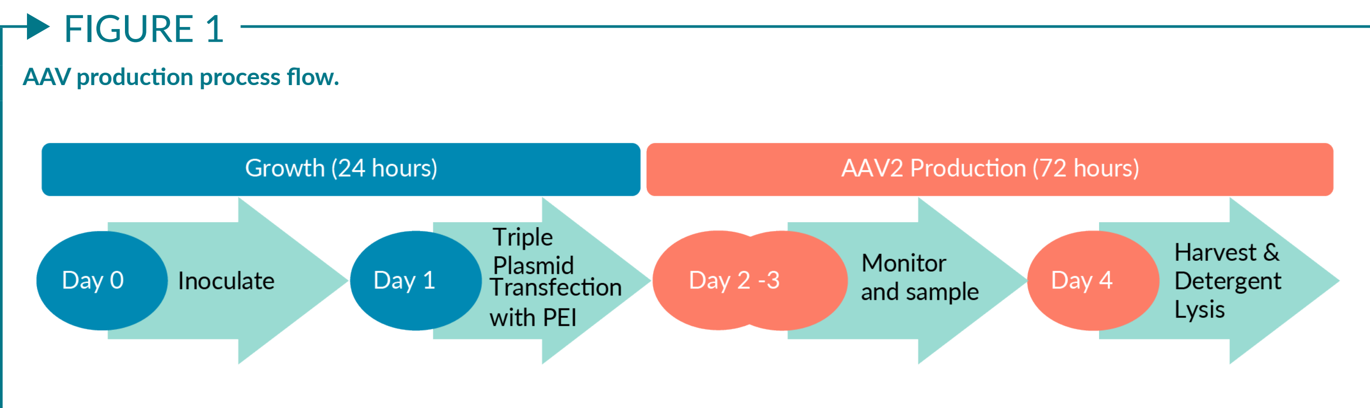
Figure 2 demonstrates the genome titers obtained from the baseline production process. The transfection process was performed over multiple passages. Overall, the cell line shows consistent productivity over increasing passage number, demonstrating process robustness with the shake flask production conditions. (It is suspected that the data point for passage 31 is an experimental outlier). Additionally, capsid titers were measured but are not discussed here.

Prior to scaling up the virus production process, it was necessary to develop the bioreactor parameters for cell growth in bench-scale bioreactors. Agitation and pH set points were selected as the parameters for study. Figure 3 lists the set points tested and the summaries of the cell growth profiles from these optimization studies. The cells were inoculated at 0.6 million viable cells/mL in Mobius® 3-L Bioreactors and cultured in batch mode. The first graph shows data for the pH study. The pH set point/range of 7.1 ± 0.1 led to the highest viable cell density (VCD) of 12 million viable cells/mL. The graph bellow summarizes the data for the agitation study. It was found that agitation rates corresponding to 20, 30, and 40 watts/m³ led to similar peak VCDs of roughly 12 million cells/mL. These data supported the premise that the bioreactor settings used were sufficient for virus production, and these parameters will now be utilized for scale-up development in the Mobius® 50-L Bioreactor.

With the baseline parameter settings established, AAV2 production in the bench-scale bioreactors commenced. A transfection protocol and process flow similar to what was followed for shake flask production was used. Figure 4 summarizes genome titer for multiple AAV2 production runs in the Mobius® 3-L Bioreactors; the orange and blue bars represent the titers from the shake flask controls and bioreactor vessels, respectively. Utilizing the baseline transfection protocol, it was possible to obtain 3-8×10^{9} genome copies/mL (gc/mL) for AAV2 production. We also tested an alternative transfection reagent, a cationic polymer, for AAV2 production and the data is shown in the 'Trial 5' column. The genome titer obtained using the alternative transfection reagent resulted in over 1×10^{10} gc/mL in the 3-L bioreactor. A consistent trend observed was higher genome titers obtained from bioreactor production when compared to shake flask production.

FIGURE 1

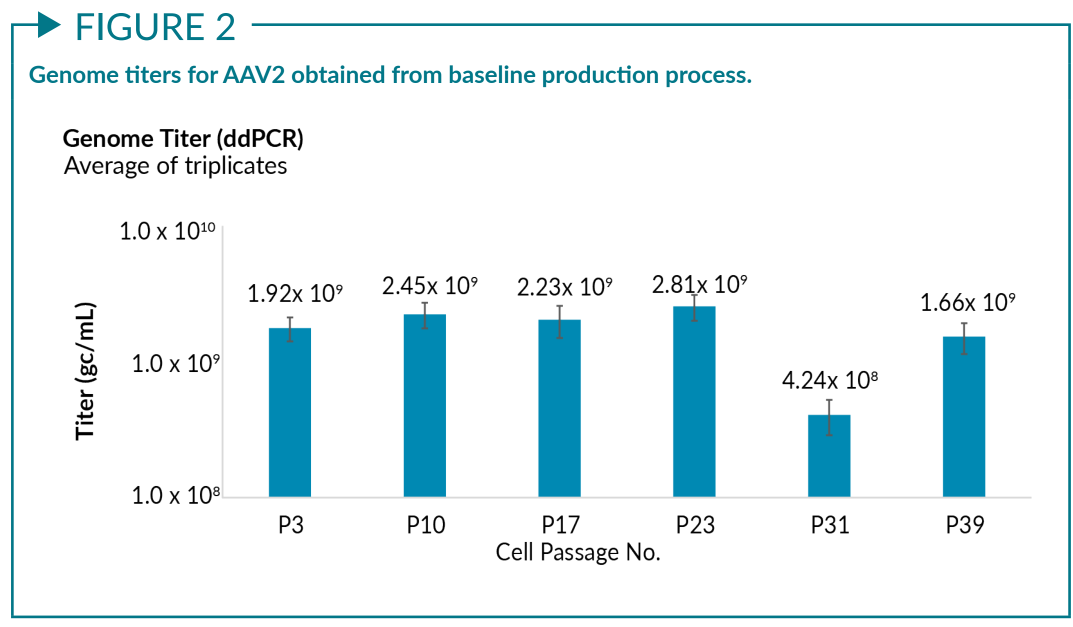
AAV production process flow.



 DOI: 10.18609/cgti.2022.074

FIGURE 2

Genome titers for AAV2 obtained from baseline production process.



After demonstrating the robustness of the baseline process for AAV2 upstream production using the VirusExpress® 293 AAV Platform, the capability for producing other AAV serotypes was tested - specifically AAV5 and AAV6. For these experiments, a transfection protocol and process flow like that used for AAV2 production was utilized, but with the introduction of the serotype-specific Rep/Cap plasmid in each case. Figure 5 summarizes the genome titers obtained in shake flask production for AAV5 and AAV6 serotypes. For AAV5, we observed genome titers that were a log higher than for AAV2. For AAV6, the genome titer was more than twice that of AAV2. Furthermore, capsid titers for these AAV production processes were measured to be able to estimate the percent full capsid at time of harvest. For both the AAV5 and AAV6 serotypes, a considerably higher percentage of full capsids was observed compared to AAV2 (data not shown).

To further demonstrate scalability of the VirusExpress® AAV Production Platform, AAV5 production was performed in 3-L bioreactors. Figure 6 summarizes the genome titer obtained in Mobius® 3-L Bioreactors and for the shake flask controls. The genome titers achieved in the 3-L bioreactors were approximately twice those achieved in the shake flask process – a similar trend to what was observed for AAV2 production in the bioreactors.

Figure 7 summarizes the genome titers obtained for three different transfection reagents tested for AAV2 production: PEI, a cationic polymer, and a lipid polymer. This study was conducted in shake flask production using the baseline protocol. Higher genome titers, approximately 1×10^{10} gc/mL, were observed using the alternative transfection reagents. We anticipate even higher genome titers could be achieved with additional process optimization. However, it is important to consider the impact of the alternative reagents on other product quality attributes and downstream unit operations.

A scale-up strategy utilizing equivalent power per unit volume, for agitation, and volume of gas per unit of liquid volume (vvm), for gas flow, was employed for a cell growth run in the Mobius® 50-L Bioreactor. The purpose of performing this study was to mimic the process flow that will be used for the production scale. Specifically, the Mobius® 50-L Bioreactor will be used for cell expansion (N-1 stage) prior to the virus production (N-stage). Additionally, this study provided an opportunity to conduct any required bioreactor control tuning for the specified process flow. Figure 8 shows the cell growth profile for the scale-up run in the 50-L bioreactor. Included for comparison is the cell growth performance of the shake flask controls. In summary, this was a successful scale-up cell growth

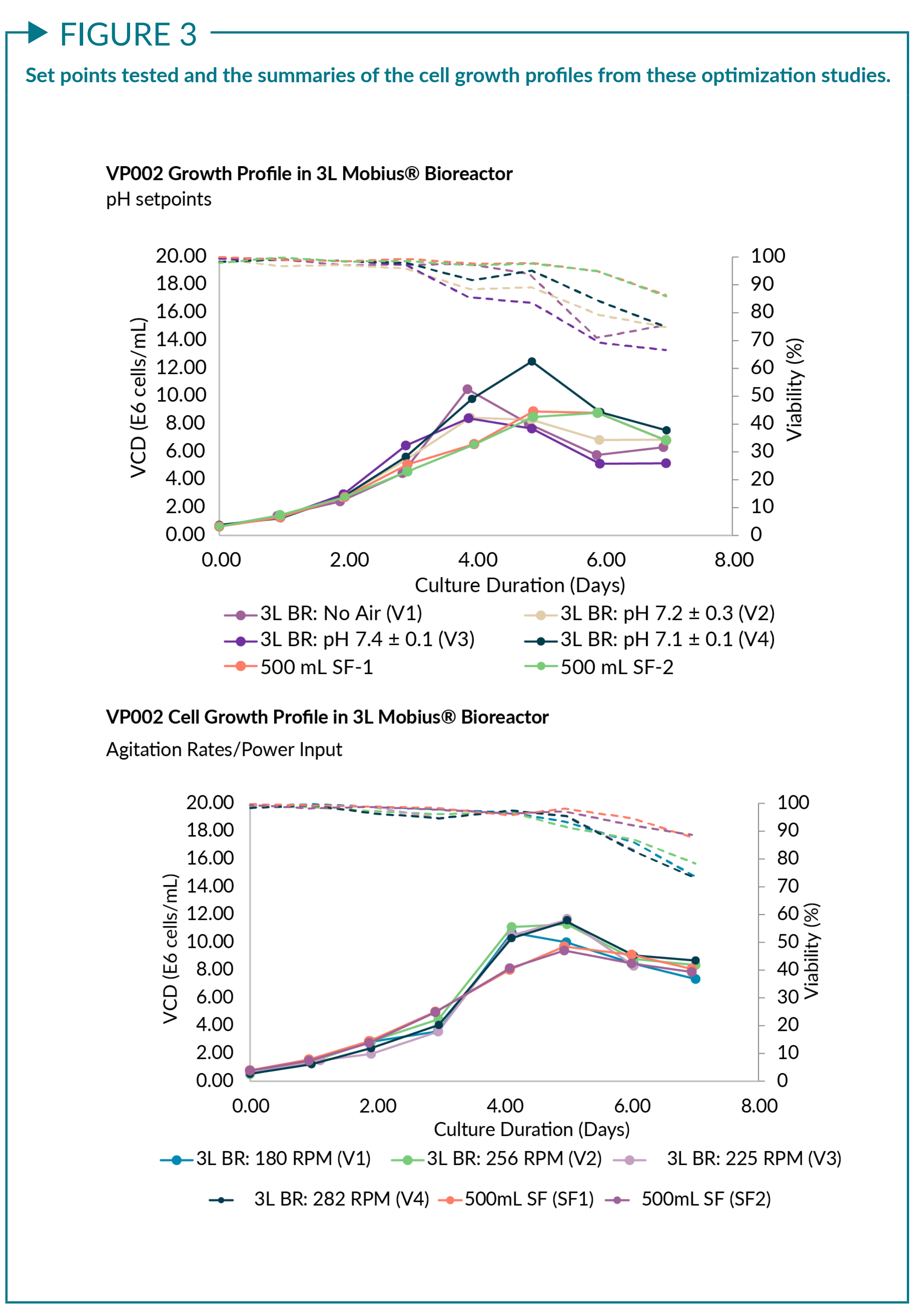


FIGURE 3

Set points tested and the summaries of the cell growth profiles from these optimization studies.

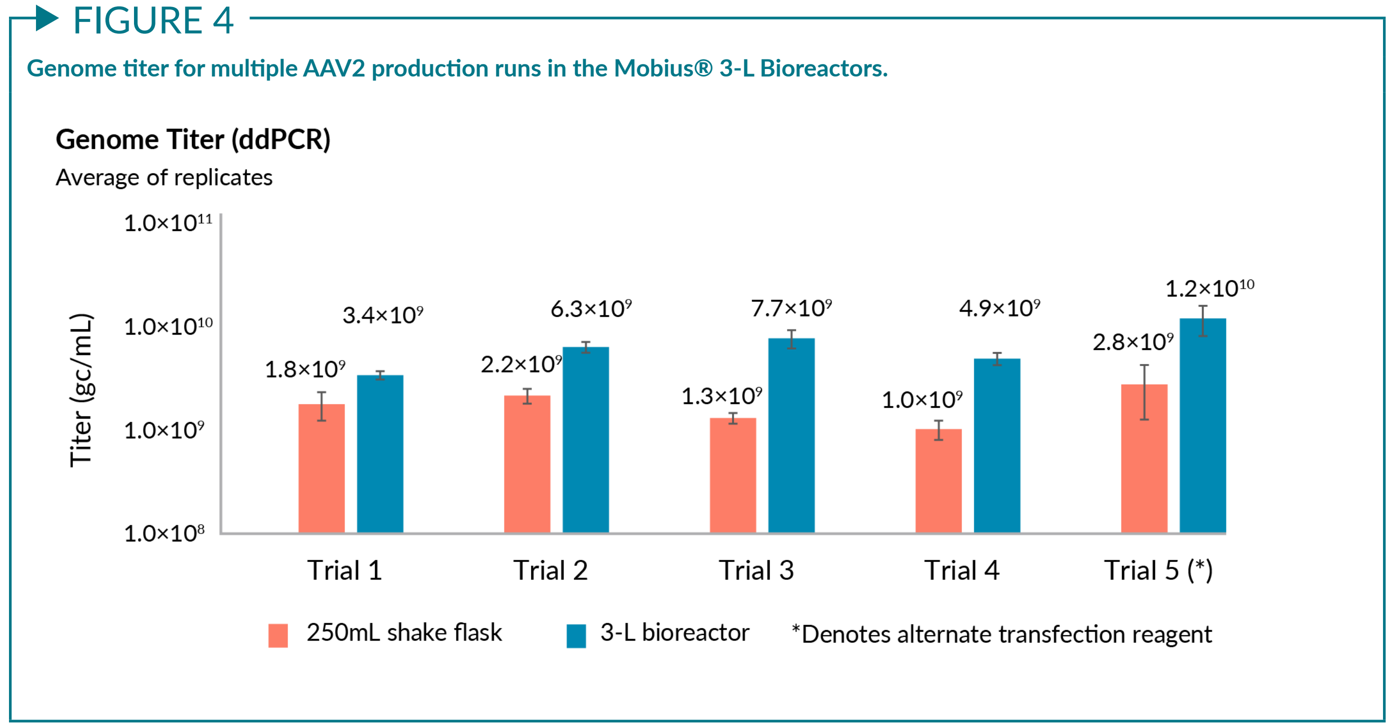
run with the VirusExpress® 293 AAV cells. The cell growth for the cell expansion stage was comparable to the shake flask controls and the target viable cell density for transfection was achieved. Further optimization may be required to improve cell growth at the virus production stage.

Overall, robust process performance for AAV2 production was demonstrated in the Mobius® 3-L bioreactors, as was the platform's capability for production of multiple AAV serotypes. Furthermore, the compatibility of the platform for use with multiple transfection agents was shown, and scalability

DOI: 10.18609/cgti.2022.074

FIGURE 4

Genome titer for multiple AAV2 production runs in the Mobius® 3-L Bioreactors.



with good cell growth in the Mobius® 50-L Bioreactor was demonstrated.

Activities aimed at further improving the platform are ongoing. These include a Design of Experiments (DOE)-based approach for transfection optimization and bioreactor parameter development, specifically for virus production. These improved processes will be scaled up in Mobius® 50-L and 200-L Bioreactors. Additionally, plans are in place for continuous improvement of the VirusExpress® AAV upstream production platform. One of the key observations from the upstream development activities described above was

FIGURE 5

Genome titers obtained in shake flask production for AAV5 and AAV6 serotypes.

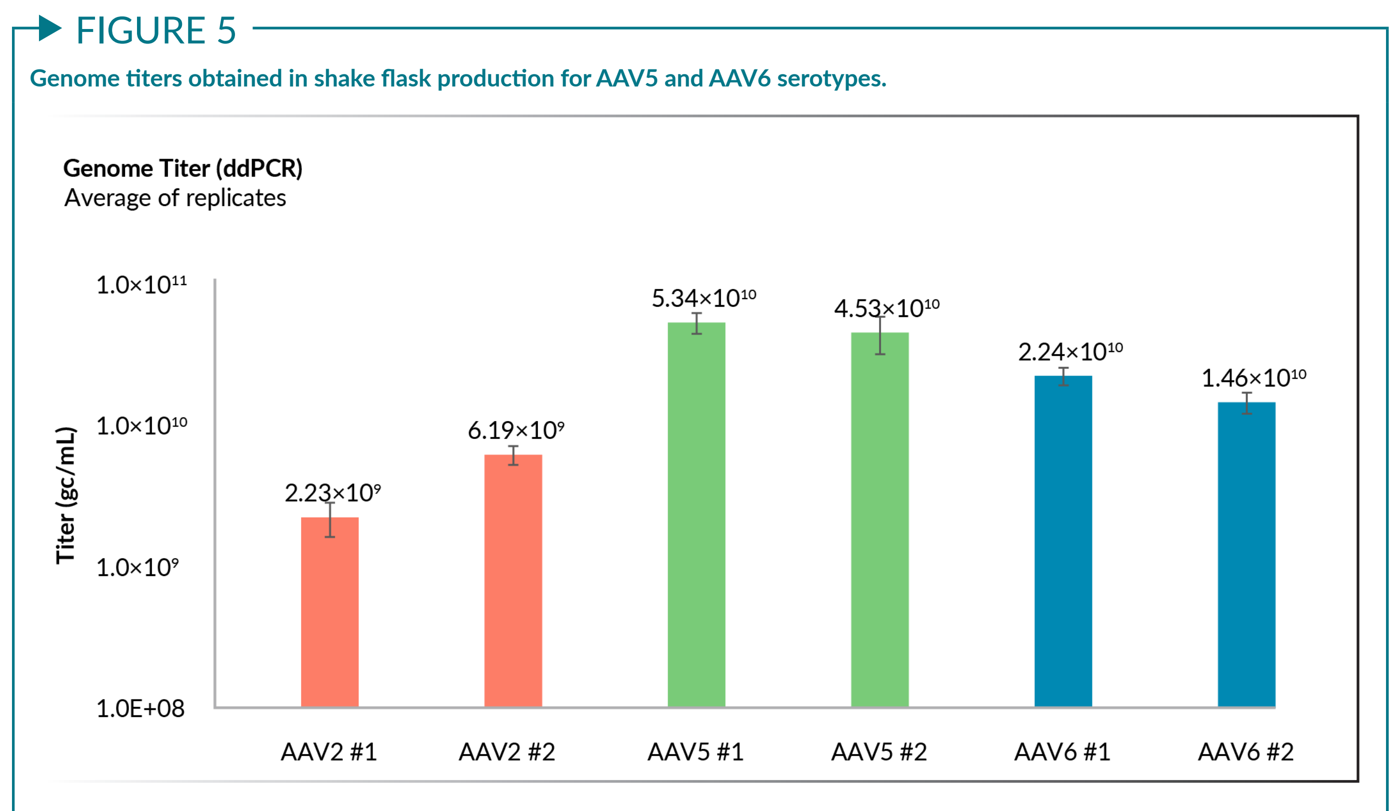
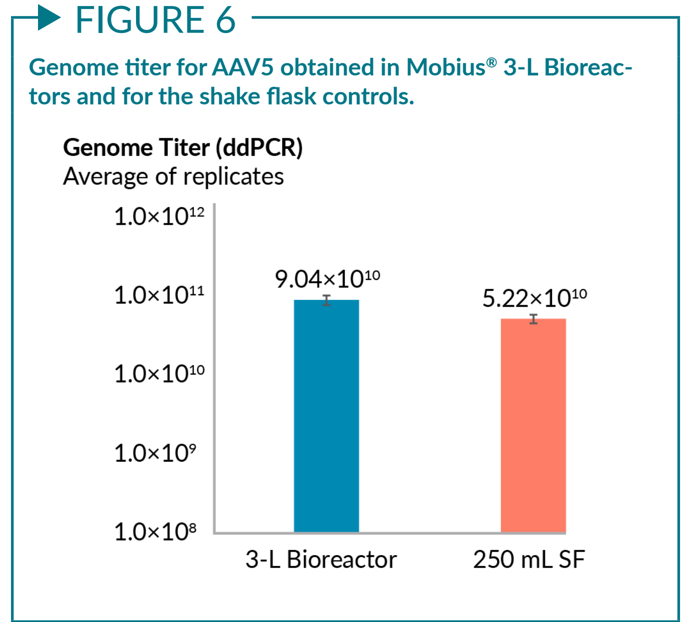


FIGURE 6

Genome titer for AAV5 obtained in Mobius® 3-L Bioreactors and for the shake flask controls.



consistently higher genome titers from bioreactor production compared to shake flask production. We have plans to implement the Ambr® 15 bioreactor for future development work to mimic the bioreactor environment and for more efficient process development.

Focusing on transfection, there are many parameters to consider. A major advantage of using a DOE approach is efficiency; by using statistical software for experimental design and data modelling, it is possible to study multiple parameters simultaneously and their potential interactions. This minimizes the number of experiments and time required when comparing it to a one-parameter-at-a-time approach.

Following some preliminary experiments to help narrow down the parameter list and ranges for transfection optimization, an experiment was conducted to study the effect of a lower DNA concentration with two different transfection agents; reagent A, which was PEI, and reagent B, which was the cationic polymer. In Figure 9, the graph on the left summarizes the genome titers obtained. It was found that for a PEI-based transfection, lowering the DNA concentration in the transfection led to a 1.8× increase in genome titer. The highest genome titer was observed with the use of the cationic polymer and the higher DNA concentration. These constitute some promising results for process improvements.

Leveraging the feed material obtained from the upstream development activities, downstream process development (DSP) could proceed.

FIGURE 7

The genome titers obtained for three different transfection reagents tested for AAV2 production.

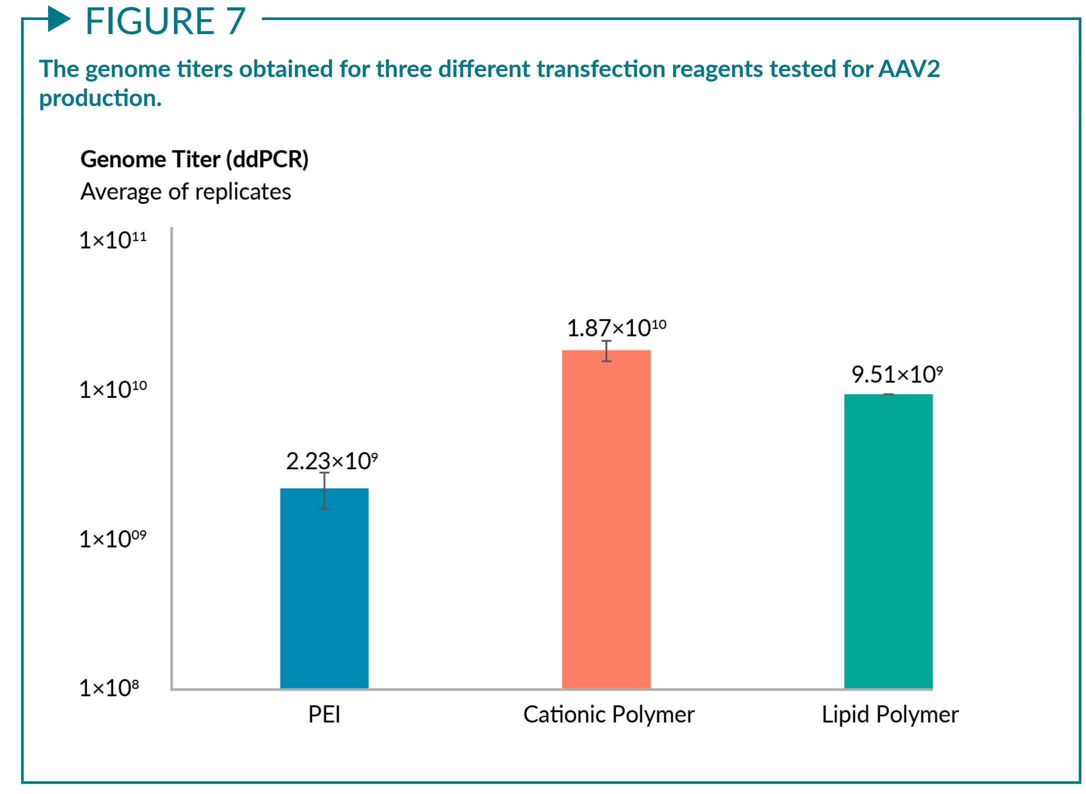


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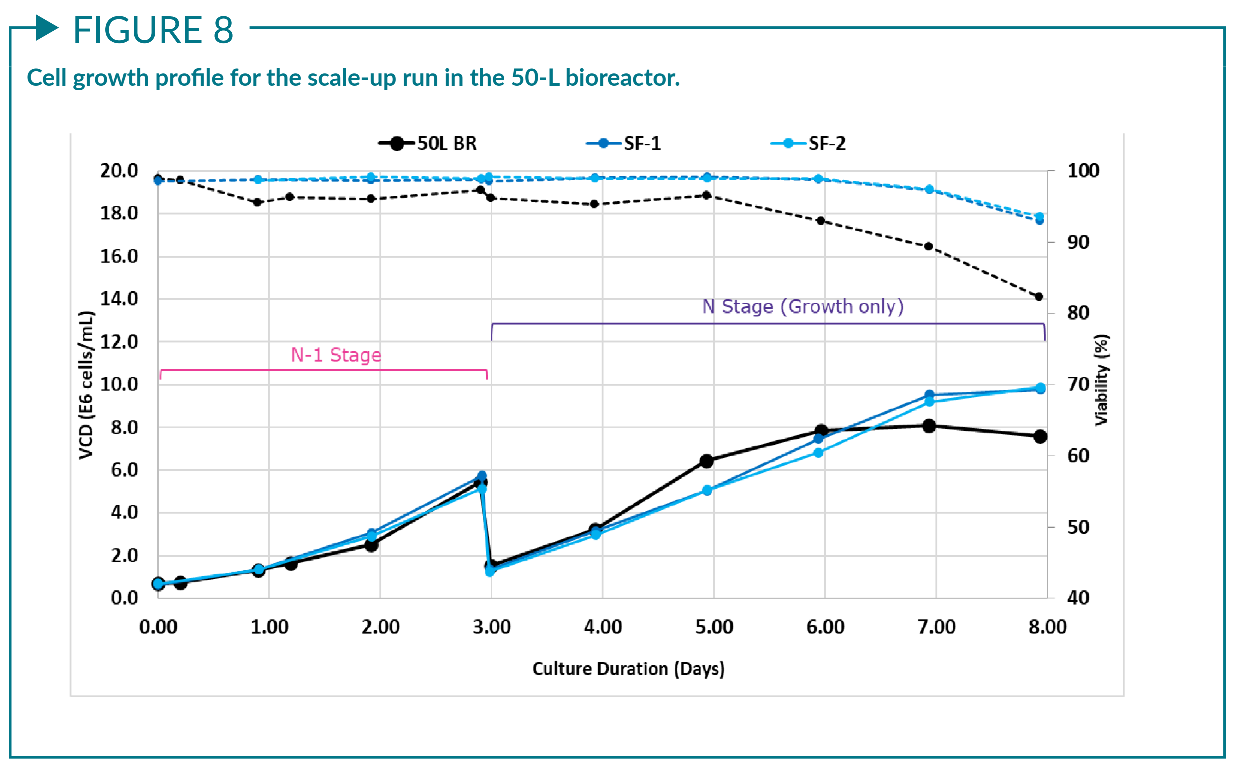
FIGURE 8

Cell growth profile for the scale-up run in the 50-L bioreactor.



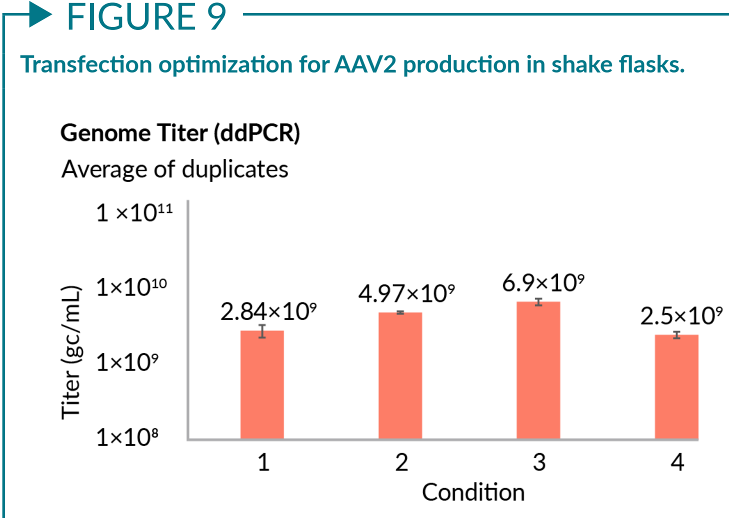
DEVELOPMENT OF CLARIFICATION & TFF UNIT OPERATIONS

As previously discussed, alternative transfection reagents were tested in the upstream development. Figure 10 shows the process flows for two different AAV2 feeds. Feed stream A is the control feed, which was generated using a PEI-based transfection. Feed stream B is the process flow where a cationic polymer was used as the transfection reagent. All other steps of the harvest were similar. The objective of these studies was to identify the clarification train and develop the TFF unit operation for feed A and B. More specifically, would the upstream process change of using a different transfection reagent impact the performance and recovery of these downstream process steps?

Including a TFF step post-clarification addresses the following: reducing the loading time for affinity chromatography and

FIGURE 9

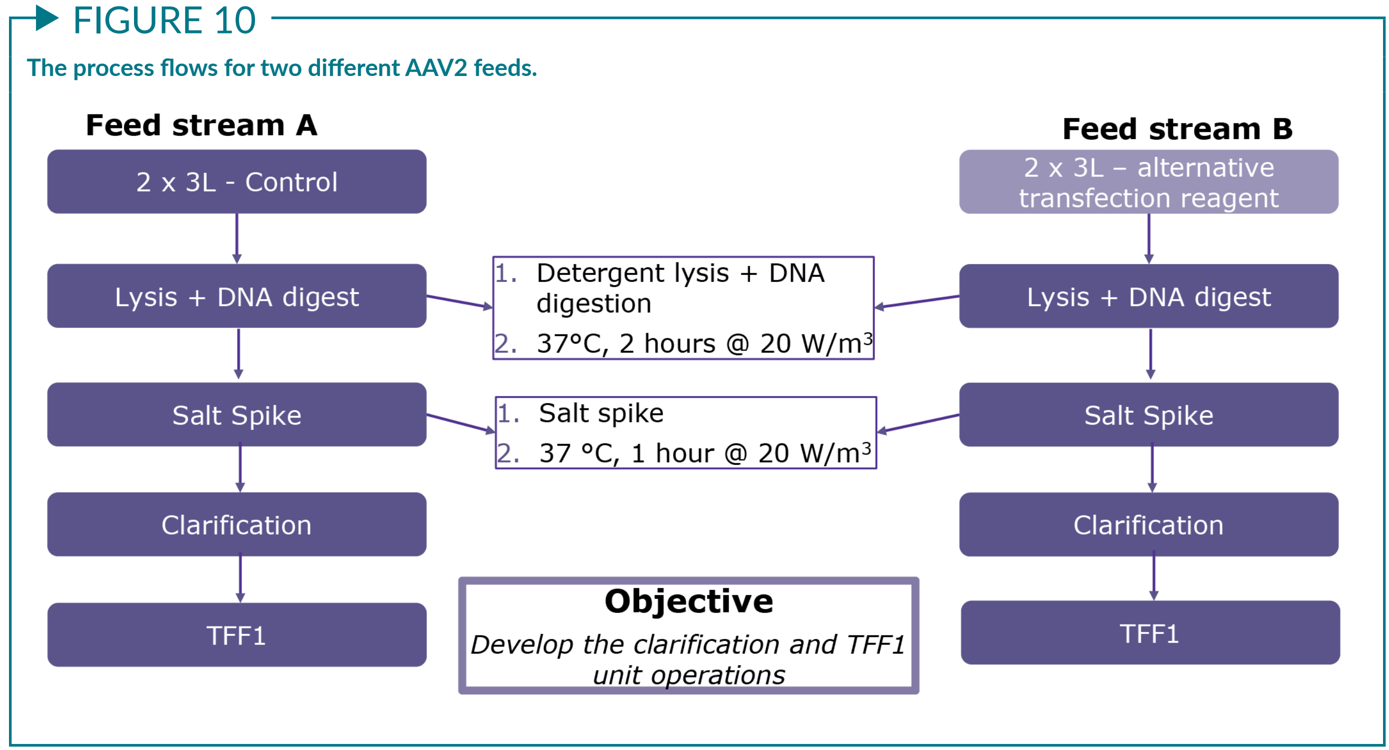
Transfection optimization for AAV2 production in shake flasks.



Condition	Transfection Reagent	DNA Conc. (µg/mL)
1	A	2.2
2	A	1.1
3	B	2.2
4	B	1.1

FIGURE 10

The process flows for two different AAV2 feeds.

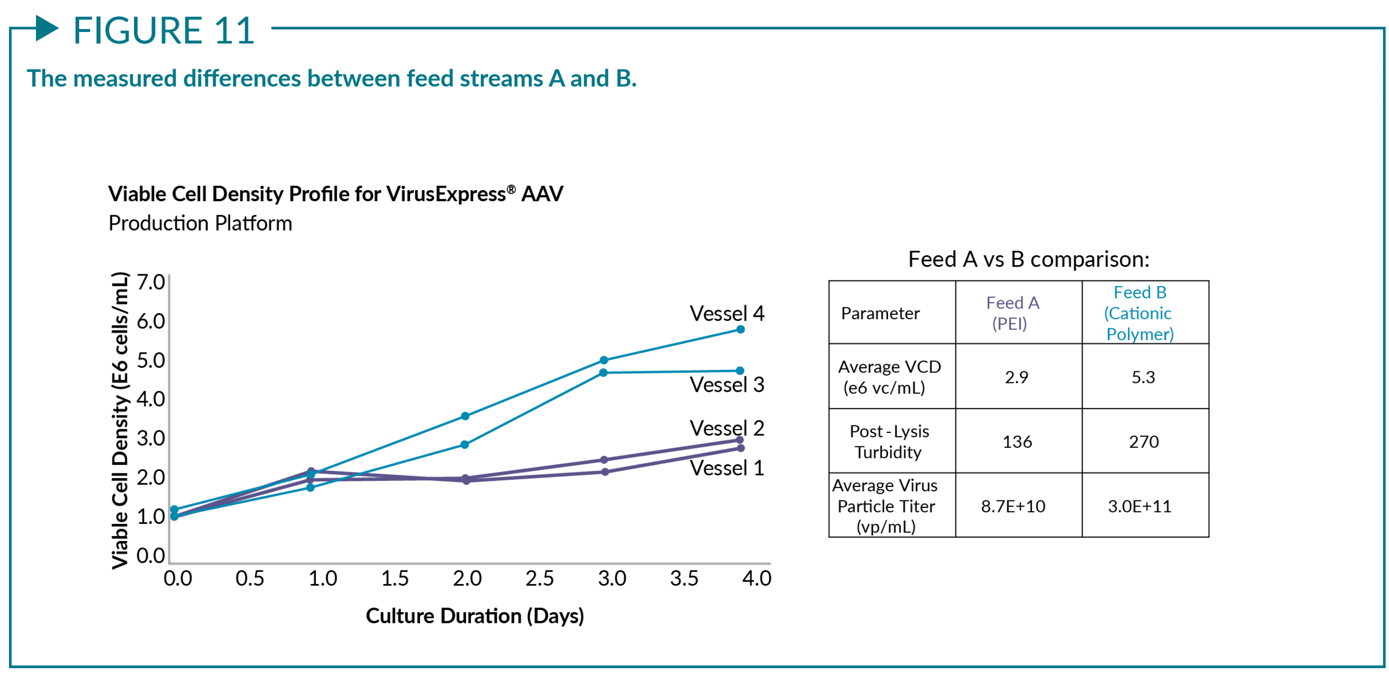


process flexibility by reducing the volume of the clarified harvest for a hold step prior to further downstream processing. To illustrate the point for the affinity chromatography loading time, for an unconcentrated clarified harvest from a 200-L batch size, an estimated affinity column size of 1 L and a column loading flow rate of 0.5 column volumes per minute (equivalent to a two-minute residence time), the estimated loading time would be 8 hours. By incorporating a TFF step, loading time could be reduced by a factor 10–20×.

Figure 11 summarizes the measured differences between feed streams A and B. The table on the right shows the average values for the experimental duplicates. In summary, feed B had a 2× higher viable cell density at the time of harvest, as summarized in the graph to the left in Figure 11. Additionally, a 2× higher

FIGURE 11

The measured differences between feed streams A and B.



Parameter	Feed A (PEI)	Feed B (Cationic Polymer)
Average VCD (e6 vc/mL)	2.9	5.3
Post-Lysis Turbidity	136	270
Average Virus Particle Titer (vp/mL)	8.7E+10	3.0E+11

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post-lysis turbidity and approximately 3.5× higher virus particle titer were observed for feed B. Feed B had a 2× higher genome titer than feed A (data not shown).

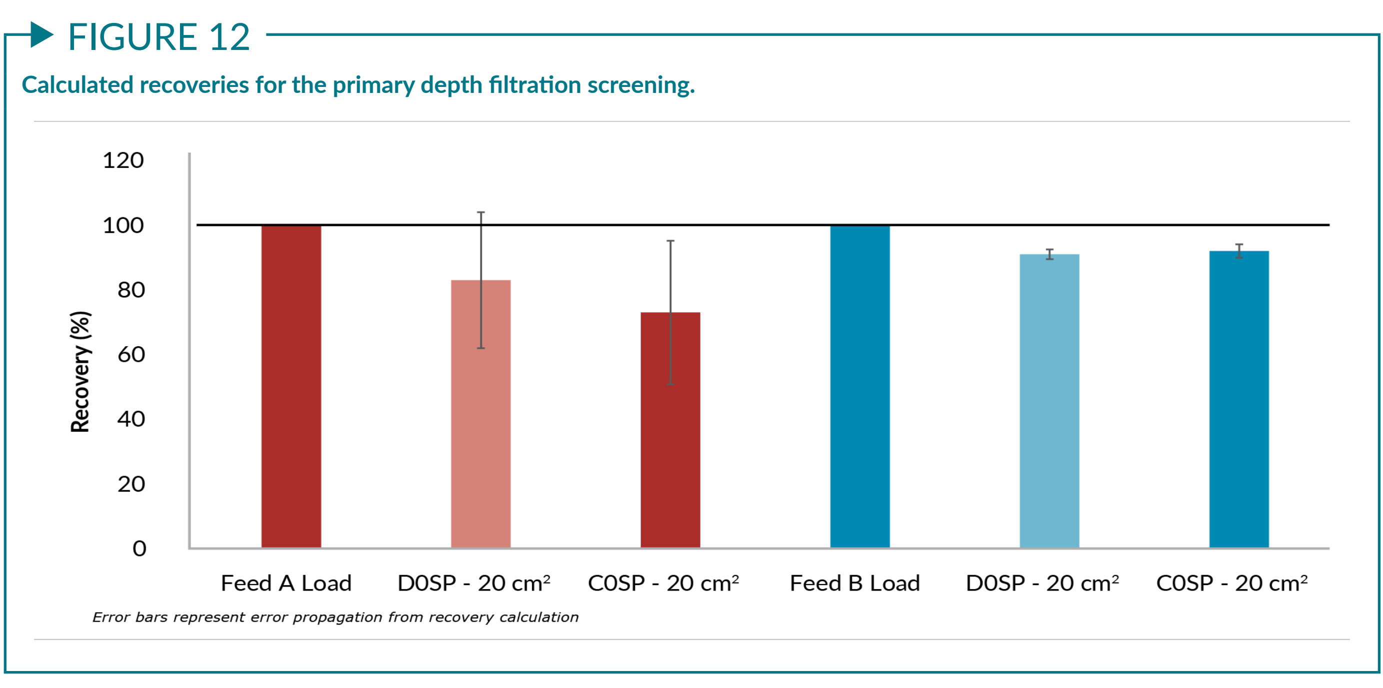
Selecting filters to screen can be overwhelming, as there are many from which to choose. Leveraging the AAV clarification experience of our Process Development Sciences team, the parameters for consideration are the following: depth filter loading, bioburden filter capacity post-depth filter, and product yield. The filters chosen for the depth filtration screening were the Millistak+® C0SP and D0SP based on their performance for the parameters previously mentioned for filtration of AAV containing cell lysates.

Once the depth filters were identified, the filter screening experiments were conducted using the P_{max} constant flow rate test using with a constant flux of 300 L/m²/h. From these studies, the throughput (or the volume of feed process per surface area of filtration membrane) for each filter type was determined for feeds A and B. It was decided to report the filtration throughput at a specific pressure of 10 psi so that the filters could be sized appropriately for safe operation. The filter throughputs for feed A were 800 and 300 L/m² for the D0SP and C0SP filters respectively, while for feed B, the throughputs were 450 and 300 L/m² respectively. An additional attribute of the filtrates was the turbidity measurement. For feed A, both filtrates had turbidity values between 5–7 NTU (nephelometric turbidity units) while for feed B, both filtrates had turbidity values between 17 and 23 NTU. The graph in Figure 12 shows the calculated recoveries for the primary depth filtration screening. The data values for feed A and B are reported as the red and blue bars, respectively. The reported recoveries were based on viral particle titer. Recoveries of > 80% were obtained for both feeds across both depth filters, factoring in assay variability.

As part of the clarification train, we wanted to include a bioburden reduction filter. Two filters were identified for this study: the Millipore Express® SHC 0.5/0.2 µm and the Milligard® PES 1.2/0.2 µm. Both devices are dual-layer PES membranes with a 0.2 µm final layer, but the Milligard PES final layer is a 0.2 µm nominal pore size whereas the Express SHC is a 0.2 µm absolute pore size or sterilizing grade membrane. Although the Milligard PES is a 0.2 µm nominal pore size, it has demonstrated greater than six log removal of challenge bacteria. For the bioburden filter screening, the primary clarification filtrates for feeds A and B were the load

FIGURE 12

Calculated recoveries for the primary depth filtration screening.



Error bars represent error propagation from recovery calculation

material. The study was performed using the Vmax constant pressure method to measure the decrease in flow as a function of volumetric throughput. The pressure used for the study was 15 psi. Figure 13 shows the filter performances based on the predicted minimum filtration area required per 50 L of feed, with no safety factor applied. The data values for feed A and B are reported as the red and blue bars, respectively. As expected, feed B primary clarification filtrates required a larger surface area as these filtrates were more turbid. For perspective, the sizes of several Opticap® Millipore Express SHC filters are shown below the predicted minimum area graph. The thick line displayed at 0.25 m² surface area corresponds to the area of an Opticap® XL5 capsule. From the data in the predicted minimum surface area graph, the C0SP and D0SP clarified lysates for feeds A and B do not require a large filtration area for a 50-L batch size.

Figure 14 summarizes the recoveries for the 0.2 μm bioburden filtration study. All trials resulted in high recoveries.

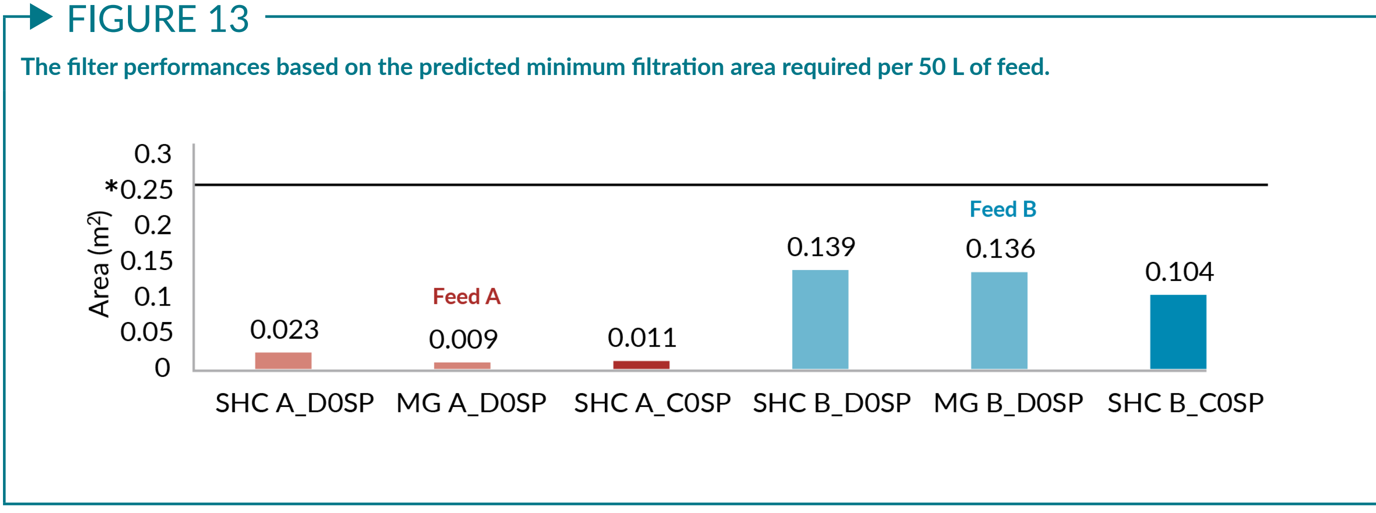
To summarize the clarification studies, good recoveries and filter throughputs were observed for the for feeds A and B. We chose to move forward with D0SP for scale-up activities. For the bioburden filter, both filters tested had good recoveries and reasonable predicted surface areas for a 50-L batch size. The Millipore Express® SHC filter has been chosen for scale-up activities because it is a sterilizing-grade membrane filter.

As previously mentioned, a post-clarification TFF step was incorporated to offer process flexibility; reducing the volume of the clarified harvest would shorten the loading time for affinity chromatography. For the TFF experiments, Pellicon® cassettes with the Ultracel® membrane, C-screen configuration and a nominal molecular weight cut-off of 100 kDa were selected. We targeted a loading factor of 50 L/m², a processing time of four h, utilized a three-stage process flow (4x initial concentration, 5DV buffer exchange, 5× concentration to reach a target 20× concentration factor) and operated in constant permeate flux mode. Critical flux excursion studies were performed for the Feeds A and B to determine the operational fluxes for each stage of the TFF process prior to performing the process simulation run. Recoveries for the simulation run were determined using viral particle titer. Figure 15 summarizes the process parameters and recoveries for the process simulation runs. Different final concentration factors were achieved for Feeds A and B. In summary, high recoveries were achieved for the process simulation runs and we have parameters that can be used for our scale-up activities.

Utilizing the feeds produced from the VirusExpress® AAV Production Platform, we developed the clarification and TFF unit operations for our AAV2 model virus. Our next steps are to demonstrate the scalability of the platform with a virus production run in the Mobius® 50 and 200-L bioreactors and developing additional downstream unit operations.

FIGURE 13

The filter performances based on the predicted minimum filtration area required per 50 L of feed.



 DOI: 10.18609/cgti.2022.074

FIGURE 14

The recoveries for the 0.2 mm bioburden filtration study.

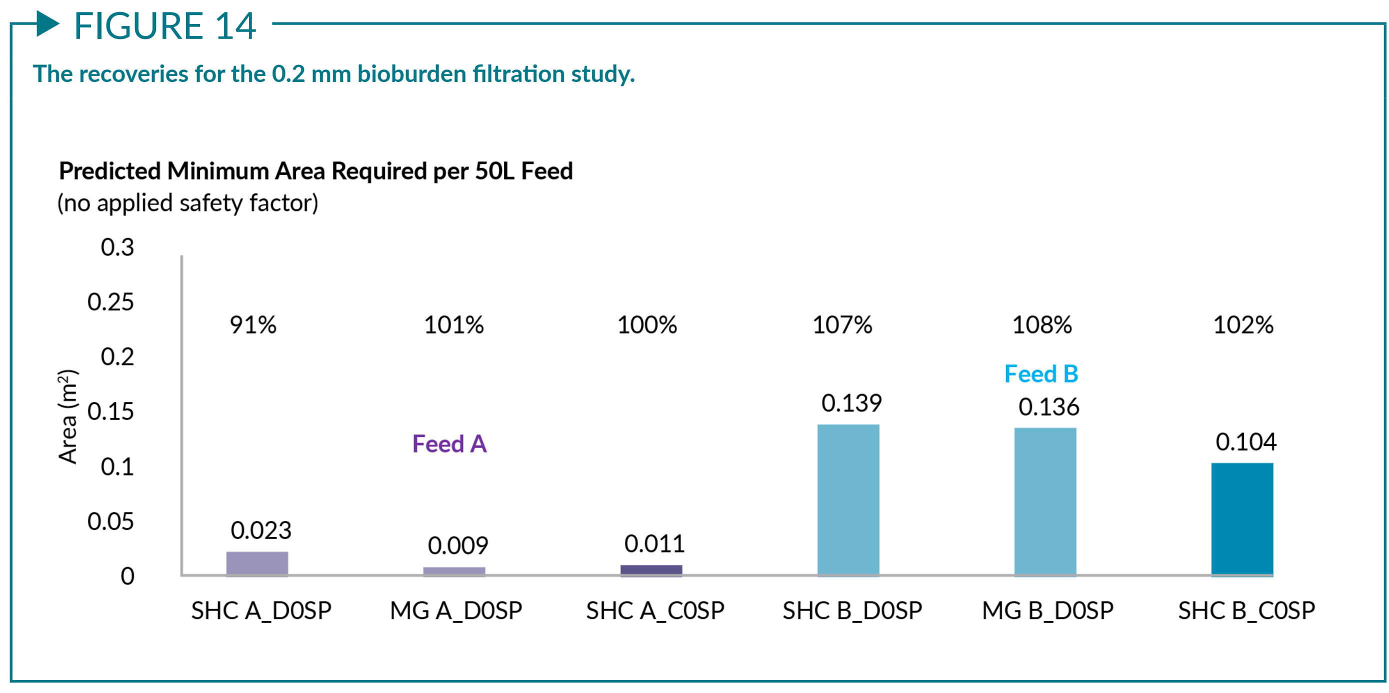
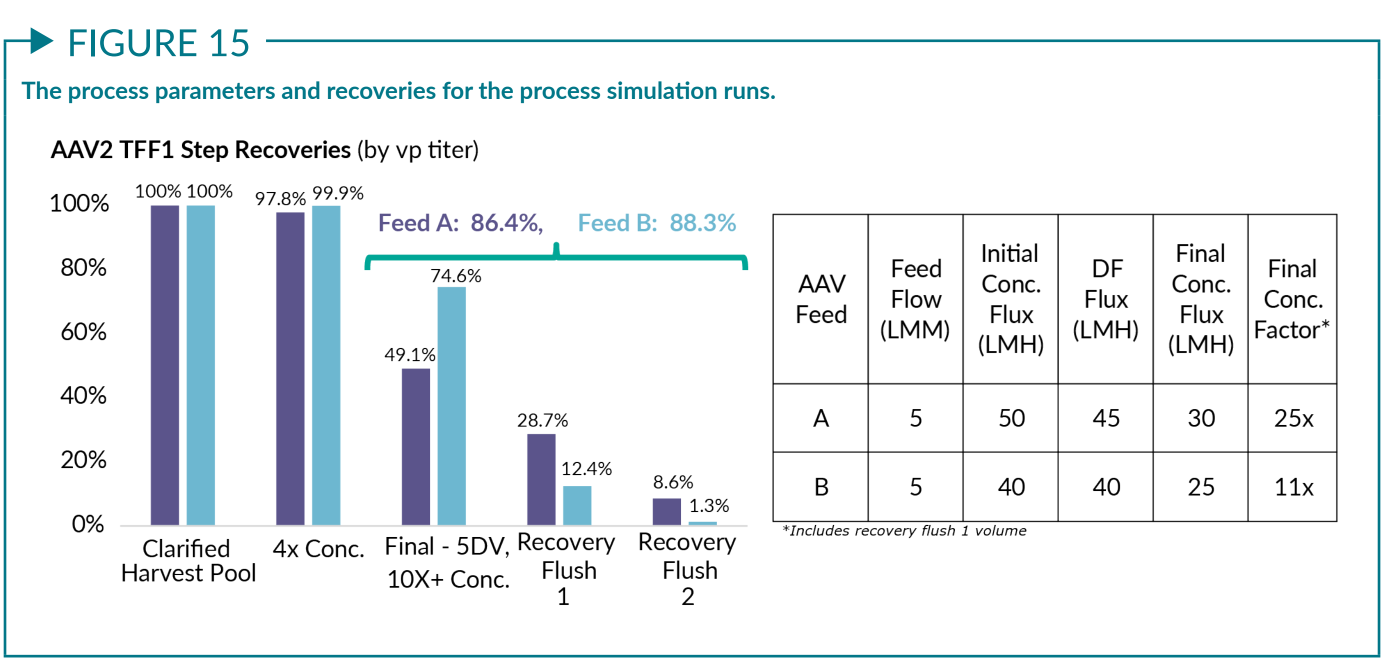


FIGURE 15

The process parameters and recoveries for the process simulation runs.



AAV Feed	Feed Flow (LMM)	Initial Conc. Flux (LMH)	DF Flux (LMH)	Final Conc. Flux (LMH)	Final Conc. Factor*
A	5	50	45	30	25x
B	5	40	40	25	11x

*Includes recovery flush 1 volume

Q&A with Eva Fong

David McCall, Editor, *Cell & Gene Therapy Insights*, speaks to **Eva Fong**, Principle Scientist, Virus and Gene Therapy Bioprocessing

EVA FONG received her BS in Chemistry and Biological Sciences from the University of California, Irvine. Since graduating, she has held roles in multiple organizations conducting pre-clinical, process development, engineering, and manufacturing activities for monoclonal antibody and viral vector therapeutics. At MilliporeSigma, she leads a team responsible for all upstream and downstream scale-up R&D to develop best-in-class lentiviral and AAV manufacturing products and templates.

Q What transfection efficiency did you achieve, and is it indicative of titer performance?

EF: We are observing 50% transfection efficiency at 24 h and 60% at 48 h post-transfection. Because we are basing the transfection efficiency on expression of the reporter gene that's contained in the transfer plasmid only, a high transfection efficiency may not be indicative of high titer.

Q How did you select the filters that you used? What would be required for a 50-L batch?

EF: We took advantage of our process development team's AAV filtration experience to help with selecting which filters we would test. From our studies, we did test two different feeds, and the filter sizing was different for the two feeds, but for a 50-L batch of the higher cell density feed stream, we would need approximately 0.5 m^2 of the primary clarification filter (D0SP) and about the same surface area of the bioburden reduction filter (Millipore Express® SHC sterilizing-grade filter).

AFFILIATION

Eva Fong
Principle Scientist
Virus and Gene Therapy Bioprocessing

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AUTHORSHIP & CONFLICT OF INTEREST

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FASTFACTS

Detecting residual host cell DNA with Droplet Digital PCR technology

Mark White, Associate Director of Biopharma Product Marketing, Bio-Rad Laboratories

Ensuring the removal of residual host cell DNA from cell and gene therapy products is crucial for keeping patients safe and avoiding costly consequences. Droplet digital PCR (ddPCR) technology is a sensitive, specific, accurate and easy-to-use technology that delivers reproducible results and analysis for detecting, sizing, and quantifying residual host cell DNA.

Cell & Gene Therapy Insights 2022; 8(3), 443; DOI: 10.18609/cgti.2022.065

RESIDUAL HOST CELL DNA TESTING

Testing for residual DNA content is a key step in the viral manufacturing process for cell and gene therapy products. Traces of host cell DNA must be removed from cell and gene therapies to avoid oncogenic risks, as well as loss of raw materials and batch products. Regulatory guidelines state that host cell impurities in cell and gene therapies must be limited to <10ng/dose and <200bp/fragment, but common methods, such as qPCR and BioAnalyzer technologies, have low accuracy at those levels.

Detecting residual DNA with ddPCR involves compartmentalizing host cell DNA into droplets. One advantage of this is that samples in complex matrices require no DNA extraction and are directly used in ddPCR after sufficient dilution. This allows close to 100% recovery to be achieved at a 1:75 dilution, saving time and money. These ddPCR assays also show resistance to inhibitors.

HEK293 RESIDUAL DNA TESTING

Bio-Rad offers a variety of kits and a GMP Supermix for detection of residual host cell DNA. This includes residual HEK293 kits for both sizing and quantification. The commonly used method of qPCR for HEK293 residual DNA detection has a cumbersome workflow and low accuracy with high false positive rates. Laboratory developed ddPCR for HEK293 residual DNA detection is usually done on purified samples, increasing workflow burden.

The ddPCR HEK293 residual DNA testing kit from Bio-Rad is the first digital PCR based sizing kit validated to meet regulatory guidance requirements. It provides specific and reproducible sizing solutions, with a sensitivity to quantify HEK293 DNA with LOD of 0.1pg/3 wells. It is easy to use and analyze, as it is an extraction free method. The quant and sizing kits are compatible with AAV and CAR-T workflows. The performance of the quant and size kits is described in **Table 1**. The sizing kit is able to distinguish genomic DNA fragment size of </>200bp. Both kits are validated on AutoDG and QX ONE with a capacity to run 100 reactions.

Table 1. HEK293 kits performance.

Characteristics	Performance metric
***Vericheck ddPCR* HEK293 Residual DNA Quant Kit**	
Sensitivity	0.1 pg /µL (3 wells)
Specificity	99.99% with 4 closely related DNA species
Reproducibility	CV% < 10%
Dynamic range	1pg–80ng at R^2 = 0.998
***Vericheck ddPCR* HEK293 Residual DNA Size Kit**	
Sensitivity	2pg/well (3 well)
Specificity	>99.99% specificity
Reproducibility	CV% < 10%
Dynamic range	2pg–300ng at R^2 = 0.996/0.997

Bio-Rad offers a whole range of ddPCR contaminant testing kits, including a CHO kit, an *E. coli* kit, and a mycoplasma kit, as shown in **Figure 1**.

Figure 1. Manual steps required to perform Ella and ELISA immunoassays.

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VECTOR BIOPROCESSING

SPOTLIGHT

INTERVIEW

David McCall, Editor, *Cell and Gene Therapy Insights* speaks to **Hugo Rojas,** Upstream Process Lead Scientist at uniQure

Exploring commercial upstream processing of AAV

HUGO ROJAS's home-country is Mexico, where he did his BSc in Biotechnology Engineering at the National Polytechnic Institute, with a thesis on characterization of cyanobacteria growing in bioreactor. After finalizing his studies, he worked for a year at the local biopharmaceutical Probiomed as technician in analytical development. Then he pursued a MSc in Life Sciences and Technology and PhD in the group of Cell Systems Engineering at Delft University of Technology focusing on metabolic engineering of yeast. After his studies, he started his career as Fermentation Scientists at INVISTA UK, working on development of gas fermentation and valorization of waste streams. Then moved on to a role in Janssen (Johnson & Johnson) as USP Development Scientists in the department of Bacterial Vaccines Development. Currently, he works at uniQure as USP Lead Scientist, relentlessly pursuing a next-generation AAV production process with insect cells, which has taken his scientific interests towards investigating scale-down models in high-throughput platforms, cell line development, process modeling and rational process intensification leveraging information obtained through PAT. His hobbies are videogaming, animals, playing guitar and acting.

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DOI: 10.18609/cgti.2022.055

What are you working on right now?

HR: I am a subject matter expert on bioreactors and the whole upstream process (USP) within uniQure. Everything from the thaw of the cryovials, whether it's the baculovirus or insect cells that we use, all the way to cell lysis and clarification, is considered to be USP. From that point onwards, we start doing the purification through chromatography and the cleaning processes.

My position as such has two different purposes. On the one hand, I service the clinical products by means of helping advance development of Phase 1, Phase 2 and Phase 3 processes, and supporting our knowledge generation for our current commercial process. On the other hand, there is also technology development. This means staying up to date with the new advancements in USP processing, where we are going in the future as a company, and what we want to implement in our USP.

These days, my time is dedicated 60% to the technology development and 40% to the support of protocols.

How would you frame the key challenges relating to scalability facing the viral vector manufacturing field at the moment? Where are the most pressing issues?

HR: Firstly, I want to mention bioreactor control. Typically, in other USP processes, you have a much more direct way to measure your product. When it comes to adeno-associated virus (AAV) gene therapies using insect cells, the human embryonic kidney (HEK) platform, and other platforms, the only way to do it right now is using quantitative PCR (qPCR). However, that does not tell you much about the product itself. Determining times of harvest and the optimal bioreactor operation can sometimes be challenging. It becomes an interesting challenge in the clinic when it comes to figuring out how to get the optimal amount of AAV and how to get proper growth of your cells after transfection or after infection.

Another thing is the DSP (downstream process). Although for many AAV companies this is somewhat straightforward, there is still work to be done to establish how to properly complete viral clearance, and to ensure that you have a concentrated product that is as pure as possible. The chatter in the field cites things like how to start separating empty from full capsids, which is an interesting challenge.

> "Determining times of harvest and the optimal bioreactor operation can sometimes be challenging."

The final thing to mention is clarification and cell lysis. Once you start moving up to 2,000 L bioreactors, it becomes interesting to see how you are going to implement clarification and cell separation techniques, and how this is going to impact the processing, because in some cases, things like sedimentation or

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filtration are acceptable. But will it still be acceptable at a certain volume or for certain cell densities? That is yet to be shown.

What can you tell us about the ongoing development of the baculovirus platform? How has it evolved and with what impact on scalability, productivity, and quality?

HR: uniQure has been in the business for 20 years. Since I joined, we have been doing a lot of the groundwork. There are a couple of advancements that we already have a patent for surrounding technologies to try to improve the baculovirus constructs.

There is work around how to package the AAV essential feeds within the baculovirus genome. We are working on this to try and generate better genetic circuits to be able to increase the yield and decrease empty-full ratio (meaning an increase in the amount of full particles).

When it comes to processing itself, there are ongoing efforts to deepen understanding of our process. As we have been doing this process for quite a while, we have generated some interesting data. We are trying to leverage all the knowledge we have to increase how rigorous we are with our quality.

Another thing we are currently working on, which we also have intellectual property (IP) around, is cell line development. We are trying to develop our own cell line which we believe will be the next evolutionary leap in the race towards better scalability, higher productivity, and safer product.

With more and more vector manufacturing facilities designed for >2,000 L production capacities in the planning or coming online, what are the chief technological solutions that enable such genuinely industrial scales? And where are further technology improvements required in this regard?

HR: The keyword in our case for all platforms, not only insect but also HEK, is process intensification.

We should start to go for much more concentrated product. Right now, the trend is to go up in volume, but eventually, we are going to hit a wall. The largest single-use bioreactor I know is about 10,000 L, meaning we could not go higher than that, even if we are searching for AAV gene therapies that require 20,000 L bioreactors. Either the bioreactor manufacturers will go for much larger single-use bioreactors, or we (the companies developing processes) will go for more intensified processes. Either way, both are very interesting challenges that come with big hurdles.

On the one hand, for the HEK platform, we need to ensure proper transfection. To generate transfection reagents, you either have plasmids or DNA that you need in huge scales. The more you propagate in a flask method, the more errors you will have in the structure, which also creates a problem.

The same goes for the baculoviruses. How are you going to ensure there will be consistency in your batches? At much larger scales, the bioreactors will not have sufficient power to supply the correct amount of oxygen, for instance. It will be much harder to control.

That is the future that we see ahead in this endeavor, which is quite exciting yet a lot of work.

What are the pros and cons of automation in viral vector processing at the moment, and where is it having the greatest impact on your work?

HR: I am a big, big supporter of process automation. However, I am against automation for the sake of just for the sake of 'wow' factor. Automation requires in-depth knowledge of your process and understanding of which steps you urgently need to control and remove human intervention.

The benefits include having much greater consistency and reproducibility, because a robot does not get tired. For example, if I repeat an experiment, my variability is ~10% coefficient variation. If I use a liquid handling robot, this variability drops to 1–5%. That tells you that even if you have experienced people, if you have a robot that decreases the variability of the process, it is really valuable because your results are more trustworthy and the data is much more relevant. You remove operator variability from the equation. This also comes with increased safety: the less human operation or handling, the less chance of contamination of the product or the bioreactor, and this also means reduced exposure of the operators to the AAV itself.

The disadvantages include flexibility. If you are stuck automating a process that is far from optimized, then improving that process will be much harder. That is why you must choose carefully where you apply automation. I am all in favor of starting the automation of certain steps in process development. Together with the original equipment (OE) approaches, it has shown itself to be quite empowering for everyone.

When it comes to the actual process and where to start, I suggest automated sampling. This means automation of sample handling from the moment it is taken from the bioreactor to when it is in position to be measured. In addition, we should to try to leverage inline probes as much as we possibly can. That information will allow better control of your bioreactor.

When it comes to automating other process steps, I struggle to see how to compromise in that trade-off of between retaining flexibility and implementing automation at the moment.

Where do you see viral vector USP heading in future years as demand for increased capacity continues to grow? What technological and strategic trends are you expecting to see unfold?

HR: This ties back to the previous question about 2,000 L production. There is a clear need for contract manufacturing organizations (CMOs) to produce preclinical and

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clinical material. Often, small companies have a brilliant idea for a gene therapy that in the initial experiments and concepts works beautifully. But when it comes to start generating the material and taking it to commercial, or even to large-scale manufacturing, it's quite difficult. They struggle to find a reliable partner, or a reliable way to go about the manufacturing process.

> "Once there is a new technology or advancement in terms of processing, we immediately want to implement it..."

How do we ensure that scale-up delivers consistent material to be able to meet future demand?

HR: I hope in the future we see things such as more online parameters. My assessment right now is that the gene therapy world is where we used to be around 20 or 30 years ago with monoclonal antibodies. In the monoclonal antibody world, we have leading competitors right now who have implemented things such as online monitoring, semi-continuous processing, and things like perfusion.

There was a huge learning curve in that space that could potentially be applied in our world in gene therapy. There are brighter minds than mine working hard on that not only in uniQure but within many other companies. I see that those examples of process intensification will be coming in the next few years.

It goes without saying that there are scientific challenges in achieving this – for example, in achieving the semi-continuous processing of a transfected cell when that cell dies in the making of your product. In that sense, the people in the monoclonal antibody world had it a bit easier because the cells making monoclonal antibodies are still alive. It will be quite a challenge to implement the process intensification, and to be able to deliver consistent product in much larger volumes.

What are your key goals and priorities both for yourself in your own role and for uniQure as a whole over the next 12–24 months?

HR: I will be supporting the partnership with CSL Behring, which requires tech transfer. When it comes to our clinical pipeline, we are going to start a Phase 3 trial. We need to supply material. I am not directly involved, but I am acting as a subject matter expert and trying to help the team advance in the process development.

The focus for the next 6 months to a year is technology development, and how to implement those new technologies for new programs coming up.

Our main commercial programs right now are for Huntington's disease, refractory temporal lobe epilepsy and Fabry disease. We have other new potential gene therapy that we are currently working on, assessing whether they work at the preclinical stage at least. Once there is a

new technology or advancement in terms of processing, we immediately want to implement it in, or at least see if it strategically makes sense to add it to, our platform – this will help us to advance any of these programs.

AFFILIATION

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AUTHORSHIP & CONFLICT OF INTEREST

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Manufacturing of RCA-free adenoviral vectors

Nico Scheer, Senior Consultant Business Development, CEVEC Pharmaceuticals

Adenoviral vectors are frequently used as delivery tools for vaccine and gene therapy applications. Once such vectors have infected a target cell, it is of utmost importance that they do not spread further in a patient's body. Therefore, avoiding the occurrence and further propagation of replication-competent adenovirus (RCA) during production is crucial. Since the risk of RCA occurrence depends on the adenoviral vector and the cell line used for virus stock generation and manufacturing, selecting an appropriate cell line and production platform is critical.

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ADVANTAGES OF ADENOVIRAL VECTORS FOR MEDICAL APPLICATIONS

Adenovirus is an intensively studied vector offering simple vector construction, efficient transduction and high stability, which enables simple handling and long-term storage. Adenoviral vectors are increasingly being developed for both gene therapy and vaccine applications. Focusing on vaccines in particular, adenoviral vectors offer a number of advantages including the stimulation of a robust immune response, and the fact that the use of a strong promoter can result in more persistent duration of antigen expression when compared to vaccines based on recombinant protein or inactivated viruses. In addition, recently-approved adenoviral vaccines for COVID-19 represent a strong precedent for the high suitability of these vectors.

IMPORTANCE OF AVOIDING RCA IN THE MANUFACTURING PROCESS

The formation of RCA is highly undesirable during vaccine and biotherapeutic development, as it can trigger adverse immune responses and spread in the human body. Consequently, regulators such as FDA and EMA have issued guidance on maximum levels of RCA. Preparations must be tested for RCA and discarded if they exceed the allowed threshold, which can lead to significant additional cost and delays in clinical development and supply to the market.

ACHIEVING RCA-FREE PRODUCTION BY DESIGN WITH THE CAP® AD PLATFORM

Usually the early region 1 (E1) in the viral genome, which is necessary for replication, has been deleted in adenoviral vectors, resulting in so-called AdΔE1 vectors. In order to produce a vector that lacks an essential component for its own replication, a complementary cell line that carries a stable insertion of the E1 region is needed. However, common cell lines such as HEK 293 carry the risk of spontaneous RCA formation due to the presence of two large homology regions and the resulting probability of homologous recombination **(Figure 1)**.

The CAP Ad platform has been specifically designed to address this as CAP cells contain only one very short and additionally inverted homology region, thereby minimizing risk of RCA formation **(Figure 1)**.

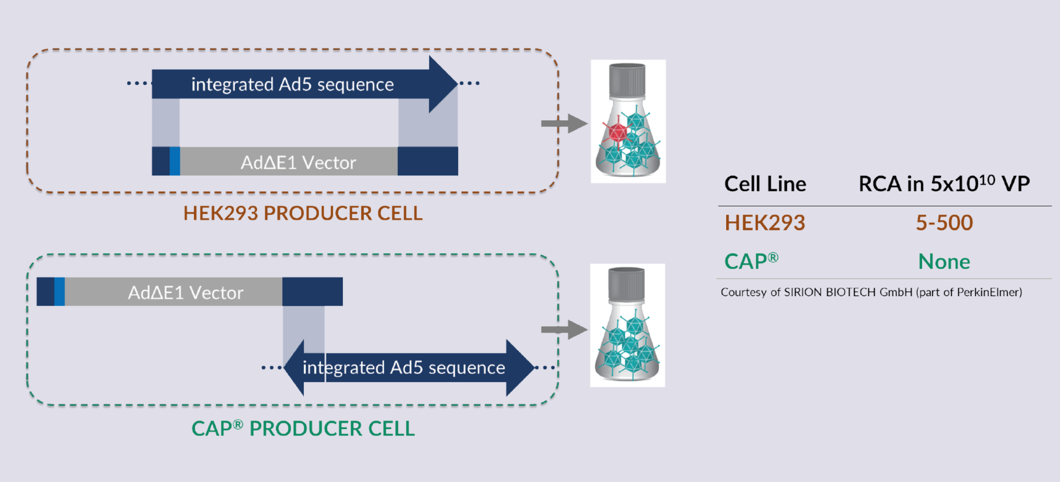
INDUSTRIAL SCALE MANUFACTURE WITH THE CAP AD PLATFORM

CAP cells are an ideal producer cell line for the development of biotherapeutics. The cell line is derived from human amniocytes, fully documented and available as a GMP bank. A Biologics Master File has been deposited with the FDA and can be referenced.

CAP cells can be cultivated in all common forms and scales of bioreactors using serum-free, chemically defined media and cell densities reach up to 15–20 million cells/mL in fed-batch, with high viabilities and productivities.

In summary, the CAP Ad platform provides a unique solution for industrial scale manufacture of adenoviral vectors at high titers, while minimizing the risk of RCA formation and therefore complying with recent regulatory standards **(Figure 2)**.

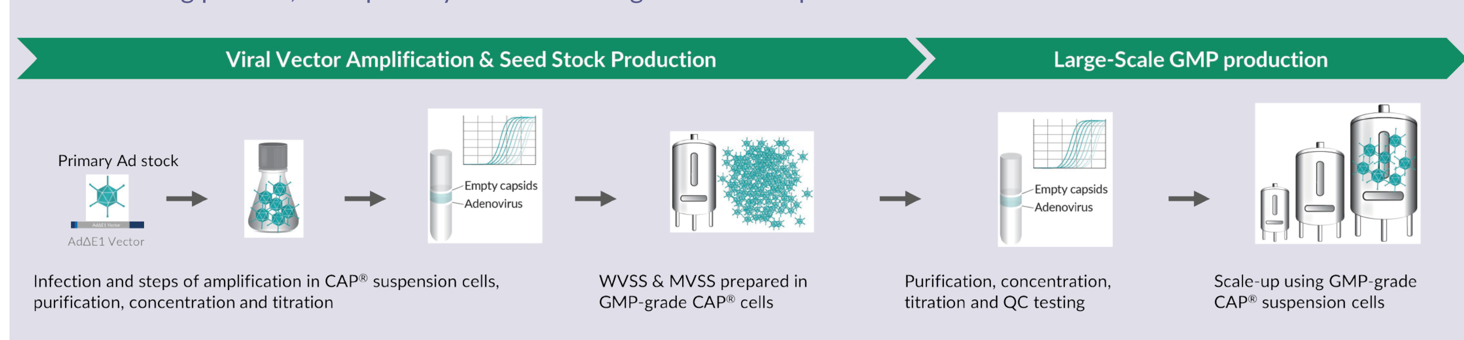
Figure 1. RCA-free production by design in CAP cells. CAP cells contain only one very short and inverted region of homology to the AdΔE1 vector. No RCAs were detected in CAP cell derived material.



Cell Line	RCA in 5x10¹⁰ VP
HEK293	5-500
CAP®	None

Courtesy of SIRION BIOTECH GmbH (part of PerkinElmer)

Figure 2. CAP Ad platform: Industrial scale manufacturing with minimized risk for RCA formation throughout the entire manufacturing process, from primary Ad stock to large scale GMP-production.



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Accelerating cell & gene therapy workflows with next-generation analytical tools

Chris Heger

Modern medicines call for modern technologies. Revolutionary cell and gene therapies offer significant promise to treat life-threatening diseases. However, getting therapies to market quickly and efficiently requires accurate testing of critical quality attributes, including accurate viral vector analysis and cell characterization. This article discusses how a variety of innovative analytical tools can aid in the automation and scalability needed on the cell and gene therapy road to discovery, including rapid and accurate analysis of cells, molecules, and contaminants, along with consistent, high-quality data across project phases. These next-generation analytical solutions are also assessed for the ease with which they fit into current workflows and their adaptability to changing needs.

Cell & Gene Therapy Insights 2022; 8(3), 463–473

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NEXT-GENERATION ANALYTICAL TOOLS

Bio-Techne has a broad portfolio of manufacturing and analytical solutions for biopharmaceutical and cell and gene therapy (CGT) applications. These include a wide variety of good manufacturing practice (GMP)-grade reagents including cytokines, growth factors, small molecules, media, and supplements. A wide variety of critical research reagents including industry-leading immunoassays, antibodies, proteins, and *in situ* hybridization (ISH) technologies are also available. Additionally, Bio-Techne provides various automated analytical instruments through the

Protein Simple brand. There are four analytical platforms in the context of these workflows: Simple Plex, Simple Western, Maurice, and Micro Flow Imaging (MFI).

SIMPLE PLEX

Simple Plex assays run on the Ella platform can provide an automated alternative to ELISA within C> analytics. Ella runs ELISA assays in 1.5 hours and requires only 25 µL of sample volume per well. The cartridges come in a variety of formats to provide sample, input, and analyte flexibility, and are supported by a broad assay menu. These cartridges come with built-in standard curves for analytes, saving assay space for important samples and imparting a high level of standardization. The platform has high sensitivity and a wide dynamic range, with excellent lot-to-lot consistency. Simple Plex assays are also easily transferrable to other sites.

US Pharmacopeia (USP) 1047 for gene therapy products states that "When the biological function of the expressed transgene exhibits a broad range of activities or only generates semiquantitative results the ELISA or other immunological or biochemical readouts can be used as a surrogate potency assay." There are currently two Simple Plex viral titer assays to address this part of the workflow, for both AAV2 and p24, a human immunodeficiency viral (HIV) protein found in lentivirus.

Specificity and robustness to matrix efforts are major advantages of ELISA, although it has long turnaround time, moderate throughout, and labor-intensive sample preparation.

Bio-Techne has partnered with Progen to use their antibodies in the Simple Plex assay cartridges to produce an AAV2 assay. It can run up to 72 samples per cartridge, with a built-in standard curve to allow the addition of any extra samples that may be required. This assay has good correlation with the Progen ELISA, providing confidence in the Simple Plex to measure viral titer.

Figure 1 shows the standard curve with a detection range between 3×10^6 capsids/mL and 1.3×10^{10} capsids/mL and a dynamic range of 3.6 logs. The assay also performs well on precision assays and can accurately measure AAV as a complex sample. This can be performed over a range of dilutional linearities for several commonly encountered sample matrices including clarified lysate, column eluates, and bulk product.

Similarly, a Simple Plex assay is available for measuring p24. Quantification of p24 can be used to estimate lentivirus titer. There is a built-in calibration curve with limit of detection of 0.67pg/mL, and the assay has strong performance with inter-assay coefficients of variability (CVs) under 10%, and intra-assay CVs under 7.1%, while also showing good linear recovery in several sample types.

Characterization of cultured immune cells following activation, gene transfer, and expansion is complex and leverages both cell surface markers and soluble secreted biomarkers. Simple Plex's multi-analyte cartridges run on the Ella instrument provide an ideal platform for fast and accurate quantification of secreted markers and cell culture supernatants. Customizable assay patterns eliminate the need to run multiple ELISAs, and assays with up to 4 logs of dynamic range allow markers of varying abundance to be measured from a common sample dilution. These panels include a T cell and natural killer (NK) cell activation panel, a cell cytokine secretion panel, as well as a chemokine secretion panel.

To examine T cell activation with Simple Plex, an experiment was conducted using the TcBuster™ transposase non-viral gene editing technology to introduce a CD19 CAR into T cells. These T cells were then used in a luciferase-based assay against two cell lines – K562 myelogenous leukemia cells that do not express CD19, and Nalm-6 acute lymphoblastic leukemia cells that do. T cells that can recognize the CD19-positive cells become activated and release granzyme B, TNF-α, IFN-γ, and IL-2, as measured on Ella **(Figure 2)**.

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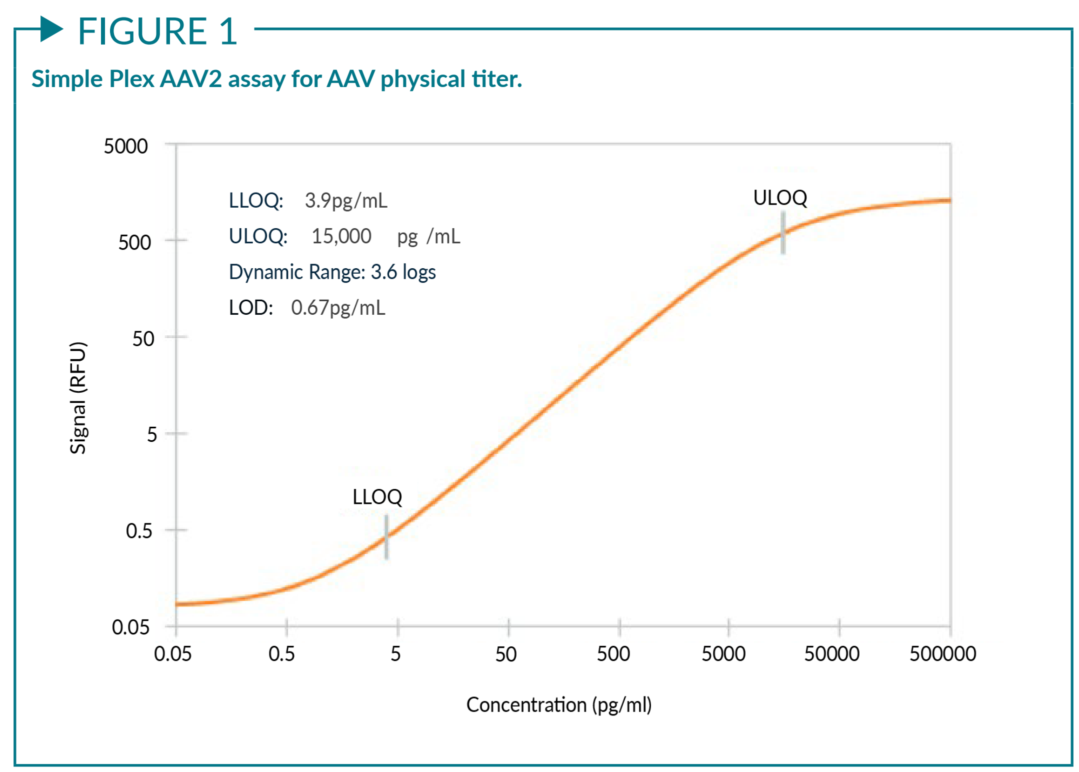
FIGURE 1

Simple Plex AAV2 assay for AAV physical titer.



SIMPLE PLEX HEK293 HCP 3G ASSAY

Bio-Techne has also partnered with Cygnus to bring an HEK293 host cell protein (HCP) assay to Simple Plex. The Simple Plex HEK293 HCP 3G assay features a 3-log dynamic range with a lower limit of quantitation (LLOQ) of 1.64 ng/mL. The assay has excellent correlation to the Cygnus ELISA but is much faster, with better reproducibility. The assay exhibits excellent spike and recovery across a wide variety of process samples. Importantly, the assay performs well with different sample matrices and has good dilutional linearity across the five most encountered samples.

SIMPLE WESTERN: GEL-FREE, BLOT-FREE, HANDS-FREE WESTERN ANALYSIS

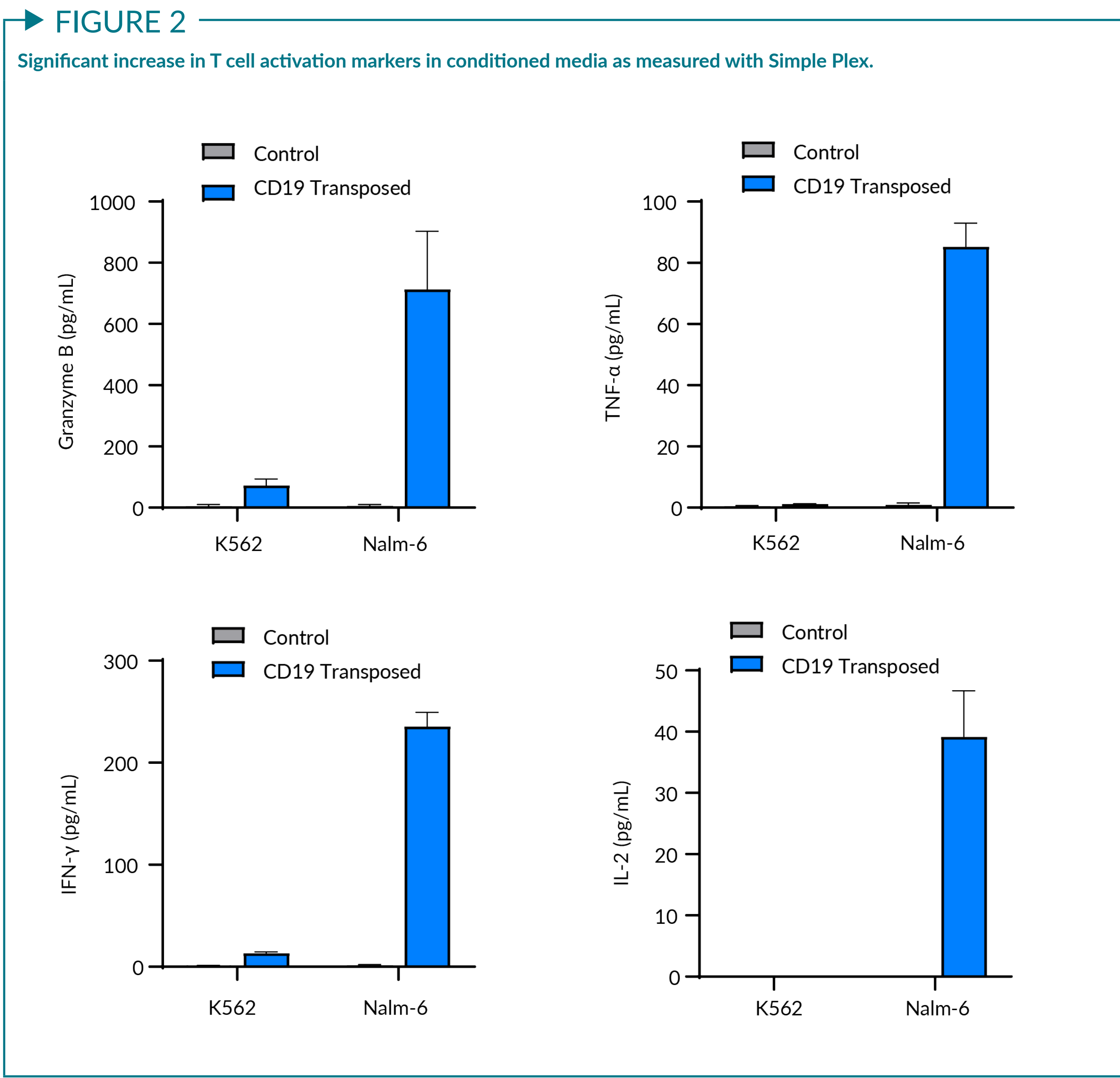
Simple Western is a fully automated capillary-based immunoassay that requires as little as 3 µL of sample to perform highly reproducible and quantitative Western analysis. As an open platform, conventional Western blot antibodies may be used in multiple detection modes, including chemiluminescence, 2-color NIR/IR fluorescence, and total protein detection. Simple Western is highly sensitive, generating assays with a wide dynamic range of detection. In addition, Simple Western features RePlex™ on Jess™ and Abby™ instruments, which performs two sequential immunoassays in the same capillary to double the amount of data per run. Because Simple Western is fully automated, it is reproducible and can easily be transferred to other sites.

In CGT applications, Simple Western is a multi-attribute platform for monitoring the critical quality attributes (CQAs) of viral vectors like AAVs, providing six assays on one platform: purity, identity, capsid protein ratio, empty-full status, physical viral titer, and protein expression potency. Many of these attributes can be measured in a single run with multiplex, RePlex, and total protein detection.

Figure 3 shows an example of a quantitative AAV assay on Simple Western that simultaneously provides identity and purity measurements. The first probing cycle of RePlex looks at the identity of 4 AAV samples with an anti-VP1/2/3 antibody. These results show that AAV1 and AAV4 are not recognized by

FIGURE 2

Significant increase in T cell activation markers in conditioned media as measured with Simple Plex.



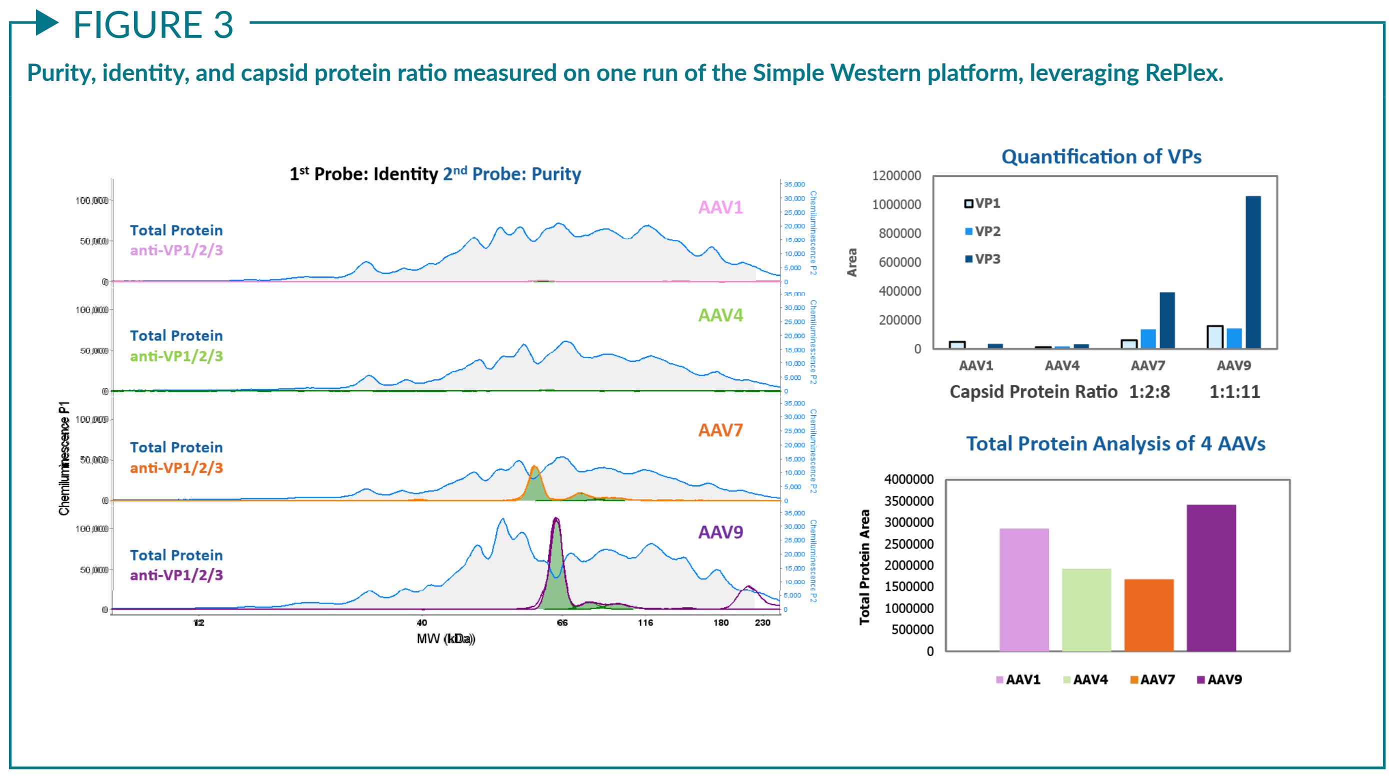
the antibody, but AAV7 and AAV9 are detected. Additionally, AAV7 and AAV9 appear to have VP capsid proteins of different sizes. From these data, it is possible to measure VP1:VP2:VP3 stoichiometry, which was 1:2:8 and 1:1:11 for AAV7 and AAV9, respectively. In the second cycle of RePlex, total protein can be detected on the same sample, which reveals the presence of non-AAV protein. These data are quantified automatically by Compass for Simple Western software for protein expression normalization or purity estimation.

Recently, a Simple Western assay was developed to quantify the content ratio, or the ratio of full to total AAV particles in a sample. In the Simple Western assay, denatured AAV vectors are run on Jess using RePlex and interrogated with an anti-DNA antibody in the first probing cycle and an anti-VP1/2/3 antibody in the second probing cycle. This analysis shows the detection of VP1/2/3 at the expected molecular weight range, and DNA in the upper MW region that increased with increasing % full AAV sample **(Figure 4)**. The signals from these two antibodies are used

 DOI: 10.18609/cgti.2022.066

FIGURE 3

Purity, identity, and capsid protein ratio measured on one run of the Simple Western platform, leveraging RePlex.



to calculate VP1:VP2:VP3 stoichiometry and content ratio simultaneously. Because Simple Western uses only 3 µL of starting material and provides rapid, sensitive, and reproducible analysis on crude and purified samples alike, it is anticipated that Simple Western can provide multi-attribute analysis that scales with AAV manufacturing workflows.

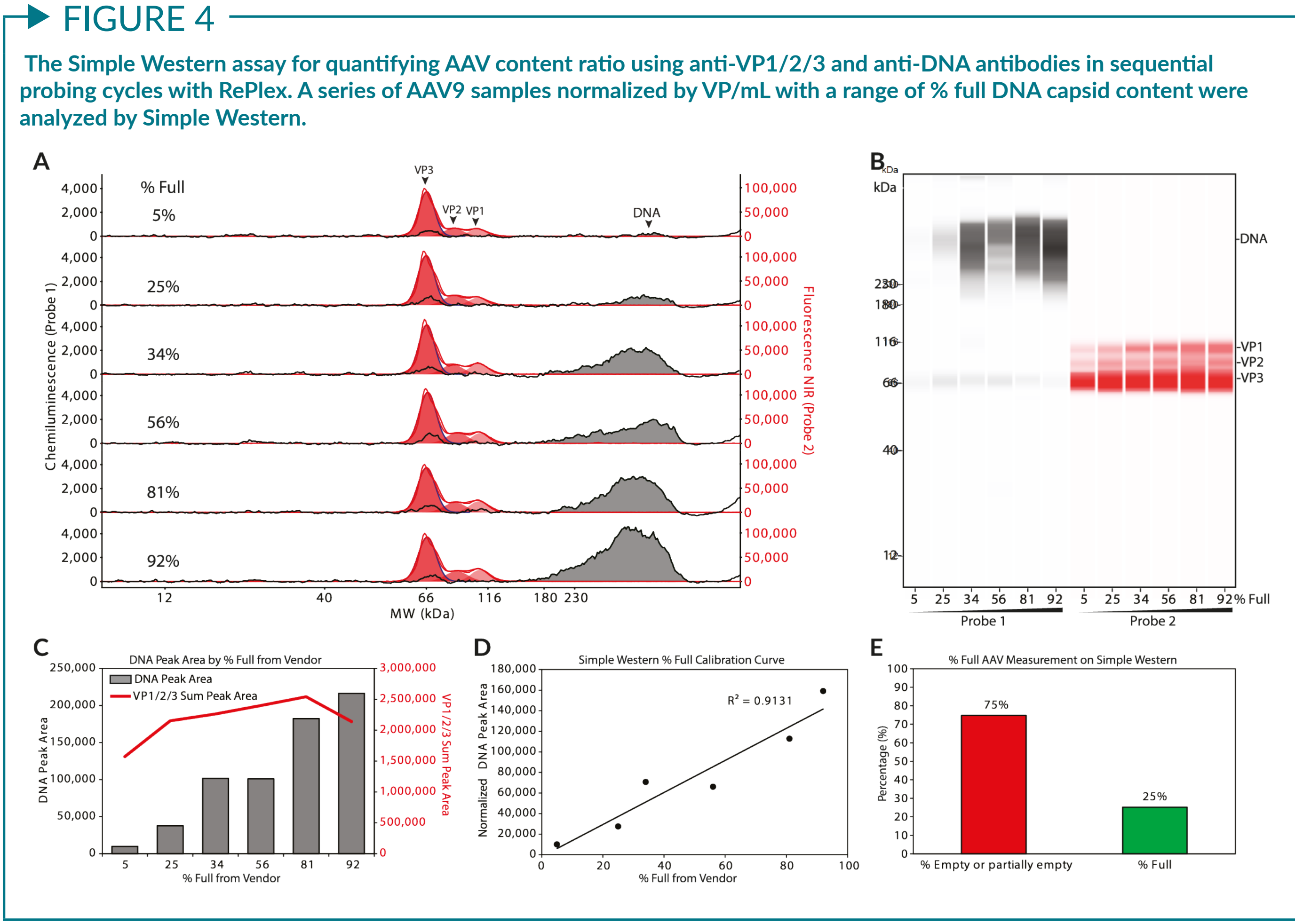
MAURICE: THE NEXT-GENERATION CAPILLARY ELECTROPHORESIS PLATFORM

Maurice provides the gold standard image capillary isoelectric focusing (icIEF) for charge heterogeneity analysis of proteins with absorbance and native fluorescence detection. In addition, Maurice has capillary electrophoresis sodium dodecyl sulfate (CE-SDS) for direct detection and purity analysis of proteins, which is useful for AAV capsid ratio and purity. Maurice can provide data within 10–15 minutes per sample, thus enabling faster decisions and rapid method development. The instrument is easy to use and has cartridges that dictate the mode run. The software is 21 CFR part 11 compliant with optional Empower™ control, and methods on Maurice are easily transferred to other sites. Maurice is an ideal late-stage viral vector characterization tool, best suited to support viral vector characterization and CQA monitoring with purity, identity, capsid protein ratio, empty/full, and stability assays.

AAV product purity is a particularly important CQA to monitor, and Maurice CE-SDS is a direct SDS-PAGE replacement in this context. Four pre-clinical AAVs from two masked serotypes from an industry collaborator, named “M” and “S”, were studied. There are several peaks observed in addition to the core capsid viral proteins (VPs) VP3, 2, and 1, shown in Figure 5. These peaks, down to a 0.1% impurity, can be quantified and used to assess overall product purity. For these four samples, purity was greater than 91.9%. The average capsid ratio was 6:1:1, which was the expected result. In addition to the purity assessment and capsid protein ratio, a novel peak was identified in Sample S2, named peak 5, at a 0.8% total peak area level. These data show an example of how Maurice can support identity in addition to purity and VP ratio, all in the same run.

FIGURE 4

The Simple Western assay for quantifying AAV content ratio using anti-VP1/2/3 and anti-DNA antibodies in sequential probing cycles with RePlex. A series of AAV9 samples normalized by VP/mL with a range of % full DNA capsid content were analyzed by Simple Western.



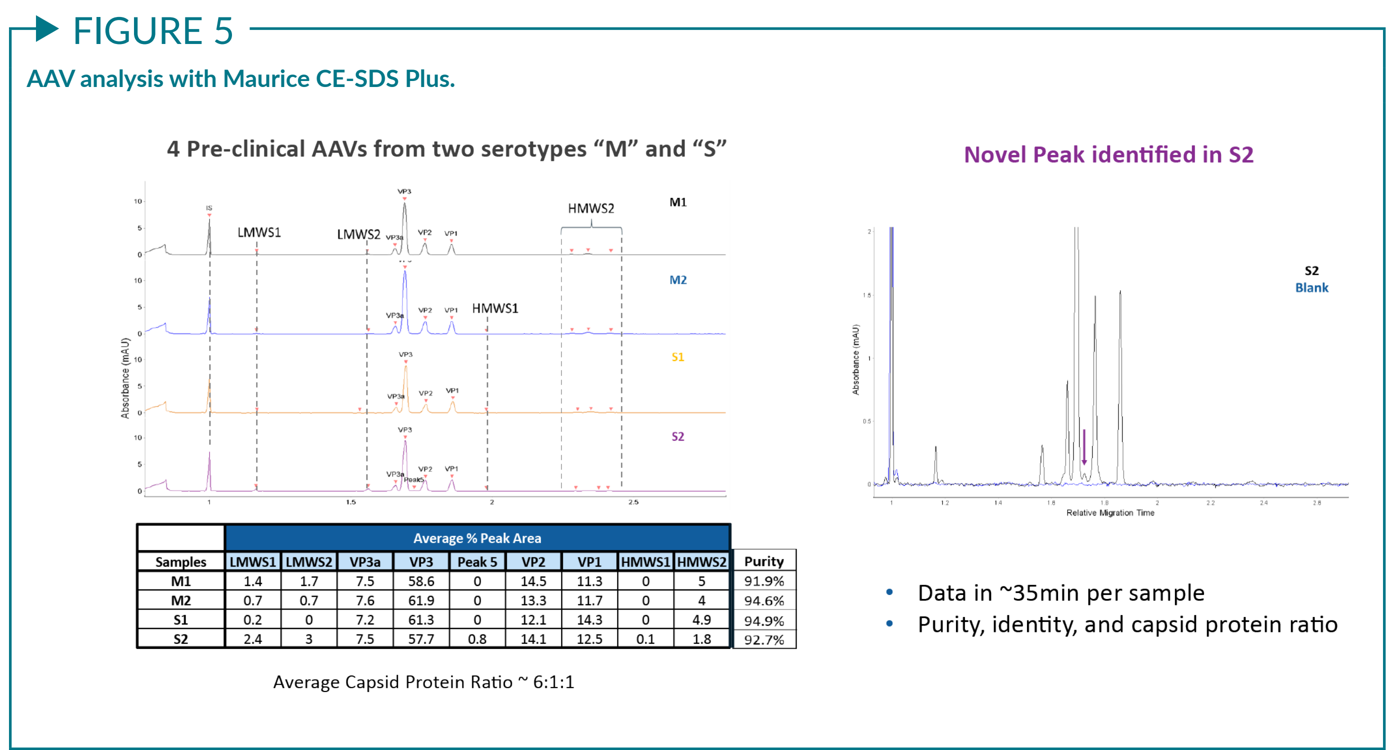
Imaged cIEF, also known as icIEF, is a powerful identity tool. Bio-Techne has developed two methods to look at AAVs. The first is a capsid protein icIEF method, which can be used for identity and stability indication, and the second one is an intact protein method that can be used as an identity assay. An evaluation of a panel of AAV serotypes using the capsid protein icIEF assay is shown in Figure 6. These samples were denatured and diluted to 4×10^{11} GC/mL for analysis. For all 8 serotypes, a minimum of 3 peaks can be seen, and in several samples, up to 5 peaks are readily quantifiable. Each serotype has not only its own series of peaks, but its own set of pI values and peak ratios, showing that the method can be used as an identity assay. While most of the AAVs exhibit a similar profile, AAV5 is shown to be the most acidic in this method, and AAV6 is the most basic. AAV6 and AAV1 are 99% identical, differing by only 6 amino acids. Yet, they differ significantly by icIEF. The pI of each 6-mer was calculated to show how much more basic the AAV6 peptide is, which explains the data and shows the power of isoelectric focusing.

Maurice icIEF features dual wavelength detection, which means the method's utility may extend beyond identity. Maurice provides absorbance detection at 280 nm, and these profiles are often influenced by DNA, which can significantly absorb energy at that wavelength. Maurice also has a native fluorescence (NF) filter which is centered closer to 350 nm, where the signal is exclusively from protein. If we compare the absorbance and NF signals together, we can de-convolute the DNA and protein signals coming from an AAV and our intact method. These NF signals can be quantified in addition to the absorbance signals, as the former can be used to normalize any differences in loading between the samples.

 DOI: 10.18609/cgti.2022.066

FIGURE 5

AAV analysis with Maurice CE-SDS Plus.



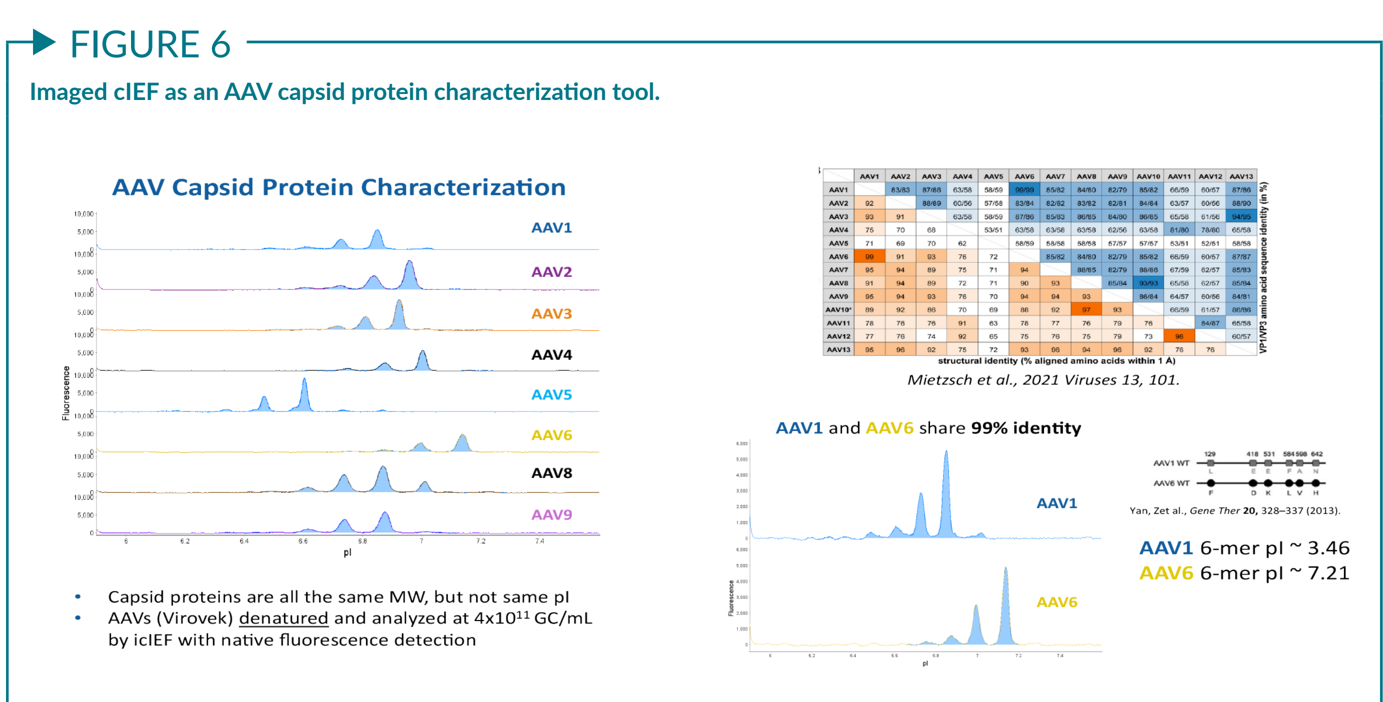
Samples	LMWS1	LMWS2	VP3a	VP3	Peak 5	VP2	VP1	HMWS1	HMWS2	Purity
	Average % Peak Area									
M1	1.4	1.7	7.5	58.6	0	14.5	11.3	0	5	91.9%
M2	0.7	0.7	7.6	61.9	0	13.3	11.7	0	4	94.6%
S1	0.2	0	7.2	61.3	0	12.1	14.3	0	4.9	94.9%
S2	2.4	3	7.5	57.7	0.8	14.1	12.5	0.1	1.8	92.7%

MICRO FLOW IMAGING (MFI)

MFI is a next-generation particle imaging platform to support CGTs. MFI can support the United States Pharmacopeia (USP) and the US Food and Drug Administration (FDA) mandated particle counting for biopharmaceuticals, namely the USP 787/788, and is commonly used for counting biopharmaceutical product aggregates. The MFI features direct, dynamic sample imaging of particles between 1 and 300 µm, with high-powered CFR 21-part 11 compliant analytical software. Particle data is available in 6 minutes per sample, and this MFI platform and method can be easily transferred to other sites.

FIGURE 6

Imaged cIEF as an AAV capsid protein characterization tool.



MFI can support CGT by monitoring particles within workflows. In addition to being an ideal subvisible particle aggregation tool, MFI has been shown to indicate stability, as well as being useful for monitoring impurities in cell therapy workflows.

Cloudz™ are dissolvable microparticles that can be used for cell activation and expansion. Figure 7 shows data from a study of the human NK cell expansion kit. The Cloudz feature CD2/NKp46 microspheres and readily dissolve in the presence of the 1x Release buffer.

When it is time to dissolve the Cloudz, one can look at both before and after the addition of release buffer. Because MFI can easily distinguish the two particle populations in the same sample, the amount of residual Cloudz can be monitored as part of the expansion process.

CONCLUSION

In summary, Bio-Techne provides a range of innovative solutions to meet CGT research and manufacturing needs. Premium analytical instrumentation from the Protein Simple brand can analyze multiple attributes for CGT products. Simple Plex supports viral particle titer, residual host cell impurity analysis, and measures cell activation and cell expansion. Simple Western supports six assays on one platform: identity, purity, capsid ratio, empty/full and viral titer, and protein expression potency. The Maurice CE platform is a dual-mode instrument ideal for formulation and late-stage characterization, with CE-SDS and image cIEF for capsid ratio and purity, and for identity, empty/full, and potency respectively. Lastly, the MFI particle counter is an ideal imaged subvisible aggregate tool for CGT formulation development.

FIGURE 7

Cell therapy product purity assessment with MFI.

Cloudz Human NK Cell Expansion Kit

Catalog # CLD004

Dissolvable Cloudz CD2/NKp46 microspheres for NK Expansion

Cloudz Dissolve with Release Buffer

Measure NK Cells and Cloudz

Filtered Average Particle Counts

Cloudz

NK Cells

Average Counts Per Analysis

CellGenix+ FBS Medium

NK Cells & Cloudz in Medium

Medium+ 1xRelease Buffer (1:1)

NK Cells & Cloudz in Medium+ 1xRelease Buffer

Medium+ QT-HEPES Buffer (1:1)

NK Cells & Cloudz in Medium+ QT-HEPES Buffer

Error Bars = STD DEV (N=3)

NK

Cloudz

DOI: 10.18609/cgti.2022.066

Q&A with Dr Chris Heger

David McCall, Editor, *Cell and Gene Therapy Insights* **speaks to Dr Chris Heger,** Director of Applications Science Analytical Solutions Division Bio-Techne.

Q How does your empty/full assay work with novel developed capsids outside of the parental panel you have?

CH: With Simple Western, we have so far looked at one pharmaceutical AAV that is non-wild type variant and have shown that it also works well with the empty/full method. We are encouraged by those data, and we look forward to other researchers testing the assay with their own AAVs soon.

Q Are Simple Plex assay panels fixed or can users design their own panels?

CH: The Simple Plex assay menu can be customized to what you need. With the cell therapy panel, you can choose individual cytokines, and we will build cartridges with the analytes you need all on the same cartridge.

Q Is there a list of antibodies suitable for Simple Western applications? What resources are available to guide assay development on the Simple Western platform?

CH: We have an antibody database on our website that has nearly 3,000 antibodies verified to work on Simple Western. The AAV viral protein antibodies from Progen are listed on our website with the working dilutions, and the anti-DNA antibody information will also be available on the website.

For assay development, there are many great resources available through our learning academy, which is geared towards helping customers teach themselves. We also have a fantastic field application science team to help with your assay development as needed.

Can you tell us more about Simple Western's RePlex technology?

CH: RePlex is an elegant and automated, strip and re-probe – using the same concept as done with traditional Western blotting. In this case, it is part of our automated capillary Western block workflow, and it works by adding only one additional row of reagents to the assay plate. The instrument will automatically strip the capillaries of the first immunoassay and allow for a second probing of the same capillary, giving you double the data per sample.

Which instrument is preferred for empty/full analysis?

CH: The isoelectric focusing method on Maurice and the size-based separation on our Simple Western platform are both suitable for empty/full analysis.

Both have solid strengths and are powerful tools. The Maurice method leverages direct detection and will be more amenable to downstream use, but it is isoelectric focusing, so you may have to develop a more specific method for your AAVs.

The size assay is more universal in that respect, although it does rely on immuno-detection. If your DNA-packaged material does not react with the antibodies, or the VP antibody does not detect one of your VPs, these could be considerations on why you would choose one or the other.

BIOGRAPHY

CHRIS HEGER, PhD Director of Applications Science for the Analytical Solutions Division of Bio-Techne, received his doctoral degree in Pharmacology from Cornell University and completed his post-doctoral training at the National Cancer Institute in Antibody Development, Purification and Technology Evaluation. Chris then joined Protein Simple, a Bio-Techne Brand, where he has worked for nearly 10 years. Chris currently leads the Applications Science group, chartered with application development, creating scientific collateral, fostering collaborations, and training. Chris is an expert in immunoassays, capillary electrophoresis, protein and antibody purification, and chromatography.

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For more information
on Bio-Techne cell and gene therapy solutions,
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CELL & GENE THERAPY INSIGHTS

VECTOR BIOPROCESSING

SPOTLIGHT

Considerations for demonstrating comparability of AAV processes

Laura Giersch
Lysogene

"...it is important to try to stockpile material during different virus productions in order to ensure that if a comparability study is required one day, the requisite material will be available."

VIEWPOINT

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Lysogene is a gene therapy company working on AAV-based projects for diseases of the CNS. We have a pipeline targeting neurodegenerative diseases, including mucopolysaccharidosis type IIIA (Sanfilippo syndrome A), GM1 gangliosidosis, Fragile X syndrome, and a therapy for both Gaucher and Parkinson's diseases. These projects range from the preclinical stage up to pivotal trial stage.

We are a virtual company, so we exclusively rely on contract development and manufacturing organizations (CDMOs) and contract research organizations (CROs) for our research and production projects. Thus, we need to be properly skilled in managing CDMOs, and the process development, analytical work, and clinical production required for both early and late-stage clinical trials. We have internal experience using various adherent processes and are working on building our suspension-based process at Lysogene.

I have worked at Lysogene for the past 7 years in AAV process development, GMP production, analytical development, validation, and quality control. Prior to joining Lysogene, I worked at Avalanche Biotechnologies on AAV-based process development.

KEY TRENDS IN A MATURING GENE THERAPY FIELD

The gene therapy field is currently evolving towards treating a broader range of indications through new ways of treating disease, such as new routes of administration. This includes diseases that are not rare or orphan, for which a greater amount of vector material is required. Production is key to ensure this can be achieved. Much optimization of processes has been performed by industry over the past few years, allowing us to try to reach these new disease targets.

This is something we are seeing at Lysogene, with the recent launch of a Fragile X syndrome development program and an investigational therapy that is applicable to Parkinson's disease, for which there are obviously many eligible patients. Furthermore, it is a trend that stretches beyond localized administration and treatment of diseases (e.g., retinal diseases) which is not so highly demanding in terms of material. Now we are seeing more projects targeting the central nervous system (CNS) or other organs, which brings with it an additional need to increase the amount of vector produced.

We are also seeing internalization of vector manufacturing by those companies that can afford it, in order to better control the supply chain.

CHALLENGES IN SCALING UP AAV PROCESS

Over the past few years, the industry has matured significantly, particularly with the use of HEK293 cells for AAV production. The use of HEK293 in the adherent cell culture setting brings up the issue of scale-out, as opposed to scale-up with suspension culture.

Most CDMOs and biotechnology companies are now switching to a HEK293 cell-based suspension process. Suspension is possible with baculovirus, too, but that comes with its own challenges. Generating a 200 L batch was a notable achievement a few years ago, but this is now routine in the industry. Most of the industry is now equipped with 1,000 L bioreactors in anticipation of future increases in demand and in preparation for commercial production.

Although this is now a much more developed process, it still comes with challenges. For example, there is a need for a producer cell line adapted to suspension. Most of the CDMOs are currently generating the proprietary clones, which brings forth issues of intellectual properties.

There also is an emerging trend of moving to a platform model. This model allows faster development by adapting a proven process to the sponsor's specific AAV serotype and transgene. Some CDMOs are now proposing their own platform process only, without any room for optimization or alteration of steps. While this does allow the CDMO or the biotech to

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master its process and thereby increase the robustness, it comes with new intellectual property (IP) challenges for those processes and the cell lines used. It can also increase the level of complexity in terms of compatibility, if one is going to bring the platform process in for late-stage development phases. Finally, it introduces a lot of constraints and/or challenges regarding the supply of plasmid, which needs to be anticipated and comes with increased cost. The field of plasmid production needs to adapt to those increased demands in time to allow the processes to deliver. Of course, scale-up requires increased volumes of other raw materials such as culture media, which also needs to be anticipated and closely monitored to ensure production is not delayed.

When switching to a suspension process, one must ensure that the downstream process is also properly scaled up. This is less of a challenge, but still something to pay attention to – if the downstream process is not streamlined, future process changes may result.

KEY CONSIDERATIONS WHEN TRANSITIONING TO A SUSPENSION CULTURE-BASED AAV PRODUCTION

When transitioning to a suspension culture-based AAV process, one key consideration is selection of the correct cell line. Selection criteria include productivity, robustness, IP, and the needs of the company in general. For example, there are many companies working on proposing suspension cell lines, but these come with IP restrictions. The best strategy for each company needs to be established, whether it is developing its own cell line or using an existing one. Many companies have already developed highly productive packaging cell lines, and the next generation of packaging cell lines are already coming to the market. Soon, there will no longer be a need for plasmids, which will reduce cost of goods (COG).

When selecting a cell line, it is important to look at its history, including the clonality status, to ensure it is properly characterized. The regulatory authorities will scrutinize the cell line closely and a sub-optimal cell line could be rejected for use in clinical trials, or down the line in commercialization.

The scale of the process should also be properly designed to encompass potential future scale requirements for later stages of development, which could trigger the need for new comparability studies. If one is producing vector at a smaller scale during Phase 1, it is important to ensure the downstream process is designed to be scaled up easily for the future stages of the project. It is also important to have a proper development plan in place for the entire process.

When shifting to suspension, one must re-evaluate all of the transfection parameters. If possible, it is important to design the process with a design of experiments (DoE) approach described in ICH Q8.

The stage of transition to a suspension base is also important and should be evaluated well in advance. It is better to start with the suspension process from the beginning, but the industry has evolved very quickly, and many current processes did not have the option to wait for a suitable suspension process to become available. It is an important decision for a company, and it should be taken using a risk assessment approach to understand all implications of the changes to the process. This will help with the identification of risks and how to mitigate them, and also assist in subsequently defending the process change to one's own company and Board of Directors, if necessary.

Of course, these changes will require a major comparability exercise that should be planned as early as possible.

APPROACHING COMPATIBILITY COMPONENTS IN AAV PROCESSING

It is important to understand the changes that are implemented between the initial process and the modified process, and potentially, changes to the product critical quality

attributes (CQAs). The CQAs should be defined before one begins to consider comparability exercises.

Firstly, it is important to know if the changes are process-related or analytics-related. We are seeing an evolution in the analytical methods employed for product release, with a shift to newly developed and less variable methods. Changes to those analytical methods should also be evaluated and taken into consideration in the comparability exercise. Risk assessments should be performed involving both the internal process development department and external expertise in order to review potential preclinical, clinical, and regulatory impacts.

Following the risk assessment, it is important to establish a comparability plan. The comparability plan should include the comparability study design (e.g., whether side-by-side analysis or head-to-head analysis is required, and the potential need for toxicology studies or even clinical studies). It should not be limited to drug product but should also include as much in-process control as appropriate. Analytical methods employed and their qualification/validation statutes should be laid out in the plan. It is also important to understand if any method changes occurred between the two processes and to evaluate them. Process performance evaluation can also be included in the comparison between the initial and modified processes.

Additional product characterization may be advised to ensure a complete understanding of the product and various orthogonal methods, up to and including the release testing. To cite one key current example, the industry and regulators are paying greater and greater attention to the empty-full ratio of AAV capsids, and various methods may be employed to measure this ratio. The stability of the product should also be demonstrated, and the results should be compared to ensure equivalent stability between the two products and processes.

The acceptance criteria for concluding on the comparability of the product should be set out in the plan using appropriate statistical methods. Many biotechs will not have this capability in-house, making it necessary to seek the help of a biostatistician to select the appropriate statistical tools and set the acceptance criteria.

One of the challenges in conducting comparability studies is the material availability. The industry is currently very limited in terms of material availability from production batches, especially for adherent processes. Head-to-head studies may be limited based on this challenge. Therefore, it is important to try to stockpile material during different virus productions in order to ensure that if a comparability study is required one day, the requisite material will be available. A further parameter to consider here is the stability of the product: if the product was produced a long time ago, the stability is not always demonstrated – another challenge in conducting robust comparability studies. It is important to anticipate this need and if possible, to qualify a primary reference standard that can be used for comparability testing purposes.

It is key to communicate with the regulatory agencies about comparability. There are various ways to interact with the regulators, and it is important to present a company's intentions regarding comparability demonstration upfront. The agencies can help to assess if what is proposed is satisfactory, or if the company needs to take further action.

Finally, it is valuable to look for available workshops and congresses regarding comparability. This is a topic that is emerging in the industry and consequently, there are a number of knowledge exchange opportunities out there. Everyone is facing the same challenges at the various stages of development.

BIOGRAPHY

LAURA GIERSCH is Director of Technical Operations at Lysogene where she is working for the past 7 years and has experience in AAV process development, GMP production and analytical development and quality control. Laura has a background as pharmacist (PharmD) and previously, she assumed different roles in quality organization at the cell

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and gene therapy unit of La Pitié-Salpétrière Hospital and process development at Avalanche Biotech (now Adverum), bringing a total of a bit less than 10 years in the industry.

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CELL & GENE THERAPY INSIGHTS

VECTOR BIOPROCESSING

SPOTLIGHT

Future trends in viral vector process & product development

Ramji Krishnan
Blue Spring Consulting

"As the program portfolios of companies mature, there is a need for appropriate scale-up or scale-out of viral vector manufacturing strategies."

VIEWPOINT

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THE 'NEW NORMAL' IN GMP VECTOR MANUFACTURING

As the program portfolios of companies mature, there is a need for appropriate scale-up or scale-out of viral vector manufacturing strategies. Currently, there is a significant supply crunch with respect to key raw materials, access to robust manufacturing platforms, and scalability to commercial requirements. The initial approval of therapies in this space focused on rare and ultra-rare diseases.

Companies are now expanding from that space and focusing on unmet needs across a wide variety of disease indications. To address these needs and the potential supply challenges, many manufacturing unit operations are being optimized.

From an upstream perspective, companies are looking to move away from transient transfection and adherent expression platforms to alternate expression platforms such as stable cell lines and suspension cells respectively. Based on the current cost of goods structure for large-scale transient transfection, the cost of raw materials is prohibitive, especially for high doses and larger numbers of patients. From a downstream perspective, companies are focusing on yield improvement and removal of potential process-related impurities (e.g., empty/full ratios). These are areas where one can anticipate some level of improvement.

From a facility standpoint, there is a crunch in available manufacturing space. Most products are currently in early-stage exploration or Phase 1 trials, and will appropriately transition to a regulatory package for pivotal studies and approval. Correspondingly, manufacturing platforms will transition from current 50–200L to commercial scale. Sponsors will need to plan for commercial requirements through either a scale-out or a scale-up approach to accommodate this need.

Additionally, Adeno-Associated Virus (AAV)-driven gene therapy companies have a pipeline of products that include multiple serotypes. This means there are multiple programs moving forward through the sponsor's facility. Manufacturing units need to figure out how to optimize the throughput and the operational aspects of upstream manufacturing with respect to downstream processing to support program requirements and corporate desires. Facilities will need a more modular approach to scaling all units – upstream processing, harvest, clarification, and purification through to final filling.

Another area of development in the viral vector space is the adaptability to disposable manufacturing components. From the downstream perspective, companies are actively exploring affinity and/or ion exchange purification strategies to replace ultra-centrifugation. There is much learning to be leveraged in this space. Many of these unit operations have been optimized in the monoclonal antibody (mAb) space; how soon that knowledge can be leveraged for viral vector manufacturing remains to be seen.

There is also a trend where larger companies are leveraging their facilities to address vector manufacturing supply challenges. There is active collaboration between smaller start-up biotech entities and established pharmaceutical facilities to ensure optimal utilization of resources.

PROGRESS IN ENABLING VIRAL VECTOR PROCESS INTENSIFICATION & STREAMLINING PRODUCTION

Companies need both a short-term strategy and a long-term strategy. Smaller gene therapy biotechs, in particular, should evaluate supply chain challenges especially when capacity could be a limiting factor. They should consider how to initiate Phase 1 studies in the appropriate platform as well as develop a long-term strategy for the amount of viral vector genomes needed for pivotal and commercial success. The desire to scale-up and increase vector titer and yield needs to be balanced with understanding the regulatory landscape and aligned to agency requirements.

When moving from Phase 1 to pivotal studies, even a minor change can have an impact on the safety and efficacy (potency) of the viral product being generated, necessitating a demonstration of comparability.

For example, to support yield through increasing cell density, figuring out when it is most appropriate to infect the cells to generate the amount of virus one wants is key. This can have an impact on product and process impurities. Similarly, any change to

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downstream buffers to optimize capsid content can have an impact on product quality. Such steps to enrich for full capsids can result in co-packaging non-specific plasmid material or host-cell DNA. In all such instances of change, however, comparability is necessary to demonstrate equivalency of final material. Companies must consider clearance of non-product related material and characterize process and product impurities. In other words, it must be a detailed characterization of product quality.

Additionally, it is important to bear in mind that the analytical methods employed will be needed for identification, characterization, and for batch-to-batch consistency and control.

Finally, in-line analytics have advanced significantly in the mAb field. Although still in its infancy in viral vector manufacturing, one can expect these tools to assist in manufacturing decisions.

THE PATH TO COMMERCIAL & REGULATORY SUCCESS

Understanding both the process and the product is very important. Given the nascent nature of manufacturing in the cell and gene therapy space, companies are optimizing many unit operations. As a result of limited long-term safety data and the risk due to process changes on product quality, it is key to align with regulatory expectations early and develop a manufacturing process with a commercial strategy in mind. Along with process development, detailed analytical methodologies to understand process and product is a must. For example, potency assays take a significant amount of time to develop and implement. Viral vector potency assays are inherently complex due to the nature of cell lines used and difficulties in linking their output to the mechanism of action. However, a robust potency assay is key to establishing comparability for any process-related changes

There is going to be an explosion in analytical tools and technologies similar to that which occurred in the mAb space. In addition to the above, characterization of raw materials is critical to boost confidence in the CMC data, especially as one moves towards pivotal studies. Everything begins with the raw materials. Any change in the plasmids or the cell line is going to have an impact on comparability. Standardization of apheresis from pre-clinical to clinical is an area for development.

Understanding all these interrelated elements will significantly expedite any process development activity and ensure alignment with agency expectations.

BIOGRAPHY

RAMJI KRISHNASAMY has worked in the CMC/process development/commercialization space for the past 20 years. He has expertise in biologics process development, process validation, and the regulatory space. Ramji was the Global Regulatory CMC lead for Opdivo. His initial exposure to cell and gene therapy came through Celgene/Juno, working on Lisocel. He subsequently joined Rocket Pharmaceuticals, where he led all of process development, analytical development, manufacturing, and CMC supply chain, along with supporting the Regulatory/Quality functions for cell therapy and gene therapy assets. At Rocket, Ramji also spearheaded the construction of a manufacturing site for gene therapy, successfully executing multiple internal runs at clinical scale and planning for commercial scale-up.

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CELL & GENE THERAPY INSIGHTS

VECTOR BIOPROCESSING

SPOTLIGHT

INNOVATOR INSIGHT

Process development innovations to improve gene therapy manufacturing

Jessica Hillmoe & Michael Shen

As viral vector-based gene therapies move toward indications with larger patient populations, the pressure to improve manufacturing efficiency is rising. To support efficient manufacturing, therapeutic developers must begin by putting a robust process development (PD) plan in place. The industry has been working towards a robust PD plan using innovations such as templated processes to improve yield whilst reducing costs of goods sold (COGS) and time-to-clinic. Templates will help, but there will always be a need for PD to optimize the upstream/transfection, as well as downstream due to differences in the gene of interest (GOI). In this article, we will discuss the PD innovations that Merck's teams have worked on to improve manufacturability, robustness of analytical testing, and scale-down models, all with a view to meeting the needs of large-scale viral vector manufacturing.

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CURRENT STATE OF THE GENE THERAPY INDUSTRY

The gene therapy field has entered what might be termed a modern-day 'gold rush' era, with high levels of interest and activity from the pharma industry and spinout biotech companies alike. Despite the COVID-19 pandemic, 2021 was a record-breaking year for investment in the space, with US$23 billion invested (a 16% increase on 2020).

The majority of clinical trials and investments occur in the USA – however, both are increasing around the world, especially in the Asia Pacific region. The industry is projected to reach a valuation of approximately US$40 billion by 2024.

The most popular vehicles for gene delivery today are adeno-associated viral (AAV) vectors (particularly serotypes 2, 8, and 9) and lentivirus.

Much of the growing confidence in the field stems from several US FDA approvals of cell and gene therapy products in recent years. With more than 1,000 investigational drugs currently in the gene therapy R&D pipeline, the FDA itself recently forecast that by 2025, there will be 10–20 product approvals per year (although the FDA has indicated it will most likely be at the lower end of that range).

The increasing development and investment in the field, alongside the desire to use gene therapy to serve larger indications, means that more companies will be looking to manufacture at large-scale to allow commercialization. A comparison between hemophilia and Duchenne muscular dystrophy (DMD), two key target indications for the industry at present, illustrates the increase in demand for large-scale production. This is both to meet the requirements of indications with larger patient populations, and to enable the ongoing migration towards systems of delivery that require greater quantities of viral vector (Figure 1).

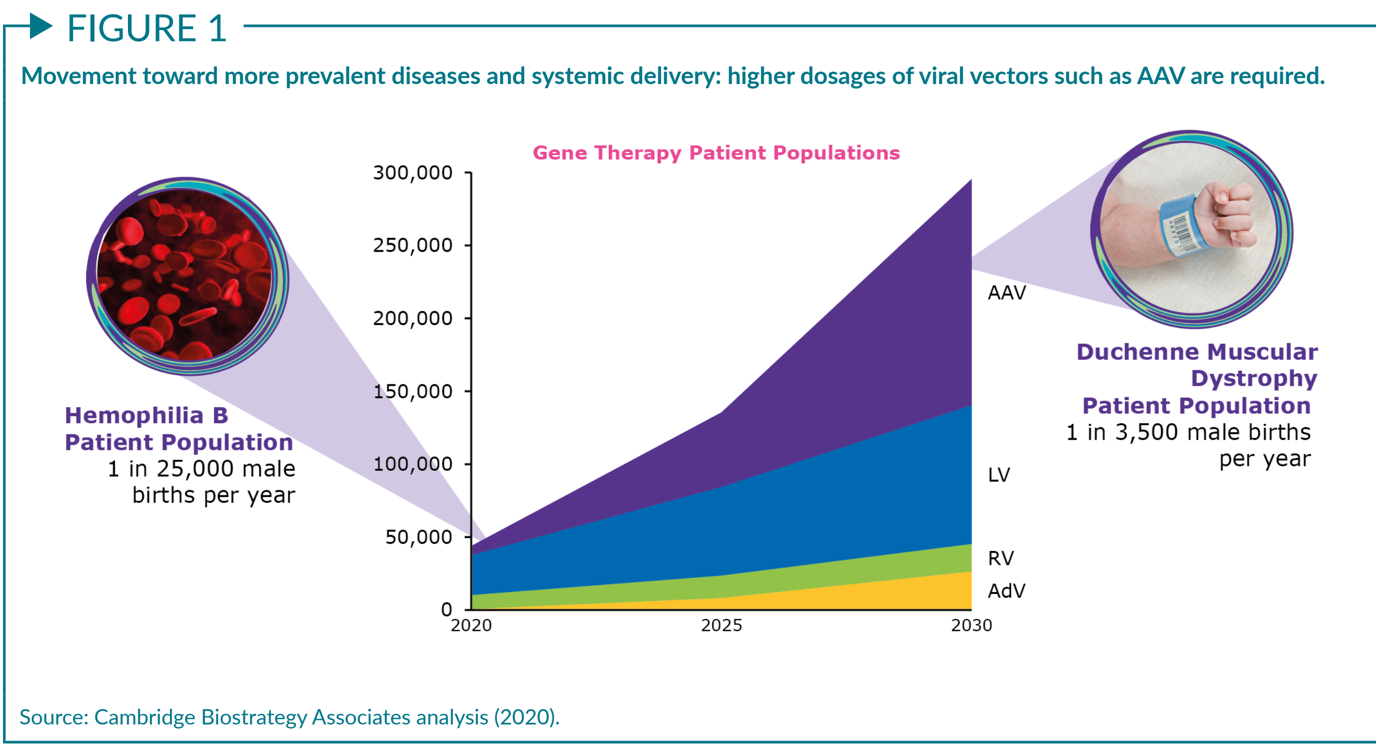
In order to fully realize the potential of gene therapy to address more prevalent diseases such as DMD, it is imperative to industrialize technology for both upstream and downstream manufacturing. Doing so will ultimately drive the reduction of timelines, operational risks, COGs, and enhance patient safety.

IMPROVING & STREAMLINING MANUFACTURING FOR RAPID PROCESS DEVELOPMENT

The sheer number of ongoing gene therapy clinical trials means it is likely that in some cases, multiple sponsors will be pursuing the same therapeutic targets. Additionally, the curative nature of AAV vector-driven treatments, and the rarity of the target indications in many cases, contributes to an increasingly competitive scenario for the industry. The onus is on gene therapy developers to accelerate clinical material generation and

FIGURE 1

Movement toward more prevalent diseases and systemic delivery: higher dosages of viral vectors such as AAV are required.



Source: Cambridge Biostrategy Associates analysis (2020).

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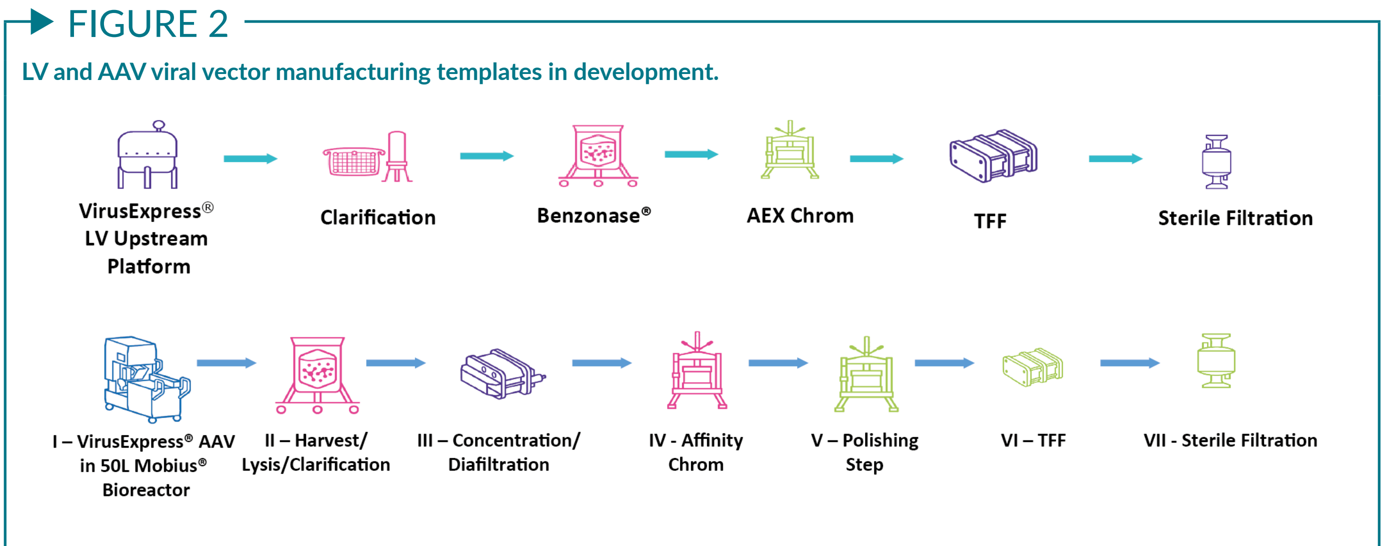
FIGURE 2

LV and AAV viral vector manufacturing templates in development.



advancement through the various phases of clinical development, and to try to be the first to market.

A platform technology approach similar to those commonly used in the traditional monoclonal antibody space can expedite development, providing a pathway to high-quality clinical materials whilst addressing the pressing requirement for speed to market. For example, our VirusExpress® Production Platform includes clonal GMP cell banks of HEK293 cells and HEK293 T cells, both of which have been fully adapted to Ex-Cell® CD chemically defined media. A set of plasmids for the transient expression of viral vector products (currently the predominant mode of expression in the industry) has also been created.

In addition to these physical products, standard template processes for both lentiviral (LV) and AAV vector production have been designed (Figure 2). This enables the rapid development of processes from cell bank thaw through transient cell culture production, to clarification of the cell culture harvest with stepped filtration and Benzonase® endonuclease treatment. This is generally followed by purification steps, including either a polishing step or both affinity and polishing chromatography, to further enrich the viral vector product. Finally, concentration and buffer exchange precede final fill-finish.

This templated platform approach offers a 'plug-and-play' option, whereby the given gene of interest (GOI) may be introduced into the plasmid system and GMP-compliant

FIGURE 3

By using a templated process, activities including cell banking, tech transfer, much of process development, engineering runs, and Custom Bill of Material and Batch Record creation can all be avoided.

Activity | 1 2 3 4 5 6 7 8 9 10 11 12 | 1 2 3 4 5 6 7 8 9 10 11 12 | 1 2 3 4 5 6 7 8 9 10 11 12

Custom
Cell Bank — MCB, WCB
Tech Transfer — Gap/Risk Analysis
Process Development — Limited, Extensive, Confirmation
Engineering — BOM/BR, Eng. Run
Clinical — GMP1, Testing, ▲ Release to clinic

Template
PD — Limited, Confirmation
Clinical — GMP1, Testing, ▲ Release to clinic

clinical materials quickly generated. Advantages to gene therapy developers include the fact that they will not need to generate their own GMP cell banks. Tech transfer and documentation for GMP material production are also streamlined and made consistent. This combination of speed to clinical materials with improved quality through standardization may in certain scenarios result in potential time savings in the delivery of GMP clinical materials of 14–18 months (Figure 3).

It is important to note that this type of platform approach, whilst it provides speed to clinic, may not be optimal for every application. Therefore, it is important to conduct what we call a manufacturing gap analysis (MGA) at the outset of any new project. The MGA is essentially a collaborative paper exercise designed to identify a gene therapy company's specific development and manufacturing needs. Based on this exercise, the optimal path forward may be agreed upon with the developer for their specific project, whether it be simply introducing a GOI to the templated platform process, or tech transferring a fully developed process for manufacturing. Equally, a more customized approach may be preferred over the templated platform process for some projects – for example, where improvements in productivity and yield are sought. It is critical to maintain a degree of flexibility in order to accommodate all of these different scenarios.

TAKING A CUSTOMIZED APPROACH TO PROCESS DEVELOPMENT

An example of a customized approach would be utilizing high-throughput technology, such as the Ambr® bioreactor system, to effectively screen for process parameters and optimize process performance.

In this instance, a Design of Experiments (DoE) approach would typically be employed to statistically generate a design, with the actual experiment being conducted in the Ambr high-throughput bioreactor system. Changes in transfection parameters may be studied, as well as various media formulations. Based on the statistical DoE outcome, the process investigation would then be transitioned into 3 L bench-scale Mobius® bioreactors where process performance may be further confirmed at that scale. This serves as a good scale-down model to inform further scale-up work into production-scale vessels.

This approach delivers an understanding of the full process performance, whilst also subscribing closely to Quality by Design (QbD) principles. QbD is favored by regulatory bodies including the US FDA because they require sponsors to have a good understanding of their processes and in particular, to understand the overall design space of the process capability.

LEVERAGING COMMERCIAL PRODUCTION SCALE-UP KNOWHOW

As discussed previously, with the viral vector-driven gene therapy field taking on larger indications by patient population, there can be a projected need for tens of thousands of liters of cell culture material to produce a sufficient quantity of final vector product. The CDMO community is responding: for example, our viral vector CDMO facility in Carlsbad, CA recently went through an expansion whereby an additional 10,000 sq. ft. of manufacturing space, fully equipped with bioreactors up to 1,000 L in scale, is being brought online.

In the past, considerable experience has been gained in scaling viral vector manufacture from 50 L to 200 L production scale. As work continues towards scaling up to 1,000 L Single Use Bioreactors (SUBs), it is a great benefit to be able to rely on our overall, synergistic technical capabilities and bioprocessing expertise. For example, the company's monoclonal antibody CDMO arm has historically been able to scale its processes directly from the 3 L bench-scale bioreactor to 2,000 L SUB production. This expertise is invaluable for viral vector production.

In addition to this scaling knowledge, leveraging technological know-how from other

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areas of the organization can support the transition to large-scale production and the improvement of process performance. For example, the biopharma process solutions group recently tested the use of Pellicon® technology for tangential flow filtration (TFF) operation, which has been shown to offer good performance and provide a viable alternative to the traditional hollow fiber TFF filters. In a further example, the viral gene technology team works on expression technology – a task that involves testing a wide variety of different transfection reagents and modes to try to improve overall expression levels.

ADDRESSING THE NEED FOR CUTTING-EDGE ANALYTICS

Successful process development must be complemented by robust analytical methodologies that can inform on the quality of the process development work.

As part of the aforementioned Carlsbad site expansion, a broad spectrum of modern analytical technology is being introduced into the viral vector process development arena. Powerful tools including capillary electrophoresis and ddPCR will allow us to take viral vector analytical development into the future, and if one looks at the direction in which analytical methodology is evolving in the industry, high-performance liquid chromatography (HPLC) is likely to play an increasingly prominent role in process development, informing on product quality as well as impurity levels.

In addition, the Carlsbad facility will be equipped with a new pilot lab, in which up to 1,000 L single-use bioreactors (SUBs) will allow proof of concept process testing prior to transfer into large-scale manufacturing.

Q&A with Michael Shen

David McCall, Editor, *Cell and Gene Therapy Insights* **speaks to Michael Shen,** Associate Director, Process Development, Merck

What is your typical yield for a process using a template?

MS: For the LV VirusExpress® template process, we have previously used a model virus (GFP) to produce some initial titer results. We have seen levels of productivity around the high 10^7/low 10^8 transduction unit (TU)/mL level. However, based on our experience and observations, we know that the yield tends to be variable depending on the specific GOI, so it is relatively product dependent.

For the AAV template process, we have tested several different serotypes ranging from AAV2 to AAV6. Using the GFP model virus, we have seen titers in the range of low 10^7 to mid-10^8 TU/mL. Again, there is variability depending on the specific GOI.

How will process development and analytical technology evolve in the next 5–10 years?

MS: I think the consensus of opinion across the leading minds in the field is that speed is currently king. Moving forward, speed will continue to be important, but there will also be a growing focus on innovation that can impact Cost of Goods (COGs). Cost will be a major driver, especially for rare disease indications. But for the larger patient population indications, too: process economies are only realized after a certain scale, and past a certain point, there will be diminishing returns.

Sponsors will always be looking to increase titer/productivity, of course, and there is a lot of discussion around the current transient transfection methods versus using producer cell lines – what happens there will impact process economics in the future. And from a process design point of view, we will also want to leverage automation more and more in order to increase throughput - again, primarily with a view to reducing COGs.

Looking to further ahead, 10 years from now, I think the two things that are of paramount importance to regulators – efficacy and safety – will continue to drive process and analytical innovation. For example, full-empty capsid ratio is a hot topic at the moment, but moving forward, I would expect that the quality of the capsid itself will be of growing concern to regulators, as will the quality of the product within the capsid. It will be necessary for process technologies to be able to deliver the requisite quality on a consistent basis, and for the analytical side to support with tools of sufficient sensitivity and robustness.

What full-empty capsid ratio can you achieve with the template process?

MS: Using the current transient transfection methods mentioned earlier, we generally obtain a full-empty ratio in the low teens percentage range. I don't believe we are unique in this – the whole industry is currently looking for ways to improve the full-empty capsid ratio.

BIOGRAPHIES

JESSICA HILMOE

Technical Leads Manager, Manufacturing and Operations of Viral Gene Therapies, Carlsbad, California, USA, an affiliate of Merck

Jessica Hilmoe is a Technical Leads Manager for the Manufacturing and Operations of Viral Gene Therapies at Merck. She brings 18 years of experience in the biotechnology industry

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with 16 years focusing on Viral Gene and Cell therapies. Her background is in Manufacturing Operations, Process Development, and Technology Transfer. She managed the Technology Transfer group at the Carlsbad facility for 3 years before taking on the Technical Leads group covering global territory. She is currently involved in evaluating proposed commercial processes, helping early phase clients with designing scalable manufacturing processes, and later phase clients with creating a road map of process validation activities to enter the commercial lifecycle. Prior to joining the team, she worked for Genzyme Gene Therapy Operations in San Diego, CA, focusing on upstream process development and technology transfer, and Shire in San Diego, CA leading Manufacturing Tech Support analytical group. She received BS in Cellular and Molecular Biology from San Diego State University and an MBA from the University of Maryland.

MICHAEL SHEN, PhD

Associate Director, Process Development, Carlsbad, California, USA, an affiliate of Merck

Michael is the Associate Director of Process Development at the company's new state-of-the-art viral vector CDMO facility in Carlsbad, CA. In this role, he leads a team responsible for the development of viral vector production processes and analytical methods. Prior to his current role, he held various positions in process development and manufacturing of monoclonal antibody and protein therapeutic products, with experience in taking projects from cell line development to FDA filing. He holds a PhD degree in Chemical Engineering from the University of California, Irvine.

The life science business of Merck operates as MilliporeSigma in the USA and Canada.

AUTHORSHIP & CONFLICT OF INTEREST

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You've developed your viral vector gene therapy and now it's time to embark on the voyage to manufacturing. But gene therapy production is uncharted territory and you need someone to help navigate through the process.

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Pharma & Biopharma
Manufacturing &
Testing Services

APRIL 2022

Volume 8, Issue 3

CELL & GENE THERAPY INSIGHTS

LATEST ARTICLES:

FASTFACTS

Reducing processing risks & operational challenges for low volume fluid transfer

Troy Ostreng and Jayanthi Grebin, Colder Products Company (CPC)

Users looking to making sterile small flow path connections typically will use one of two methods for joining their tubing: either making open connections under a laminar flow hood, or using traditional tube welding that cuts and uses thermal heat to connect two ends together. Each option is viable but comes with inherent disadvantages that can be addressed by converting to sterile connectors.

Cell & Gene Therapy Insights 2022; 8(3), 353; DOI: 10.18609/cgti.2022.054

CHALLENGES & RISKS IN LOW VOLUME FLUID TRANSFER

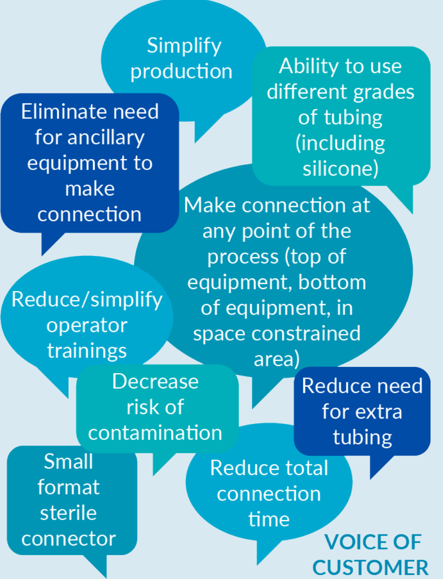
Potential issues with traditional connections include:

- **Efficiency/complexity:** tube welding can be a complicated process involving a dozen steps
- **Size/footprint:** whether you are using a laminar flow hood or a tube welder, both are relatively big and bulky equipment pieces, which can take up valuable cleanroom space.
- **Speed/time:** depending on the specific process application, tube welding or laminar flow hood connections can take several minutes to complete
- **Cost:** in addition to the extra tubing and space involved with welding methods, there are also costs associated with training, equipment, validation, maintenance, and downtime.
- **Risk/reliability:** The risk of operator error brings related repeatability and reproducibility issues.

Sterile connectors help streamline your process and reduce cost, while protecting the process from the external environment.

We surveyed 50+ end users from across the cell therapy, gene therapy and biopharma fields. These are some of the key needs they highlighted relating to connector technology (Figure 1).

Figure 1. Single-use connector needs for cell and gene therapy applications.



BENEFITS OF STERILE CONNECTORS FOR SMALL FLOW APPLICATIONS

MicroCNX® sterile connectors represent a compelling alternative to tube welding for cell therapy and gene therapy process applications – an option that is already well validated in the biopharma space (Table 1).

APPLICATIONS

MicroCNX connectors are being applied today across the advanced therapies space (Figure 2).

With cell and gene therapies, the materials used are precious. Every drop counts when working with patient cells, and loss of genetic material can be extremely costly in both human and financial terms. Sterile connectors offer advantages over traditional connection methods including gresater speed, ease of use, and sterility – all of which combine to reduce risk for low volume fluid transfer applications across the cell therapy and gene therapy fields.

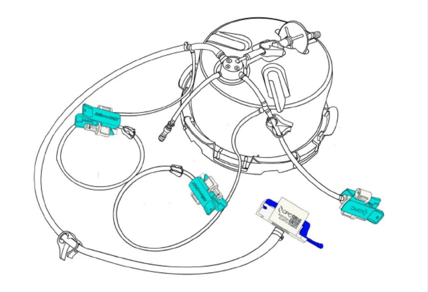
Table 1. MicroCNX connectors: features and benefits.

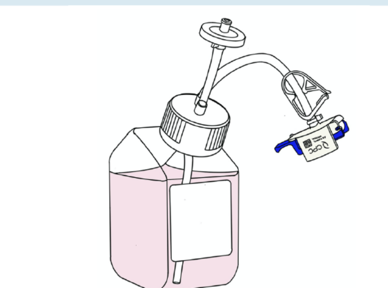
Features	Benefits
Pinch–Click–Pull: a simple, three-step sterile connection process requiring minimal operator training and no additional equipment	Three-step connection process, reduces risk of operator error
Easy-to-use	Consistent, reliable connections
Genderless	Audible confirmation of assembly with no additional hardware required
CPC audible click	Fits well with current Good Manufacturing
1/16", 3/32" & 1/18" ID tubing	Process trends
Compact size	
Minimal weight	
Gamma & autoclave-able	

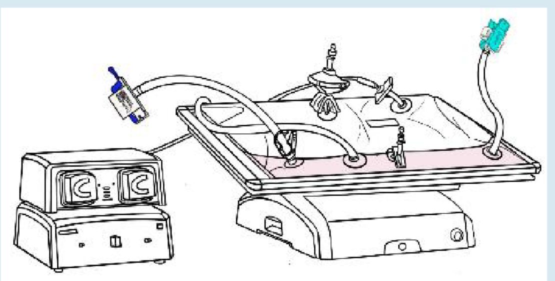
Figure 2. Applications for MicroCNX series connector products in advanced therapies.

MicroCNX connectors for scale up and transfection

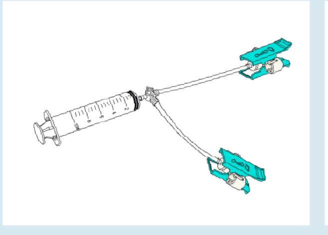
MicroCNX connectors on bioreactors

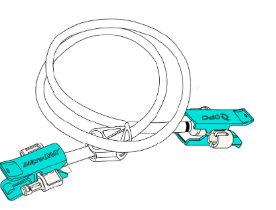


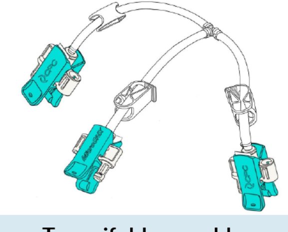


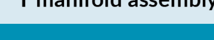


MicroCNX connectors for sterile transfer


Syringe mixer


Transfer or pump line


T manifold assembly



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In partnership with:

FASTFACTS

Laying the foundations for success with scale-down models

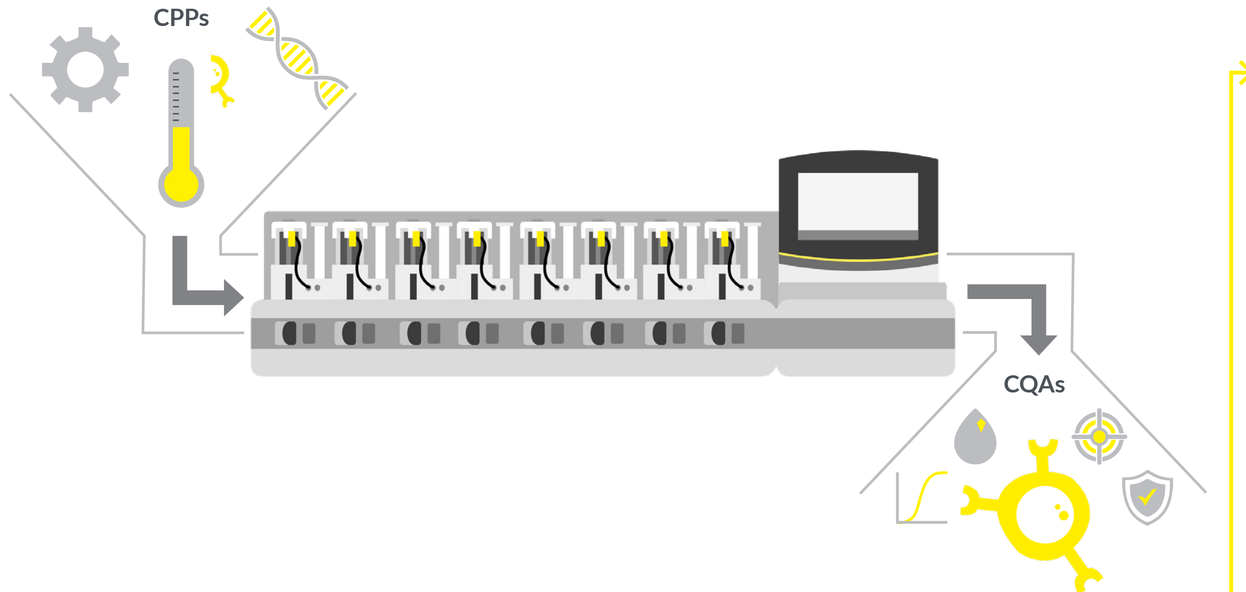
Rohit Saklecha & Andres Castillo

Cell & Gene Therapy Insights 2022; 8(3), 431; DOI: 10.18609/cgti.2022.062

WHAT TYPE OF PLATFORM CAN HELP ACCELERATE TIME TO MARKET AT REDUCED COGS?

Scale-down models are low-volume systems that mimic clinical-scale manufacturing. They're an essential tool in process development — you can use them to build high-throughput, multiparallel experiments that reduce variability and to design workflows that are more robust and consistent.

Ambr® 15 and Ambr® 250 bioreactors are instrumental for scale-down models for cell therapy process development. Combining process parameter control with cell analysis and advanced analytics, these tools help you rapidly identify and correlate CPPs and CQAs.



HOW DOES AUTOMATION FACILITATE PROCESS KNOWLEDGE DEVELOPMENT?

A successful scalable process leverages process knowledge related to the mechanisms driving CQAs. Ambr® 15 and Ambr® 250 bioreactor systems support DoE studies, so you can explore CPPs and gain insights into clinically relevant systems to determine how changes in process parameters affect corresponding quality attributes.

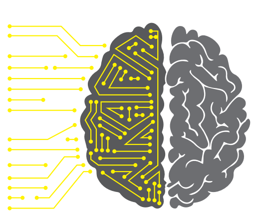
Once your parameters are set, you can establish optima and maxima for each parameter before clinical-scale manufacturing by setting design space, ensuring successful scale up of your cell therapies.

WHY WOULD THIS TYPE OF PLATFORM BENEFIT MY PROCESS DEVELOPMENT?

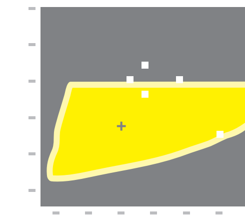
Identifying parameters that impact quality attributes is complex, labor-intensive, and a cost driver in cell therapy development. Scale-down models can help you save time and reduce raw materials consumption by supporting parallel evaluation of parameters like DO, stirring speed, pH, and temperature.

Compared to static flasks, automated scale-down models reduce variability and support rapid optimization of culture conditions. With greater efficiency, you can use scale-down models to improve your timelines in process development and to set the foundation for clinically transferrable protocols.

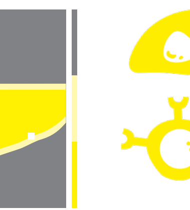
Identify CPP & CQAs



Analyze the data to understand the correlation between CPP and CQAs



Design space to minimize the risk of failure once you scale up



Happy cells

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CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

The digital revolution: technological innovations to enable automation in cell therapy manufacturing

Sean Chang, Bruce Greenwald & Krish Roy

Cell therapy manufacturing workflows typically involve multiple complex steps, requiring extensive hands-on and labor-intensive interventions. They also typically involve several open processes, spanning a multitude of different products. As emerging therapeutics move through the clinical pipeline, scale and regulatory compliance have come to the forefront of the discussion. Closed, modular systems can help overcome some of the current cell therapy manufacturing challenges associated with lack of flexibility, maintenance of sterility, and a lack of standardization. A key to addressing these challenges and facilitate scalability lies in both process automation and digital automation. In this article, experts discuss how a fully automated cell therapy manufacturing process, which addresses digital connectivity and instrument-to-instrument compatibility, can increase quality of the final product and reduce manufacturing failure rates.

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SECTION 1: Sean Chang discusses Thermo Fisher's solutions for CAR T manufacturing & introduces DeltaV

Considerations & benefits of a closed, modular cell therapy workflow

The key issues in cell therapy manufacturing today can be illustrated within the field of autologous T cell therapy. The manufacturing process is complex, labor-intensive, and requires many open manipulations. It is also difficult to synchronize different instruments and products to make the workflow traceable and compliant with regulatory requirements.

To solve these issues, we propose three main solutions. Firstly, a closed system will minimize contamination. Secondly, a modular system will maintain flexibility. Third and most importantly, automating the process will reduce labor and human error. It is in this third aspect that digitalization plays a particularly vital role.

To support cell therapy manufacturing, Thermo Fisher Scientific is building closed, modular and automated instruments, which can be incorporated into a cell processing platform and controlled by the DeltaV™ distributed control system **(Figure 1)**. Digital integration will allow fully automated process management and maintain data integrity. Consequently, delivering a software solution that provides direct connection to the DeltaV is a key step in the platform's ongoing development.

Thermo Fisher solutions for CAR T manufacturing

Instrumentation for each part of the workflow has been developed to address challenges

FIGURE 1

Instrument solutions for every step of the T cell therapy workflow.

1. Leukapheresis
2. Selection
3. Activation & gene transfer
4. Cell expansion
5. Infusion

Rotea — DynaMag (automated version in development) — Rotea — Xenon EP system — HyPerforma controller & rocker — CryoMed controlled-rate freezer — Heracell Vios CR incubator — Rotea — DeltaV

 DOI: 10.18609/cgti.2022.053

across the entire end-to-end process, beginning with cell processing (wash, concentrate, buffer exchange) using the CTS Rotea™ system followed by isolation and activation using the Invitrogen Dynabeads magnetic separation products, which include the CTS™ DynaMag™ magnet. For the cell engineering step, the newly launched CTS™ Xenon system™ for large-scale electroporation can be used, followed by cell expansion, employing a high-throughput mode rocker and controller for dynamic culture. At the end of the process, cells can be harvested and processed for fill and finish using the Rotea, and cryopreservation can be performed using the CryoMed® control rate freezer. Throughout the manufacturing processes, incubators designed and certified for cleanroom use for static culture are available for use. All of these instruments are being adapted and designed to have the capability of connecting to DeltaV systems directly, allowing them to be controlled and managed in the same network within the same interface.

Proof of principle studies performed using this platform demonstrate the high efficiency of peripheral blood mononuclear cells (PBMC) and T cell isolation using the Rotea and DynaMag at the beginning of the process, and show high quality and potency of the manufactured CAR-T cells after cryopreservation.

Digital strategy: off-the-shelf connection between instruments and DeltaV controller

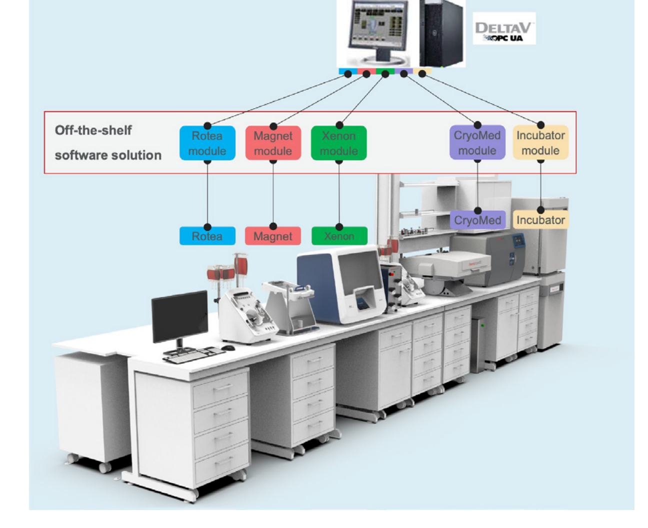
The end goal of a mature manufacturing environment is to manage the instrument layer through a distributed control system (DCS), enabling the integration and management of workflows, and ensuring traceable, repeatable, and secure data connectivity through manufacturing execution systems (MES) up to enterprise level (ERP). The DeltaV DCS controller is one of the most reliable and widely used DCS controllers in the biopharmaceutical industry. However, there is no current 'off-the-shelf' software solution between the instrument and the DCS layer. This connection requires coding, engineering, and configuration to allow the DCS layer to talk to the instruments directly. Furthermore, customized software development projects provided by tools providers or DCS vendors are usually costly and time consuming.

Thermo Fisher Scientific's new 'off-the-shelf' software product will allow customers to operate all of the modular instruments in the same DeltaV network, using the same operator interface **(Figure 2)**. Utilizing DeltaV's capabilities, this software product allows the user to create batch recipes to control different workflows across various unit operations, and to collect and store historical data in compliance with regulations. The user will also be able to retrieve data to produce batch reports through third-party batch reporting packages.

The software includes an OPC-unified architecture (UA) interface module, an equipment module, and phases (the building blocks of batch recipes) which run in the DeltaV controller. The interface module maps the data between DeltaV and the instruments, whilst the equipment module executes commands to the instruments. The equipment

FIGURE 2

The five software modules included in Thermo Fisher's software product, providing DeltaV connectivity to Rotea™, the next-generation magnetic separation system, Xenon™, CryoMed™, and incubator modules.



module can be controlled by higher level batch recipes using phases. The DeltaV Batch Executive is used to create batch recipes for different workflows and collect data from all instruments. The software interface includes batch banners that can be used for messages and prompts from both equipment modules and workflow phases, and an equipment module faceplate that can be used for manual control – for example, to start or stop a protocol.

SECTION 2: Bruce Greenwald disscusses DeltaV architecture & system start-up costs

Building your digital plant with DeltaV

The BioPhorum Operations Group has created the Digital Plant Maturity Model (Figure 3) to define the stages of maturity from paper-based plants to fully automated and adaptive plants. DeltaV and the Emerson product line can support customers all the way from the pre-digital plant level to the adaptive plant level, without replacing system components.

System start-up costs: integrating islands of automation

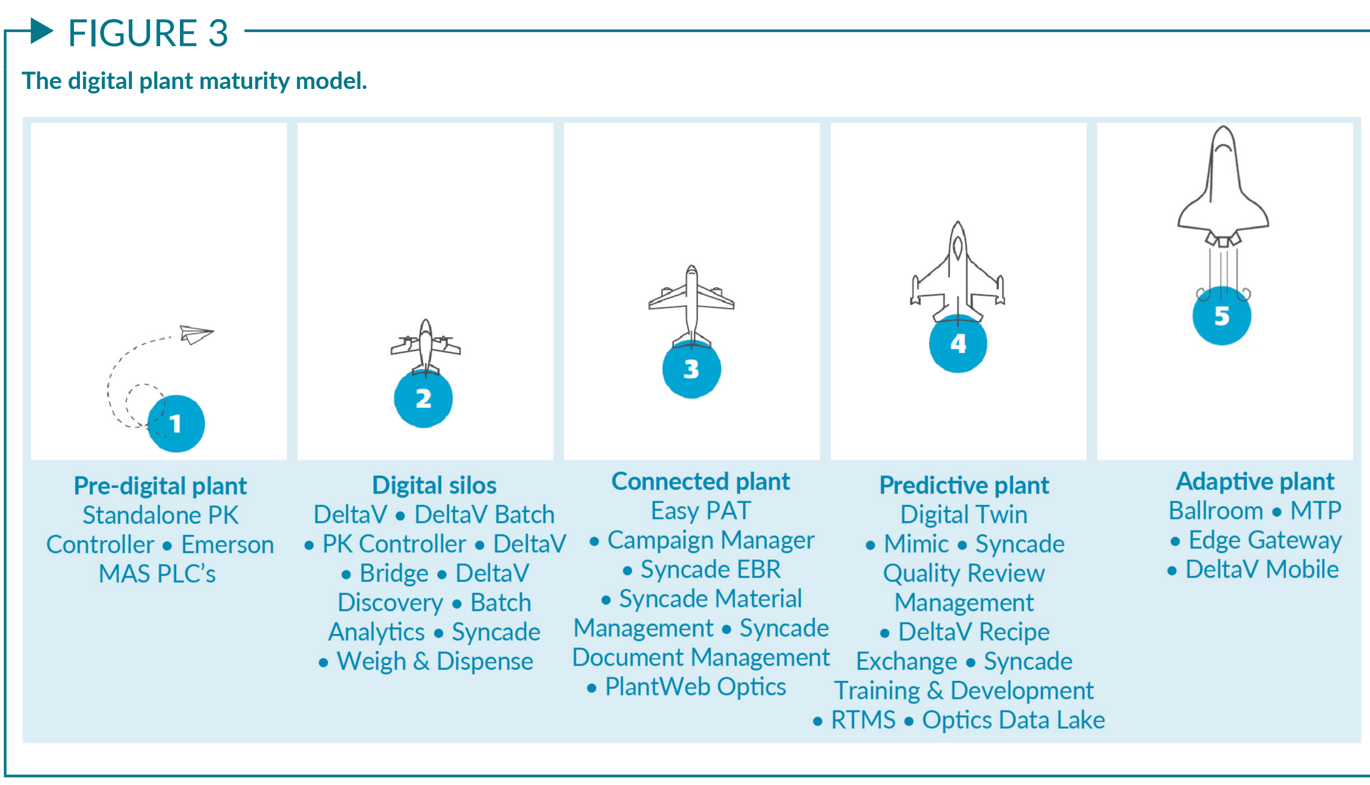
There is a hidden cost of integrating at an individual level. Using individual unit operations to piece together a solution to reach the historical data level requires engineering and validation at each touchpoint. This can be very costly and can severely impact the time to market. Often, layers get skipped, causing intermediate gaps that lead to paper-on-glass solutions that can impact time and cost, and make the actual day-to-day operations more complex.

DeltaV architecture

Since its release in the late 1990s, DeltaV has established the concept of 'Easy', due to its inclusion of 'off-the-shelf'-type technologies. It is designed to be easy to use for the engineers who develop the system, as well as for the operators to control and maintain the system, and it is easy to get the information out of the system. DeltaV was deployed on Microsoft operating systems and off-the-shelf PCs, because in the long-term, being able to use the 'off-the-shelf'

FIGURE 3

The digital plant maturity model.



 DOI: 10.18609/cgti.2022.053

technologies' embedded functionalities would make things easier for automation engineers and production personnel. DeltaV has embedded advance process control and a built-in ISA-S88 batch process control infrastructure.

The overall DeltaV architecture is easy, flat, and simple (Figure 4). Peer-to-peer architecture with DeltaV is used, which is not dependent on client server architecture. This allows embedded nodes to publish information, which can either be consumed by other embedded nodes or consumed at the workstation level, making system configuration easy. DeltaV provides a single, integrated, automated solution for immuno-oncology subsystems, controllers, user management, operations experience, advanced process control, and recipe management. DeltaV's use of a single database architecture means you only have to go to a single application when additions or modifications to your system are required.

Data contextualization at the runtime level is a large part of the DeltaV solution. The system allows real-time alarm, event, and batch data to be contextualized, making it easy to share and store data in standardized databases and use industry standard tools to move data to higher levels within the system from an MES and ERP perspective.

DeltaV's integrated capability for meeting electronic records management (ERM) and data integrity requirements in process automation applications is described in a white paper published in April 2017 [1]. This paper explains the configuration requirements, the real-time runtime environment, and the historization requirements, and how DeltaV complies to both US FDA 21 CFR Part 11 and EU Annex 11.

OPC-UA enables digital transformation and allows embedded nodes to function as OPC-UA servers that feed their data directly into DeltaV. DeltaV can then feed that information to third-party historians, cloud applications, reliability applications, and other analytical tools to evaluate the data that has been collected and harmonized at the DeltaV level.

Within the overall workflow, the DeltaV system sits at Level 2 (Figure 5). At Level 3, there is the laboratory information management system (LIMS), and an MES such as Emerson's Syncade. At level 4, there is the business network where the ERP systems reside, and advanced analytics and scheduling can be performed and passed down in real-time to the DeltaV system.

FIGURE 4

DeltaV architecture.

SECTION 3: Krish Roy discusses the quality control in automation in cell therapy manufacturing, & cell manufacturing of the future

Quality-by-design-driven scalable manufacturing of therapeutic cells

From the discovery-centric perspective, cell therapy is thought of as an interaction between multiscale dynamic complex systems. The starting material is highly variable and highly dependent on prior therapies, as well as the age, sex, lifestyle, and environment of the patient (and the donor, in the case of allogeneic cell therapy). The material is used in a complex manufacturing process in which any manipulation impacts the properties and potentially the function of the cells. The product is hugely complex compared to anything the biopharmaceutical industry has ever manufactured before. Furthermore, once the engineered cells are delivered to the patient, the patient's own microenvironment shapes their properties, behavior, and function.

The complexity from the basic donor side to the patient side poses a tremendous data challenge. How to create models to predict whether particular cells are going to be functional in a specific patient with a specific disease remains a mystery. This is where digitization, data integration, and data processing are of tremendous value.

There are two key areas where large data processing and integration, and thus digitization, are needed. Firstly, in the identification of critical process parameters (CPPs) and monitoring of early quality attributes of the manufacturing process to ensure a consistent, reproducible product within specific parameters. Secondly, in the identification of the multivariate parameters of a product that are the most predictive of patient outcome. Both require an understanding of data manipulation, analytics, data sciences, and data collection for very large data sets.

FIGURE 5

Purdue model for process suites to business systems integration.

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In-line or at-line process & product analytical technologies

In- and at-line process analytical measurement testing and product quality control (QC) will be the future of the cell manufacturing industry. Right now, processes are very fixed and recipes are repeated, despite the starting material being so varied. (Even for allogeneic therapies, the starting material will differ from donor to donor).

In-line or at-line process analytical technologies during R&D and process development allow discovery of early product critical quality attributes (CQAs) to predict end-product quality, and discovery of CPPs that control end-product CQAs. During manufacturing, process analytical technology (PAT) allows the early identification of batch failure, monitoring of microbial contamination, and monitoring of CQAs and CPPs to ensure optimal CMC compliance. Feedback controls, data management, data integration, and digitization become critical.

Cell manufacturing of the future: product is the product

In this vision of the next generation of cell manufacturing, the product is the product, rather than process being the product. Within the next decade, bioreactors should have multiple sensors and measurement tools, and digitalization and data input will become critical in supply chain management and logistics.

The field will see digital models of both a centralized and a distributed cell manufacturing network, and capacity planning tools that select the optimal locations and manufacturing capacities. Impacts of reagent supply disruptions and labor shortages on patient access and capacity utilization must be considered. A hybrid cost model including activity-based costing and parametric costing will be needed. This will be used to assess cost implications and return-on-investment of technological innovations. Data-driven manufacturing, PAT, supply chain management, artificial intelligence (AI), machine learning (ML), and automation will all be critical elements to be integrated within the digitalization and digital infrastructure process.

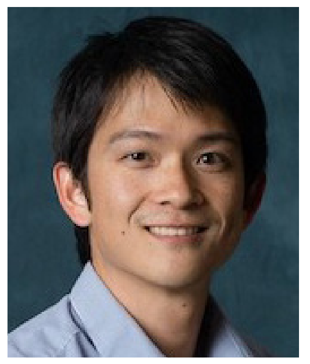
Data infrastructure needs to be layered into both the physical and the human infrastructure, alongside the need for an integrated conduit between the three that includes sensor-controlled automation, a collaborative environment between industry and academia, preclinical data, pilot manufacturing, predictive analytics, and supply chain understanding.

REFERENCE

1. DeltaV™ Capabilities for Electronic Records Management and Data Integrity. Emerson. 2017.

Ask the experts

Elisa Manzotti speaks to (from left to right) **Sean Chang**, **Bruce Greenwald** and **Krish Roy**, who answer your questions about how a fully automated cell therapy manufacturing process that addresses digital connectivity and instrument-to-instrument compatibility can increase quality of the final product and reduce manufacturing failure rates.



Q How does DeltaV provide data integrity for configuration data?

BG: DeltaV contains a tool known as version control audit trail (VCAT). VCAT allows us to version every object in the configuration database. Every time someone needs to make a change, that object is checked out, the change is made, the object is checked back in, and a new version is automatically created. From a management perspective, you can see each of the different versions and who made the change. You can also compare the versions both visually and textually automatically using the VCAT tool for validation and management of change requirements.

How would digitization and end-to-end data integration help accelerate development of cell and gene therapy (CGT) products?

KR: It would help in multiple ways. In the scientific lens, it starts at the discovery and process development stage, where you can look at large datasets and create decision processes about quality attributes and clinical process parameters. This leads to interfacing with clinical trials and understanding mechanisms of action and the critical quality attributes that are predictive of patient outcomes. Integrating process analytics, supply chain data, and cost modelling components would greatly improve the process and the product quality and reproducibility, reduce batch failures, and drive down cost.

I remember listening to a talk by the former president of Intel, whose processes are incredible. They have been making microchips for decades now. They have a process model, and data for every manufacturing run is fed back into the process model to further improve it. We do not do that in the biopharmaceutical industry anywhere. We need to make that move towards full digitization.

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Q Can Thermo Fisher's instruments connect to other non-DeltaV DCS systems?

SC: Yes. At Thermo Fisher, we make sure all our cell therapy instruments will be equipped with the OPC-UA which is the standard interface to allow this instrument to exchange data with other platforms or control systems. With OPC-UA, the Thermo Fisher instrument has the capability to connect to other systems.

However, the 'off-the-shelf' product I introduced only provides the codes to directly connect to DeltaV. If the customer wants to connect to other DCS systems from other vendors, there is some software engineering that the customer will need to figure out with those vendors. Themo Fisher can provide support by providing the OPC-UA document manual or related information from the instrument support teams.

Q With the connections to other levels within my organization, how does DeltaV manage cyber security?

BG: DeltaV can provide a bubble around your entire control system. We are compliant and allow end users to achieve ISASecure SSA Level 1 certification for their control system from a cyber security perspective. The up and out communications go through our secure Emerson smart firewall. Using industry standards like OPC-UA, we also have web services tools that will be used for connecting to the ERP or MES layers.

Q What areas do developers and vendors working on digitization need to pay attention to?

KR: When I see folks working on digitization, a lot of focus is on the manufacturing and GMP end. That is great, but we need to bring this concept down to the discovery and product development sides. Digitization needs to start much earlier if we are to understand CQAs and how product behaves under different manufacturing scales.

We are an academic consortium of eight or nine universities, and one of the things we are trying to do is create digitization in each laboratory. This feeds into a cloud platform and allows us to do analytics, bringing the power of many experiments together to make decisions and understand the data and manufacturing variabilities better. Vendors are not there yet, but that is where the biggest long-term impact in the cell and gene therapy field will be.

By the time we have the process transferred to our manufacturing, we are too late. It takes many years for the company to then identify quality attributes, critical process parameters and MOAs. If we can bring that process up, even to the graduate student level, we will be much better off.

Does Thermo Fisher Scientific have other 21 CFR Part 11 compliant software solutions without a DCS system?

SC: All of our cell therapy instruments have another standalone software product to support 21 CFR Part 11, or any regulatory requirements in terms of the digital integration. We call it the Security, Audit and Electronic signature (SAE) solution. We use the same interface and functions of the existing REO software, but further support the security settings, audit trails, and e-signature functions. It is a great start-up solution for a customer who does not have a DCS system.

Why connect to DCS and not to MES?

SC: At Thermo Fisher Scientific, we believe in providing comprehensive digital integration to customers. We believe in the DCS layers supporting, managing, and operating instrument layers, and supporting data integrity. We want to follow the architecture of this digital integration, so right now, we are mainly focused on connecting the DCS to instrument layers.

BG: As previously mentioned, having a harmonized layer between the MES and the field instruments in the unit operations provides a common interface. It can also automate a lot of the tasks built into their implementation with DeltaV, as opposed to having MES and unelectronic workflow instructions that are only partially automated.

Within the DeltaV, what is the feedback loop timeframe?

BG: From a closed loop control, it is as fast as 25 milliseconds from a modular execution perspective. With the interface to unit operations at the Thermo Fisher Scientific level we were performing supervisory control, which typically would execute in the one-second timeframe. DeltaV is acting as supervisory control, passing down commands, and then the Rotea and other devices are doing the heavy lifting.

How soon will the Thermo Fisher digital platform be available for cell and gene therapy manufacturing operations?

SC: The first module to connect Rotea to DeltaV will launch in early Q2 of 2022. We have five different modules to connect to DeltaV that will be launched at different times. However, once you purchase one, you get access to the connectivity of all five different software modules. The newer modules will be released as an update of the whole software solution.

Do you see the field moving to in-line testing, and will technology make this possible?

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KR: We need to bring in in-line or at least at-line process testing – it is unrealistic to have all in-line, but we should have rapid at-line process testing at least. As we move into more complex products, especially induced pluripotent stem cell (iPSC)-derived products where the manufacturing timeline is four to eight weeks, you cannot just rely on end of process testing. It does not make business sense, let alone scientific sense.

We should start with the existing technologies for pH, glucose, and lactate. But most of the sensors on the market today were developed for other purposes and do not fit our purpose for cell and gene therapy – here needs to be significant innovation in this space. I think optical sensors have a lot of potential here. Most of the work we are doing at the moment involves multiplex measurements with wireless data transmission capabilities, with those two elements combining for a process control capability.

Especially in the autologous setting, our input – the raw materials, the cells – is different every time we manufacture something. Each time we manufacture something, if we put it in the current fixed and uncontrolled process, we will inevitably end up with a different product. In-line testing is a way to be able to understand what a product is going through, where it is going in terms of its differentiation and expansion process, and to allow tweaking to get consistent product parameters. In that sense, PAT is one of the most critical things that we can pursue as a field.

Q What is the data historian used by the DeltaV platform?

BG: There are three different types of data and data historian. For continuous data, we have our DeltaV continuous historian. For any alarms and operator events, we have a sequel based, which is an OPC-alarm and events (A&E)-based database. A third separate database is the batch historian, which is sequel-based for all batch information automatically collected within DeltaV. Those three databases comprise the historians within DeltaV, and then provide views, OPC-historical data access (HDA), and OPC-UA to remotely query information.

Q Most cell and gene therapy innovation occurs in small organizations that will not have access to these tools for a decade or two. How should we effectively manage innovation and product process development in this context?

KR: This is why I advocate heavily towards collaboration with academia and government laboratories, that are also focusing on this mission. There are a number of existing consortia. Many of our industry partners want to collaborate and extend their capabilities and reach because not everything can be done in-house, especially for small companies and businesses. We just signed a contract with NIH to become an in-depth cell characterization hub for their medicine innovation program trials. This kind of consortium partnership

is critical, especially for small and medium businesses, where they do not want to build these capabilities in-house.

BIOGRAPHIES

DR SEAN CHANG is the Early Innovation Manager in the Cell and Gene Therapy business at Thermo Fisher Scientific and is responsible for the integration and automation of next generation cell therapy manufacturing workflow." Prior to this role, he was a Process Development Scientist, leading manufacturing process optimization of an allogeneic CAR-T product. Sean also gained extensive experiences in new product development in his previous career role as an R&D Scientist, where he identified novel T cell genome editing and non-viral delivery solutions, and worked on new closed modular instruments. Sean received his Ph.D. in Integrative Molecular and Biomedical Sciences from Baylor College of Medicine, where he focused on the mechanisms underlying therapeutic resistance of breast cancer.

BRUCE GREENWALD is the DeltaV Platform Business Development Manager for Emerson Automation Solutions, located in Austin, TX. In his current role, Mr Greenwald assists customers in understanding the features and benefits of DeltaV to improve their automation experience. Mr Greenwald is a 1979 graduate of the University of Kansas with a degree in Chemical Engineering. He started his career with the Dow Chemical in Freeport, TX and joined Fisher Controls in 1983 as a systems engineer. Mr Greenwald joined the RE Mason Company, an Emerson Impact Partner, in 2000, executing PROVOX and DeltaV projects. He held several positions at RE Mason, and in 2011, rejoined Emerson. His 4 decade-long career has been focused on process control.

DR KRISHNENDU (Krish) Roy received his undergraduate degree from the Indian Institute of Technology (India) followed by his MS from Boston University and his PhD in Biomedical Engineering from Johns Hopkins University. After working for 2 years at Zycos Inc., a start-up biotechnology company, Dr. Roy left his industrial position to join the Biomedical Engineering Faculty at The University of Texas at Austin in 2002, where he was most recently Professor and Fellow of the Cockrell Chair in Engineering Excellence. He left UT-Austin in July of 2013 to move to Georgia Tech. where he is the Robert A. Milton Chaired Professor in Biomedical Engineering. At Georgia Tech, he also serves as the Director of the newly established NSF Engineering Research Center (ERC) for Cell Manufacturing Technologies (CMaT) and The Marcus Center for Cell-Therapy Characterization and Manufacturing (MC3M) – as well as the Director of the Center for ImmunoEngineering. He is also the Technical Lead of the NIST/AMTech National Cell Manufacturing Consortium (NCMC), a national public-private partnership, focused on addressing the challenges and solutions for large scale manufacturing of therapeutic cells. Dr. Roy's research interests are in the areas of scalable cell manufacturing, Immuno-engineering, stem-cell engineering and controlled drug and vaccine delivery technologies, with particular focus in biomedical materials. In recognition of his seminal contributions to these fields, Dr. Roy is elected Fellow of the American Institute for Medical and Biological Engineering (AIMBE) and the Biomedical Engineering Society (BMES). In addition, Dr. Roy has received numerous awards and honors including Young Investigator Awards from both the Controlled Release Society (CRS) and The Society for Biomaterials (SFB), NSF CAREER award, Global Indus Technovator Award from MIT, the CRS Cygnus Award etc. He is also the recipient of Best Teacher Award given by the Biomedical Engineering Students at UT-Austin and the best advisor award given by bioengineering students at Georgia Tech. He serves as a member of the Editorial Boards of the Journal of Controlled Release, the European Journal of Pharmaceutics and Biopharmaceutics, the Journal of Immunology and Regenerative Medicine, all from Elsevier, as well as the AIChE Journal of Advanced Biomanufacturing and Bioprocessing. He is a member of the Forum on Regenerative Medicine of the National Academies of Science, Engineering and Medicine (NASEM), and a Board Member of the Standards Coordinating Body (SCB) for Cell and Regenerative Therapies.

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AUTHORSHIP & CONFLICT OF INTEREST

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Rapid translation of a cellular therapeutic from research to clinic

Ashish Patel, PhD Clinical Senior Lecturer & Consultant Vascular & Endovascular Surgeon, The National Institute for Health Research (NIHR) Guy's & St Thomas' Biomedical Research Centre (BRC) and **Joseph Candiello,** PhD, Senior Product Manager, RoosterBio Inc.

Cell & Gene Therapy Insights 2022; 8(3), 445 • DOI: 10.18609/cgti.2022.012

Cell therapies face regulatory and manufacturing challenges long before they are accessible to patients. Even as therapies prove effective and safe, raw material and manufacturing decisions made during early development can either stall progression or rapidly accelerate it.

Utilizing scalable manufacturing processes and translation friendly raw materials during product development of MSCs and extracellular vesicle (EV) therapies will enable cell therapy developers to accelerate leading candidates into clinical trials, ultimately enabling more curative treatments to reach patients.

CELL-BASED THERAPY MANUFACTURING PROCESS DEVELOPMENT

Developing a cell-based therapy manufacturing process requires varying focus and expertise, as demonstrated in **Figure 1.** The critical quality, scale and expertise needs change as developers move from research and development (R&D) to product development, to clinical implementation. There are key development requirements that can accelerate programs through this journey are described in **Figure 2**.

RoosterBio Cell and Media Systems are engineered to make mesenchymal stem cells (MSCs) and extracellular vesicles (EVs) easy to establish within new programs, and easy to scale for product and process development **(Figure 3)**. The overall goal is to make MSCs and EVs easy to translate into the clinic. Strategically choosing the right supply chain partner can remove years of time and millions of dollars from standard product development timelines.

INDUSTRIALIZED SUPPLY CHAINS & RAPID TRANSLATION

An industrialized supply chain can enable rapid translation of a cellular therapeutic in a number of ways. Where possible, leveraging 'off-the-shelf' products with built-in processes will reduce development timelines. This is because reducing internal resources in cell manufacturing allows a more targeted focus on your IP. Building manufacturing scalability into process development is of great importance, as process development is cyclical. Scalability will enable a smooth transition to larger lot sizes, thus rapidly accelerating therapeutic program development. High-quality fit-for-purpose materials can reduce regulatory hurdles. Development grade materials with cGMP analogs backed by regulatory packages support a seamless transition from pharmaceutical product development (PPD) to the clinic.

THE MONACO CELL THERAPY TRIAL: A CASE STUDY

Initial results from the MONACO study (**Mon**ocytes as an **A**nti-fibrotic therapy after **COVID**-19), which uses RoosterBio Cell and Media Systems in MSC production, have been encouraging and signal towards efficacy. RoosterBio's "off-the-shelf" solutions enabled regulatory compliance as well as rapid translation, which were critical in meeting the escalated need for COVID -19 treatments. With encouraging clinical data and a robust, scalable manufacturing process, Dr Ashish Patel is now working towards a large Phase 1b/2a trial.

Figure 1. Industry standards in the cell-based therapy manufacturing process

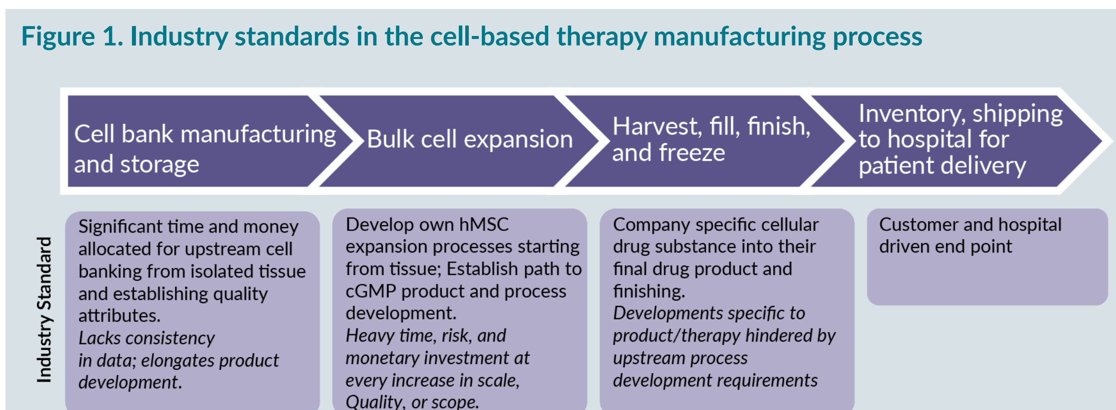


Figure 2. Key requirements in rapid translation of a product

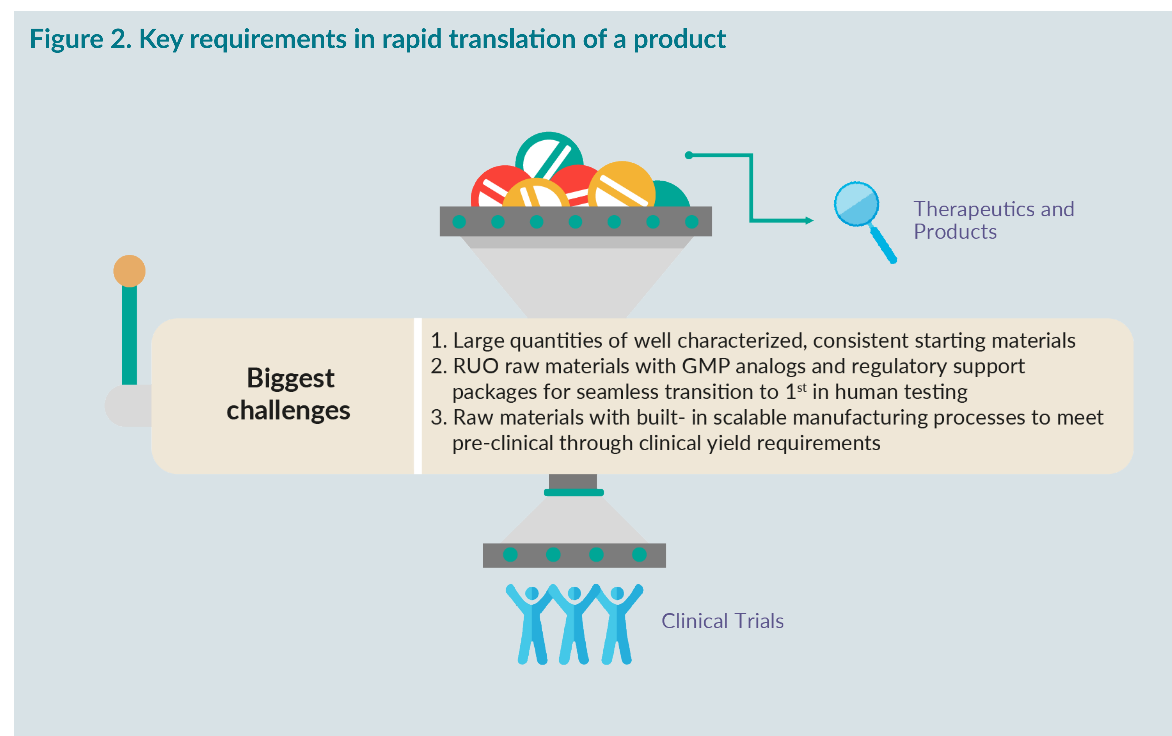
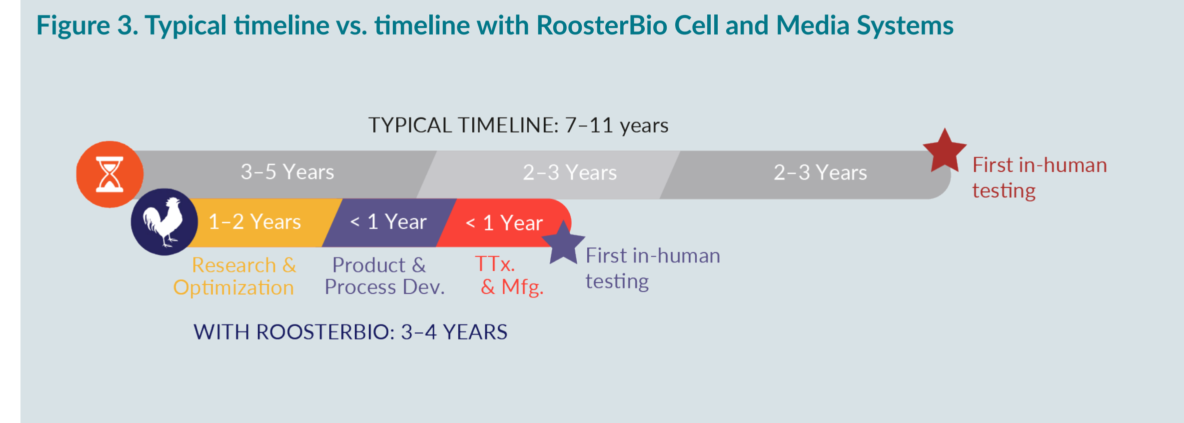


Figure 3. Typical timeline vs. timeline with RoosterBio Cell and Media Systems



CELL & GENE THERAPY INSIGHTS

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In collaboration with:

Supply chain efficiency and sourcing for scaling your gene therapy operation

Celine Martin and Don Young

Scale-up for clinical and commercial gene therapy manufacturing impacts both process and operations. In the wake of SARS-CoV-2, supply chains have been significantly challenged and raw materials are in short supply. As the potential of gene therapies is being realized, long-term sustainable manufacture needs to be considered.

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Manufacturers of viral vector-based gene therapies implemented a single-use strategy early when setting up new manufacturing facilities. However, format, packaging, shipment, and storage are all key elements to ensure efficient operations and mitigate supply chain disruption risk moving forward. Moving from standard media and single-use technologies to configurable solutions will help future sourcing and increase operations performance.

Thermo Fisher Scientific offers a number of configurable packaging and mixing technologies, alongside various cell culture media formats. These configurable solutions solve many problems in manufacture scale-up, such as logistical space management challenges.

CONFIGURABLE PACKAGING

There are many BPC design options including varied tubing, connectors, and clamps. BPC totes (Figure 1) are important for ease-of-use and handling. Reusable outer support containers hold liquid-filled BPCs.

Thermo Fisher Scientific has the largest and only truly networked single-use manufacturing organization in the world. The network approach consists of 5 networked sites, 7 regional assembly centers for manufacturing redundancy, and 3 Centers of Excellence. We work closely with our customers to troubleshoot or innovate to find a way to meet even the most unique requirements.

Figure 1. BPC totes.

Plastic totes for 10–20 L 2D BPCs	Plastic cylindrical tanks for 50–250 L 3D BPCs	Plastic 4-sided totes for 100–1000 L 3D BPCs	Steel 4-sided totes for 100–3,000 L 3D BPCs
Stackable Easy tube set access	Shipping qualified Options for top and bottom drain	Shipping qualified Options for top and bottom drain	Jacketed totes, load cells, hoists optional - Options for top and bottom drain

CELL CULTURE MEDIA

When it comes to media, choice of format is key to both increasing efficiency and de-risking the supply chain. Pre-made liquids, Advanced Granulation Technology (AGT), and dry powder media each have their own pros and cons (Figure 2). However, dry formats are generally preferred for large-scale manufacture due to their smaller footprint. Gibco's AGT format offers the advantage over standard dry powder of a simplified, one-part media formulation, as well as flexibility and stability benefits.

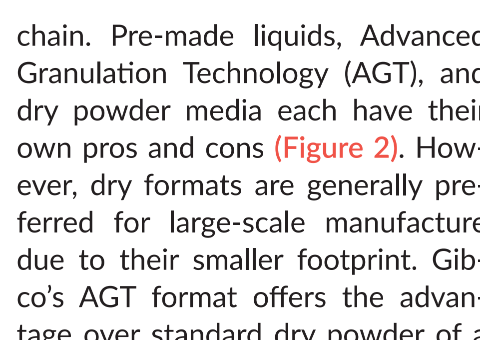


Figure 2. Media format pros and cons.

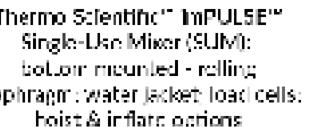
Premade liquids & concentrates	Advanced granulation technology (AGT) format	Dry powder media
- Ready to use Suitable for aseptic processing - 'Dry' facility	- One-part media supporting large scale media preparation - Flexible choice of liquid container Longer stability - Safety stock	- Options for supply redundancy Flexible choice of liquid container Longer stability
- Higher storage footprint Shorter stability - Cold shipment	Non aseptic WFI infrastructure - Higher control of QC/QA	pH/osmolality adjustment required - Dust generation Multiple part media

MIXING TECHNOLOGIES

Choice of mixer should be dependent on current and future process needs. Thermo Fisher caters to a variety of mixing needs (Figure 3). Considerations include BioProcess Container (BPC) deployment, room dimension, temperature control, cost-benefit, and scale-up volumes.

Your mission is our mission. As your supplier, we strive to exceed expectations and provide stability during disruption so you can keep pace with evolving market needs. This includes offering end-to-end supply chain resiliency by investing in capacity, capabilities, and quality systems.

Figure 3. Thermo Scientific™ mixing technologies.



Thermo Scientific™ ImPULSE™ Single-Use Mixer (SUM): bottom mounted - rolling diaphragm; water jacket; load cells; hoist & inflate options

Thermo Scientific™ HyPerforma™ Single-Use Mixer (SUM): top mounted - marine impeller; water jacket; load cells; hoist options

Thermo Scientific™ HyPerforma™ DS 300 Single-Use Mixer (SUM): docking; top mounted marine impeller

Thermo Scientific™ HyPerforma™ Mixtainer System: stir bar impeller

Q&A

What if we cannot increase our storage space?

Celine Martin: When it comes to media, it depends on how your facility has been designed. If a buffer or media preparation area has been built-in, then going for dry format can save space. If it has not been built-in, we offer a GMP storage service that can help you increase your storage space while you develop other facilities.

Don Young: There is a program in place known as Forward PO, which is another way to maximize storage space on site. We do not build or deliver until right before it is needed. We work closely with the customer to plan a delivery date. We build 30 days beforehand, and the product arrives 48 hours before it is needed in the production area cleanroom, ready for use.

How do you manage the shortage of single-use plastic for biopharmaceutical use?

Don Young: This challenge is COVID-related. The choice of materials and suppliers is critical – we encourage multiple sourcing. We encourage sourcing materials on a material construction basis rather than a specific part number. Rather than supplying a specific filter, we would encourage you to look at membrane-based options to give longer term flexibility in suppliers.

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FASTFACTS

POROS CaptureSelect AAVX Wash Optimization Study for Optimized Recovery and Purity of AAV6 Capsids

Jenny England, Thermo Fisher Scientific

The growing use of AAV in the field of gene therapy has emphasized the need for an effective downstream process to generate high titer, purity, and recovery of AAV capsids. Affinity purification shows great promise to overcome downstream processing challenges due to its high platformability, productivity and scalability, and low complexity. This poster describes a wash optimization study for the POROS™ CaptureSelect™ AAVX affinity resin to identify optimized recovery and purity of AAV6 capsids from HEK293 cell culture.

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A series of studies were carried out to determine optimum AAVX wash conditions. After loading onto the AAVX resin, the impact of different wash buffers on recovery and purity was tested. The conditions tested included variations in pH, additions of high and low salt, and the addition of arginine.

OPTIMIZING WASH CONDITIONS

The samples were first clarified with diatomaceous earth (DE) and filtered with a 0.22 µm PES vacuum filter. After clarification, samples were loaded onto the POROS™ GoPure™ AAVX pre-packed 1 mL column. The experimental steps are summarized in Table 1.

Figure 1. Manual steps required to perform Ella and ELISA immunoassays.

	Run 1: 50 mM Tris pH 7.5	Run 2: 50 mM Tris pH 7.5 +0.1 M NaCl	Run 3: 50 mM Tris pH 7.5 +0.1 M NaCl (D)	Run 4: 50 mM Tris pH 7.5 +1.5 M NaCl	Run 5: 50 mM Tris pH 7.5 +0.25 M Arginine	Run 6: 50 mM Tris pH 9.0	Run 7: 50 mM Tris pH 9.0 +1.5 M NaCl	Run 8: 50 mM Tris pH 9.0 +0.25 M Arginine	Run 9: 50 mM citrate pH 5.5	Run 10: 50 mM citrate pH 5.5 + 1.5 M NaCl	Run 11: 50 mM citrate pH 5.5 + 0.25 M Arginine
Recovery	74%	87%	79%	90%	68%	64%	*100%	51%	27%	57%	73%
Column load	2.42×10¹⁴	1.70×10¹⁴	4.31×10¹³	2.52×10¹⁴	4.03×10¹³	3.21×10¹³	1.35×10¹⁴	4.70×10¹⁴	5.61×10¹⁴	3.38×10¹³	1.73×10¹⁴
Elution	1.80×10¹⁴	1.48×10¹⁴	3.41×10¹³	2.27×10¹⁴	2.76×10¹³	2.06×10¹³	1.35×10¹⁴	2.41×10¹⁴	1.54×10¹⁴	1.91×10¹³	1.26×10¹⁴

Table 1. Experimental summary.

Step	Buffer	Column volumes	Residence time (min)	Flow direction
Equilibration	50 mM Tris pH 7.5+100 mM NaCl	5	1	Downflow
Load	Clarified lysate	100	1	Downflow
Wash	Variable	5	1	Upflow
Elution	50 mM citrate pH 2.5	5	1	Upflow
Regeneration	100 mM phosphoric acid	5	3	Upflow
Cleaning	6 M Guanidine HCl	5	3	Upflow
Storage	20% ethanol	5	1	Downflow

A total capsid ELISA was used to examine column load and elution and determine the total % recovery, shown in Figure 1. Tris at pH 7.5 or 9.0 with 1.5 M NaCl resulted in the greatest recoveries. The addition of arginine and the flow direction had limited impact on recovery. Citrate washes resulted in lower recoveries than Tris, but recovery could be improved by 25–50% with the addition of 0.250 M Arginine.

Host cell protein removal was achieved at an average log10 reduction value (LRV) of 4.5, independent of wash buffer. Host cell DNA removal showed a similar trend, with an average LRV of 3.2 achieved independent of wash buffer.

COMBINATION WASH STUDY

The wash conditions with the best % recoveries in the initial experiments were selected for additional combination wash purifications, with three extra wash steps as outlined in Table 2. AAV was eluted in a smaller elution pool (1 column volume [CV]) with combination wash purification.

Table 2. Experimental summary for combination wash study. Box shows additional steps.

Step	Buffer	Column volumes	Residence time (min)	Flow direction
Equilibration	50 mM Tris pH 7.5+0.1 M NaCl	5	1	Downflow
Load	Clarified lysate	100	1	Downflow
EQ Wash 1	50 mM Tris pH 7.5+0.1 M NaCl	10	1	Upflow
Wash 2	50 mM Tris pH 9.0+1.5 M NaCl	5	1	Upflow
Wash 3	Variable	5	1	Upflow
EQ Wash 4	50 mM Tris pH 7.5+0.1 M NaCl	10	1	Upflow
Elution	50 mM citrate pH 2.5	5	1	Upflow
Regeneration	100 mM phosphoric acid	5	3	Upflow
Cleaning	6 M Guanidine HCl	5	3	upflow
Storage	20% ethanol	5	1	Downflow

Approximately 80% recovery was achieved, independent of the wash buffer used. A ten-fold increase in host cell protein was observed in the elution for citrate versus Tris wash, while similar levels of host cell DNA removal were achieved for all wash buffers.

KEY FINDINGS

- Tris at pH 7.5 or 9.0 with 1.5 M NaCl followed by a low salt wash resulted in the greatest % recovery and host cell protein removal
- Citrate wash buffer resulted in the lowest recovery, but the addition of 0.250 M Arginine improved the recovery by 25–50%.
- The combination of four washes results in a smaller elution pool volume (1 CV vs 2–3 CVs) compared with a single wash.
- An average LRV of 4.5 and 3.2 was achieved for residual host cell protein and host cell DNA removal.

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VECTOR BIOPROCESSING

SPOTLIGHT

INNOVATOR INSIGHT

From cells to purified capsids: How to develop a scalable rAAV process

Åsa Hagner McWhirter

Adeno-associated viral (AAV) vectors are widely used for gene therapy, with multiple serotypes and several different synthetic capsid variants targeting different tissues. The number of AAVs in clinical trials has increased over recent years and the serotypes primarily used have changed, from AAV1 and AAV2 initially to AAV8 and AAV9 today.

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Within our AAV process at Cytiva, on the upstream side, we use triple plasmid transfection and HEK293 cells in suspension and AAV expressing green fluorescent protein (GFP) as a model system. We use scalable, single-use bioreactors. In the downstream process, steps include cell lysis and DNA fragmentation, clarification, concentration and buffer exchange, capture, polishing, formulation and finally, sterile filtration. We have a large analytical package including various assays for infectious virus titer, total virus titer, and viral genome (VG) titer. We also utilize different assays to measure full/empty ratio of samples and host cell impurities, and for vector characterization.

This article describes the production and purification process of several AAV serotypes and the optimization of each process step, with a particular focus on ensuring

high overall yields of full capsids, empty capsid reduction, and efficient impurity removal. We will explore common pitfalls for rAAV processing and ways to overcome the challenges presented. We will also discuss how full/empty AAV capsid separation for AAV2, AAV5, AAV8, and AAV9 serotypes can be significantly improved with a single chromatography resin and one protocol.

UPSTREAM CELL CULTURE & VIRUS PRODUCTION

Transient transfection optimization

We used a design of experiment (DoE) approach to optimize and develop a new transfection protocol for production of recombinant AAV5 (rAAV5). We confirmed that DNA concentration affects vector genome (VG) titer and percentage full capsids. As DNA concentration increases, the VG titer also increases, whilst the percentage full capsids decreases. This poses some difficulty, because it is desirable to have both high VG titer and high percentage full capsids. In order to balance these variables, we used 0.75% µg/mL of DNA. We also discovered that a short incubation time of the transfection mix prior to application to the suspension cell culture significantly increased the % full capsids.

Based on our DoE results, we developed a workflow for rAAV5 production. The transfection parameters we selected included a viable cell density of 1×10^6 cells/mL, a polyethylenimine (PEI) transfection reagent in a 2:1 ratio with DNA, a transfection volume of 5% of total volume, and an incubation of 15 min at room temperature with a DNA plasmid ratio of 1:1:2 (rep/cap:helper:transgene GFP).

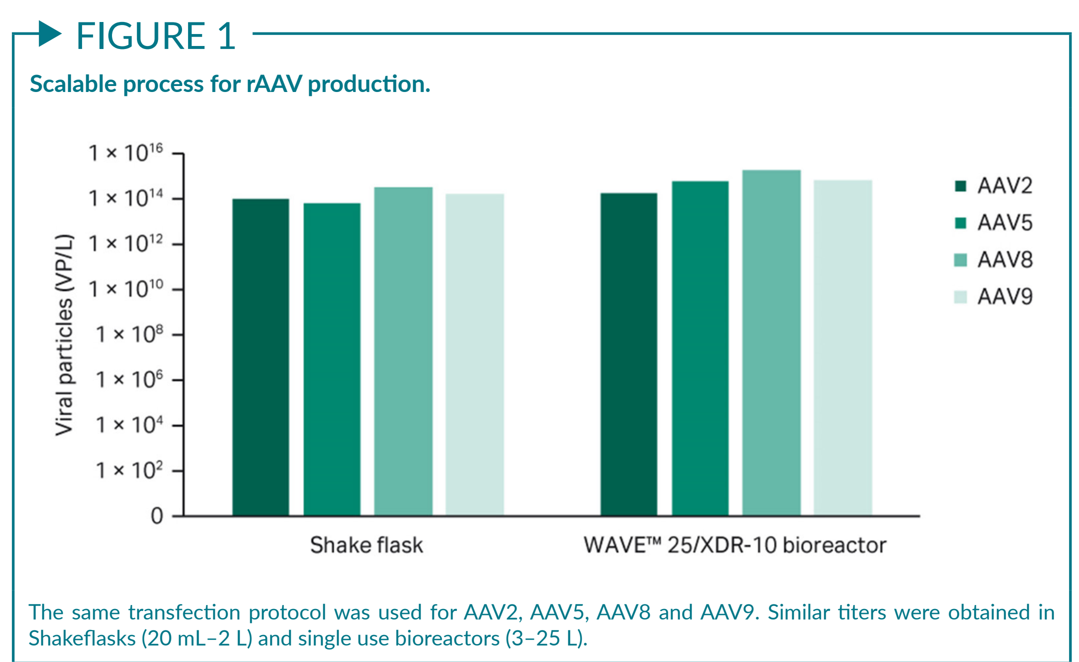
AAV production in a single-use bioreactor

We performed three 10 L rAAV5 production runs in a stirred tank bioreactor, which were reproducible both with VG and viral particle (VP) titers. The percentage full capsids were all above 10%.

As can be seen in Figure 1, this AAV production process is scalable from 20 mL to 25 L and is consistent across various AAV serotypes. We reached approximately 10^{14} VP/L and 10^{13} VG/L. The range of percentage full capsids was 10–40%.

FIGURE 1

Scalable process for rAAV production.



The same transfection protocol was used for AAV2, AAV5, AAV8 and AAV9. Similar titers were obtained in Shakeflasks (20 mL–2 L) and single use bioreactors (3–25 L).

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DOWNSTREAM PURIFICATION PROCESS

Harvest & filtration

To develop the purification process for recombinant AAV5, we firstly considered the harvest and filtration steps. The harvest was completed by cell lysis, DNA fragmentation and clarification, using Tween™ 20, followed by nuclease treatment directly in the bioreactor. Filtration was performed with three different filter capsules. We achieved recovery of up to 80% over the harvest and clarification steps. Concentration and buffer exchange was completed by tangential flow filtration using hollow fibers. We achieved efficient impurity removal with low loss of virus using 300 KDa molecular weight cut-off (MWCO).

We performed affinity capture chromatography with the Capto™ AVB, HiTrap™ column. The elution was different for AAV2 and AAV5, with AAV5 recovery being negatively affected by salt in the elution buffer, and we used a lower elution pH compared to AAV2. However, using the respectively optimized protocols the performance was similar, with a recovery of up to 90%. The binding capacity was 2–5×10^{14} VP/mL with a 100-fold or higher concentration. Even without salt in the eluate, we confirmed with analytical sequencing that aggregation was below 1%.

Full/empty capsid separation

The resultant highly concentrated, pure AAV sample, containing both full and empty capsids, required a polishing step using ion exchange chromatography to reduce the empty capsids. Full capsids have a lower pI than empty capsids (5.9 versus 6.3 on average), and this charge difference can be used in anion or cation exchange with salt elution to separate the capsids. In our experience, anion exchange is preferable for this process. At higher pH, the net charge is negative, and the less-charged empty capsids will elute first in a salt gradient.

During anion exchange to reduce empty capsids for AAV5, we discovered that the $MgCl_2$ concentration was critical for the separation. We used bis-tris propane buffer at pH 9 to compare the separation results using 0, 5, 17, or 20 mM $MgCl_2$ at a constant concentration, and then applied a linear NaCl gradient. We identified that high concentrations of 15–20 mM of $MgCl_2$ were optimal to maximize separation.

The final protocol we used for scaling up the AAV5 polishing step with Capto Q ImpRes resin to 51 mL HiScale™ column is shown in Figure 2. We used 20 mM tris pH 9 with a constant 18 mM $MgCl_2$ concentration and a linear NaCl gradient up to 200 mM.

The empty capsids eluted in an elution hold step with 18 mM $MgCl_2$ before the linear NaCl gradient, in which the full capsids eluted.. VG recovery was measured using quantitative PCR (qPCR). The capacity was approximately 1–3×10^{13} VP/mL resin.

We achieved approximately 60–70% VG recovery and 40–65% full capsids. The result varies depending on the fractions that are pooled, as there is a tradeoff between VG recovery and percentage full capsids.

From our three repeated 10 L AAV5 process runs, we saw virus titer results of approximately 10^{15} VP/L in the final samples. Percentage full capsids was determined in three different ways: qPCR/ELISA, qPCR/surface plasmon resonance (SPR), and anion exchange chromatography (AIEX) high-performance liquid chromatography (HPLC). The result varied depending on the method used, ranging from 42–71%. Regulatory purity targets were met, as the host cell protein (ELISA), host cell DNA (qPCR) were below 100 ng and 10 ng per dose (10^{11}–10^{12} VG) respectively, and residuals levels (nuclease and Tween 20) were below the limit of detection.

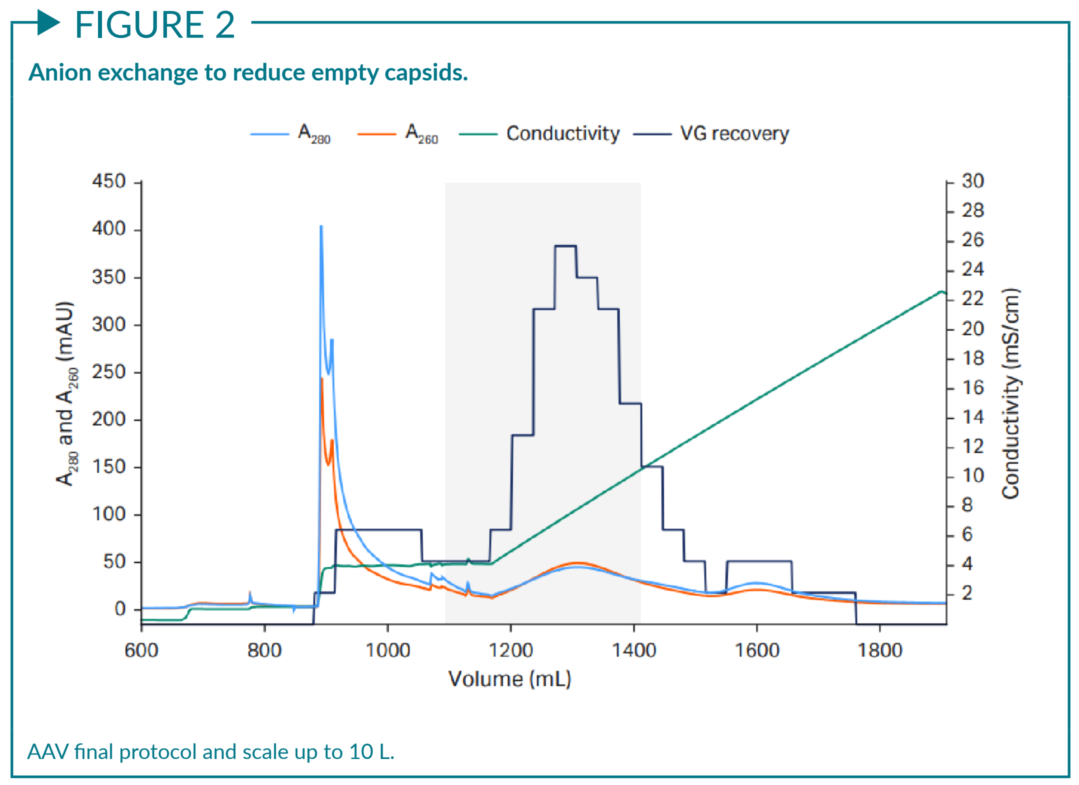
IMPROVING THE AAV POLISHING STEP

Dextran extenders and $MgCl_2$

We further explored the AAV polishing step to attempt to improve the percentage full capsids and VG recovery.

FIGURE 2

Anion exchange to reduce empty capsids.



AAV final protocol and scale up to 10 L.

We found that dextran extenders enhance full/empty capsid separation (Figure 3a). Without dextran extenders, the peaks overlapped. When present on the resin, the dextran extenders enhanced the separation of the peaks. We also found that higher constant concentration of $MgCl_2$ also enhanced the separation and resulted in earlier elution and base line separation (Figure 3b).

Next, we wanted to investigate isocratic step elution with NaCl at high constant concentration of $MgCl_2$ (Figure 4).

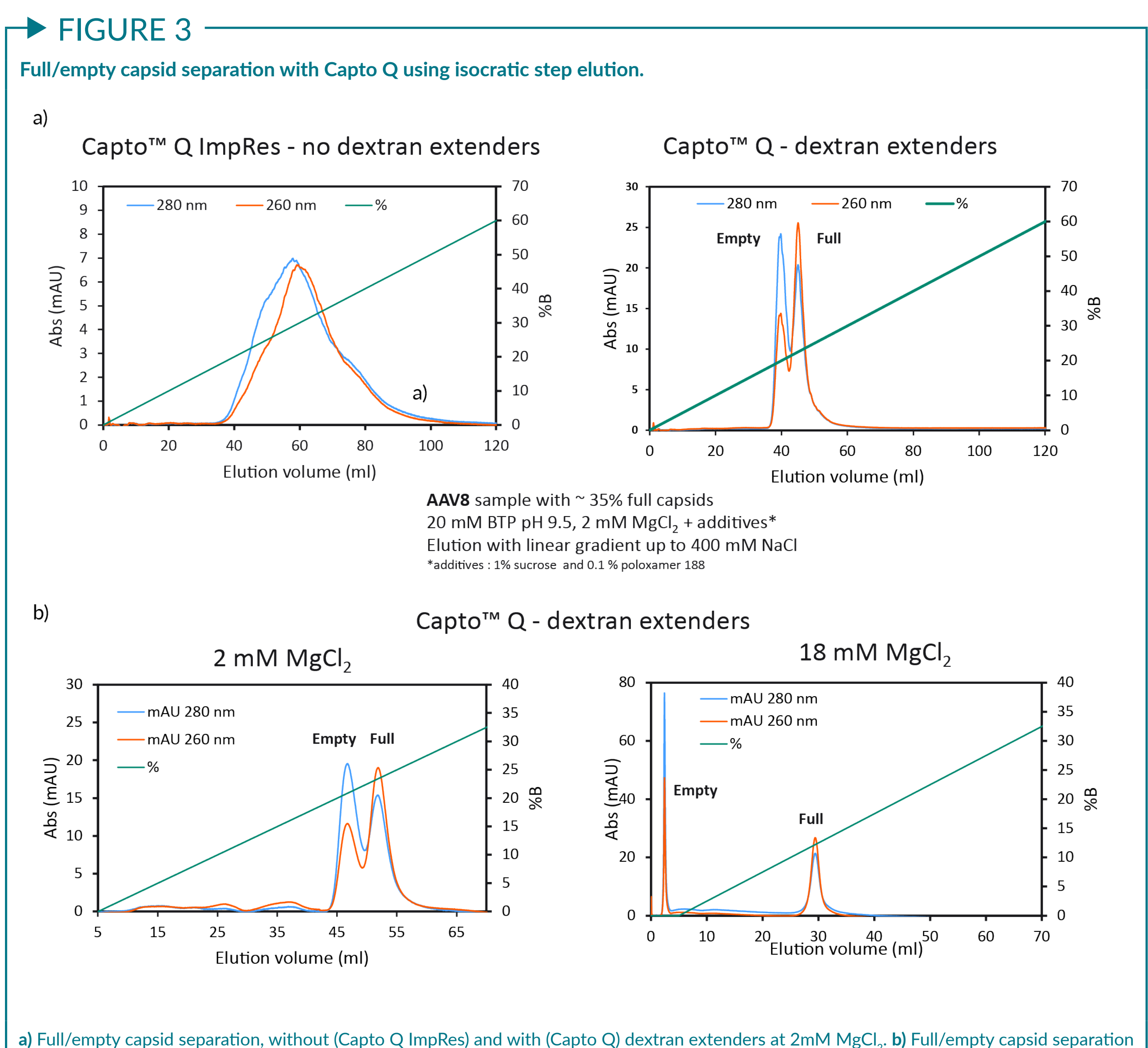
MULTI-SEROTYPE PROTOCOL FOR POLISHING WITH CAPTO Q

Capto Q (with dextran extenders) equilibrated with 20 mM Na-acetate, pH 9 containing 2 mM MgCl2 (buffer A) and after sample application (up to approx. 10^{13}VP/ml resin and conductivity 1–3 mS/cm) a buffer B containing 250 mM Na-acetate was used to elute empty and full capsids respectively. To identify the optimal elution conditions for all serotypes studied (AAV2, AAV5, AAV8, and AAV9), a pre-screening procedure with 5% B buffer incremental steps was performed (Figure 5). This enabled the selection of the %B for the first elution step in the final 2 step protocol. The % B buffer at which the first empty peak eluted was selected for elution step 1, eluting the empty capsids, followed by the second elution step with up to 100% B buffer to elute the full capsids.

The final protocol that was selected for all serotypes is shown in Figure 6. Between all four serotypes, we saw variation of the optimal %B buffer for step one. The data show the binding strength to the anion exchange resin as follows: AAV2 > AAV5 > AAV8 > AAV9. AAV9 binding was the weakest, seen by the decreasing % B buffer required to elute the empty capsids. 40% B buffer was required for eluting AAV2 empty capsids and only 5% B buffer was needed to elute AAV9 empty capsids.

Step one is prolonged over 20 column volumes, to maximize the elution of empty capsids before the elution of full capsids. This protocol can be used for most capsid types including engineered variants, if the pre-screening procedure is firstly used to determine the exact %B buffer needed for the step 1 eluting the empty capsids. The UV ratios indicating the full to empty capsid

 DOI: 10.18609/cgti.2022.094



a) Full/empty capsid separation, without (Capto Q ImpRes) and with (Capto Q) dextran extenders at 2mM $MgCl_2$. **b)** Full/empty capsid separation with Capto Q, with dextran extenders, at 2 or 18 mM $MgCl_2$.

ratio have also been confirmed with qPCR and ELISA. The results are summarized in Table 1.

ANALYTICS

Analytics are critical for a successful AAV process development and manufacturing but time consuming. Knowledge about the detection range is needed to know the compatibilities with samples of different concentration. Assay variation, including inter- and intra-assay reproducibility, is required in order to know if and how small differences in the result may be significant and/or reliable. Controls and references should be included in every analysis run to determine accuracy, and any assay inhibiting or enhancing effect from the process buffers must be checked to minimize matrix or sample buffer interference. Orthogonal methods for full and empty capsid analysis are also critical, as qPCR and ELISA ratios can be variable. Finally, any simplification and/or automation where possible will reduce throughput time. We recommend

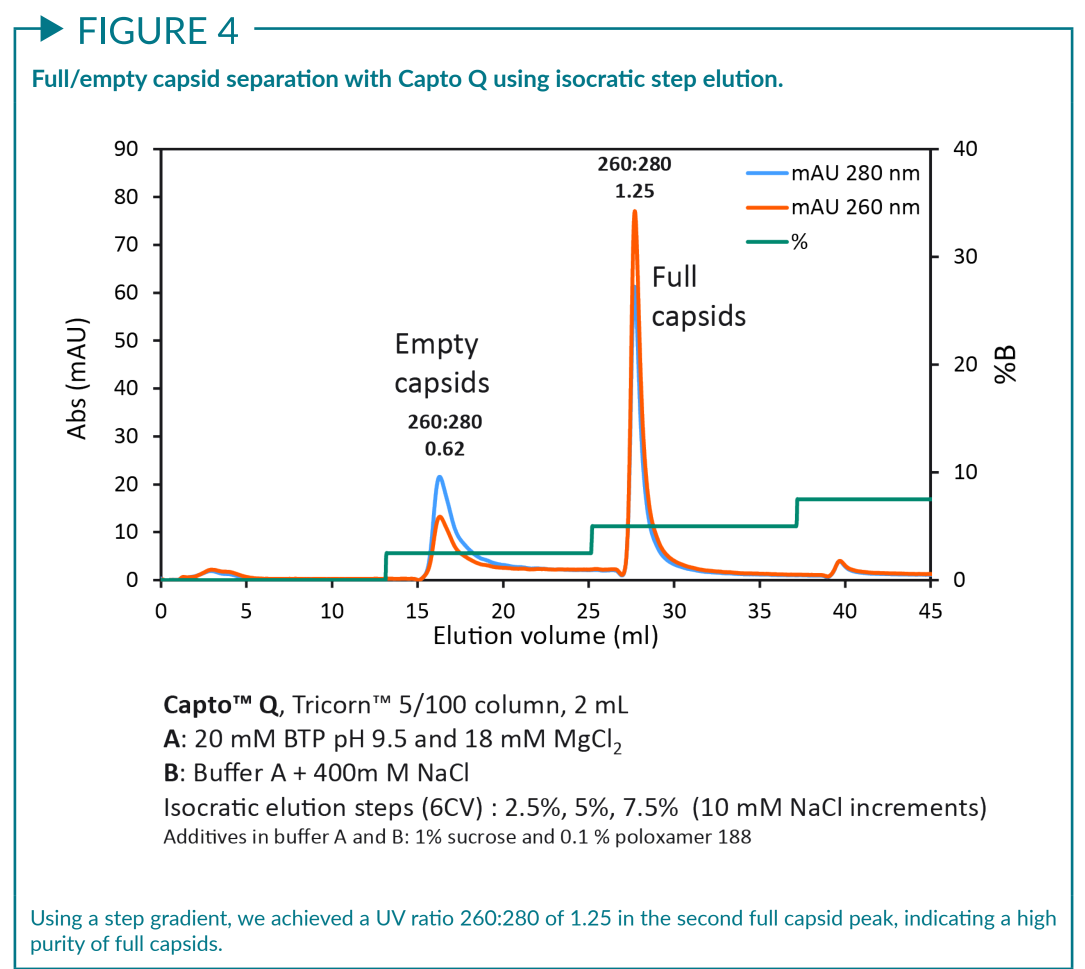


FIGURE 4

Full/empty capsid separation with Capto Q using isocratic step elution.

Using a step gradient, we achieved a UV ratio 260:280 of 1.25 in the second full capsid peak, indicating a high purity of full capsids.

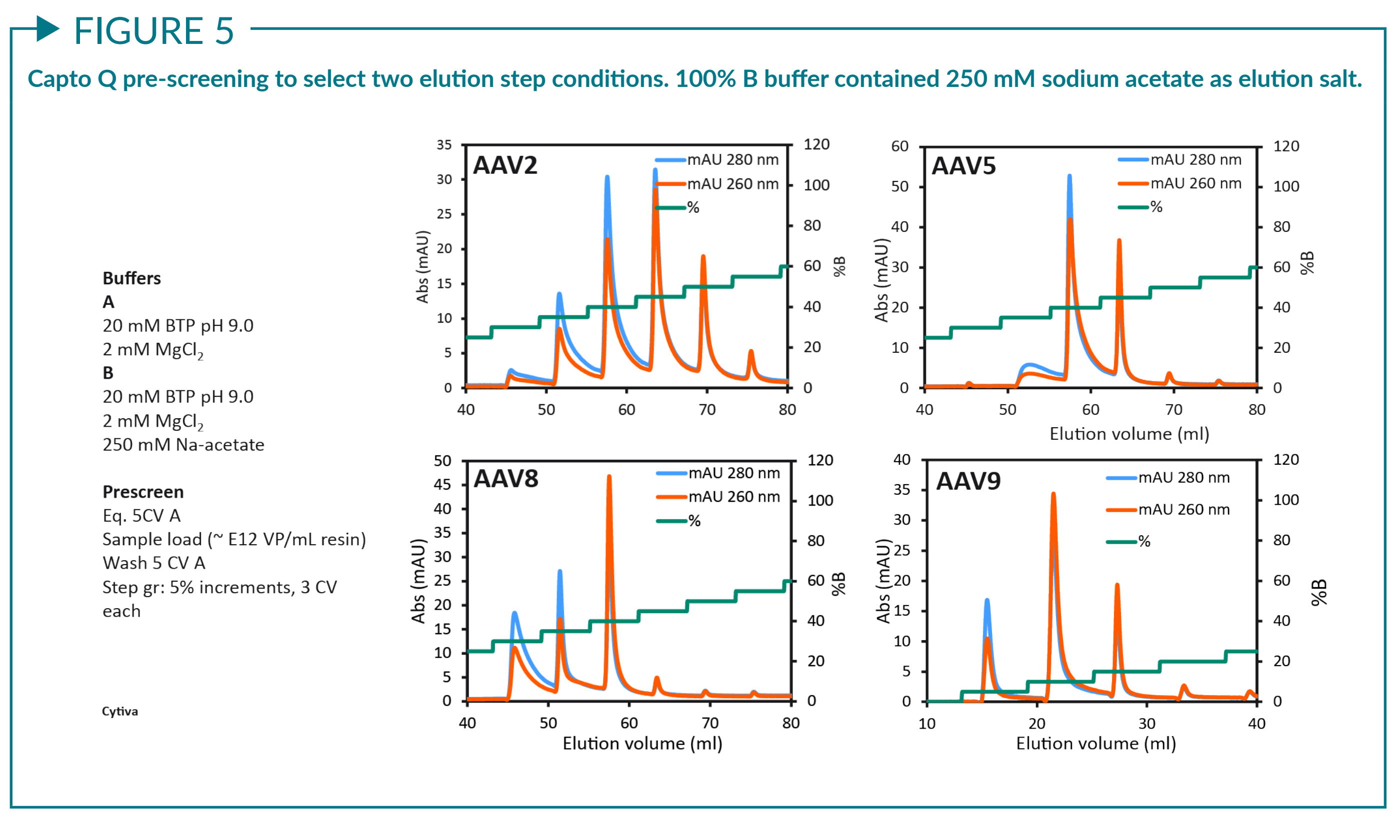


FIGURE 5

Capto Q pre-screening to select two elution step conditions. 100% B buffer contained 250 mM sodium acetate as elution salt.

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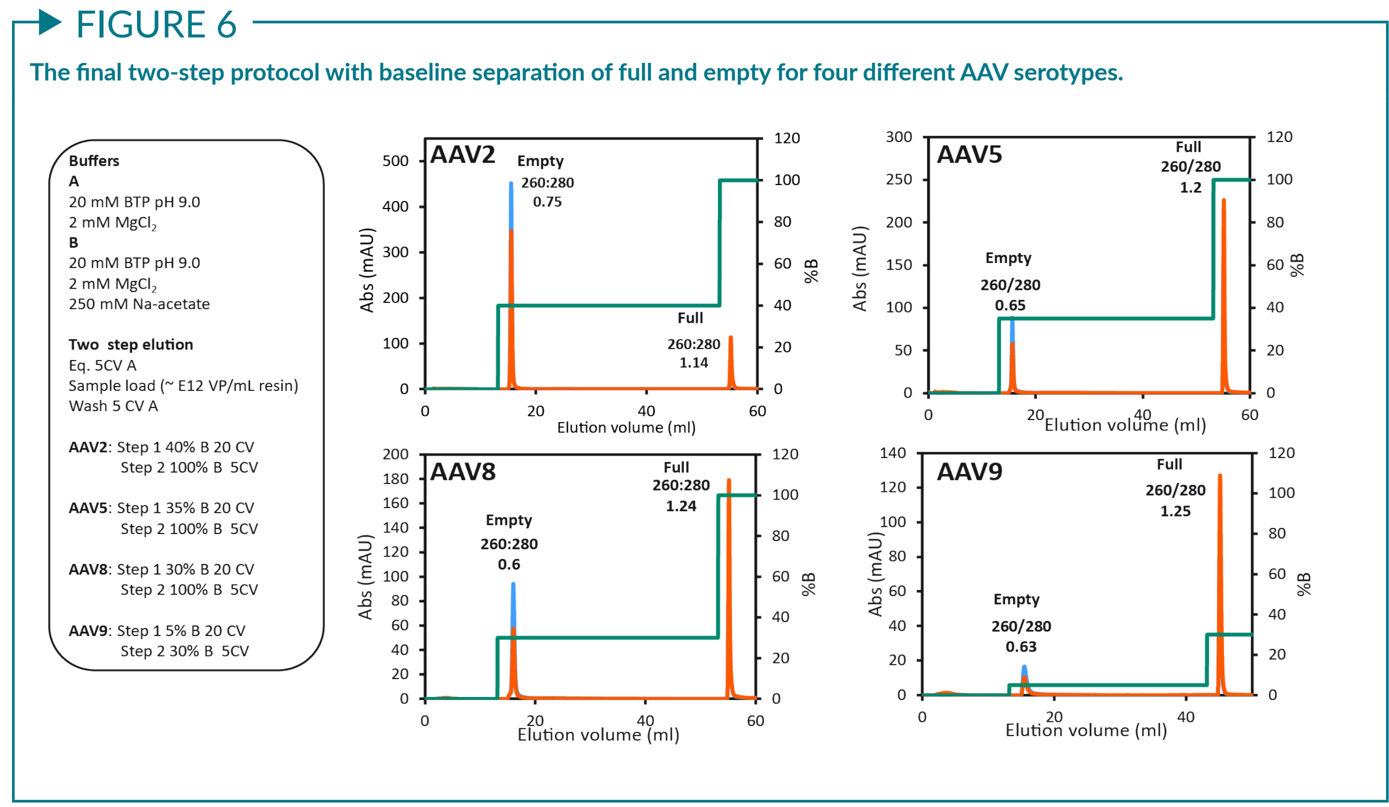

FIGURE 6

The final two-step protocol with baseline separation of full and empty for four different AAV serotypes.

spending time for assay validation as it will facilitate process optimization and bring confidence in the process quality and productivity.

We have developed antibody-based Biacore SPR assays for AAV2 and AAV5, which are compatible for AAV samples from cell culture to final product. The assays are simple and easy to use, with improved precision and a higher degree of automation compared to ELISA.

SUMMARY

We have a scalable HEK293 suspension cell culture AAV production process, with animal-derived component-free medium in single-use bioreactors. Our triple plasmid transfection protocol shows high productivity of viral genomes and high percentage full capsids, and is suitable for use with AAV2, AAV5, AAV8 and AAV9 serotypes. We have a scalable, start-to-finish AAV5 production process and a polishing protocol with anion exchange using Capto Q. Orthogonal methods determining percentage full capsids are needed and we have developed reproducible, easy-to-use Biacore assays for AAV2 and AAV5. This start-to-finish AAV process is suitable for large-scale, clinical-grade production.

TABLE 1

Summary of final two-step protocol results with each AAV serotype.

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

Q&A with Åsa Hagner McWhirter

David McCall, Editor, *Cell & Gene Therapy Insights,* talks to **Åsa Hagner McWhirter,** Principal Scientist, Cytiva

ÅSA HAGNER MCWHIRTER holds a PhD in Medical Biochemistry from Uppsala University in 1999. Åsa has been with the company since 2003, working as a Downstream SME, with a broad and deep understanding biopharmaceutical application challenges in the area of viral vectors and vaccines as well as general protein purification and analysis.

Q How closely do the empty/full ratios via qPCR or capsid ELISA correlate with the area under the curve (AUC), and other analytical methods for determining empty/full ratios?

AHM: It is important to use more than one assay, because qPCR and ELISA assays can vary. A ratio between them can give uncertain values, but you cannot use analytical ultracentrifugation or cryo TEM on all your samples during process development due to high cost and long time to results. You need to do some selection of samples for those high-end analysis. There are many different methods you can use to more quickly confirm those qPCR and ELISA results, such as analytical anion exchange or size exclusion combined with UV 260:280 detection for example.

How do you determine infectious titer by flow cytometry?

AHM: This can be done using a cell-based assay that measures transduction.

The insert here is GFP. When the cells are transduced, GFP will be expressed. You incubate different dilutions of your virus sample with the cells, detach the cells, and then run them in flow cytometry. This allows you to quantify the GFP expression as a measurement of the activity of the virus and give you a functional titer.

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Was the Capto Q protocol tested with any other AAV serotype?

AHM: Yes - we have collaborated with customers to try out this protocol, and it looks promising. We definitely think that it will be possible for other serotypes, including AAV6 and engineered capsids. However, right now, the data is preliminary and has not yet been published.

AFFILIATION

Åsa Hagner McWhirter, PhD
Principal Scientist, Cytiva

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CELL & GENE THERAPY INSIGHTS

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SUPPLY CHAIN CHANNEL: Best practices for ensuring cell & gene therapy supply chain scalability

April 2022
Volume 8, Issue 3

PODCAST INTERVIEW

Precisely for CGT: automating aseptic filling for lowest volumes
Barbara Fischer
403–408

FAST FACTS

Key factors in cell cryopreservation and impact on manufacturability of cell and gene therapies
Robert Newman
433

INTERVIEW

A collaborative approach to cell & gene therapy supply chain management
Anthony Johnson
435–441

CELL & GENE THERAPY INSIGHTS

BEST PRACTICES FOR ENSURING CELL & GENE THERAPY SUPPLY CHAIN SCALABILITY

PODCAST INTERVIEW

Precisely for CGT: automating aseptic filling for lowest volumes

"...do not be afraid of digital transformation. Follow the opportunities that pharma 4.0 offer for your process and facility."

Roisin McGuigan, Editor, Bioinsights, **speaks to**
Barbara Fischer, Process Consultant, Single Use Support

Cell & Gene Therapy Insights 2022; 8(3), 403–408

DOI: 10.18609/cgti.2022.059

Q The number of approved cell and gene therapies is increasing. However, prices remain incredibly high – from several hundred thousand to millions of US dollars. What are the most important factors to consider when looking to make significant cost savings?

BF: There are two major factors that could substantially contribute to lowering the costs of producing regenerative medicines and gene therapies: automation and

standardization. On one hand, standardization is essential. In cell therapies, for example, scalable and reproduceable culture conditions are required to maintain cellular function during *ex vivo* culture. Furthermore, large capacity automated bioreactor systems have the potential to reduce costs effectively, particularly for allogeneic therapies.

Autologous cell therapies are by their nature currently mainly produced manually, in very small-scale and in dedicated suites. Reimagining this process with clear regulatory framework in the background could be an option to continue culturing the cells for other patients to reuse them if they are suitable. In any case, CGTs require skilled and expansive personnel. Unfortunately, manual intervention is amongst the leading causes for deviations, resulting in significant delays of production, product release, or even batch losses. These points should be addressed by the automation of processes, and I would also stress the importance of pharma 4.0 and digital transformation. Standardized, replicable, and automated processes with high output lower costs.

Q What are the biggest hurdles that need to be overcome to address the issues of standardization and scale-up, and speed up this process?

BF: Currently, most processes are carried out literally as manufacturing, meaning many steps are done manually. Standardization of process control and monitoring is a key factor, and it starts with the effective monitoring of cells. Microscopic examination for assessing morphological and functional properties of cell cultures is the routine method used for the evaluation of cell cultures. I see a need for automation with the prerequisite of developing machine learning algorithms and artificial intelligence. In general, the higher the degree of automation and digitalization, the higher the potential to own a standardized and controllable process for all steps from cell bank to filling.

One hurdle may be the investment. As a manufacturer, you need to ensure the systems you are using are scalable and flexible enough to meet rising demands and changing process requirements when switching from one product or process to another. You must not only consider the process flow and variable volumes involved, but also different monitoring points and critical process parameters. It is a choice between investing time to find a suitable solution or embracing the possibility of making additional significant investments.

For cell therapy products, it is essential to have robust cell lines that can undergo as many divisions as is needed for large scale manufacturing. This must be addressed and tested during development to avoid a rude awakening during scale-up or commercialization.

Another hurdle is time; finding the time to make a detailed plan as part of the development process. This plan should include the potential of the product with respect to volumes and batches per year, and the highest possible degree of automation. This does not only include the process and equipment used but also primary packaging, considering all potential options from vials to single-use bags. The question is: which of these primary packages are suitable for all process steps and unit operations, and flexible enough to be used from early development to scale-up? I see it as a bit like planning a kitchen or the configuration of a new car. You should consider all the nice-to-haves from the beginning and then rate them with respect to criticality

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in the form of a risk-assessment. If necessary, deselect options that are rated as non-critical or with low criticality.

“The question is: which primary packages are suitable for all process steps and unit operations, and flexible enough to be used from early development to scale-up?”

Q What specific trends are you seeing currently in the selection of primary packaging?

BF: Common container types used in this sector include cryovials with screw caps, plastic or glass vials, and single-use bags. Primary packaging needs to provide the robustness and physical properties to ensure product quality and safety during multiple handlings across visual inspection, labelling, packaging, cooling, freezing, and thawing. Containers for drug product solutions in particular have to withstand a lot.

Each solution comes with its own advantages and disadvantages. For example, cryovials have a long history, especially in master working cell banks, and are well known and convenient when it comes to handling. Additionally, they are cost-effective, which may be linked to their broad and year-long usage, that facilitates optimized production processes and a decrease in prices. On the other hand, there are clear disadvantages. In most cases, operating with vials means operating with open systems and increased potential for product contamination. Consequently, they need to be filled in an isolator or a filling line situated in a conventional clean room. Both are very costly with additional risks of deviations. Also, the vials have quite limited volumes per dose.

On the other hand, we also see a constant increase in the usage of single-use bags in this area. They also have clear advantages, such as their ability to operate in closed systems, customized options, and the ease of adaptability to changing requirements of system setups. Usually, the systems are designed to minimize manual interventions, thus preventing human errors which can result in quarantined or even rejected batches. Unlike vials, there is no need to operate in a clean room or isolator, minimizing tasks with respect to room requalification, specific monitoring, and extensive cleaning and decontamination procedures. This in total leads to higher throughput. Single-use systems are also more versatile, allowing scale-up and scale-out without total redesign of the equipment – or even the facility.

On the other hand filling and draining of single-use containers requires specific equipment, a one-time CapEx investment that should be considered. There is a clear trend towards the usage of single-use bags in the biopharmaceutical industry. Manufacturers find this technology to be both agile and cost effective. Many newly-established facilities are designed to be used with single-use technologies, and more and more are being reconfigured. This technology has proven to be reliable, especially in the relatively new field of commercial production of regenerative medicine and gene therapies, with high personalization and individualization. We are only at the beginning of a new era of therapeutic possibilities, and there is an opportunity to implement these innovative processes using state of the art technologies from the beginning.

What are the key challenges and limitations when using single-use bags in small volume manufacturing?

BF: Single-use bags that are filled with small volumes of 100 ml, 50 ml, or even below 10 ml are available and easy to use. They are already in use, but mostly in 100% manual handling. A challenge for manufacturers could be finding the right filling unit which also provides the required accuracy.

So, what are the general requirements that a filling unit ideally needs to satisfy for filling single-use bags? The system should be fully automated to provide a 'plug and play experience' for the operator. This includes not only the filling of multiple bags but also the sampling and the sealing. Sealing especially can be tricky and time consuming when working with small volumes. If the whole process is done by the system, then the operator only needs to push the start button and come back when the filling is done to collect the bags. Automation comes with standardization, repeatability, and traceability, substantially reducing variabilities.

Another requirement is to have a completely closed system. This is the best prevention against contamination, and it also addresses costs. When a filling operation does not require a conventional Grade A clean room but can be done in a Grade C or D clean room, it saves a lot of resources for continual environmental monitoring and requalification. This also forces the line to be idle from time to time, so throughput is increased with a closed single-use assembly system that fills single-use bags while decreasing manufacturing overheads.

Last but not least, there is the issue of accuracy. CGTs are highly potent and filling volumes are extremely low, and manufacturers need to be aware of the nominal filling values and accepted ranges. When talking to a supplier, this should be addressed and stated in the user requirement specification from the beginning, with state-of-the-art scales that communicate with a control unit. Each single-use bag should be individually weighed in a controlled manner during the filling.

What are your top recommendations for manufacturers who are transferring a process from manual to automized production?

BF: Start to make a realistic plan for scale-up, scale-out and varying demands as early as possible. Engage the quality control teams, the operators, and the validation teams early in the planning as they may be able to provide valuable input from their first-hand experience. Consider full automation from the beginning, including sampling, in-line monitoring of process parameters or, for example, reaction to pre-alarms. Stay flexible by choosing modular yet scalable solutions that support your processes with high accuracy at all stages.

My recommendation: do not be afraid of digital transformation. Follow the opportunities that pharma 4.0 offer for your process and facility. Try to make your processes and facilities fit for the present, and fit for the future.

> ““We are only at the beginning of a new era of therapeutic possibilities…”

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BIOGRAPHY

Barbara Fischer

Process Consultant, Single Use Support

Dr Barbara M Fischer holds a PhD in Biology and is currently Process Consultant at Single Use Support. In this role, she is working together with established pharmaceutical manufacturers and start-up companies to find the most suitable solution to sustain the product quality through its journey ensuring optimized utilization of resources. Barbara has in-depth experience in low bioburden and aseptic GMP manufacturing of liquid and powder formulations from downstream processing to fill finish.

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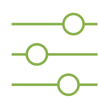
Manual handling for dispensing volumes less than 100mL into single-use bags has never been the gold standard. There is now a reasonable way to turn your back from manual operations and to enter a new filling experience for small volumes:



Automized Plug & Play
Full automation whilst providing options to fill multiple bags, but also sampling and sealing in one.

Closed system
Best prevention against contaminations paired with high cost-efficiency and increased throughput

Accuracy
Controlling filling accuracy is even more important at low volumes, such as cell and gene therapies

Automation
Comes along with standardization, repeatability and traceability

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FASTFACTS

Key factors in cell cryopreservation and impact on manufacturability of cell and gene therapies

Robert Newman PhD, Chief Scientific Officer, FUJIFILM Irvine Scientific

There are three key factors in cell cryopreservation: the cryoprotectant, the buffered media, and protection from osmotic stress. Slow freezing of cryopreservation reagents and protocols is of great importance to cell and gene therapies.

Cell & Gene Therapy Insights 2022; 8(3), 433; DOI: 10.18609/cgti.2022.054

SLOW FREEZING PROCESSES

Most cell therapies utilize a slow freezing process. Immune cell therapies, such as use of mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs), T cells or natural killer (NK) cells, require delivery of 100 million to a few billion cells per dose. Somatic cell therapies such as these require much larger volumes of cells than embryonic or germ cell therapies, which commonly undergo flash freezing to avoid ice crystal formation. With larger volumes of cells, rate of heat transfer becomes a challenge, and slow, controlled rates of cooling (~1oC/min) must be used. Low levels of cryoprotectant are used to minimize toxicity.

Cells dehydrate during the slow freezing process, as illustrated in Figure 1. Water flows out of cells, and ice crystals form extracellularly. Intracellular ice crystal formation is detrimental to the viability of cells, as the ice crystals can puncture the cell membrane. Extracellular ice crystals are less harmful to cell viability. Dehydration during the slow freezing process leads to a higher concentration of salts on the inside of cells which can be problematic. Thus, cryoprotectant selection is important.

CRYOPROTECTANT FORMULATION SELECTION

There are two types of cryoprotectants: penetrating and non-penetrating, as shown in Figure 2.

Figure 1. Cells undergoing the slow freezing process.

Water flows out

~-135°c

Extracellular ice crystal formation

Penetrating cryoprotectants, such as dimethylsulfoxide (DSMO), enter the cell through the lipid bilayer to take effect. DMSO forms hydrogen bonds with water and can take the place of water to reduce the overall water content within the cell. This reduces the freezing point of the cells to help avoid ice crystal formation. Non-penetrating cryoprotectants are often low molecular weight sugars, which will stabilize the cell membrane to reduce the harm of the dehydration process.

Another major part of formulation selection is the buffer, which protect against changes in pH caused by environment, time, and exposure to atmospheric air. The buffer chosen depends on the conditions the cells will be kept in. Components that protect against osmotic stress are also included in the media formulation.

Figure 2. Cryoprotectant selection.

Cryoprotectant

Penetrating cryoprotectant

H_2O

CPA

Dehydration

Nonpenetrating cryoprotectant

Solute

Buffered media

Useful pH range of buffers

3 4 5 6 7 8 9 10 11

Acetate buffer 3.6–5.6

Phosphate buffer, potassium buffer 5.0–8.0

MES 5.5–6.7

Bis-Tris (5.8–7.2)

PIPES (6.1–7.5)

MOPS (6.5–7.9)

HEPES, TES (6.8–8.2)

Tris (7.0–9.0)

Tricine (7.4–8.8)

CHES (8.6–10.0)

CAPS (9.7–11.1)

Protection from osmotic stress

Shrink

Swell

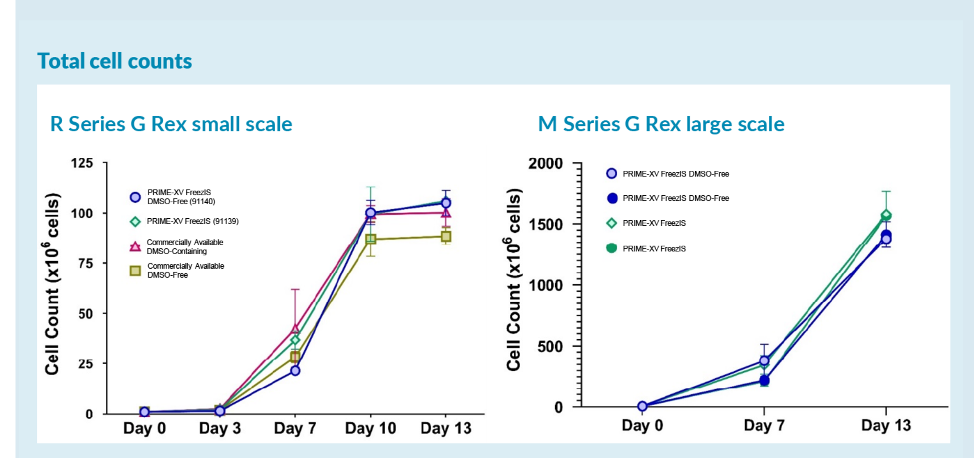
Osmotic stress-protecting molecules such as proteins

TOTAL CELL COUNT

In addition to viable cell density, total cell count is also important. In Figure 3, the small-scale R series bioreactor shows 100 million cells at day 13. The large-scale M series bioreactor can scale-up to a few billion cells. In both examples, no significant difference was seen between DMSO-free and DMSO-containing media with respect to cell expansion.

Having a DMSO-free solution is a huge advantage to cell therapy companies looking to move away from some of the cytotoxic and epigenetic effects that have been reported in literature, including changes in methylation patterns, acetylation patterns, and chromosome structure.

Figure 3. Clinical-grade DSMO-free cryopreservation media for T cells.



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BEST PRACTICES FOR ENSURING CELL & GENE THERAPY SUPPLY CHAIN SCALABILITY

CHANNEL CONTENT

INTERVIEW

A collaborative approach to cell & gene therapy supply chain management

David McCall, Commissioning Editor of *Cell & Gene Therapy Insights*, **talks to** Anthony Johnson, Head of Supply Chain & Warehouse, Cell and Gene Therapy Catapult

ANTHONY JOHNSON is an Operations leader with experience in manufacturing, facilities, and supply chain management, with over 15 years experience in the pharmaceutical sector. Since 2021 Tony has worked for Catapult Cell & Gene Therapy as Head of Supply Chain & Warehouse. Prior to this he worked in Operations and Manufacturing leadership roles, for GSK, Bio Product laboratories and Catalent. He was responsible for leading teams in manufacturing of micronised respiratory and ODS API's, IGG and CF blood products and modified release technologies.

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What are you working on right now?

AJ: We have a little bit of a different setup at Cell and Gene Therapy Catapult, in that we don't actually manufacture any products ourselves. Instead, companies are able to lease a module from us that allows them to start their clinical manufacture, and then get themselves through to the point of commercial manufacture, where they have a product that can go to market. The fundamental idea behind the Catapult is to enable these smaller biotechs

to be able to grow in a more cost-effective manner, rather than having to establish their own manufacturing facility.

When it comes to the supply chain side, apart from those goods that we have to purchase in for our own use, our biotech collaborators will purchase all of their own materials and have their own approved suppliers. Our role is to look after those materials for them within our own warehouse.

That in itself presents a number of problems, because the space we have is at a premium. Our collaborators don't necessarily have a large amount of space allocated within our warehouse, which means we have to operate on a 'just-in-time' basis. Consequently, a lot of our partners need to have collaboration agreements with third-party logistics (3PL) companies – other warehouses where they can store their items locally in much larger quantities, then call them off when they are required. One of the things I'm working on at the moment is helping collaborators identify the arrangements and 3PLs that would work best for them, talking their specific requirements into consideration.

When materials are moved into the grade C cleanroom area, they have to go through various cleaning steps. From a small biotech company's point of view, you want your operators actually manufacturing products, which is the value-add part, rather than spending their time cleaning materials. Consequently, we are also engaged in working with a couple of local 3PL companies on the preparation of cleanroom kits for our biotech collaborators.

The idea is these 3PLs will hold all of the materials for the collaborator. The collaborator will then receive kits at the appropriate time based on their process requirements, which will contain all of the materials they need for that particular stage of the process. These kits will have been put together in a grade C environment and then hermetically sealed, so that upon receipt, rather than having to clean each material down individually, the operators only need to clean the outside of the bag before opening it and transferring the materials into the process. This means that a task that usually takes around an hour to complete becomes one that takes about five minutes. We are currently working with a couple of sample kits that have been provided to us by collaborators with the specific materials they need. We are at the stage of getting those kits made so that we can trial the process, and do some sampling regimes to make sure they are fit for purpose from a cleanliness/contamination perspective. If they pass this test, we will aim to have these kits in place for a couple of our collaborators in April of this year.

> "The fundamental idea behind the Catapult is to enable these smaller biotechs to be able to grow in a more cost-effective manner, rather than having to establish their own manufacturing facility."

Q Your career path took you to a number of different sectors before you joined Cell and Gene Therapy Catapult – what drew you to the cell and gene therapy space?

AJ: I started in pharmaceuticals 2006, and I kind of fell into it by chance. However, I found that I enjoyed the challenges

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it brings – the difficulties you have to overcome around regulations, for instance. I've worked in several different subsets of the pharmaceuticals space – modified release technologies in the form of lyophilized tablets, blood products (which has some similarities to cell and gene therapy), micronization of respiratory products, and natural API manufacturing.

There were a few things that really stood out to me when the opportunity to work in cell and gene therapy arose. Firstly, the thing I like about working in pharmaceuticals generally is that the products you are making actually matter to someone – that what you're doing actually makes a difference to someone at the end of the supply chain. I had mainly worked in manufacturing of products that you could say were 'nice to have': they do make differences in people's lives, but you can live without them or find an alternative elsewhere. Here, we enable our collaborators to make patient-named batches that will truly change someone's life, or save it, in many cases. That in itself is quite a draw to this field.

I also love the science around pharmaceuticals, and cell and gene therapy is right at the cutting-edge of medical innovation. And thirdly, I had always mainly worked in manufacturing and operations background roles, and this was my first role that really focused on supply chain and specifically looked at working with collaborators, which is quite a different model in itself. There is a lot of relationship building and influencing you need to do in order to achieve your end goal, which is not necessarily the case in other sectors.

What is your take on the key challenges that cell and gene therapy specifically presents to supply chain management?

AJ: I think there are several special challenges this sector and this role bring with them. One is that our collaborators tend to be very highly risk averse. For example, when you are running a supply chain for a normal grade C cleanroom environment, there wouldn't necessarily be as many sterile items required in the way of gowning, gloves, etc. Most people would go for the option that meet requirements and is most cost effective, which is generally a non-sterile option. However, from my experience in cell and gene therapy, our collaborators want to treat the cleanroom areas almost like aseptic filling suites. It's perhaps not quite to the same extent, but certainly, they want to have sterilized and irradiated gowns and gloves. They want to keep the areas as clean as possible, so that there's no chance of any potential contamination. Also, because we have different collaborators working in the building with different products, there is much greater concern over the risk of cross-contamination. In most cleanroom environments, you would typically re-use gowns for a full day, but in cell and gene therapy, the strategy we have around cross-contamination dictates that gowns are discarded to be either re-cleaned or disposed of after every single use. So the sheer volume of waste we generate because of this cautiousness is a challenge.

Another issue, which stems from the fact that this is still a relatively nascent sector, is that there are not necessarily a lot of manufactures out there that make the materials and consumables you need. This makes your portfolio of potential suppliers much smaller and less diverse. And because this is a rapidly growing sector, you have a lot of other companies going to those same suppliers as well. The suppliers are trying to build their capabilities and capacity to be able to meet demand, but you do find the lead times for a lot of items are very long. In the UK, that

> "...the fact we use the same PPE consumables as hospitals and other stakeholders battling the pandemic is one of the biggest issues we have faced through the COVID era"

particular issue has been exacerbated by Brexit, so obtaining things like plastic consumables has become more difficult.

The extended lead time for items is probably the biggest thing, for me. It makes it really important to have a good forecast and identify what you need as far in advance as you possibly can. You also need to try to keep waste to a minimum.

Where specifically are the greatest pressure points in cell/gene therapy materials supply at the moment, from your own experience?

AJ: It's mostly fairly basic items like pipette tips, sterile gloves, sterile disposable gowns. We have actually switched recently to laundered gowns because of the difficulty in sourcing disposables. That seems to be more of a COVID-related issue. And as I mentioned, plastic consumables – tubing kits, for instance – are the items that are probably the most difficult for our collaborator cohort to source currently. We are working together to try to enable multiple collaborators to pool their buying power – this will make the contracts more meaningful to suppliers and therefore, more likely to be fulfilled in a timely fashion.

Are there any other particular issues that the COVID-19 pandemic has presented, and can you tell us more about how you have gone addressing those issues?

AJ: Obviously, the fact we use the same PPE consumables as hospitals and other stakeholders battling the pandemic is one of the biggest issues we have faced through the COVID era. That does seem to be a problem that is now dissipating now. However, there are still problems for supply chain – for example, you might be reliant on a particular item coming from another country, but if that country's pandemic response dictates that they require that same item themselves at any point, they will basically stop exporting it.

Our approach to addressing that particular challenge has been to look at all of our processes, and take a more pragmatic approach to what is required and where. For example, if we see that we don't really need sterile items for a given application, we will put a non-sterile version in. I mentioned the shift to laundered gowns earlier, which is another example of this sort of approach. (Laundered gowns bring other benefits, including being generally better for the environment, costing less, and also increasing the robustness of the supply chain because you have a contract with a provider that they have to fulfil on a weekly basis).

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You have mentioned Brexit already – can you expand on its repercussions as you have experienced them?

AJ: To be perfectly honest, Brexit is becoming less and less of a problem as people get their heads around what to do. It was a strange situation from the outset because COVID-19 and Brexit hit at more or less the same time, which meant that it wasn't necessarily clear which one was causing the bigger problem. You just had to firefight. Building any robustness into the supply chain and trying to do some meaningful forecasting has been tremendously difficult over this period, because there just hasn't been a stable foundation from which to work.

I would say that it's probably only from the beginning of this year that we have started to see stability returning to the sector, and it's coming slowly, but it is definitely getting back to normal now. This is enabling us to do more forward planning and providing the opportunity to really build relationships rather than having to hop between different suppliers.

What would you pick out as the key considerations for your role regarding scalability?

AJ: At Catapult, we aim to not just work as a business entity that looks inwardly all the time at what we need to do ourselves – we try to look at the sector as a whole and to reach out to other stakeholders such as the 3PL companies, cleanroom kitting providers, or plastic consumables suppliers we've discussed previously. We actively reach out to other players within the broad pharmaceutical sector, and sometimes even outside of it, and ask them if cell and gene therapy is an area they have considered, We have forward-looking conversations with them and try to entice them into the space, which we see as a key part of building a much stronger foundation for the industry as a whole.

The scalability part of it for us really starts with working with the partners we have to build those relationships. A lot of our biotech collaborators are new to industry, so with them it's about helping to develop an understanding of how to build a robust supply chain, how to build a good S&OP (Sales and Operations Planning) model, etc. And because all of these companies are in the clinical phase, they are running with very high cost but no profits – helping them to realize the importance of sticking to timelines for their regulatory submissions, or keeping their total waste down to a minimum, will benefit them in the long-term.

Do you have any general advice or best practice you can share with the cell and gene therapy sector and developers seeking to scale their supply chains robustly and cost effectively?

AJ: When it comes to materials supply, where it makes sense to do so, use single-use items by all means, but if you can build in reusable options, they do tend to be more cost effective.

Regarding low-cost general consumables, seek to build a good relationship with one of the larger suppliers like VWR, Fisher or SLS. You know with those companies that if they don't have the exact item you are looking for, they will likely have another version of it that will do. And having such a big presence in the market means they are generally able to supply you in a repeatable pattern. For your high-ticket items, you are best off going direct to the manufacturer (you can generally get a better cost), and then try to identify a secondary source to mitigate risk.

But the thing that brings everything together is building a really good S&OP forecasting model that will give you the information you need to be able to go to these suppliers and negotiate from a position of strength. You can then say to the supplier, 'this is our forecasting model and this is how well we do with adhering to it. What this means is we can supply you with the information looking out to 18–24 months ahead, and we'll then review it on a quarterly or half-yearly basis to give you an update of where we are at.' Having that sort of open discussion with your suppliers will help ensure they buy-in more to what you are doing.

Finally, can you sum up some key goals and priorities for your work over the next 12–24 months?

AJ: For me, the key thing over the next year will be getting back into more of a day-to-day drumbeat in terms of how the supply chain should run. Really drilling down into what our normal usage should look like.

Looking a bit further ahead, it's about putting together good relationships with common suppliers, so that I can help take our biotech collaborators through the process of onboarding with the surety that these suppliers have a genuine interest in what our collaborators are doing, and will always do their utmost to supply them with what they require as a consequence.

AFFILIATION

Anthony Johnson
Head of Supply Chain & Warehouse
Cell and Gene Therapy Catapult

"For me, the key thing over the next year will be getting back into more of a day-to-day drumbeat in terms of how the supply chain should run. Really drilling down into what our normal usage should look like."

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Regulatory Trends

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REGULATORY PERSPECTIVE

Current global regulatory landscape for biodistribution & shedding assessment of rAAV gene therapies & recommendations of the IMI ARDAT consortium on future directions

Natalie Anne Schmidt, Jon Giblin, Timothy K MacLachlan, Shera Dandapat, Gabor Veres, Tatiana Anna Reimer, Martin Schulz, Eva Hatzmann, Andeleeb Dahy, Gregory LaRosa, Mimoun Azzouz, & Christopher J Mann

377–394

EDITORIAL

Unproven stem cellbased interventions & harm to existing & future patients

Flora Naylor & Kirstin RW Matthews

397–401

CELL & GENE THERAPY INSIGHTS

Regulatory Insights

REGULATORY PERSPECTIVE

Current global regulatory landscape for biodistribution & shedding assessment of rAAV gene therapies & recommendations of the IMI ARDAT consortium on future directions

Natalie Anne Schmidt, Jon Giblin, Timothy K MacLachlan, Shera Dandapat, Gabor Veres, Tatiana Anna Reimer, Martin Schulz, Eva Hatzmann, Andeleeb Dahy, Gregory LaRosa, Mimoun Azzouz, & Christopher J Mann

An understanding of the biodistribution and shedding profile of a gene therapy product following *in vivo* administration is an important element of the development program. Recommendations for biodistribution studies have been issued by various regulatory authorities with the most recent draft International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use guideline S12 'Nonclinical biodistribution considerations for gene therapy products' released for public comment on 3 June 2021. In this paper the Innovative Medicines Initiative, Accelerating Research & Development for Advanced Therapies consortium provides an overview of the current regulatory landscape for conducting shedding and biodistribution studies and makes a call for harmonization across regions. In addition, over the last three decades, a significant body of literature on biodistribution and shedding of AAV-based gene therapies has amassed, and we describe herein the initial stages of construction of a formal database of published biodistribution

and shedding data. The outputs from the database could be leveraged by Sponsors of AAV programs in regulatory submissions. This would reduce the need for unnecessary duplicative studies, streamline nonclinical development and expedite the arrival of this important class of novel medicines into clinic.

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Advanced therapies represent an important class of medicinal products where recent clinical successes have translated into large increases in the number of clinical trials, and investments in the field. In recent years there has been an increasing call to action for a more harmonized approach to requirements for developing gene therapies both among EU member states and between global regulatory agencies [1,2]. One such example of a move toward harmonization is the 2021 release of the draft International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) Guideline S12 "*Nonclinical biodistribution considerations for gene therapy products*" [3]. This draft guideline provides recommendations for the overall design of nonclinical biodistribution assessments, while also offering considerations for the interpretation and application of biodistribution data to support a nonclinical development program and clinical trial design. Whilst this is a welcome step toward harmonization, it is recognized that there are further opportunities to leverage existing data to reduce animal usage [4]. In general, scientific practice has shown that a lack of agreement on concepts, practices, standardized terms and definitions can hamper collaboration and alignment [5]. The Innovative Medicines Initiative (IMI; imi-europe.org), ARDAT (Accelerating Research & Development for Advanced Therapies) consortia was formed in early 2020 to fund five years of intensive research into AAV biology in the hopes that lessons learned will facilitate the development of AAV therapies. Over the last three decades, a significant body of research has been generated as developers of AAV gene therapies and academic researchers publish data from biodistribution and shedding studies. ARDAT proposes that, for AAV serotypes where biodistribution and shedding data is publicly available, regulatory applications could utilize the plethora of published literature instead of duplicating nonclinical studies. However, leveraging published data should also take into consideration the biological relevance of the animal species used to investigate biodistribution and/or shedding to the investigational product (e.g., virus-host interaction and tropism). In addition, assessments of transgene expression should consider the nature of the promoter, including tissue-specificity.

Our ongoing work to build the first formally constructed database of published biodistribution and shedding data on AAVs has revealed inconsistencies across the field in many aspects regarding the reporting of data (e.g., terminologies used, units of measurement), experimental design (analysis timepoints and tissue types analysed) and analytical methods used (e.g., lack of detailed information on method protocols, validation and detection limits). These inconsistencies could potentially limit the extrapolation of the data obtained from such studies to support regulatory submissions of vectors based on the same, or similar, serotype. The identification of these inconsistencies in reporting biodistribution and shedding data during database construction will facilitate future proposals for minimum data standards (including minimum analytical method validation) in this area.

The creation of a publicly available database of AAV biodistribution and shedding data

aims to enhance regulatory convergence and facilitate nonclinical development of AAV-based therapies. By allowing developers of such therapies to focus nonclinical studies to only product-specific issues, animal use with the associated time and investment costs will be reduced. In this way, it is hoped that the ARDAT biodistribution and shedding database will accelerate the development of these important therapies so that they become available as soon as possible to those patients who are waiting for the potentially life-changing benefits they offer.

REGULATORY AGENCY EXPECTATIONS FOR BIODISTRIBUTION

Biodistribution, as defined in the draft ICH Guideline S12, is the *in vivo* distribution, persistence, and clearance of a gene therapy product at the site of administration and in target and non-target tissues (including blood, cerebrospinal fluid and vitreous fluid), in biologically relevant animal species [3]. Data on biodistribution for a gene therapy medicinal product (GTMP) collected during nonclinical development will contribute to the design of nonclinical safety studies and can also inform dose decision, dosing schedules and monitoring plan for subsequent early-phase clinical trials.

The current regulatory guidelines discussing biodistribution requirements are summarized in Table 1. These clearly state that the biodistribution profile should be determined for a gene therapy product that has not previously been administered to humans and is proposed for a first-in-human (FIH) clinical trial. Under certain circumstances, biodistribution studies may also be conducted during later-phase clinical trials. For biodistribution studies, as well as nonclinical studies in general, incorporation of the 3Rs principles (reduce/refine/replace) regarding animal use [6] are recommended to eliminate the conduct of redundant studies.

In 2015, regulators from the International Pharmaceutical Regulators Programme (IPRP) Gene Therapy Working Group presented the expectations of various international regulatory authorities for nonclinical biodistribution studies [1]. Briefly, the importance of collecting biodistribution information early in product development to guide design of nonclinical toxicology studies and inform decisions on the need for additional nonclinical studies was emphasized. Specific requirements for biodistribution study design were discussed, including the use of a dosing protocol that mimics the proposed clinical protocol with appropriate safety margins (usually highest dose), assessment of all relevant organs, and extension of the analysis interval until the gene therapy product is not detected or a plateau phase is reached. Further considerations were made on inclusion of a relevant animal species and the use of different detection methodologies, including quantitative polymerase chain reaction (qPCR), immunohistochemistry, *in situ* hybridization, fluorescent protein expression, or *in vivo* imaging. The importance of collecting biodistribution data for new gene therapy vectors was discussed.

It was also acknowledged that nonclinical biodistribution studies have some limitations, such as the inherent differences between animals and humans (e.g., differences in organ size, receptor distribution, and physiology/pathophysiology) and that appropriate justifications for the choice of animal species/model would be required on a product-specific basis.

Importantly, there was also a recognition by some regulators that the use of shared or existing biodistribution data could facilitate development of gene therapy products of the same vector class by reducing or eliminating redundant nonclinical studies. However, the relevance of existing data should be justified on a case-by-case basis taking into consideration vector design, manufacturing process, dose, route of administration and disease. Furthermore, in the context of integration and germline transmission, a need for regulatory harmonization in approaches to study design and assessment was suggested.

A summary of biodistribution data submitted to support EMA and FDA marketing

TABLE 1

Summary of global biodistribution guidelines[1].

Health Authority	Guideline	Summary of recommendations on biodistribution
European Medicines Agency (EMA)	Guideline on quality, nonclinical and clinical requirements for investigational advanced therapy medicinal products [7]	▶ Data should be available to provide information on the persistence, duration of effect, and target organs to support the design and duration of safety study(ies). ▶ Extrapolation might be possible case by case with exemption to replication competent viral vectors where nonclinical biodistribution studies are expected prior first clinical trial.
	Guideline on the nonclinical studies required before first clinical use of gene therapy medicinal products [8]	▶ Studies should provide data on all organs, whether target or not. ▶ Observation time should cover persistence of signal (i.e., duration of transgene expression and activity) and include time-points for which there is no signal detection, if applicable. ▶ The dosing should mimic the clinical use with appropriate safety margins.
	Guideline on nonclinical testing for inadvertent germline transmission of gene transfer vectors [9]	▶ The biodistribution studies should be performed using the final vector construct with the gene of interest, with two dose levels at minimum, in at least two species, one of which should be a non-rodent species. The study should be conducted using both sexes. If no positive and persistent signal in gonads is detected in biodistribution studies, this might exclude the need for further nonclinical germline transmission studies. ▶ As a worst-case scenario, biodistribution studies should also be carried out using the intravenous route of administration with a dose per kg body weight at least 10-fold higher than the one to be administered to subjects/patients.
	Guideline on the risk-based approach according to annex I, part IV of Directive 2001/83/EC applied to Advanced Therapy Medicinal Products [10]	▶ Biodistribution data is identified as important for the identification/evaluation of risk factor-risk relationships translated later into support for MAA.
	Guideline on scientific requirements for the environmental risk assessment of gene therapy medicinal products [11]	▶ Nonclinical biodistribution and shedding data can be used to define which tissue samples are to be taken and the timing of sampling pre- and post-administration. For example, urine, faeces or mucosal nasal swabs, could be analysed as a part of a biodistribution study for the presence of the GMO.
	Reflection paper on quality, nonclinical and clinical issues related to the development of recombinant adeno-associated viral vectors [12]	▶ The guidance states that non-clinical biodistribution data of a human serotype-derived vector in animal models may not correlate with human biodistribution and there may be a scientific justification in some cases for the use of serotypes specific to the animal model used. ▶ Transgene expression should also be investigated. ▶ Study design should include where possible assays for the detection of co-packaged plasmid DNA to assess distribution and persistence. ▶ Impact of concomitant treatments (e.g., immuno-suppression) on biodistribution should also be considered. ▶ Germline transmission studies should be conducted before first administration to humans.
US Food and Drug Administration (FDA)	Guidance for Industry Pre-clinical Assessment of Investigational Cellular and Gene Therapy Products [13]	▶ The characterization of vector presence, persistence, and clearance profile can inform the selection of the GT product dosing schedule, the monitoring schedule for various activity/safety parameters, and the animal sacrifice time points in the definitive pre-clinical studies. ▶ Biodistribution data, coupled with other pre-clinical safety endpoints such as clinical pathology and histopathology, help determine whether vector presence or gene expression correlates with any tissue-specific detrimental effects in the animals.
	Guidance for Industry Long Term Follow-Up After Administration of Human Gene Therapy Products [14]	▶ Nonclinical studies to assess persistence are recommended to inform the potential risk of delayed adverse events and to aid in planning for long-term follow up in clinical studies ▶ To determine vector persistence, PCR assay is recommended – persistence is indicated by the presence of detectable levels of vector sequences above a threshold level (≥ 50 copies/μg genomic DNA) and the absence of a downward trend over several timepoints. ▶ Nonclinical data from similar gene therapy products may be used to support conclusions with regard to persistence (e.g., a vector with identical route of administration and final formulation that only differs in the coding sequence for the proposed therapeutic gene product) ▶ Biodistribution studies can be conducted as 'stand-alone' or as part of pharmacology or toxicology studies. ▶ Detailed recommendations for animal study design are provided, which include considerations regarding the use of final product formulation, number of animals required of each sex per timepoint, use of intended clinical route of administration, dose levels, characterization of product distribution and clearance kinetics. ▶ A minimum panel of tissues to be sampled in biodistribution studies is recommended along with general considerations for tissue collection. ▶ Recommendations for qPCR assays are made including demonstrated limit of quantitation and use of spike controls to determine assay sensitivity.
PMDA	Quality and Safety Assurance for Gene Therapy Products and Human Cell-based Products, 9 July, 2019 [15]	▶ Biodistribution of the gene therapy product or human cell-based product in suitable animals should be presented as basic data for evaluating the safety and efficacy of the product. Analysis of biodistribution can clarify distribution not only to the intended tissues but also to non-target tissues and germ cells, making it possible to identify which organs to focus on when evaluating safety and the risk of inadvertent integration in humans. ▶ Clarifying aspects of vector persistence such as distribution and elimination will yield information on suitable timing for analysis in humans. ▶ Biodistribution data may be useful when considering the toxicological significance of abnormal findings specific to certain tissues in toxicity studies. ▶ If biodistribution studies are not performed before starting clinical trials of a new gene therapy product or human cell-based product, the reason for this must be explained. ▶ When analysing biodistribution, tissue, blood and other materials should be collected at defined intervals after administration of the gene therapy product or human cell-based product, and the vector copy number should be measured using qPCR or similar methods. In addition, measuring changes in the vector copy number over time will yield information on the fate of the vector. ▶ If expression constructs are found in specific tissues, bodily fluids, etc., expression of the target gene from these expression constructs should be analysed.
IPRP[2]	Reflection paper – "Expectations for Biodistribution (BD) Assessments for Gene Therapy (GT) Products" [16]	▶ Overarching focus on the need, design, conduct and analysis of gene therapy biodistribution studies. Details are provided for study design. Suggestions on implementation of data into design of FIH clinical trials. Considerations on when additional biodistribution studies are needed.

[1] Note: ICH guideline S12 "Nonclinical biodistribution considerations for gene therapy products" was released for public comment on 3 June 2021, but has not been included in the table as the recommendations may change in the final document.
[2] The International Pharmaceutical Regulators Programme (IPRP) is a consortium of international regulators from Australia, Brazil, Canada, China, European Union, India, Japan, Singapore, South Korea, Switzerland, Thailand and United States
EMA: European Medicines Agency; FDA: Food and Drug Administration (United States); GMO: Genetically modified organism; GT: Gene therapy; IPRP: International Pharmaceutical Regulators Programme; MAA: Marketing Authorization Application; PMDA: Pharmaceuticals and Medical Device Agency (Japan); qPCR: Quantitative polymerase chain reaction.

authorizations of AAV-based GTMPs is provided in Table 2.

REGULATORY AGENCY EXPECTATIONS FOR SHEDDING

From the regulatory perspective, shedding is defined as the dissemination of virus/vector through secreta (e.g., urine, saliva, nasopharyngeal fluids), excreta (faeces) or through the skin (pustules, sores and wounds) of the patient [22,23]. The selection of sample types to be collected for shedding assessment are dependent on a variety of factors such as route of administration, virus tropism, and the natural route of transmission of the parental virus.

General regulatory expectations with regard to shedding data to support clinical trial applications (CTAs) and applications for marketing authorization in the EU, and investigational new drug applications (INDs) and biologics license applications (BLAs) in the US, are described in Table 3. Considerations for environment risk assessments are summarized in Table 4, highlighting relevant differences between the EU and US.

In the European Union, the EMA guideline "*Nonclinical Studies Required Prior to Clinical Use of Gene Therapy Medicinal Products*" states that an investigation of GTMP shedding is a component of the minimum requirements for nonclinical studies before first use in human subjects [8]. Furthermore, the incorporation of shedding studies in an animal model during nonclinical development is also recommended in the EMA guidance on "*Environmental Risk Assessment of Gene Therapy Medicinal Products*" [11] to support the environmental risk assessment (ERA) required for marketing authorization. Complying with GMO requirements at the time of CTA is complex, varies significantly across EU Member States and is leading to delays to clinical trials with ATMPs [2].

In the United States, an environmental assessment is not required at the start of clinical trials for investigational new drugs, except under special conditions. A full report of clinical shedding in the target patient population(s) is expected in the Biologics License Application (BLA) for a GTMP in order to address the potential for transmission to untreated individuals [23]. Clinical shedding reports should include a summary of nonclinical findings (if conducted), rationale for study design and assay development, details of the data collection/sampling plan, procedures for sample handling, collection and storage, description of assays, an analysis of shedding data, and an estimate of the potential for transmission to untreated individuals [23].

Of note, a process of regulatory harmonization with respect to shedding was initiated in 2009, as the topic "*Virus and Gene Therapy Vector Shedding and Transmission*" was the subject of an ICH concept paper [30] and an ICH Considerations document [24], which addressed the general principles to be considered when designing nonclinical and clinical shedding studies, including analytical methods, sampling profiles and schedules. The ICH Considerations document emphasized that data obtained from nonclinical studies of shedding can aid the design of clinical shedding studies by providing an estimation of the likelihood and extent of shedding in humans. 'Stand-alone' nonclinical studies of shedding are not required and shedding analyses can be incorporated into toxicity and/or biodistribution studies, for example by the analysis of urine, faeces or mucosal nasal swabs for the presence of GTMP [11,24]. However, two years later, the ICH steering committee concluded that harmonization on this topic could not be supported due to "*the current state of the science and related resource allocation*".

The extent of shedding data required by regulatory authorities to assess the potential risk to third persons or the environment is dependent on the biological properties of the viral vector. As summarized in Table 5, the biological properties of AAV vectors support the conclusion that they represent a very low shedding risk.

As summarized in Table 6, according to publicly available information, clinical

TABLE 2

Summary of biodistribution data submitted to support EMA marketing authorization of AAV-based GTMPs.

Product / Reference documents	Vector subtype (transgene) Posology / Route of administration	Therapeutic indication	Biodistribution data to support marketing authorization
Luxturna® (voretigene neparvovec) [17,18]	AAV2 (hRPE2) 1.5×10^{11} vg/eye Subretinal injection	Treatment of adult and paediatric patients with vision loss due to inherited retinal dystrophy caused by confirmed biallelic RPE65 mutations and who have sufficient viable retinal cells.	Biodistribution of Luxturna was evaluated at three months following subretinal administration in non-human primates. The highest levels of vector DNA sequences were detected in intraocular fluids (anterior chamber fluid and vitreous) of vector-injected eyes. Low levels of vector DNA sequences were detected in the optic nerve of the vector-injected eye, optic chiasm, spleen and liver, and sporadically in the stomach and lymph nodes. In one animal administered with Luxturna at 7.5 x 10^{11} vg (five-times the recommended per eye dose), vector DNA sequences were detected in colon, duodenum and trachea. Vector DNA sequences were not detected in gonads.
Zolgensma® (onasemnogene abeparvovec) [19,20]	scAAV9 (SMN1) 1.1×10^{14} vg/kg Intravenous infusion	Treatment of patients with 5q SMA with a bi-allelic mutation in the SMN1 gene and a clinical diagnosis of SMA Type 1 or patients with 5q SMA with a bi-allelic mutation in the SMN1 gene and up to 3 copies of the SMN2 gene	The biodistribution and SMN transgene expression profile of ZOLGENSMA were evaluated in neonatal FVB mice through 12 and 24 weeks. Following intravenous administration of 1.5 x 10^{14} vg/kg Zolgensma, the highest vector DNA concentration was detected in the heart, followed by the lung, liver, lumbar spinal cord, quadriceps muscle, brain, ovary, spleen, and testis. The human SMN mRNA transcripts had a similar tissue expression profile with highest levels in the heart, followed by quadriceps muscle, liver, lung, brain, and lumbar spinal cord. Low levels of SMN mRNA were detected in the spleen and gonadal tissues. Additionally, biodistribution was evaluated in two patients who died 5.7 months and 1.7 months after infusion of Zolgensma at the dose of 1.1 x 10^{14} vg/kg. Both cases showed that the highest levels of vector DNA were found in the liver, followed by the spleen, inguinal lymph node and heart. Vector DNA was also detected in the muscles, peripheral nerves, kidney, pancreas, lung, spinal cord, brain, and thymus. Immunostaining for SMN protein showed generalized SMN expression in spinal motor neurons, neuronal and glial cells of the brain, skeletal muscles, heart, liver, kidney, lung, pancreas, spleen, thymus, stomach, large intestines, small intestines, and inguinal lymph nodes.
Glybera (alipogene tiparvovec) [21]	AAV1 (human lipoprotein lipase gene) Intramuscular injections in the legs, 1.5×10^{12} vg per injection site	Familial lipoprotein lipase deficiency	Following intramuscular administration of Glybera to mice, vector DNA was transiently detected in the circulation. Eight days after administration, high levels of vector DNA sequence were detected in injected muscle and the draining lymph nodes. Except for the site of injection, the highest vector DNA copy numbers were found in the liver and blood. The lowest number of copies was found in the brain, lung, heart and non-injected groups of muscle. In gonads and reproductive organs, vector DNA copies were found at low levels. After time, residual vector DNA levels remained high in the injected muscle and inguinal lymph nodes while decreasing steadily in the other organs. The levels of Glybera vector DNA found in gonads were measurable but lower than in other non-target organs. Immunosuppressant co-treatment did not influence the biodistribution pattern neither at low dose nor at high dose in mice. The biodistribution pattern was very similar in the other tested species (cats and rabbits).

Note: [1] The marketing authorization for Glybera expired in 2017, following the decision of the marketing authorization holder not to apply for a renewal due to a lack of demand for the product.
AAV: Adeno-associated viruses; SMA: spinal muscular atrophy

shedding data was presented to support marketing authorization of both AAV-based GTMPs currently on the market; Zolgensma and Luxturna, as well as for the withdrawn product Glybera. It is noteworthy that nonclinical shedding data did not appear to be presented for any of the aforementioned products.

In the case of Zolgensma, the possible expression of transgene (or partial/nonvector related sequences arising as viral packaging impurities from the manufacturing process), induction of immune responses against capsid proteins, and vector mobilization were identified in the ERA as the potential hazards related to shedding and third party transmission. The likelihood of shedding was considered to be high, considering clinical trial data that showed that vector shedding occurred in urine, saliva and faeces. Due to the replication-deficient nature of the vector, no infectious particles were expected to be shed (although this was not formally shown in clinical studies), and therefore the environmental consequences of transmission to non-target individuals to occur were expected to be limited [20].

The presence of replication-competent AAV (rcAAV), arising due to either impurities in the manufacturing process or complementation by co-infection with wild-type AAV, was considered to pose a negligible risk. In particular, the limitations on packaging capacity for AAVs do not permit the therapeutic transgene (SMN1) and the genes required for helper virus-mediated replication (Rep and Cap) to exist in the same viral particle.

Shedding of vector particles was not specifically identified as a potential hazard in the ERA for Luxturna, due to the transient and low level of shedding reported in clinical studies [17].

The ERA for Glybera contained an assessment of the potential risks associated with third-party transmission to healthy persons due to the reported shedding of viral vector from patients through urine, faeces, saliva, and seminal fluid [21]. Several aspects were considered including; i) the effect of transgene over-expression, ii) possible non-site specific AAV integration and insertional mutagenesis, iii) possible adverse effects associated with certain vector elements (e.g., tumorigenicity risks associated with the Woodchuck hepatitis virus post-transcriptional regulatory element [WPRE] present in the viral genome), iv) presence of replication-competent AAV by recombination events occurring during manufacturing or after administration to patients, v) Incorporation of shed DNA by other animal or plant species; vi) Germline transmission. EMA and the national competent authorities responsible for GMO regulation agreed with the Applicant's conclusion that Glybera was a negligible risk to human health (of third parties) and the environment.

Given that the biological properties of AAVs suggest that the potential risk to third parties via shedding is generally low, ARDAT proposes that for serotypes where biodistribution data is publicly available, regulatory applications utilize the plethora of published literature instead of duplicating nonclinical AAV biodistribution studies. This is consistent with the observation that nonclinical shedding data did not appear to be presented at the time of Marketing Authorization Application for AAV-based medicinal products currently approved in the EU.

REGULATORY AGENCY EXPECTATIONS FOR BIOANALYTICAL METHODS

The current 'gold standard' for the measurement of specific DNA or RNA corresponding to vector genome or transgene expression products in tissues and biofluids is qPCR for DNA and quantitative reverse-transcriptase PCR (qRT-PCR) for RNA. These assays are used to assess both biodistribution and shedding. As stated in ICH S12, qPCR-based assays have the advantage that they are sensitive, reproducible, and rapid [3].

In (non)clinical biodistribution studies other techniques that can be used to quantitatively assess vector or expression product biodistribution include enzyme-linked immunosorbent assay (ELISA), digital droplet PCR, flow cytometry and other *in vivo* and *ex vivo* imaging techniques. Other techniques such as immunohistochemistry (IHC), Western blot, in situ hybridization (ISH) can be used for a qualitative assessment of transgene expression. A comprehensive description of the methodology and a justification for the

technique used should also include the performance parameters of the method [3].

There is also a recognition that the standardized requirements for bioanalytical method development and validation may be difficult to apply for these types of assays and in the absence of specific regulatory recommendations for qPCR / qRT-PCR method validation it remains a challenge for the field to develop standardized methods for the analysis of biodistribution and shedding [31]. A recently published article provides some orientation with regard to those aspects of qPCR-based method development and validation which should be considered; extraction efficiency of the AAV-based product in each matrix, inhibitory effects of the matrix on PCR components (e.g., DNA polymerase), and primer design and selection (e.g., including at least a portion of the inserted transgene to avoid false positives arising from the presence of wild-type AAV) [32].

While the guidance documents on bioanalytical method validation released by EMA, FDA and ICH are comprehensive, their focus is on the detection of small molecule drugs and therapeutic proteins by chromatographic and ligand-binding assays in a limited set of biological matrices such as serum, blood, plasma, and saliva [33–35]. There is no specific mention of nucleic acid amplification techniques such as qPCR in those documents.

The FDA draft Guidance for Industry on the Preclinical Assessment of Investigational Cellular and Gene Therapy Products states that qPCR assays should be used to determine the number of vector copies per microgram of genomic DNA in tissues/biological fluids, but there is no mention on whether validation is required [13]. Likewise, FDA does not require validation of qPCR assays used to assess shedding, only that the assay should be qualified to meet minimal performance capabilities (specificity, sensitivity, reproducibility, and accuracy) and be suitable for the intended purpose [23].

In contrast, EMA guidance states that the methods of analysis used in nonclinical studies should be validated with the test article in the appropriate matrix [36]. It is noteworthy that during the review of the MAA for Luxturna, the lack of validation of the assays used to detect the AAV-based viral vector to an acceptable standard meant that the nonclinical biodistribution data obtained was not considered definitive by the Agency [17]. This was not believed to be an issue in the FDA's Pharmacology-Toxicology Review for Luxturna, which stated that the report of the qPCR analysis for the evaluation of biodistribution and shedding in AAV2-hRPE65 studies was reviewed and deemed adequate by CMC reviewers.

While method validation is not explicitly mentioned in the newly released draft ICH guidance on nonclinical biodistribution studies, the establishment and documentation of the sensitivity and reproducibility of the quantification method is recommended [3]. The draft guidance also states that spike and recovery experiments are required to demonstrate the ability to detect target. Furthermore, the Gene Therapy Working Group of the International Pharmaceutical Regulators Programme also stated that method validation should be considered in the 2018 reflection paper "*Expectations for Biodistribution Assessments for Gene Therapy Products*" [16].

It should be noted that, to some extent, cross-validation of qPCR-based methods used for release characterization (e.g., viral particle quantification) may also be applicable in the context of bioanalytical methods used to detect viral genomes in biodistribution and shedding studies.

According to the ICH Considerations document on virus and vector shedding, PCR and infectivity are the two assays typically used for the detection of shed virus/vector. qPCR-based assays to detect viral genetic material are recommended. However, assays based on nucleic acid detection do not differentiate between intact (and potentially infectious) viral vector and non-infectious degraded or fragmented virus. Therefore, infectivity assays may be required for adequate assessment of the potential risk for transmission to third persons [24].

TABLE 3

Summary of general regulatory considerations for shedding.

Authority issuing guideline	Name / reference of guidance document	Summary of key considerations
ICH	General Principles to Address Virus and Vector Shedding [24]	**Quality** ▶ For replication-incompetent GTMPs, potential replication-competent recombinants that may arise during manufacturing should be characterized. **Nonclinical** ▶ Although shedding profiles obtained from nonclinical studies may not directly correlate with the human situation, the data obtained can be used to estimate the likelihood and extent of shedding in humans. ▶ Animal disease models may be most appropriate to assess shedding. ▶ 'Stand-alone' nonclinical shedding studies are not necessary, shedding endpoints can be incorporated in biodistribution and/or toxicology studies. ▶ A range of excreta and secreta samples can be collected, most commonly urine and faeces. To obtain sufficient sample size/volume, the pooling of samples collected from several animals receiving the same dose is suggested. **Clinical** ▶ The design of clinical shedding studies should consider the biological properties of the parental virus/vector, replication-competence of the product, dose, route of administration and patient population. ▶ The sampling schedule is usually more frequent in the initial period post-administration and should continue until multiple negative samples are obtained. ▶ The potential for a second round of shedding in the case of replication-competent vectors and the possible impact of immunosuppressive regimens should be taken into consideration when designing the study. ▶ The potential for transmission to third parties (e.g., family members and healthcare workers) should be evaluated if clinical shedding is observed. **Analytical methods** ▶ The use of qPCR to detect viral genetic material in shed samples is recommended and the use of an infectivity assay is considered important to accurately assess the potential for transmission of shed material. ▶ Assay interference from the biological matrix is an important consideration and sample dilution may be necessary.
EMA	Guideline on nonclinical studies required before first clinical use of gene therapy medicinal products [8]	Biodistribution studies should include an investigation of shedding as one of the minimal requirements for nonclinical studies on GTMPs before first use in human subjects.
	Guideline on Scientific Requirements for the Environmental Risk Assessment of Gene Therapy Medicinal Products [11]	▶ Shedding data from both nonclinical and clinical development (data from one or more clinical trials) may contribute to the ERA. ▶ Analysis of urine, faeces and mucosal swabs for the presence of the GMO are suggested, using sensitive and state-of-the-art methods. ▶ The presence of vector genome in shed samples is assumed to represent a potential for transmission into the environment. Assays to demonstrate non-infectivity of shed material should be as sensitive or more sensitive than the assay used to detect vector genome. ▶ Shedding of a GTMP in itself is not considered an adverse effect for the environment, but is rather a factor which is used in the evaluation of the likelihood of a particular environmental adverse effect – i.e., increased shedding resulting in higher environmental exposure only leads to a high risk if significant consequences have been identified (e.g., potential of transmission to third persons and/or other species).
	Guideline on Safety and Efficacy Follow-up – Risk Management of Advanced Therapy Medicinal Products [25]	Shedding data can be used to inform the preparation of the risk management plan and measures to mitigate the potential risk to close contacts of treated patients
	Guideline on Environmental Risk Assessments for Medicinal Products containing, or consisting of Genetically Modified Organisms [26]	The extent of shedding by target patients should be considered in the evaluation of the likelihood of an identified adverse environmental effect.
	Guideline on the Risk-based Approach According to Annex I, part IV of Directive 2001/83/EC applied to Advanced Therapy Medicinal Products [10]	Although shedding is not specifically mentioned, a stated risk associated with the clinical use of ATMPs was disease transmission (presumably to third parties) – as such, shedding data may be useful for determination of the relevance of a particular risk during risk identification. Risk identification can also be supported by reference to relevant literature data.
	Reflection paper on quality, nonclinical and clinical issues related to the development of recombinant AAV [12]	▶ Analysis of the shedding of co-packaged plasmid DNA sequences arising from the manufacturing process should be considered in nonclinical studies ▶ If vector DNA is detected in shed material (e.g., saliva, serum, urine and semen), ideally samples should be followed up for infectious virus quantification; data derived from nonclinical and early clinical studies can be used to assess the likelihood of transmission and to justify the extent of viral shedding evaluation in later clinical studies. ▶ All available data on viral shedding should be used in the ERA. ▶ In clinical studies, samples to be collected and timing of collection for shedding analysis should be justified on the basis of nonclinical data and/or the profile of the parental virus, practical feasibility and ethical justification of sampling. Examples of samples that could be collected include blood/serum, tears, urine, serum, buccal swabs/sputum, lung lavage and faeces.

AAV: Adeno-associated viruses; EMA: European Medicines Agency; ERA: Environment Risk Assessment; FDA: Food and Drug Administration (United States); GMO: Genetically Modified Organism; GTMP: Gene Therapy Medicinal Product; ICH: International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use; LOD: Limit of Detection; PMDA: Pharmaceuticals and Medical Devices Agency (Japan); qPCR: Quantitative Polymerase Chain Reaction.

TABLE 3 (CONT.)

Summary of general regulatory considerations for shedding.

Authority issuing guideline	Name / reference of guidance document	Summary of key considerations
FDA	Guidance for Industry – design and analysis of shedding studies for virus and bacteria-based gene therapy and oncolytic products [23]	**Nonclinical** ▶ Nonclinical data cannot substitute for human shedding studies on the basis that animals may not adequately predict the shedding profile in humans, particularly with respect to patient-specific factors such as differences in immune status at the time of product administration. ▶ Nonclinical shedding data may possibly be requested for replication-competent GTMPs in certain cases (e.g., no previous human exposure to product, changes in route of administration). ▶ Shedding data can be collected from nonclinical studies designed to assess safety or biodistribution. Such shedding data may inform the types of samples to be collected during clinical shedding studies. **Clinical** ▶ Recommendations for the timing of shedding data collection during different phases of clinical development are given; for replication-competent products shedding data should be collected from Phase I onwards, while for replication-incompetent or deficient products shedding data should be collected during Phases II and III of clinical development, after a dose and regimen have been determined. ▶ Modifications of the administration route, dose regimen and indication may cause alterations in shedding profile – shedding data from pivotal studies should be collected. ▶ Comprehensive advice on shedding study design and reporting is provided: ▶ Frequency of sample collection - sampling of shed material should begin immediately after product administration, irrespective of replication competence. Frequent sampling during the initial weeks post-administration is advised to ensure the shedding pattern is accurately captured. ▶ Duration of sample collection - Sampling should continue until three consecutive data points are obtained at or below the LOD of the shedding assay or if a decreasing trend reaches a plateau for at least three consecutive data points. Monitoring periods may need to be longer for patients who are immune-compromised or are receiving immunosuppressive regimens. ▶ Type(s) of samples collected – types of clinical samples collected to assess shedding are dependent on a range of factors including administration route, vector tropism, natural route of transmission and data obtained from nonclinical biodistribution/shedding studies. ▶ Sample storage – to minimize degradation of product-specific nucleic acids and to ensure no loss of product-specific infectivity appropriate storage conditions for different types of samples need to be established. ▶ Overall analysis of shedding data should address the potential for transmission to untreated individuals due to shedding (i.e., the nature of the shed material and the extent of shedding). ▶ Analytical Methods ▶ A quantitative assessment of shedding is recommended (e.g., number of genome copies or infectious units). qPCR is commonly used due to high sensitivity and practicality (e.g., ease of assay standardization). If product is replication-competent detection of nucleic acids should be followed up with infectivity or growth-based assays. ▶ The effect of different biological matrices on assay performance (including selectivity, specificity and sensitivity) should be well understood, particular in the case of shed samples rich in complex organic matter (e.g., urine and faeces).
PMDA	Quality and Safety Assurance for Gene Therapy Products and Human Cell-based Products [15]	The risk of human transmission to of GTMPs should be evaluated, including the risk of a vector administered to a patient being transmitted to a third party other than the patient with specific reference to the "ICH Consideration Document: General Principles to Address Virus and Vector Shedding" [24]

AAV: Adeno-associated viruses; EMA: European Medicines Agency; ERA: Environment Risk Assessment; FDA: Food and Drug Administration (United States); GMO: Genetically Modified Organism; GTMP: Gene Therapy Medicinal Product; ICH: International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use; LOD: Limit of Detection; PMDA: Pharmaceuticals and Medical Devices Agency (Japan); qPCR: Quantitative Polymerase Chain Reaction.

The general recommendation is for a 'step-wise' or 'tiered' approach, whereby the presence of viral genome is detected by qPCR in the first instance, followed by a suitable infectivity assay if viral DNA is detected above a certain threshold [23]. The ICH Considerations document suggests that an assessment of infectivity would be necessary if vector DNA in shed samples is detected at a level greater than the detection threshold of the infectivity assay [24]. Of note, nuclease treatment of clinical shedding samples followed by PCR amplification or amplification of the full-length viral genome from the intact virus particles has also been suggested by FDA as an alternative method for detection of infectious virus [23].

There is relatively little regulatory guidance with regard to infectivity assays. The ICH Considerations document includes an acknowledgement that assays to measure the extent of shedding have the additional complication that detection of infectious virus should be optimized in several different matrices, some of which are rich in complex organic matter (e.g., urine and faeces) and/or genomic material from organisms forming part of the body's natural flora (e.g., saliva and nasal swabs). These matrix effects can affect assay performance, possibility resulting in an underestimation of shedding or a false negative result [24]. For the detection of infectious virus, the FDA recommend assays that measure infectivity in terms of Tissue Culture Infectious Dose 50 ($TCID_{50}$), plaque-forming units (PFU) or focus-forming units (FFU) [23].

In the AAV-specific context, if the presence of replication-competent recombinants or intact virus particles are suspected in shed material an infectivity assay should be developed. These assays can be problematic as AAVs do not induce a cytopathic effect in cell culture and may not be infectious despite internalization [32]. A suggested approach is to treat a susceptible cell line with shed material in the presence or absence of helper virus followed by assessment of viral internalization by qPCR [32].

Storage conditions for samples obtained for shedding and biodistribution analysis also need to be taken into consideration, especially given that requirements may vary due to differences in product stability in different matrices. Multiple aliquots may also be required for different tests (e.g., qPCR for nucleic acid detection and infectivity assays) [23]. FDA guidance suggests that degradation of viral or bacterial nucleic acids in enzyme-rich clinical samples can be accounted for by spiking of mock/donor samples with a reference standard shortly after collection in order to determine the percentage recovery – such samples should be collected, stored, shipped, and extracted in the same way as the 'test' samples.

APPROACHES FOR A HARMONIZED APPROACH FOR THE ASSESSMENT & REPORTING OF BIODISTRIBUTION & SHEDDING

Since 2008, the US National Institutes for Health have supported the National Gene Vector Biorepository (www.NGVBCC.org) as a means to collect data from pharmacology/toxicology studies, archive reagents and samples from nonclinical studies, and in some cases, offer core analytical tests typically employed in nonclinical and clinical studies for gene therapies [37]. The organization has distributed over 1,000 reagents and collected over 36,000 specimens from nonclinical viral gene therapy studies. Furthermore, it maintains a searchable database of gene therapy pharmacology/toxicology studies ultimately resulting in 114 publications since 2018. As of 2020, the pharmacology/toxicology database contained information from 52

TABLE 4

Summary of expectations for shedding in the context of environmental risk assessment (EMA)/environmental assessment (US).

	EU	US
Legislation	**Pharmaceutical legislation** ▶ Directive 2001/83/EC (as amended by Directive 2009/120/EC) **Environmental legislation** ▶ Directive 2001/18/EC "Deliberate Release" ▶ Directive 2009/41/EC "Controlled Release" ▶ Directive 2000/54/EC - protection of workers from risks related to exposure to biological agents at work	The FDA must comply with the National Environment Policy Act of 1969 when considering both IND and BLAs
Relevant guidance document(s)	▶ Environmental Risk Assessment for Medicinal Products containing, or consisting of, Genetically Modified Organisms (GMOs) (Module 1.6.2) [26] ▶ Good Practice on the assessment of GMO related aspects in the context of clinical trials with AAV clinical vectors [27]	FDA Guidance for Industry - Determining the Need for and Content of Environmental Assessments for Gene Therapies, Vectored Vaccines, and Related Recombinant Viral or Microbial Products [29]
CTA / IND expectations	▶ In accordance with the environmental legislation, an environmental assessment is required for GMOs such as investigational GTMPs to ensure compliance with GMO legislation (either "Deliberate Release" or "Contained Release" Directives, depending on Member State). ▶ Authorization is granted on the basis of the submitted environmental assessment ("GMO application") by the relevant environmental authority before a clinical trial can begin in each Member State[1] ▶ Good Practice documents and Common Application Forms, adapted to the specific characteristics of AAVs are available, and have been endorsed by a majority of Member States. In the context of AAVs, specific requirements with regard to shedding data are not detailed. However, potential environmental hazards, including to human and animal health, are identified, which could arise from unintended transmission of clinical vectors ▶ The common application form for AAVs contains a section where detailed data on clinical vector shedding should be included to ensure an appropriate evaluation of environmental risk ▶ In the context of GMO applications for AAVs, if there is no prior clinical experience with the vector, a discussion of the potential for shedding based on nonclinical data should be provided according to the common application form for investigational medicinal products for human use that contain or consist of AAV vectors [28]. In addition, clinical experience from related clinical vectors can be included to support the environmental risk assessment – the relevance should be justified based on dose and route of administration in particular.	A "categorical exclusion" can be claimed for IND submissions for GTMPs, based on the assumption that any potential effect on the quality of the environment would not be significant due to close monitoring and a limited number of treated patients. Therefore, no environmental assessment is generally required to support an IND submission.
MAA / BLA expectations	▶ An Environmental Risk Assessment (ERA) must be included in a MAA for all medicinal products and the legislation (Directive 2001/83/EC, as amended by Directive 2009/120/EC) contains specific requirements for information on the shedding of GTMPs due to their potential for transmissibility / infectivity. ▶ The ERA should include an assessment of the potential for transmission to third persons and clinical studies should include an analysis of shedding to address product excretion. ▶ In addition to a potential adverse effect on human health through transmission to third persons, shedding is also considered a factor that could produce an adverse effect on the environment through transfer to other species or possible interference with other prophylactic or therapeutic medical treatments (e.g., transfer of antibiotic resistance genes), and data on shedding is therefore required to appropriately evaluate environmental risk and mitigate it if necessary [26]. ▶ ERA submitted with a MAA is evaluated in consultation with the national bodies responsible for the release of GMOs into the environment	▶ For BLAs, any GTMP which is not considered to occur naturally in the environment (i.e., any GTMP expressing one or more protein coding sequences from a genus different from the organism expressing the sequence) should include an Environmental Assessment (EA) in the BLA. ▶ FDA recommends that an analysis of shedding demonstrating the release of vector DNA and / or infectious virus should be included in the EA – based on these data the potential effects on the environment are then considered. For example, if no infectious virus is detected in shed material, a justification can be made that only the environmental effects of vector DNA should be considered. ▶ The shedding of potential variants, which may pose greater environmental risk, should also be considered in the EA (e.g., in the context of AAV, the presence of replication-competent virus due to recombination events during the manufacturing process).

Note: 1 For further information and discussion regarding the variability in the timelines and application of the GMO legislation between EU Member States, which can result in delays in clinical development, particularly in multi-centre studies, please refer to a recent review by EFPIA [2].
AAV: Adenoassociated Virus; BLA: Biologics License Application; CTA: Clinical Trial Application; EA: Environmental Assessment; EU: European Union; FDA: Food and Drug Administration (US); GMO: Genetically Modified Organism; IND: Investigational New Drug; MAA: Marketing Authorization Application.

anonymized nonclinical studies and is intended to foster data sharing between sponsors in the interest of comparing data, such as biodistribution, of similar viral serotypes and routes of administration. While this has been an outstanding effort to collect a broad array of information from gene therapy sponsors, we propose to take a deeper dive into the details of nonclinical biodistribution and shedding studies, further collating study design aspects as well as analytical output. Such outputs would enable comparison between studies and could permit extrapolation of existing data for use in regulatory submissions of similar class vectors, thus reducing animal usage and ultimately accelerating gene therapies into the clinic.

In November 2020, the Accelerating Research & Development for Advanced Therapies (ARDAT) consortium began. It represents a collaboration between academia, small and medium-size enterprises (SMEs) and the European Federation of Pharmaceutical Industries and Association (EFPIA) members funded by the European public/private partnership Innovative Medicines Initiative (IMI) and EFPIA 'in kind' contributions. The project involves 12 industry members, 7 SMEs and 16 academic institutions to conduct five years of intensive research into AAV biology in the hopes that lessons learned will facilitate increased and more effective AAV therapies in the clinic. The project is organized in five 'work Packages', each with a particular focus including immune responses to AAVs, the metabolism of the AAV genome after cellular transduction and the development of a biobank of human samples from both AAV and non-AAV clinical trials, which will feed into these research questions. The final work package will focus on engagement with regulatory authorities to ensure that the data generated by the project will support recommendations for regulatory harmonization and create predictable regulatory pathways for innovation.

One initiative within the regulatory work package is to gather information on biodistribution and shedding in the public domain with the intent of collating data from various studies in a database. By formally organising biodistribution and shedding data in this way, developers utilizing the same viral serotype, route of administration, dose levels, etc., could leverage this data to accelerate product development. It is hoped that this leveraged data may be sufficient to satisfy regulatory expectations for the description

TABLE 5

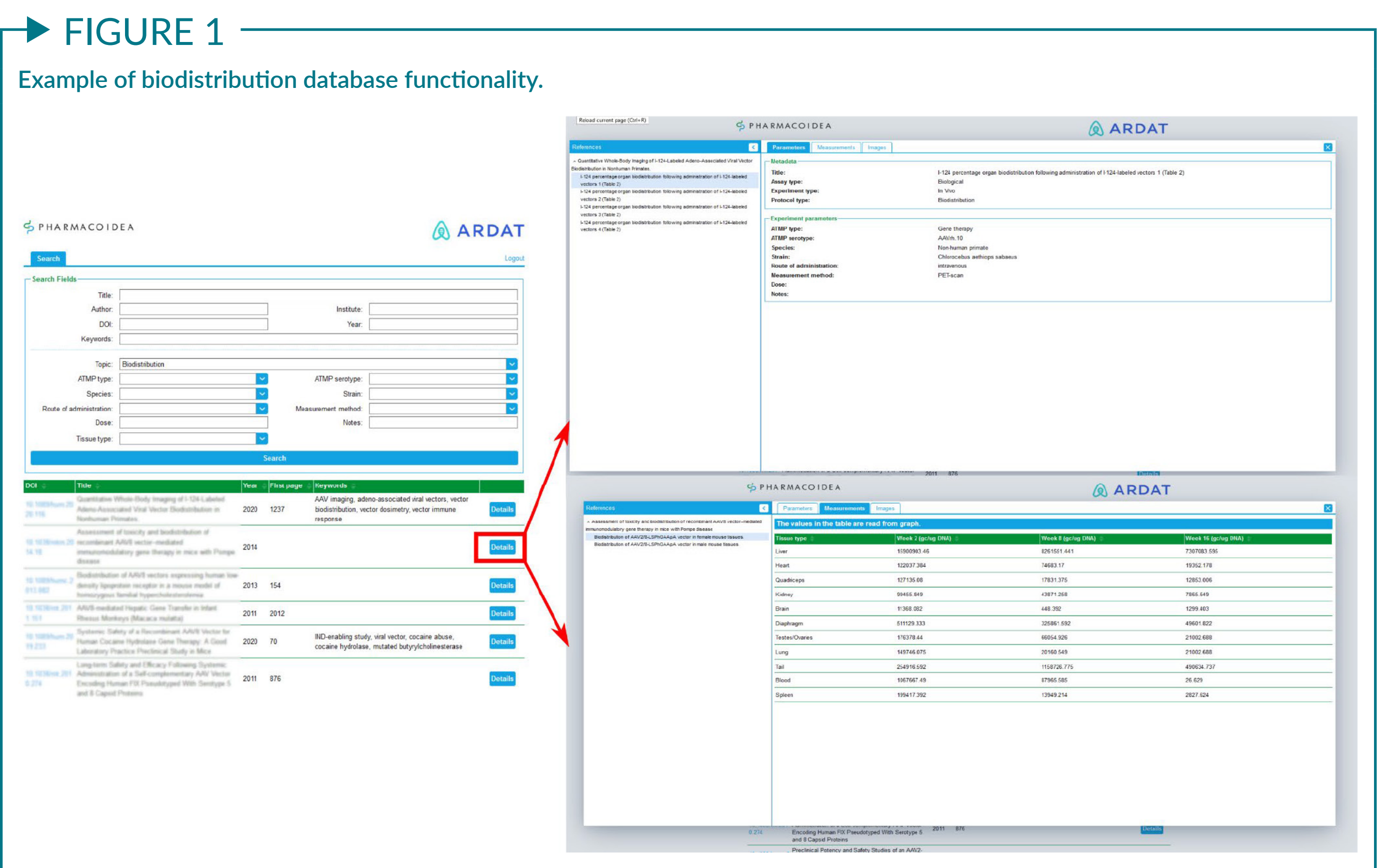
Application of shedding requirements to AAV vectors.

Biological property	Consequences for shedding	Considerations for AAV
Replication competence	Replication-competent vector may persist in the patient for extended periods and may increase in amount over time. Shedding potential of replication-competent viruses can be higher, resulting in a higher probability of transmission	Wild-type AAVs are naturally replication deficient and require co-infection with helper viruses to replicate. Furthermore, recombinant AAVs also lack the rep and cap genes, which are typically replaced by the therapeutic transgene. Consequently, rAAV-based vector shedding is predicted to be of short duration. Replication-competent recombinants potentially produced during rAAV manufacturing may need to be considered.
Duration of infection / vector persistence	Short-term infection and/or rapid viral clearance due to immune response may reduce the duration and extent of vector shedding	Long-term infection and therefore shedding may occur in patients receiving immunosuppressive regimens, a second peak of shedding may occur if the regimen is discontinued.
Properties of parental viral vector (e.g., route of transmission)	There could be an increased risk of transmission in excreta or secreta corresponding to the natural route of transmission	For AAVs, there is potentially more risk of transmission from nasopharyngeal secretions rather than urine or faeces

AAV: Adenoassociated virus; rAAV: Recombinant adenoassociated virus.

FIGURE 1

Example of biodistribution database functionality.



In the left panel headed "Search Page" the user has input search parameters to obtain literature articles which contain data regarding AAV9 biodistribution in non-human primates. After clicking on the "Details" button (indicated by the red square), information regarding a specific article can be obtained, including detailed experimental parameters and experimental data.

TABLE 6

Summary of shedding data submitted to support EMA marketing authorization of AAV-based GTMPs.

Product/Reference documents	Vector subtype (transgene)	Therapeutic indication	Posology/Route of administration	Shedding data to support marketing authorization
Luxturna (Voretigene neparvovec) [17]	AAV2 (hRPE2)	Treatment of adult and paediatric patients with vision loss due to inherited retinal dystrophy caused by confirmed biallelic RPE65 mutations and who have sufficient viable retinal cells.	1.5 × 10^{11} vg/eye Subretinal injection	**Nonclinical** ▶ Viral shedding was not described in animals. **Clinical** ▶ In Phase I and Phase III clinical studies, shedding of AAV2-hRPEv2 in tears was reported in approximately 55% (17/31) of treated patients. Shedding was transient in nature, with the majority of positive samples were seen between one and three-days post-administration. ▶ Low levels of vector were also detected in serum of some patients, up to 14 days post-administration.
Zolgensma (onasemnogene abeparvovec) [20]	scAAV9 (SMN1 gene)	Treatment of patients with 5q Spinal Muscular Atrophy (SMA) with a bi allelic mutation in the SMN1 gene and a clinical diagnosis of SMA Type 1 or patients with 5q SMA with a bi allelic mutation in the SMN1 gene and up to 3 copies of the SMN2 gene	1.1× 10^{14} vg/kg Intravenous infusion	**Nonclinical** ▶ No nonclinical shedding data was included in the dossier, data on shedding in humans based on the published literature was presented, which was considered sufficient. **Clinical** ▶ Data from clinical studies showed that the vector was primarily cleared from the body in stool for up to 60 days post-administration. Low levels of shedding in urine and saliva were reported at 1-day post-dosing. **Environmental risk assessment** ▶ The risk associated with shedding of viral particles and potential third-party transmission was considered to be low; the SMN1 transgene was not considered immunogenic or toxic, and AAV infection would be asymptomatic. ▶ The worst-case scenario was considered to be the spread of replication-competent AAV expressing SMN1 arising from recombination during manufacturing or co-infection with wtAAV in the patient's cells. However, negligible risk was assigned to this scenario due to the lack of evidence for a direct effect of SMN1 on viral biology and pathogenicity, and the limited capacity of AAVs which precludes packaging of rep, cap and SMN1 genes in the same vector.
Glybera (alipogene tiparvovec)[1] [21]	AAV1 expressing human lipoprotein lipase	Familial lipoprotein lipase deficiency (LPLD)	Intramuscular injections in the legs, 1.5 × 10^{12} vg per injection site	**Nonclinical** ▶ Shedding was not addressed in nonclinical studies **Clinical** ▶ Shedding was assessed in the clinical studies by collecting saliva, urine and semen. In one clinical study faeces was also collected. After administration of Glybera to the participants, the highest vector DNA concentrations were detected in the serum, with clearance by one to two logs per week. In saliva vector DNA was still detectable up to 12 weeks; in urine up to 10 weeks and in semen up to 26 weeks. All but two patients received immunosuppressants for 12 weeks. There is the theoretical risk that the co-administration of the immunosuppressant regime leads to longer persistence of virus DNA in serum and as well to longer shedding in saliva, urine and semen. High levels of vector DNA were observed up to 12 months after dosing in the target tissue for Glybera, injected leg muscle, but not in non-injected muscle.

Note: 1 The marketing authorization for Glybera expired in 2017, following the decision of the marketing authorization holder not to apply for a renewal due to a lack of demand for the product.
AAV1: Adeno-associated virus type 1.

and communication of biodistribution and shedding data to health authorities, or at least drive the design of more focused studies that could be reduced in size, scope and length.

Methodological aspects for the construction of a database to collate biodistribution & shedding data

While more recently new AAV viruses with either novel or ancestral capsid proteins have shown enhanced targeting and high selectivity for key tissues [38], for many years the core set of AAV capsids – 2, 5, 8 and 9 – were used in a variety of research and development programs. In addition, the routes of administration, dose levels and species have generally remained within a small range of choices. It has already been suggested that the field of AAV gene therapy could take advantage of this plethora of information and leverage published data for a particular serotype, route of administration, dose level and species to permit the reduction or elimination of further nonclinical biodistribution assessments, thus minimising animal usage and streamlining nonclinical development programs [4].

We describe here the initial stages of the construction of a database of published biodistribution and shedding data for AAVs. By collecting a core set of metadata relating to how the study was performed as well as the experimental data, it is hoped that the database will formalize the currently existing data on biodistribution and shedding of AAVs.

An example of biodistribution database functionality is shown in Figure 1. Such information could be made publicly available to developers of AAV-based therapies where data could be leveraged in regulatory applications, and depending on the depth of information available, potentially permit dedicated biodistribution analysis for particular products to be waived. As the field evolves and understanding of AAV biology progresses, we anticipate that newly generated data for the aforementioned 'core' serotypes will be complemented by data on new serotypes or routes of administration as the database grows.

To initially populate the database, a search was conducted on the PubMed database (https://pubmed.ncbi.nlm.nih.gov) using the search terms 'AAV' AND 'biodistribution', which yielded 122 potentially relevant abstracts. A complimentary second search with the terms 'AAV gene therapy' AND 'biodistribution' was conducted and yielded 107 potentially relevant abstracts. After screening of each abstract for relevance by members of the ARDAT consortium, a total of 102 relevant articles were identified. These articles were uploaded into a shared reference manager (https://sciwheel.com) where consortium members conducted a detailed review of each article and identified the data to be uploaded to the database.

Our first priority was to identify and collect quantitative data on vector distribution (e.g., genome copy (gc) amount, gc/µg DNA or gc/mg tissue), which on most occasions was found within tables and figures. After consultation with ATMP experts within the ARDAT consortium, a series of fields for data entry were designed to capture all relevant information with regard to study parameters. The main general parameters include ATMP class, ATMP serotype, Species, Strain, Route of Administration. Parameters specific for biodistribution and shedding include Measurement method and Tissue type. These parameters appear in the browser interface as dropdown lists to filter search results with the possibility to select any number of parameters. To recover exact numerical data from plots and images, we used a web-based application (https://apps.automeris.io/wpd/). The topics and their parameters are flexible, such that database can be expanded, for example with immunogenicity information in the future.

Need for data standards & minimum requirements for reporting & publishing studies

During construction of the database, we identified inconsistencies across the literature which may limit the usefulness of extrapolation of these data to support regulatory submissions of similar class vectors.

Areas of inconsistency include, for example, the units of measurement used, e.g., results presented in vector genome/μg (vg/μg, or any other mass) and results given in vg/diploid genome or vg/*x* amount of DNA make comparisons difficult across the literature. There are many less frequently used units (vg/cell, vg/eye) employed without referencing mass of DNA used to calculate cell number. Similarly, the AAV terminology is reported in various ways, for example, an AAV 2 rep gene with AAV 5 cap gene is reported as AAV2/5 [39], AAV5 [40] and AAV2.5 [41], which causes confusion.

Another major inconsistency is in the type of tissue analysed and the timepoints and which measurements are taken. For example, measurement of biodistribution can vary between whole tissue or many parts of a single tissue type [42–44]. A standard set of tissues for biodistribution measurements would allow these data to be referenced in regulatory submissions and prevent duplicative nonclinical work. The most recently recommended tissue list from health authorities is outlined in the IPRP reflection paper on conduct of biodistribution studies [16]. In addition, standard tissues expected to be collected are also outlined in the current draft version of ICH S12 [3]. It is unlikely that there will be further granular guidance from health authorities on topics such as how tissues are collected, fractionated and stored.

We noted that data from infectivity studies are rarely reported, and where it is reported there is not always a description of methods.

Finally, our work revealed discrepancies in the reporting of the bioanalytical methods used, including a general lack of reported validation parameters e.g., limit of detection. There were also varied approaches to the reporting of sampling profiles and schedules. Such variations in reporting render the experiments difficult to interpret and reproduce.

In an attempt to illustrate this point, we conducted an exercise from the point of view of a hypothetical developer of an AAV9-based gene therapy intended for intravenous administration in the clinical setting. Using the search filters 'AAV9' for ATMP serotype and 'intravenous' for route of administration, we identified 12 articles currently in the database, which reported biodistribution data derived from qPCR analysis for AAV9-based vectors after intravenous administration.

The majority of published studies (9 of 12; 75%) were conducted in mice (either wild-type strains, namely Balb/c or C57BL/6 [7 studies] or specific disease models [one study each in Barth syndrome and dystrophin-deficient mouse models]). The remainder of the studies were conducted in NHPs (3 of 12; 25%). With regard to pre-existing immunity, none of the studies in mice included an analysis of this issue, whilst 2 of the 3 studies in NHPs included such an analysis. Only one study in NHPs included animals with and without pre-existing immunity [45].

It was noteworthy that none of the studies included information with regard to bioanalytical method validation, including the effects of different tissue matrices, nor were limits of detection stated. In general, biodistribution of vector genomes was only analysed at one timepoint, which showed a wide variation; ranging from 10 days to 5 months in mouse studies and 28 days to 2 years in NHP studies. Such wide ranges could be explained by the desire to limit animal use and study costs and the use of complementary approaches to examine transgene expression as described in the next paragraph.

A significant proportion of studies used a reporter transgene to facilitate the analysis of transgene expression over several timepoints (4 of 12 studies; 33%). Detection methods included *in vivo* imaging of live animals using luciferase [46] or analysis of secreted enzyme activity [45]. These analyses of transgene

expression complemented the vector genome biodistribution data obtained from tissues harvested at the terminal timepoint. The use of GFP transgenes also facilitated analysis of transgene expression in specific cell types / areas of tissue, for example in the study of Weber-Adrian and colleagues [47].

Vector genomes were detected using primer sets to detect either sequences in the parental vector (3 of 12 studies; 25%), transgene (7 of 12 studies; 58%) or were not stated (2 of 12 studies; 17%). It is notable that none of the studies appeared to use primer sets at junctional regions that would permit detection of both parental vector and transgene sequences.

There was relatively little consistency with regard to the units of quantification used to expressed vector genome biodistribution. Viral genome copies / vector copies / transgene copies were expressed per µg of genomic DNA / per diploid genome / per cell or per copies of reference gene. There was also a wide variation of reference genes used to quantify genomic DNA (e.g., where it was stated in the article, the following genes were used as references for genomic DNA quantification; NCAM, beta-actin, lamin B2, titin, epsilon-globin).

More consistency was seen with the panels of tissues analysed, vector genome distribution to heart was reported in all studies and liver in 11 of 12 studies (91%). Other tissues commonly analysed included lung (10 of 12 studies; 83%), kidney (7 of 12 studies; 58%), and brain (7 of 12 studies; 58%).

Once the database is constructed, the next step of this initiative will be to publish recommendations in analytical and reporting standards, such that data sharing across groups will be more universally interpretable.

CONCLUSION

Recommendations for biodistribution and shedding studies have been issued by regulatory authorities across the globe, which has resulted in points of divergence and complexities in the global development of gene therapies. Whilst the 2021 release of the draft ICH Guideline S12 is a welcome step toward harmonization, further opportunities exist. Extensive nonclinical work over the course of the last three decades has generated a plethora of literature on the nonclinical biodistribution and shedding of AAV vectors, such that regulatory applications should rely on this data set and reduce animal usage. Inconsistencies in reporting standards across the field has hampered the usefulness of extrapolation of published work to support regulatory submissions of similar class vectors. A publicly available database of biodistribution and shedding data, with established minimum data standards for reporting and publishing data will greatly facilitate regulatory convergence and nonclinical development by avoiding repetitive studies and reducing animal usage which ultimately will accelerate these important therapies to market.

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CELL & GENE THERAPY INSIGHTS

Regulatory Insights

EDITORIAL

Unproven stem cell-based interventions & harm to existing & future patients

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"To address these risks to current and future patients, companies developing SCBIs should work with policymakers and patient advocates to protect clinical research and the reputation of the field."

EDITORIAL

Over the past decade, the landscape of unproven stem cell-based interventions (SCBIs) has dramatically changed [1]. Once perceived as only operating in countries with less developed health infrastructures, clinics advertising unproven SCBIs are now located in almost every developed country and their numbers are increasing despite regulatory authorities working to close the more unscrupulous and dangerous ones [2,4]. In 2021, Leigh Turner from the University of California, Irvine, identified 2,752 clinics in the USA operated by 1,480 businesses, a 400% increase from the numbers of clinics reported in 2016 [5]. This SCBI marketplace remains largely unregulated despite evidence indicating that many interventions are ineffective and can increase risk for patients [6,8]. Moreover, providing unproven interventions threatens legitimate stem cell clinical research, delaying–if not potentially preventing–the development of therapies for a variety of conditions. To address these risks to current and future patients, companies developing SCBIs should work with policymakers and patient advocates to protect clinical research and the reputation of the field.

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Unproven SCBIs are treatments that use stem cells and stem cell-derived components that have not undergone clinical testing to prove they are safe and effective, nor have been approved by a regulatory body [6]. Currently, the only approved SCBI in the US involves hematopoietic cells used to treat patients with disease of the blood and immune system [9,10]. However, many clinics advertise using SCBIs for a variety of medical conditions in which evidence of effectiveness is lacking or inconclusive, from orthopedic injuries and/or pain, to neurological or cardiovascular conditions, aging, and even hair loss and other cosmetic issues [5]. On their websites, these clinics typically offer treatments using autologous stem cells derived from adipose tissue or bone marrow, which are easy to obtain [11]. Many of the unproven SCBIs promoted by clinics do no harm the patient but have little to no impact on the patient's outcomes. Other SCBIs carry relatively high risks and can have acute side effects. For example, a 2021 study of orthopedic patients found that the frequency and severity of complications when patients received stem cell injections were significantly higher compared to the standard care [7]. Furthermore, more than 360 adverse events from unproven SCBIs have been reported since 2004, and scholars believe this is likely an undercount since not all adverse events specify whether the procedure was unproven [8]. Patients have experienced adverse events from unproven SCBIs through infections in the product and unsafe injection practices. These adverse events were quite far from benign and included tumors (some inoperable), infections including sepsis, retinal detachment leading to blindness, heart attacks, organ failures, and death in some cases.

To recruit patients, providers of unproven SCBIs use internet- and social media-based direct-to-consumer marketing tactics to hype the benefits of stem cells for treating a variety of conditions while obscuring their risks and lack of approval. This hyping of SCBIs not only risks harming exposed patients but also erodes the public trust in stem cells and can potentially harm patients in the future by delaying clinical research. While it is difficult to identify a causal relationship between hype, public trust, and support for science, there is concern that overhyping emerging biotechnologies that fail to meet public expectation can lead to loss of public trust in that biotechnology and ultimately reduce support and funding for science in general [12]. In countries with a high level of public trust in the regulatory systems, unproven SCBIs can be viewed as safe and effective, which can facilitate hype and potentially cause confusion between future proven and current unproven SCBIs [13].

Clinics advertising unproven SCBIs can also damage the public's perception of stem

cell research and regenerative medicine by manipulating and co-opting aspects of the clinical trial process by using 'tokens of legitimacy.' Scholars define 'tokens of legitimacy' as actions and products associated with the clinical trial process and research institutions that are imbued with public trust [4]. Clinics have been found to: register their treatments as clinical trials without going through any regulatory approval process; publish questionable data without any comprehensive peer review; rent lab space near or in legitimate hospitals and research centers; and claim certification by and membership in scientific organizations to capitalize on the existing legitimacy of these markers of scientific integrity [14]. Some SCBI clinics go a step further by referencing legitimate stem cell research being conducted at prestigious universities and medical centers to promote their unproven treatment [15].

Misusing these tokens can cause confusion for patients. For example, patients might believe a clinic's SCBI has regulatory approval or is a clinical trial if it is registered on ClinicalTrials.gov, a website supported by the National Health Institute (NIH), despite the website's warning that listing a study "does not mean it has been evaluated by the US Federal Government" [15–17]. Patients may also play a role in perpetuating the hype and misunderstanding of a SCBI [4]. In public-facing crowd-funding appeals, such as GoFundMe campaigns, patients will state that the procedure is supported by the NIH or U.S. Food and Drug Administration (FDA), copying language found on unproven SCBIs clinics' websites. Patients will also cite ongoing basic and clinical research conducted at legitimate institutions to demonstrate the efficacy of the unproven SCBI they are seeking [15]. This effort, intentional or otherwise, to co-opt the legitimacy of clinical trials, causes confusion for patients and prospective research participants and can erode trust in legitimate stem cell and regenerative technologies, which could result in a negative public perception when legitimate stem cell treatments are approved for clinical use [16].

The expanding unproven SCBI market can also affect clinical trial participation and reputation. Recruitment is particularly problematic in the case of rare diseases with limited populations that can qualify for clinical trials. Patients wishing to undergo methodologically-sound clinical trials could be excluded after having received an illegitimate SCBI elsewhere since most interventions do not adequately define the procedure including detailing the number of cells used, testing the types of cells injected or follow the results using rigorous metrics [2,3] Previous procedures could taint the clinical trial's results. Recruiting patients can also become difficult when a patient can choose between either the supposed certainty of receiving some treatment from an illegitimate clinic or just the possibility of receiving one (vs. a placebo) in a legitimate clinical trial. Unproven SCBIs could impact the reputation of clinical research and lead to a loss of trust by the public in the treatments if the public confuses clinics providing unproven therapies as being legitimate clinical trials or having completed a clinical trial.

Unproven SCBI clinics harm patients and undermine legitimate stem cell clinical research. Through co-opting the tokens of legitimacy, unproven SCBI clinics pose a serious threat to the public perception of, trust in, and willingness to support stem cell medicine. More reporting and studies of the negative impacts of unproven SCBI is emerging. However, unproven SCBIs have caused confusion in the marketplace with increasing hype by both clinics and patients. As these clinics and procedures continue to expand, they will – and have started to – impact legitimate stem cell clinical trials. While closing unscrupulous SCBI clinics has proven challenging for regulatory authorities in the past, clinical and translational researchers should continue to advocate for more stringent regulation of unproven SCBIs to their local policymakers and regulators to better protect patients and promote sound research. These efforts are required to curb unproven SCBI and protect clinical research and the field.

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