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CELL & GENE THERAPY INSIGHTS

SPOTLIGHT ON
Vector processing and materials

Guest Editors
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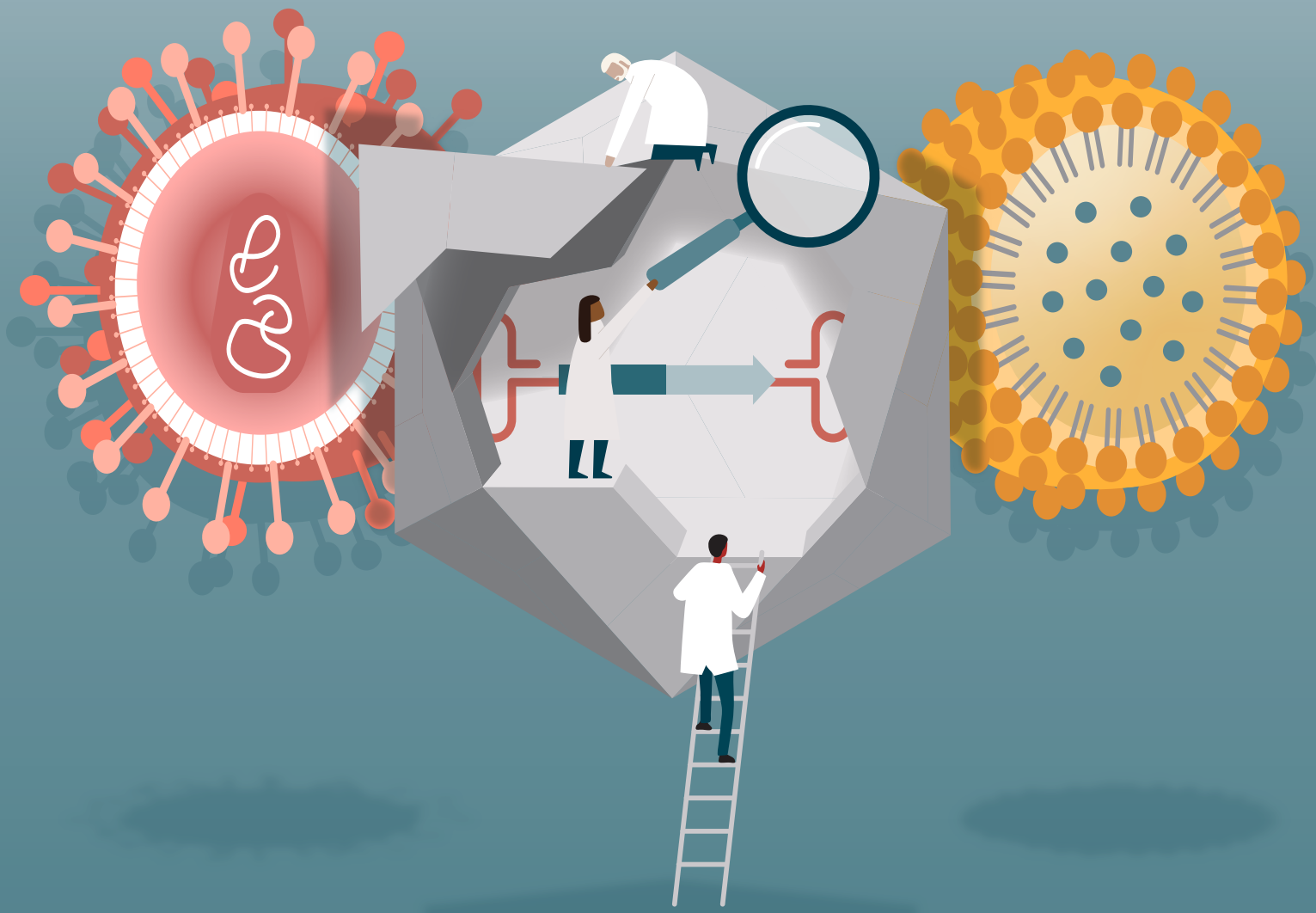

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Are we there yet? After 250+ AAV-based clinical trials, do we have a well-paved road toward first-in-human entry?

Long Chen, Chia Chu, and Francesca Vitelli

Gene therapy is a revolutionizing technology that brings hope to millions of patients suffering from rare diseases. Unlike other mature therapeutic modalities, chemistry, manufacturing, and control (CMC) is often on the critical path for gene therapy programs to initiate clinical trials and obtain accelerated approvals. In recent years, health authorities and industry leaders have recognized a need of platform approach to streamline AAV-based gene therapy and initiated programs to address this need. Here we analyze these streamlining efforts in the industry, and emphasize the collective benefit of harmonizing the CMC platform, including manufacturing, analytics, and regulatory filing strategy, for AAV-based gene therapy. This endeavor is particularly impactful for accelerating advancement of gene therapies to the clinic at lower costs, and thereby further incentivizing drug development even for ultra-rare indications. Furthermore, considerations in building a minimum CMC package for initial clinical trial applications and some opportunities for harmonization are highlighted. Finally, potential future breakthroughs to alleviate the current pain points are discussed.

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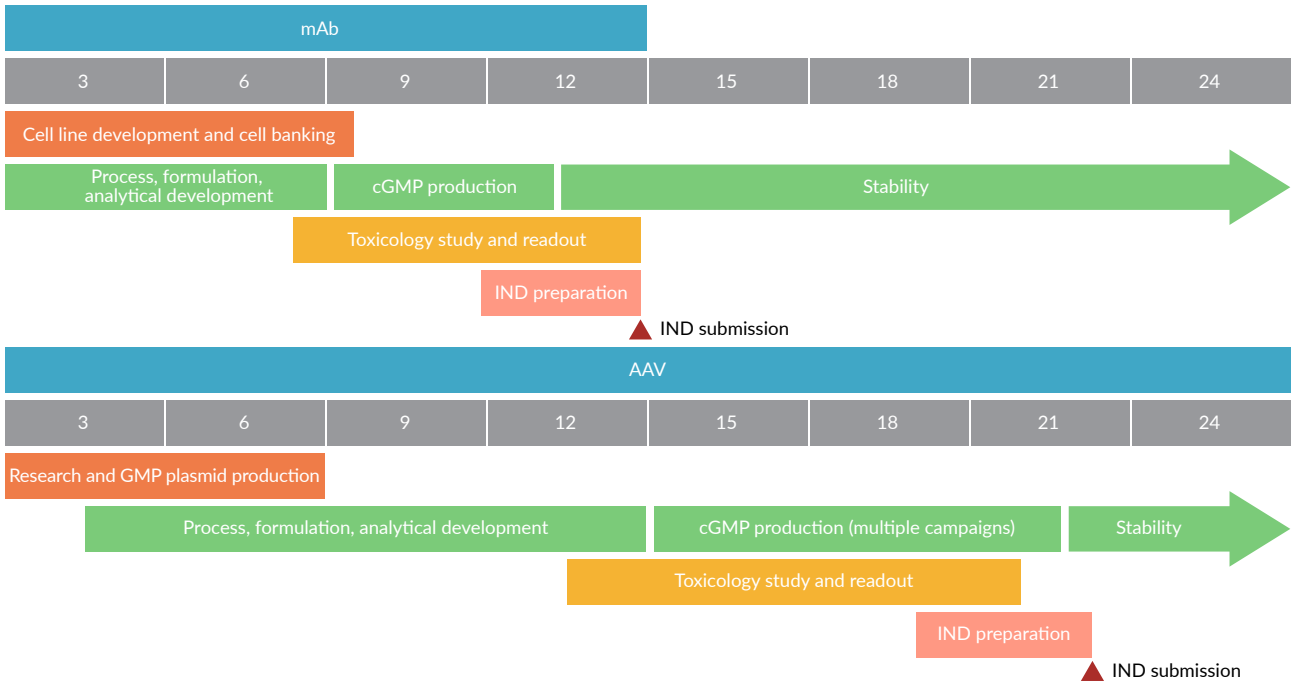
DESIRE TO STREAMLINE THE ADVANCEMENT OF GENE THERAPY CANDIDATES TO THE CLINIC

For many years now, gene therapies have been heralded as the next wave of breakthrough medicines with the promise of administering one-time treatments that may potentially lead to lifelong cures of genetic diseases and vast improvements in patients' quality of life. A surge in investments in the mid-2010s appears to have decelerated in recent years [1]. Though there were timeline setbacks globally due to the COVID-19 pandemic, the translation of gene therapy (GT) candidates from bench to bedside has not progressed as quickly due to more development challenges than originally anticipated [2]. The potential gains from compressed clinical trial timelines due to faster readouts, smaller trial size requirements, and expedited regulatory frameworks [3] have been largely offset by delays in chemistry, manufacturing, and controls (CMC) readiness. Both cost and timelines to enter first-in-human (FIH) trials far exceed those of more mature modalities, such as monoclonal antibodies (mAbs). As an illustration in Figure 1, the current industrial benchmark of bringing adeno-associated virus (AAV)-based therapies, which have emerged as a vector of choice in the past decade, from lead selection to FIH regulatory submission (such as an Investigation New Drug application, IND) is 18–24+ months while timelines for traditional mAbs can be typically achieved within 10–12 months, and as short as 5–6 months [4]. Low productivities with AAV-based gene therapy modalities have directly contributed to high costs and long timelines. The cost of producing a clinical grade batch of AAV at 500 L is estimated to be 3–5 folds higher than that of mAbs [5,6].

FIGURE 1

Example of generic timelines from lead selection to IND submission for mAb vsersus AAV.



1). Toxicity readouts for mAb can be shortened to <6-month with justification [44] while there are more considerations for AAV toxicology studies including biodistribution and genotoxicity. The associated bioanalytical assay development also adds to the AAV timeline. 2). Process, formulation, and analytical development duration for AAV varies and may be shortened for platformed applications, when the capsid, dosing, route of administration, etc. are the same.

Furthermore, complexity in clinical supply chain increases when multiple manufacturing campaigns are required to meet clinical demands, translating to execution risk.

Admittedly, maturation of the recombinant biologics field took significant time and effort, requiring a clear understanding of the fundamental biology of the therapeutic molecules, as well as building the process understanding and control strategy so production could be scaled and product quality controlled through the quality-by-design paradigm. Over the course of several decades of development and alongside the evolution of regulatory guidance on cell culture-based therapeutics, the mAb field has converged on platform processes and analytics, thus production titers have increased >100-fold [7]. The chronicle of mAb can serve as a guide for newer, more complex modalities and highlights the criticality of platform processes, platform analytical methods, and adequate productivity for early-stage programs. With hundreds of active AAV-based gene therapy clinical trials and many more to come, CMC considerations and supply of clinical grade viral vectors is consistently an underserved need often on the critical path to FIH. In response, many contract development manufacturing organizations (CDMOs) have not only added cell and gene therapy capabilities but are also offering drug developers access to gene therapy platforms that can theoretically achieve mAb-like rapid and de-risked, timelines.

PLATFORM APPROACH ACCELERATES BESPOKE GENE THERAPIES FOR RARE DISEASES

While rare diseases imply small patient populations, collectively it is estimated that between 263–446 million people suffer from some form of rare disease around the world at any point in time [8]. By 2019, the approval of leading AAV-based gene therapy products, Luxturna® [9] and Zolgensma® [10], sparked broader drive in the industry to create bespoke gene therapies for other rare diseases. However, the traditional product-specific development approach is cumbersome [11]. A simplified, clear and publicly available production and analytical platform which drug developers could easily leverage to accelerate speed to clinic is highly desirable.

Nonetheless, unlike traditional mAbs, this effort is complicated in two aspects. First, there is lack of consensus in AAV production modality, as several AAV production systems exist, each with distinguishing features [12,13]. In addition, since recombinant AAV drugs have serotype-specific features such as tissue-specific tropism conferred by the capsid protein shell, the selected AAV serotype may vary with disease indication and other criteria. The capsid serotype has a great impact on manufacturing, as yield and production scale, process unit operation design specifics, and product quality profile vary with serotype. When the same AAV serotype is utilized for different disease indications, the process of manufacturing and testing for one program could be readily leveraged to save both cost and time. Such is the aim of experimental pilot program PaVe-GT launched by National Center for Advancing Translational Sciences (NCATS) in 2019. PaVe-GT program targets four rare diseases using rAAV9 with a single manufacturing facility and process executed by the National Institutes of Health (NIH) to ensure transparency to the public [11]. While all four ongoing programs are currently at pre-clinical development stage, the experience and learnings from this government-backed platform project, including toxicology and biodistribution data, and their regulatory interactions, are to be shared with the public when they become available.

The PaVe-GT program inspired more efforts to streamline the development of gene therapies for rare diseases with less commercial incentive. Notably, Bespoke Gene Therapy Consortium (BGTC) [14], part of the Accelerating Medicines Partnership® program managed through the NIH, was

launched in October 2021 to develop platforms and standards to speed the development and delivery of gene therapies for rare disease. In recognition of PaVe-GT's valuable work, BGTC expands its goals to include AAV basic biology exploration, preclinical and product testing harmonization, regulatory submission package standardization, and clinical manual development. At a cost of over $90 million spanning 6 years, BGTC seeks to develop a gene therapy framework by bringing eight AAV-based drugs for diseases across ocular, neurological and systemic indications into clinical trials, and has selected Andelyn Biosciences, a CDMO as its rAAV manufacturing partner [15]. Of note, both PaVe-GT and BGTC initiatives were sparked by low commercial interest in ultrarare diseases, yet the resulting playbooks could equally benefit the development of AAV gene therapies for more prevalent diseases.

CDMOs HAVE ESTABLISHED CMC PLATFORM PROTOTYPES DRIVEN BY CLIENTS' BUSINESS NEEDS

The authors of this article specialize in CMC development of rAAV drug candidates. Encouraged by preliminary successes of rAAV platform initiatives, we also recognize the significant upside of streamlining a CMC platform to improve process robustness and product quality reproducibility and thus speed to clinic. In fact, BGTC addresses this in the recently published regulatory playbook by establishing minimum critical quality attributes (CQA) and analytical methods [16]. We searched public information and analyzed the platform offerings from a selection of AAV CDMOs of varying sizes, under three main categories of manufacturing, testing, and regulatory offerings (Table 1).

The above review of selected CDMOs' platform offerings indicates the HEK293-based transient transfection system is currently the most prevalent manufacturing platform of choice. Proprietary clonal, high-producing cell lines may be provided to clients at a relatively low or no cost, circumventing potentially prohibitive costs associated with platform-agnostic commercial HEK293 cell lines from other sources. Some vendors further distinguish their offering by providing productivity enhancing tools such as optimized plasmid design. For example, Oxford Biomedica's two-plasmid system integrates GOI and RepCap plasmids into a single plasmid, which along with a pHelper, reportedly yields up to 10^{15} AAV genome copies per liter (vg/L) at harvest, almost two orders of magnitude above the industry standard of 10^{13}–10^{14} vg/L [17], delivering >1 × 10^{17} vg purified AAV product per 500 L batch [18]. Several other CDMOs' platforms, such as Lonza's Xcite® AAV suspension transient transfection platform, include the proprietary know-how on helper and/or Rep/Cap plasmids design into their service package [19]. Downstream purification unit operations are currently more uniform than upstream processes, and specifically the scale-up friendly affinity and ion-exchange chromatography have mostly replaced the 'scale-out' ultracentrifugation process [12]. Given the recent advancements of AAV manufacturing platform, it is worthwhile to understand the likelihood of clients opting for a platform offering versus a customized solution. In response to the authors' query, Oxford Biomedica communicated that the vast majority of their AAV clients use their 'plug-and-play' dual-plasmid platform. Similarly, Viralgen's Pro10-based platform has been used to produce >56 clinical GMP batches and submit 19 global CTAs between the year of 2019 and 2022 [20]. If this trend is representative of the field and continues, it is reasonable to assume that platform approaches will be widely adopted for AAV gene therapy manufacturing.

Availability of phase-appropriate platform assays should also contribute to significant cost and time savings. SK pharmteco's Analytical Accelerator™ for Vector Testing purportedly shortens time to GMP batch

TABLE 1

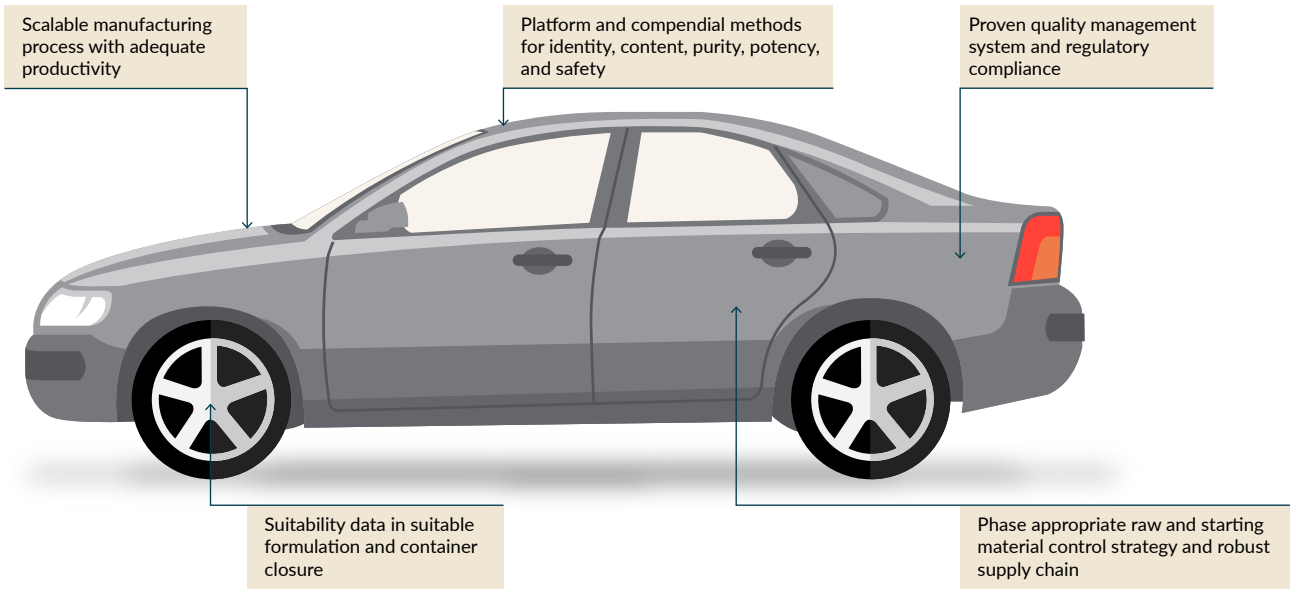
Summary of selected CDMOs offering manufacturing, analytics, and regulatory service platforms.

CDMO	Manufacturing platform	Analytics platform	Regulatory service platform
Catalent [45]	UpTempo℠ AAV platform: for serotypes 1, 2, 5, 6, 8, 9, and rh10; and Catalent's clonal HEK293 cell line, and off-the-shelf pHelper and rep/cap plasmids Speed: UpTempo℠ AAV platform delivers AAV drug product in 9 months	In-house panel of analytics, with robust and reproducible analytical methods including mass spec, bioassays, and Analytical Ultracentrifugation	Regulatory support service: Dossier Authoring Services; Regulatory Support and Advice Regulatory Publishing; and Health Authority Meetings Clinical support and clinical labelling
Forge [46]	Proprietary HEK293 suspension Ignition Cells™, royalty-free license for clients pEMBR™ ad helper plasmid	End-to-end capabilities from research to cGMP reduce the need for bridging studies: safety and quality; concentration and potency; integrity, identity, and composition purity	Review and authoring of client CMC documents; review and authoring of any regulatory documents; assist with FDA requests for information; participation in formal meetings; *ad hoc* consulting services; and eCTD compliant publishing and submission services Forge will maintain a DMF for their process with the FDA for client's CMC documentation
Fujifilm Diosynth Biotechnologies (FDB) [47]	'Off-the-shelf' cell line and plasmids: cGMP clonal suspension HEK293 cell line; Rep/Cap and helper plasmids (AAV2, 5, 6, 8, 9); and GOI backbone plasmid Speed: 16 months for cGMP batch release, and 7 months for non GMP material release	Analytical and quality control teams experienced in titer and quality analytics	Support the sponsor with content for IND drafting and CMC module writing including responses to information requests and post-approval maintenance
Lonza [48]	Xcite® AAV suspension platform: 5B8 Production Host Cell Line (clonal, suspension, scalable); and proprietary know-how on pHelper plasmid and promoter for balance Rep/Cap expression (pLHI-Helper and pLHI-RepCap)	Pre-developed and ready to rapidly qualify assays, including safety, identity, strength, purity and overall quality of the product	Provide access to regulatory information for Lonza's media, capsules and DMFs or CEPs to aid submission
Oxford Biomedica [49]	'Plug-and-Play' Dual Plasmid system: RepCap/GOI plus pHelper; >1×10^{15} vg/L out of bioreactor; and >90% full capsids after downstream purification Speed: 11 months timeline achieved from client onboarding to released GMP batch	>45 methods developed for full vector characterization quality control, including AUC. NGS, MS and stability testing	Support regulatory activities such as IND and CTA submissions; stability testing plans, shelf-life determination, and release specifications; clinical supply storage and stability study management; and support regulatory filing strategy and author clients' CMC
SK pharmteco [50]	AAV adherent platform: production cell line, plasmids manufacturing (precision platform), and established processes AAV suspension platform: a clonal GMP-qualified HEK293 cell line licensed from Asimov Inc.	Analytical Accelerator™ platform: speeds time to GMP batch release more than three-fold (from 22 to 6 weeks) Custom assay development can be performed in half the time (6 months vs 12) of *de novo* development using Analytical Accelerator™ methods	End-to-end quality assurance and regulatory affairs: Regulatory Strategy and Advisory; Regulatory Authority Submissions; and Regulatory/Quality Compliance
Viralgen [51]	Proprietary Pro10 cell line-based platform: clonal HEK293-derived suspension cell line; triple transfection with the transgene plasmid, a plasmid carrying pAAVrep2capX and a helper plasmid carrying the VA-RNA, E2A and E4 helper genes of the Adenovirus serotype 5; perfusion based bioreactor production; and speed: 5–6 months for 250 and 500 L; 6–7 months for 2000 L	Has a QC and release process available, with a high-performance analytical platform under development under the MAPAAV project with three other companies	Help with whatever level of support required for the CMC areas of each submission—from providing template articles to authoring them, and participation in information exchanges with regulators either in writing or in person

AUC: Analytical ultracentrifugation; CEP: Certificate of Suitability that confirms that a pharmaceutical ingredient complies with the European Pharmacopoeia's (EP) monograph requirements; cGMP: Current Good Manufacturing Practice; CTA: Clinical trial application; DMF: Drug Master File; eCTD: Electronic Common Technical Document; IND: Investigational New Drug; MS: Mass spectrometry; NGS: Next-generation sequencing; QC: Quality Control.

FIGURE 2

Illustrating a minimum CMC package for FIH studies (Phase 1).



Optional packages

- Program-specific process with titer enhancements
- Functional activity assay
- Demonstration and engineering runs
- Stringent raw material acceptance testing
- Forced degradation studies
- Container closure testing
- Clonal cell line for transient production systems
- Program-specific viral clearance study
- Commercial manufacturing experience

'Base model' requirements could be supplemented with 'optional packages' in a phase-appropriate manner.

release more than three-fold, from 22 weeks to 6 weeks [21]. As a result, the expected timeframe of bringing an AAV therapeutic product to clinical trial using a platform approach falls between 14–16 months (Table 1). Other CDMOs claim to achieve even shorter timelines, such as the plug-and-play platform by Viralgen offering 6–9 months with their unique Pro10™ system requiring no process or analytical development work. Catalent claims that its proprietary UpTempo℠ AAV platform yields AAV drug product in 9 months, aided by a simplified supply chain and in-house panel of analytics [22]. Understandably, such aggressive timelines require right-first-time execution of many moving parts, which along with risk-reduction, is arguably one of the most attractive advantages of a well-vetted platform approach, but does require feasibility testing to determine fit-to-platform.

AAV GENE THERAPY ROADMAPS NEED ANOTHER LAYER OF GRANULARITY

While the expansion of a range of manufacturing and analytical platform offerings, from plug-and-play to agile workflows, have been beneficial to drug developers in juggling speed, cost, and quality, drug developers seek to strike the right balance between meeting regulatory requirements and taking calculated risks to accelerate time to clinical proof-of-concept. A search in clinicaltrials.gov revealed 262 clinical studies involving the use of AAV as of February 2024. Though AAV development may still feel nascent due to the small number of programs within each institution, the combined dataset should be sufficient to generate consensus on a roadmap and requires establishing collaborative forums for open exchange in the industry. The AAV

gene therapy playbooks published by credible, non-profit organizations such as BGTC and Alliance for Regenerative Medicine (ARM) [16,23] aim to help less experienced sponsors streamline early-stage development of rare disease therapeutics. Nonetheless, what is lacking still is a clear definition of the must-haves versus nice-to-haves. While it is acknowledged that guidance documents from health authorities do contain considerations for taking a phase-appropriate approach to CMC development, in actuality, regulatory agencies have shied from providing prescriptive guidelines and prefer case-by-case assessments by advising sponsors to seek clarity through early interactions for their programs. In practice, the justification of phase appropriateness is more based on prior experience and successful precedents rather than clear rationales that are transferrable to newcomers to the field, or even new programs from established players. For instance, is it required to prove clonality of a host cell line, which adds to the timeline, in a production system that utilizes transient transfection rather than stable producer cell line? As an analogy (Figure 2): what are the minimal indispensable elements to make a car run vs. the bells and whistles that can be waived if over budget? While this cannot possibly be comprehensively addressed in this writing, below are several debatable points to prompt further discussion in the community.

Adequate productivity and product quality

A robust CMC platform is the core engine enabling the manufacturing of clinical trial material. Although required AAV dosages vary widely depending on target indication, 10^{15} to 10^{17} total vector genomes should support dosing small patient numbers that are typically in the low 10s for first-in-human studies [24], and with current advances this is feasible to produce in <1,000 L total bioreactor volumes. When adequate productivity is already demonstrated, additional resource-intensive program-specific process development to further bolster productivity might translate to diminishing returns. This is similarly applicable to product quality control. It should be emphasized that patient safety is of the utmost priority and activities to increase confidence (e.g., addressing 483 findings at manufacturing facilities) must be conducted. Without compromising safety assurance, wider specifications that do not pose higher risks of causing adverse events are likely acceptable for Phase 1/2 initiation. In-depth understanding of structural--functional relationships and establishment of assays to demonstrate mechanism of action are not necessary FIH prerequisites and can progress concurrently with clinical development. Furthermore, relevant historical data from similar programs could be leveraged as part of the platform approach to help set appropriate specifications and eliminate potentially redundant but costly and time-consuming activities, such as viral clearance studies, when there is no change in unit operations.

Timely and successful execution

Large batch-to-batch variability and high batch failure seen in the field during early manufacturing might be attributed to brute-force adoption of academic processes in industrial settings, the lack of experienced workforce and/or inadequate gene therapy-specific training. Advantages of state-of-the-art gene therapy platforms not only include process scalability and operators' familiarity with the unit operations, but also established raw material supply chains and specifications, reduced program-specific assay development and qualification, and increased confidence to omit costly demonstration and engineering runs, all of which can individually derail accelerated timelines. However, a platform approach does not eliminate all risks completely and sponsors might concede some level of confidence to maintain progress. Implementation of stringent raw material specifications and expanding the knowledge space of process parameters can increase production robustness at the added

expense of extending pre-GMP timelines beyond the required time for a replacement batch. It is therefore more pragmatic to do so when a large number of batches are planned, which is typically not the case at early stage.

Quality and regulatory compliance

Manufacturing facilities with effective quality management systems (QMS) and proven track records in satisfactory regulatory audits are a must-have for sponsors to ensure successful batch release and disposition, particularly when filing in EU countries where a qualified person (QP) release process is required. Pharmaceutical QMS enforces quality standards, mitigates risks, and promotes continuous improvement. Facilities that have successfully supported clinical trial applications in major markets should be considered as primary choices for sponsors to partner with. The lack of commercial manufacturing experience or experience interacting with rest-of-the-world health authorities should not be deterrents at the early stage. Nevertheless, both sponsors and CDMOs should actively monitor and anticipate evolving regulatory expectations and perform risk assessments to justify differing stances. An example of this is the FDA guideline for AAV product impurities. As gene therapy is relatively new, the understanding of impurities' impact to patient safety is in development. This is further complicated by multivarious routes of administration and drastically different doses per patient for AAV-based *in vivo* therapies. Current guideline acceptable impurity levels remains unclear. For instance, WHO's guidance on limiting host cell DNA (hcDNA) impurity to under 10 ng/dose for vaccines [25] is impractical to achieve in high-dose AAV therapies such as those for neurodegenerative diseases. This is because during AAV packaging, a certain level of hcDNA inevitably is encapsulated into capsids. The concern raised by ARM [26], among others [27,28], has resulted in the removal of 10 ng/dose limit from the finalized 2022 FDA guidance on Human Gene Therapy for Neurodegenerative Diseases [29]. However, this same constraint stands for other diseases using gene therapy and awaits alignment. Similarly, there is no clear guidance on the minimum level of residual packaged plasmid with bacterial components such as antibiotic resistance gene, although the industry has developed vectors with higher safety profiles, such as nanoplasmid [30] and enzymatically synthetic linear DNA with covalently closed end(s) [31,32], both free of antibiotic resistance genes. Moreover, therapeutics developers seek clarity in full capsid requirements, as separation of full capsids from empty, partially full, and overpacked capsids is a challenging step in downstream operations given the divergence and stochasticity of AAV capsid assembly [33].

Shelf life and cost-of-goods considerations

Recombinant AAV drug products are formulated in similar phosphate or Tris buffered saline solutions with poloxamer 188 as surfactant to mitigate surface adsorption [34]. Formal stability studies to support shelf-life claims are mandatory to ensure no product degradation between time of release to drug administration. However, when identical formulations and container closures are used, one could potentially justify leveraging stability data from other programs using the same AAV serotype instead of generating program-specific data. Along this line of thinking, there may also be other opportunities where other platform data can be leveraged to further trim down program costs. Additionally, developers should consider alternative to components, materials, or reagents that carry significant licensing fees where possible in the manufacturing process.

FORWARD-LOOKING BREAKTHROUGHS OF GENE THERAPY

As we consider the current pain points in AAV gene therapy development, the most

urgent unaddressed need is the development of a robust and scalable manufacturing platform. As discussed above, the maximum yield out of a HEK293-based transient transfection platform (i.e., 1×10^{12} vg/mL harvest titer, 1,000 L bioreactor scale, 30% overall recovery) is approximately 3×10^{17} total vgs. For diseases with systemic administration, the required dose could be up to 1×10^{16} vgs per patient [26]. In such scenario, a single 1,000 L batch can only supply less than 30 patient doses, creating challenges for late-phase clinical trials and also leading to record price tags for commercialized therapies. Some exciting progress has been reported recently. For one, Oxgene recently leverages the helper functionality of adenovirus within early phase of its lifecycle to boost rAAV production, while turning off the late region that is responsible for the production of adenoviral structural proteins. As a result, this Tetracycline Enabled Self Silencing Adenovirus (TESSA™) technology generates 10–30-folds increase in AAV productivity [35], while repressing adenovirus production by almost 100%. Some vendors delved into the molecular biology of host cell, and developed compounds to boost AAV yield by 2–5 times with a simple bolus liquid addition into the bioreactor. Such compounds include Virica's viral sentitizers (VSEs™) library that transiently antagonizes a broad range of cellular innate antiviral pathways [36], and Mirus' *Rev*IT™ AAV Enhancer screened from small molecule candidates spanning many classes of cellular pathway modulators [37]. To completely eliminate use of plasmids and helper viruses, stable producer cell lines achieving 1×10^{12}–1×10^{13} vg/mL harvest titers have been reported; for example, the novel helper virus-free Lonza Xcite® AAV Producer Cell Line is currently in beta testing and proposes scalability to 2000+ L, a significant reduction in cost of goods and manufacturing and supply chain complexity, with the potential to enable true industrialization of AAV therapies [38]. In fact, a CRB 2023 survey reported that 95% of survey respondents are pursuing stable producer cell lines, and 60% have reached the point of using stable producer cells in their clinical or commercial process [39].

To ensure the delivery of high quality bioproducts, multiple product quality attributes are analyzed, but the development and qualification of each individual assay is costly, time-consuming, and labor intensive. Moreover, the significant volumes required during sampling to perform those assays individually could take up to 20–40% of an AAV batch depending on the yield. For mAbs, liquid chromatography-mass spectrometry (LC-MS)-based multi-attribute method (MAM) is gaining popularity to achieve multiple attribute testing, e.g., sequence verification, post-translational modification, and impurities, through a single low-volume injection into a high-resolution mass spectrometry. Although AAVs are one order of magnitude larger in molecular weight in comparison to mAbs, scientists have recently achieved quantification of full/empty capsid ratio, capsid protein stoichiometries, PTMs and truncations specific to the full capsid with a 2DLC-MS by coupling AEX and RPLC-MS [40]. Solutions for a one-stop-shop instrument for AAV product development are emerging. For example, Unchained Labs' Stunner utilizes rotating angle dynamic light scattering (RADLS) and multi-angle light scattering (MALS) to measure genome/capsid titer, empty/full ratio, aggregation, sizing and polydispersity with only a 2-µL liquid load. Further validation to expand Stunner's use from research sample testing to clinical material testing is needed. Should MAM be successfully adopted by gene therapy modality, the tremendous time and cost savings from analytics development and qualification will truly advance gene therapies into a fast track.

Another potential breakthrough lies in AAV drug product formulation. Currently, most clinical and commercial AAV products are stored frozen below -60 °C, creating challenges for cold chain. Moreover, the potential aggregation and loss of potency from freeze-thaw cycles, which occur during

drug product manufacturing and drug product administration, poses another concern. Formulations compatible with refrigerated storage of AAVs need to be systematically investigated, but selection of proper excipients could be the key to developing a suitable buffer formulation that enables stable, safe, and efficacious AAV vectors [41]. A recent success is Oxford Biomedica's novel formulation achieving liquid product stability at 2–8 °C for 12 months at a concentration of 1×10^{14} vg/mL and 3 months at 1×10^{15} vg/mL [42]. Moreover, among the approved AAV-based gene therapy drug products, Hemgenix® is the only one stored at 2–8 °C with a shelf-life of 24 months, setting the precedent for refrigerated storage. Hemgenix® formulation buffer contains common constituents utilized in other approved AAV-based gene therapy drugs, plus the use of polysorbate 20 as a stabilizer in addition to sucrose as a cryoprotectant [43]. Overall, it seems formulation development for AAV drugs at high concentration (>1×10^{14} vg/mL) is more challenging than mid (~1×10^{13} vg/mL) or low (<5E12 vg/mL) concentrations, when stored at 2–8 °C.

In conclusion, though the development of gene therapy has not reached par with other matured biologics, it is a promising modality with several highlighted areas for improvement. While we acknowledge the distinctions and limitations, other matured modalities share CMC commonalities and can serve as a reference guide. The implementation of platform process, analytical approaches and technical breakthroughs discussed herein could potentially further accelerate development and eventual commercialization of gene therapy to transform the lives of millions of patients.

REFERENCES

1. Lohr A. 2023's Market Outlook For Cell And Gene Therapies. *Cell & Gene* (February 14, 2023) (accessed Apr 2024).
2. Kohn DB, Chen YY, Spencer MJ. Successes and challenges in clinical gene therapy. *Gene Ther.* 2023; 30, 738–746.
3. Drago D, Foss-Campbell B, Wonnacott K, Barrett D, Ndu A. Global regulatory progress in delivering on the promise of gene therapies for unmet medical needs. *Mol. Ther. Methods Clin. Dev.* 2021; 21, 524–539.
4. Kelley B. Developing therapeutic monoclonal antibodies at pandemic pace. *Nat. Biotechnol.* 2020; 38, 540–545.
5. Hosseini M, Fath S. Cutting the Cost of Gene Therapy Manufacturing. *Roland Berger* (July 6, 2023) (accessed April 2024).
6. Farid SS, Baron M, Stamatis C, Nie W, Coffman J. Benchmarking biopharmaceutical process development and manufacturing cost contributions to R&D. *MAbs* 2020; 12(1), 1754999.
7. Wurm, F. Production of recombinant protein therapeutics in cultivated mammalian cells. *Nat. Biotechnol.* 2004; 22, 1393–1398.
8. Nguengang Wakap S., Lambert DM, Olry A, *et al.* Estimating cumulative point prevalence of rare diseases: analysis of the Orphanet database. *Eur. J. Hum. Genet.* 2020; 28, 165–173.
9. Tirrell M. A US Drugmaker Offers to Cure Rare Blindness for $850,000. *CNBC* (January 3, 201) (accessed Apr 2024).
10. Pagliarulo N. Novartis Gene Therapy Approved, But Will Come at Cost of More Than $2M. *Biopharma Dive* (May 24, 2019) (accessed on Apr 2024).

11. Platform Vector Gene Therapy.

12. Capra E, Gennari A, Loche A, Temps C. Viral-Vector Therapies at Scale: Today's Challenges and Future Opportunities. *McKinsey & Company* (March 29, 2022).

13. Penaud-Budloo M, François A, Clément N, Ayuso E. Pharmacology of recombinant adeno-associated virus production. *Mol. Ther. Methods Clin. Dev.* 2018; 8, 166–180.

14. Bespoke Gene Therapy Consortium.

15. Andelyn Biosciences Selected as Viral Vector Manufacturing Partner for the Foundation for the National Institutes of Health (FNIH) Accelerating Medicines Partnership® (AMP®) Bespoke Gene Therapy Consortium (BGTC). *PR Newswire* (February 28, 2024) (accessed Apr 2024).

16. Accelerating Medicine Partnership Bespoke Gene Therapy Consortium of Foundation for the National Institutes of Health. Bespoke Gene Therapy Consortium (BGTC) Regulatory Playbook Version 1.0 2024 (accessed Apr 2024).

17. Kelly T. Novel Dual-Plasmid Transfection System Improves Titer and Product Quality in AAV Manufacturing. *Bioprocess Online* (July 15, 2022) (accessed Apr 2024).

18. Clarkson N, Yin J. Innovative Platforms for Improved Viral Vector Development and Scale-Up. *Bioprocess Online* (June 21, 2023) (accessed Apr 2024).

19. Li C-T, Tripp C, Wang J, *et al.* Ramp Up Your AAV Productivity with Novel Engineered Plasmids and Optimized HEK293 Cell Line. Lonza Cell & Gene (accessed Apr 2024).

20. Viralgen Vector Core. White paper: a proprietary mammalian suspension technology for scalable rAAV production: the Pro10™ cell line (accessed Apr 2024).

21. Analytical Accelerator™ for AAV. Center for Breakthrough Medicines (now SK Pharmteco) Brochure (accessed Apr 2024).

22. UpTempo℠ AAV Platform Process. Catalent Cell and Gene Therapy Brochure (accessed Apr 2024).

23. Alliance for Regenerative Medicine. Project A-Gene: a case study-based approach to integrating QbD principles in gene therapy CMC programs (accessed Apr 2024).

24. Au HKE, Isalan M, Mielcarek M. Gene therapy advances: a meta-analysis of AAV usage in clinical settings. *Front. Med. (Lausanne)* 2022; 8, 809118.

25. WHO informal consultation on the application of molecular methods to assure the quality, safety and efficacy of vaccines. WHO, Geneva, Switzerland, April 7–8, 2005 (accessed Apr 2024).

26. Alliance for Regenerative Medicine. Comments for FDA Docket Number: FDA-2020-D-2101 for "Human Gene Therapy for Neurodegenerative Diseases; Draft Guidance for Industry" 86 FR 549, April 5, 2021 (accessed Apr 2024).

27. Mezher M. Drugmakers suggest tweaks to FDA's neurodegenerative disease gene therapy guidance. Regulatory Focus, a RAPS publication (accessed Apr 2024).

28. Beattie SG. Facing potential chemistry, manufacturing, and control (CMC) development challenges with recombinant adeno-associated viral vectors: available regulatory guidance and recommendations. *Cell Gene Ther. Ins.* 2023; 9(6), 811–830.

29. *US Department of Health and Human Services Food and Drug Administration Center for Biologics Evaluation and Research.* Human Gene Therapy for Neurodegenerative Diseases, Guidance for Industry, October 2022 (accessed Apr 2024).

30. Aldevron. Nanoplasmid Vector Platform (accessed Apr 2024).

31. Touchlight. DNA vector and enzymatic manufacturing process (accessed Apr 2024).

32. 4basebio (accessed Apr 2024).

33. Wörner TP, Bennett A, Habka S, *et al.* Adeno-associated virus capsid assembly is divergent and stochastic. *Nat. Commun.* 2021; 12, 1642.

34. Bollenbach L, Buske J, Mäder K, Garidel P. Poloxamer 188 as surfactant in biological formulations—an alternative for polysorbate 20/80? *Int. J. Pharm.* 2022; 620, 121706.

35. Su W, Patrício MI, Duffy MR, Krakowiak JM, Seymour LW, Cawood R. Self-attenuating adenovirus enables production of recombinant adeno-associated virus for high manufacturing yield without contamination. *Nat. Commun.* 2022; 13(1), 1182.

36. Virica Biotech. White paper: the impact of antiviral defenses in viral vector production (accessed Apr 2024).

37. Reese B, Swanson J, Storck A, Juckem L. RevIT™ AAV Enhancer: rev-up AAV genome production in upstream manufacturing. Mirus Bio (accessed April 2024).

38. Sanyal S, Gu B. Lonza's AAV transient transfection and producer cell line platforms to accelerate your path to IND. 2024 Viral Vector Summit oral presentation.

39. CRB Group. Horizons: Life Sciences 2023 Report (accessed April 2024).

40. Wu Z, Wang H, Tustian A, Qiu H, Li N. Development of a two-dimensional liquid chromatography-mass spectrometry platform for simultaneous multi-attribute characterization of adeno-associated viruses. *Anal. Chem.* 2022; 94(7), 3219–3226.

41. Srivastava A, Mallela KMG, Deorkar N, Brophy G. Manufacturing challenges and rational formulation development for AAV viral vectors. *J. Pharm. Sci.* 2021; 110(7), 2609–2624.

42. Wash K, Peters T, Karpes L, Mercaldi M, Kelly T. Novel AAV drug product formulation achieving long-term liquid state stability (2–8 °C) and high titer preparations (>E15 vg/mL). 2022 European Society of Gene and Cell Therapy (ESGCT) poster presentation (October 13, 2022) (accessed Apr 2024).

43. Som M, Gikanga B, Kanapuram V, Yadav S. Drug Product Formulation and Fill/Finish Manufacturing Process Considerations for AAV-based Genomic Medicines. *J. Pharm. Sci.* 2024; S0022–3549(24), 00125–00124.

44. Chien HT, Prior H, Andrews L, *et al.* Re-evaluating the need for chronic toxicity studies with therapeutic monoclonal antibodies, using a weight of evidence approach. *Regul. Toxicol. Pharmacol.* 2023; 138, 105329.

45. Catalent Biologics.

46. Forge Biologics AAC manufacturing services.

47. Fujifulm Diosynth Biotechnologies end-to-end viral vector gene therapy CDMO services.

48. Lonza cell and gene therapies.

49. Oxford Biomedica full scope AAV services.

50. SK Pharmteco viral vector manufacturing CDMO services.

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CELL & GENE THERAPY INSIGHTS

VECTOR PROCESSING AND MATERIALS

SPOTLIGHT

EXPERT INSIGHT

Improving process efficiency to reduce cost-of-goods per dose in manufacturing of recombinant AAVs

Garima Thakur, Sheldon Mink, Hanne Bak, and Andrew D Tustian

AAV have emerged as a leading platform for *in vivo* gene delivery due to their robust safety profile and ability to effectively deliver therapeutic genes to a range of tissue and organ targets. However, a key challenge limiting access to AAV therapies is the high cost of treatment of up to US$3.5 million per dose. Reasons for high cost-of-goods (COGs) per dose include low bioreactor production yields, significant product loss during purification, limited process scalability, and expensive raw materials. Notably, even a two- to three-fold reduction in costs can bring gene therapy treatments financially in-line with well-established biologics such as mAbs. For example, recurring costs for regular doses of mAbs can range from US$10,000–50,000 per year, equivalent to US$0.3–1.5 million over a lifetime usage of 30 years. Thus, there is significant opportunity to intensify gene therapy manufacturing processes to enable these curative treatments to become the most compelling option for patients both socially and financially. In this article, we discuss several manufacturing-related approaches to lower COGs/dose of AAV-based gene therapies by improving process efficiencies.

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RISING STARS

This article is part of our 'Rising Stars' series, giving a platform to the emerging leaders of the sector. In this series, we share the perspectives of fledgling thought-leaders, chosen by our Editorial Advisory Board members as future stars in their field. Andrew Tustian, Senior Director of Viral Vector Process Development at Regeneron Pharmaceuticals, had this to share on his Rising Star nomination:

> "Garima, a rising star in AAV viral vector process development, illuminates the field with her intelligence, hard work, and innovative ideas. She is not just developing processes, she is enabling wider patient access to these potentially curative therapies."

INTRODUCTION

AAV have emerged as a leading platform for *in vivo* gene delivery due to their robust safety profile and ability to effectively deliver encapsidated therapeutic genes to a range of tissue and organ targets [1]. Gene therapy medicines offer long-term treatment or cures for formerly untreatable genetic disorders by modifying or deleting defective genes or replacing absent genes to restore functionality [1]. In the last 5 years, 13 gene therapy products have been approved in the USA, generating US$1.3 billion in sales in 2022 with projections to reach US$4.3 billion by 2028 [2]. However, despite rapid technological and commercial growth in the sector, a major challenge preventing widespread access is the cost of treatment which currently ranges up to US$3.5 million per dose to the patient [3], significantly driven by the manufacturing cost of goods per dose (COGs/dose) which are in the range of US$0.5–1 million [4].

Key raw material cost drivers required for upstream production of AAV in HEK293 cells, currently the most common manufacturing platform for AAV, include plasmids containing the required genes for production of AAV capsids and the desired gene insert, cell culture media, and transfection reagents. For downstream purification, key raw material cost drivers include endonuclease for digestion of host cell DNA and chromatographic units for affinity capture and polishing of the AAV capsids to remove impurities and enrich for full capsids. Figure 1 illustrates a typical manufacturing process for AAV production including upstream production, lysis, clarification, affinity capture, and full/empty separations using either chromatography or ultracentrifugation steps. These are followed by final concentration and buffer exchange, with optional intermediate in-process concentration steps such as prior to affinity capture. In this article, several approaches will be discussed to lower COGs/dose in the upstream and downstream AAV manufacturing processes by reducing raw material requirements or enhancing productivity.

REDUCING COGS/DOSE IN UPSTREAM OPERATIONS

Plasmid DNA production bottlenecks

Plasmid DNA is a critical raw material for current AAV manufacturing processes that rely on transient transfection of HEK293 cells.

Recent advances in producer cell line (PCL) systems for AAV production may eliminate the need for transient transfection in future and are discussed further in the next section. However, the current norm in the industry is to use a triple-transfection approach utilizing three plasmids [5]. These are:

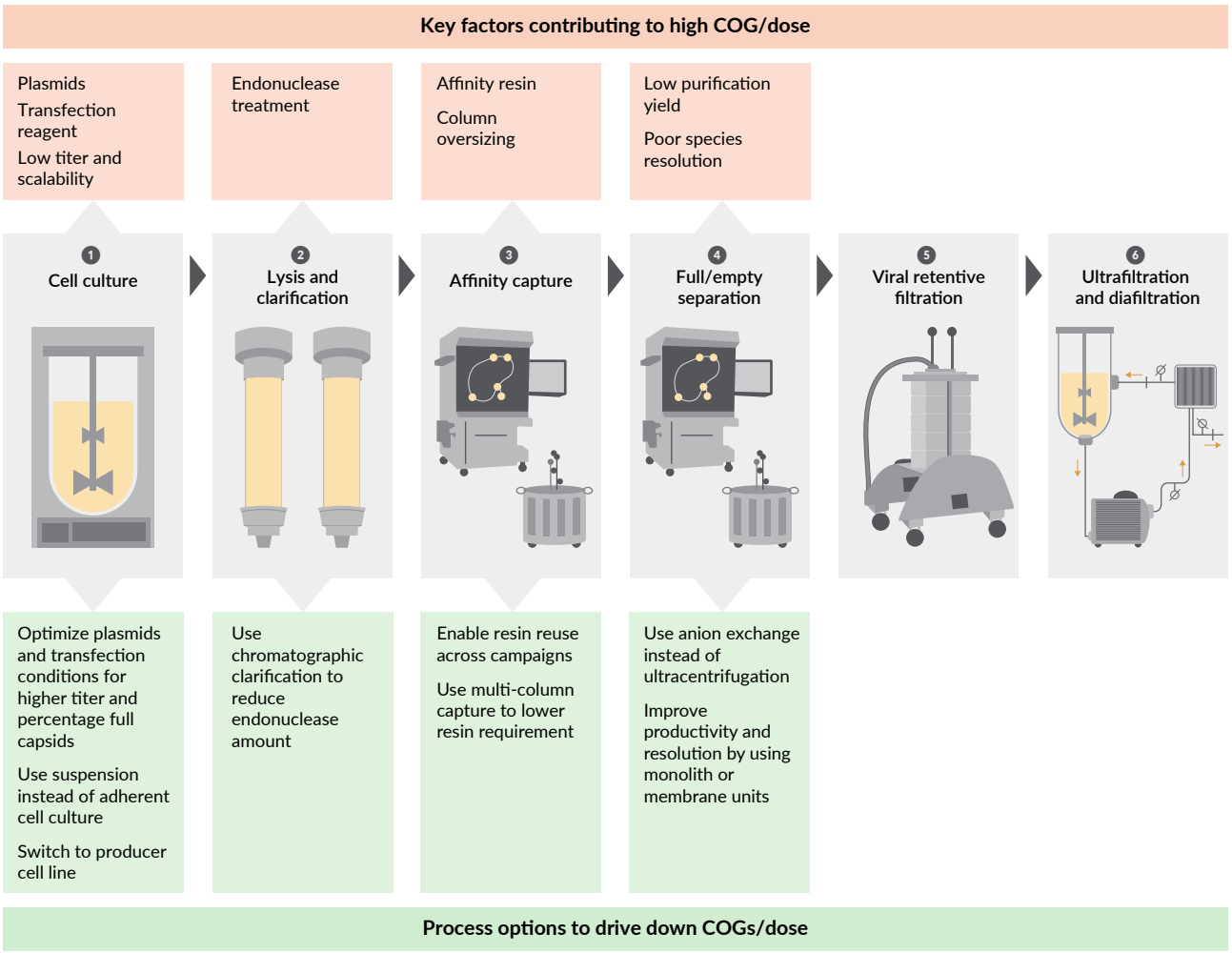
- A transgene plasmid encoding the therapeutic gene that is desired to be packaged inside the AAV capsids flanked by inverted terminal repeat (ITR) sequences;
- A rep/cap plasmid encoding proteins required for production and replication of AAV capsid proteins; and
- A helper plasmid providing minimal adenoviral genes required to support AAV replication.

These three plasmids are co-transfected in the bioreactor to initiate AAV production. Post-transfection, the cells begin to lose viability and are typically harvested within 3–4 days [6].

Thus, a constant supply of GMP grade plasmids is required for each clinical production of AAV using this approach. It is estimated that 100–1,000 g/year of each plasmid vector is required to meet production demands for a marketed gene therapy product with over 10,000 patients, though this can vary based on dosage requirements [7,8].

FIGURE 1

AAV production process including key factors contributing to high COGs/dose at each step.



Currently, plasmids are typically produced using *E. coli* bacterial fermentation and purified using a combination of clarification and chromatographic steps. With current productivities in *E. coli* ranging from 200–500 mg/L of bacterial culture [9], 2–20 kL production capacity is required per year to meet demands for a single plasmid. In addition to the scalability challenge, it is critical to mitigate lot-to-lot variability and maintain consistent quality attributes of the plasmids, as they directly impact AAV production yields and quality [10].

Intensifying plasmid production and engineering plasmids to improve AAV yields

Technological advancements by plasmid manufacturers to increase yields and lower manufacturing costs can drive down the cost of plasmids, resulting in lower COGs/dose for AAV manufacturers. In general, higher yield plasmid processes can result in lower COGs/dose, though technology advancements required to drive these higher yields can also result in higher manufacturing costs, particularly in the early stages of adoption. Plasmid processes typically include fed-batch cultivation of *E. coli*, alkaline lysis of the cell paste to extract plasmid DNA, and several chromatographic purification steps including ion-exchange and hydrophobic interaction to separate host cell DNA, RNA, and open-circular plasmids from the desired supercoiled form [11]. There has been extensive research on engineering of *E. coli* strains and plasmid vectors to reach higher productivities than the widely used commercial DH5a strain by reducing metabolic burden and intensifying cell culture conditions [9,12–16].

Engineering of plasmid characteristics can also be considered a key approach for reducing COGs/dose of the final AAV therapy [17], as it can enable higher yields, better process control post-transfection, or improved full/empty AAV capsid ratios. Engineering the *rep/cap* plasmid to mutate the *rep* genes has been shown to improve production of difficult serotypes [18], particularly with respect to the AAV2 *rep* genes which are frequently used for production of different AAV serotypes as they are the most widely characterized and understood. Notable recent work also includes comprehensive mutagenesis to map the effect of all single codon mutations in the AAV2 *rep* gene, providing a sequence-to-function map for *rep* engineering to enhance AAV production [19]. This can help to overcome potential drawbacks of using AAV2 *rep* genes for production of different serotypes, such as in the case of AAV5 where low titers are observed due to dissimilarities between the telomere resolution sites of AAV2 and AAV5 [19]. Lastly, approaches to switch from triple-transfection to dual transfection systems have shown promising results by integrating required functions from the *rep/cap* and *helper* plasmids into a single plasmid such as in the case of the pDB system [20], or more recently by integrating the *GOI* and *rep/cap* plasmids into a single plasmid such as in the case of the pOXB system [21]. These dual plasmid approaches can significantly reduce process plasmid needs.

Other plasmid engineering improvements include adding DNA sequences enabling control of the timing of expression of *rep* and *cap* genes to control transgene production and packaging. One example is a 'Tet-Cap' expression system in which the *rep* and *cap* genes are separated onto two plasmids to control the timing of *cap* expression, enabling increased capsid yields with two- to four-fold higher full capsids across a range of serotypes [22]. Another example is the use of plasmids with 'Tet-On/Tet-Off' systems for inducible gene expression to control transgene expression within a desired window of time during the AAV production process, enabling better packaging with lower cytotoxicity [23,24]. Finally, recent studies have shown the adverse impact of oversized genomes on viral genome production and packaging, as well as the impact of single-stranded versus

self-complementary ITR configurations [25]. Thus, engineering the gene of interest plasmid to be of an appropriate kilobase length can serve to improve both upstream and purification yields of full capsids.

Switching to in-house fermentation or next-generation production techniques

AAV manufacturing companies can consider moving towards developing in-house production facilities for plasmids. This would increase scheduling control and flexibility and eliminate the need to onboard different vendors which can introduce additional comparability testing needs. In-house plasmid capabilities can also enable rapid technology development in the areas of plasmid engineering, fermentation and purification tailored to future needs of the pipeline. However, building out large-scale *E. coli* fermentation facilities require significant upfront investment which may be difficult for companies in the early stages of gene therapy product development. Furthermore, such companies may lack expertise in development and scale-up of bacterial fermentation processes, requiring specialized manpower and research teams. However, the costs associated with development of fermentation facilities and expertise can potentially be made up for by future easy access to large quantities of plasmid with reduced timelines and cost-per-gram, lowering COGs/dose and reducing reliance on external suppliers.

Finally, next-generation plasmid production methods can also be considered. A recent commercial technology called 'doggybone DNA' or 'dbDNA' was introduced in 2022 by Touchlight, leveraging an in-vitro cell-free enzyme-based manufacturing process to construct synthetic plasmids without the need for *E. coli* fermentation [26]. The manufacturing process for dbDNA is at benchtop scale, in contrast to kL-scale fermentation required for *E. coli* cultivation. The process reportedly enables production, quality assurance and release of dbDNA within 50 days, in contrast of timelines for GMP plasmids which are in the range of 3–12 months [27]. A further notable benefit of dbDNA is the elimination of bacterial sequences such as antibiotic resistance genes in the plasmids, removing the risk of packing of these contaminant sequences inside the AAV capsids along with the therapeutic transgene. Though the cost at small scale is reported to be higher than that of comparable amounts of traditional plasmid, at larger scales, the costs of production progressively decrease relative to traditional plasmid costs due to elimination of large-scale bioreactors and purification facilities [26]. Notably, large-scale use of the technology for the production of mRNA was initiated by Pfizer in 2022, though similar use in plasmid applications is not yet reported [28].

Reducing COGs/dose of upstream bioreactor processes

Choice of adherent vs suspension cell culture

Upstream production of AAV in HEK293 cells is currently carried out using either adherent or suspension cell culture [29]. In adherent cell culture, cells are physically attached to an extracellular matrix, simulating a natural environment for cell growth with intracellular interactions. Alternatively, suspension cell culture involves cultivation of free-floating cells in agitated vessels such as shake flasks, rocker bags or stirred-tank bioreactors. The choice of adherent vs. suspension cell culture is a key contributor to process COGs/dose due to the impact on process yield and scalability. Adherent cell culture processes are well-established for research productions of AAV and typically result in higher cell-specific productivities of >100,000 vg/cell, compared to suspension cell culture with productivities of ~50,000 vg/cell. Reported dose sizes for AAV treatments range from 3×10^{11} to 1.2×10^{16} per patient depending on the indication and

delivery target, and low prevalence diseases (1–10,000 patients) vs. higher prevalence diseases (10,000–100,000 patients) can result in an overall annual demand in the range of 1×10^{13}–1×10^{19} vg. Thus, with adherent cell culture yields in the order of 10^{14} vg/m^2, it is challenging to generate material in the order of 10×10^{17} vg for clinical and commercial use without the use of excessive surface area such as >6000 m^2, much higher than the largest available surface area of 333–600 m^2 for commercial adherent cell culture bioreactors [30].

In contrast, suspension cell culture in single-use bioreactors (SUB) results in highly scalable processes of 500–2000 L scale with titers in the range of 10^{14}–10^{15} vg/L, suitable for commercial needs [31]. A literature article modelling operational costs associated with adherent versus suspension bioreactors estimated COGs/dose reduction of ~20% when shifting from adherent to suspension bioreactors, as well as reduction of operator requirements from 16–18 to only 2 [32]. Furthermore, the economic model predicted that the COGs/dose of both approaches were similar for lower dosages of 10^{12}–10^{13} vg/dose, but that at higher dosages of 10^{14}–10^{15} vg/dose there were clear economic advantages for suspension cell culture [32]. At the highest dose, suspension cell culture processes were estimated to result in $50–100k/dose, compared to $150–200k/dose for adherent cell culture processes [32]. However, as much of AAV early-stage development is done using adherent cell culture, it may not always be feasible to switch production systems while maintaining speed to clinic, and subsequent changes may result in variations in the product quality attributes due to essential differences in cell behavior and, morphology and metabolism between adherent and suspension cells. This may in turn require process redevelopment and new toxicology or even clinical studies in case analytical comparability of the drug product cannot be established.

Several recent approaches can be considered for reducing COGs/dose associated with adherent cell culture processes by increasing scalability. Firstly, bioreactors such as iCELLis™ (Cytiva) or Scale-X™ (Univercells Technology) bioreactor systems can enable cost-effective large-scale adherent cell culture [30,33–35]. The iCELLis system is estimated to lower COGs/dose by half compared to multi-tray adherent cell culture, comparable to the result for suspension cell culture [36], and a literature article directly comparing the iCELLis and Scale-X systems reported both to be equally efficient in production of both AAV and lentiviral vectors [30]. Another recent approach is the use of microcarrier culture, a hybrid between adherent and suspension systems where cells can adhere to microcarrier beads of 50–250 µm in diameter that are suspended in stirred-tank bioreactors [37–39]. A 500 L microcarrier culture provides equivalent surface area to 940 10-layer cell stacks, and a recent study compared titers in vg/cell and vg/mL across culture formats and showed comparable results for adherent, suspension, and microcarrier based processes in the order of 10^{11} vg/mL [39]. This suggests that microcarrier culture can enable production of large quantities of adherent cells in scalable single-use bioreactors, though data at process scale is currently limited to vaccine applications [40]. Lastly, it is important to note that, process efficiencies for adherent, suspension, and microcarrier systems can all be improved by using systems such as the NevoLine™ (Univercells Technology) system which integrates all upstream and midstream steps, including inoculation, upstream production, clarification, concentration and diafiltration to deliver a concentrated, clarified bulk ready for downstream processing [41].

Finally, optimized scale-up of suspension cell culture to improve titer and productivity are key to reducing COGs/dose by enabling production of a higher number of doses per unit volume of cell culture. Significant research is ongoing on developing optimized bioreactor parameters for higher titers and yield, including using different ratios of the transgene and packaging plasmids, and

cultivating the bioreactor to higher cell densities [42,43]. Novel transfection reagents have been recently developed which have been shown to increase genomic and capsid titer 10- to 20-fold compared to the traditional polyethyleneimine (PEI) transfection reagent [44,45]. Advances in transfection processes to reduce volumes required or improve transfection mix quality are also critical to COGs reduction, as the cost of these reagents can range up to US$100,000 per 500 L bioreactor and are known to have significant impact on yield and full/empty capsid ratios [32]. Additionally, the time-sensitive nature of the transfection process can negatively affect process reproducibility and robustness when scaling up, limiting many AAV production facilities to 200–500 L scale bioreactors. In a recent study, it was demonstrated that mixing variability in a transfection tank for a 2,000 L suspension bioreactor adversely affected transfection efficiency. Potential solutions include in-line mixing of transfection reagent and plasmid DNA to control transfection complex size within a narrow distribution [46]. Finally, further work is required to explore the relation between yield benefits and increased costs associated with these novel transfection reagents or methods to determine impact on COGs/dose.

Switching to producer cell lines and recent advancements in production systems

The availability of stable producer cell line (PCL) systems is a recent advancement in the AAV field with the potential to transform the upstream process. Development of PCL systems is challenging due to the need to stably integrate all components necessary for rAAV production, including adenovirus helper functions E1A, E1B, E2A, E40RF6, VA RNA, as well as AAV replicase, capsid sequences and a transgene flanked by the AAV inverted terminal repeats [47]. As some of the integrated components are cytotoxic, expression is typically regulated by an inducible promoter. Two currently available commercial PCL systems are the ELEVECTA™ system from Cytiva available in both human amniocyte and HEK293 cell lines [48], and the Pinnacle PCL™ from Ultragenyx based on a HeLa cell line [49], both of which have been scaled up to 2,000 L [50,51]. There are also efforts underway to develop more HEK293-based PCL systems in academia and industry, with a recent reported breakthrough by Lonza via the Xcite® AAV platform, though limited details are currently publicly available [52,53]. However, due to the technical challenges of developing such systems and the need to redevelop the cell line for each new serotype, promoter and transgene combination, as well as potential high costs associated with development and production, it remains to be seen whether such approaches will become the norm for AAV manufacturing processes, despite advantages including elimination of the need for plasmid DNA and improved scalability to suspension cultures of 2,000 L [54].

Even higher process efficiency can be achieved by coupling PCL systems with perfusion cell culture, in which cells are grown to high densities and supernatant containing the product as well as spent media is continuously removed from the bioreactor while cells are retained. This is accomplished using alternating tangential-flow filtration (ATF) combined with continuous feed of fresh media in a 'feed and bleed' approach. Such systems are only applicable to rAAV processes in which the viral particles are predominantly or exclusively secreted extracellularly, such as in the case of the ELEVECTA system where cells are not only viable for more than 3–4 days post-transfection, but also secrete over 90% of the total viral particles into the cell culture supernatant from day 4 onwards [55]. The ELEVECTA system was also demonstrated with N-1 perfusion, which is an approach utilizing continuous expansion of cell lines by attaching a cell retention device to the N-1 bioreactor to attain high cell density and viability, allowing the N bioreactor to be seeded at a higher

starting cell density and shortening the production time. Cell-specific productivities were eight-fold higher with 30–40% full particles using this approach [56]. This likely results in several-fold reduction in COGs/dose, considering the combined effects of higher cell density processes with higher cell-specific productivity along with elimination of plasmid DNA and transfection reagent requirements, though it is important to note that the process costs are likely to be impacted by factors including perfusion rate, media costs, ATF system costs, and bioreactor scale.

Finally, another recent development with the potential to reduce upstream COGs is the TESSA (tetracycline-enabled self-silencing adenovirus) system [57–59]. This is based on the 'helper' adenovirus technique, in which expression of the dependovirus, AAV, is significantly enhanced by the presence of its partner adenovirus. The use of adenovirus to enhance AAV cultures is typically undesirable due to its high infectivity in humans, leading to creation of the helper plasmid to replicate key adenovirus functions critical for AAV production. In the TESSA process, the adenovirus is genetically engineered to be self-silencing, i.e., to repress itself after its helper function is completed. The TESSA system claims several-fold higher titers with 5- to 60-fold higher infectivity across multiple AAV serotypes. Publicly available data shows moderate titers of 6 x 10^{14} cp/L with 20–30% full capsids (1.2 × 10^{14} vg/L) achieved using this system [58], though titers of 1 × 10^{16} vg/L with 66% full capsids have been reported by the technology developers [60]. Additionally, TESSA particles can be engineered to include rep/cap and transgene sequences, eliminating the need for plasmid co-transfection. Overall, the TESSA system can result in reduction of COGs/dose for the upstream step due to the combination of increased titer, higher infectivity and elimination of plasmid DNA requirements, though detailed cost analysis of TESSA processes is not available in the literature and production and purification may be complicated by the need to generate adenoviral master seed stocks and remove residual self-silencing adenovirus.

REDUCING COGS/DOSE IN DOWNSTREAM OPERATIONS

The downstream purification process for AAV includes cell lysis, clarification, affinity capture, full capsid enrichment, and ultrafiltration/diafiltration operations [61]. For industry-standard AAV processes utilizing a combination of suspension cell culture in upstream followed by affinity capture and ion exchange operations in downstream, a recent literature article estimated that the downstream process contributed 65–77% to the overall COGs/dose, two-fold higher than upstream operations with 23–35% contribution [32]. This can be attributed to a combination of high raw material costs, many process steps, and low volumetric titers at harvest, as well as the combination of unit operations used such as including intermediate concentration steps such as before affinity capture to reduce resin requirements Another key reason is the production of empty AAV capsids in the upstream bioreactor, typically comprising 50–90% of the overall capsids produced and resulting in genomic yields of 30–70% over the full/empty capsid purification step [62]. Several options for lowering COGs/dose by increasing process productivity and reducing raw material requirements across the downstream purification process are discussed below.

Reducing endonuclease requirements in lysis and harvest operations

For lysis and clarification operations, a key raw material contributing to high COGs/dose is endonuclease, an enzyme added to digest large amounts of host cell DNA (~10^4 ng/mL) that is released along with intracellular AAV from HEK293 cells during lysis [63]. Costs for this enzyme are US$~100,000 for a 500 L batch, not only driving up COGs/dose but increasing supply chain complexity due to a need for a regular supply of GMP-grade endonuclease

[64]. One approach to reduce endonuclease requirements is to adjust the process such the endonuclease treatment is carried out post-concentration of the AAV material via tangential flow filtration or affinity capture, to obtain the desired activity in units/L over a smaller overall volume. However, the presence of long strands of undigested host cell DNA in the harvest can lead to rapid fouling and thus low throughput and volumetric loss for clarification operations with traditional depth filters as well as any subsequent membranes or resins. Early removal of host cell DNA is also desirable such that continued clearance can be achieved across the following chromatographic unit operations to match stringent quality targets [65,66].

An alternative approach to host cell DNA removal is the use of chromatographic clarification techniques [67]. Next-generation depth filters such as Harvest RC™ (3M) have similar costs as traditional polypropylene depth filters but are constructed using anion exchange fibrous media that can bind negatively charged impurities such as host cell DNA during clarification [68]. Harvest RC™ filters have gained prominence in mAb processes, showing removal of 99.99% of soluble host cell DNA during clarification of CHO cell harvest, as well as 15–30% reduction in COGs/dose of the harvest step for mAbs [67,69]. Though the host cell DNA burden is 2 log higher in the case of HEK293 cell lysate as compared to CHO cell culture supernatant, recent studies on Harvest RC™ filters in AAV processes resulted in reduction of host cell DNA from ~10^4 ng/mL to <10^2 ng/mL in an endonuclease-free process, comparable to the filtrate quality obtained from traditional depth filters such as C0SP (MilliporeSigma) with loads treated with 100 U/mL of endonuclease [70].

Lowering COGs of affinity capture operations

For the affinity capture unit operation, high COGs/dose result from the use of large affinity columns packed with expensive resins carrying AAV-specific proteinaceous ligands [71]. The columns are generally loaded below their maximum binding capacity due to a need to process a large volume of low-titer AAV material over a reasonable duration, such as with <10 hours of column loading time [61]. For example, a 2,000 L process-scale AAV bioreactor with a titer of 1×10^{15} cp/L requires a 12 L affinity capture column to load ~2200 L of clarified lysate in approximately 9 hours of loading time using a standard 3-minute residence time. This results in binding of 2×10^{17} capsids per L of resin, 10-fold lower than 2×10^{18} cp/L resin which is the maximum column binding capacity at 2% breakthrough for POROS CaptureSelect AAVX, an industry-standard affinity capture resin for AAV. The cost of a 12 L affinity column packed with POROS CaptureSelect AAVX is in the range of USD ~400,000, thus there is a significant opportunity to lower COGs/dose by reducing resin requirements by 90% via the use of smaller columns loaded near to their binding capacity.

However, directly switching to 10-fold smaller affinity capture columns would result in unfeasible loading times in the range of ~100 hours. Alternative approaches to reduce the affinity capture resin requirements are pre-concentration of the material or switching to a multi-column continuous capture process [63,72]. For example, a 10-fold reduction in the clarified lysate using tangential flow filtration (TFF) would enable a 300 mL column to be used for affinity capture with the same 9-hour loading time, resulting in 90% savings in the cost of resin needed. Disadvantages of this approach include the addition of a new unit operation with associated costs in terms of membrane area, equipment setup, and yield loss, as well as an increase in operational time to accommodate a ~6-hour pre-concentration step prior to a ~9-hour affinity capture load. Alternatively, single-pass TFF (SPTFF) or hollow-fiber countercurrent dialysis can be considered to concentrate the affinity

capture load material in-line [73–76]. AAV processes are well-suited to in-line concentration due to low concentrations of AAV capsid proteins at harvest, enabling high volumetric concentration factors of 10–50 × to be readily achieved due to low viscosity and minimal concentration polarization reducing the permeate flux [75].

Column volumes can also be reduced by implementing continuous processing for the affinity capture unit operation. This has been shown to contribute to 70% lower resin requirements of the affinity capture step in mAbs processes and has been implemented at manufacturing scale by several biopharmaceutical company for affinity capture operation alone, without end-to-end process continuity. The major approach for continuous affinity capture chromatography currently implemented in the industry is via multi-column or periodic counter-current chromatography (PCC), a well-established approach in which two affinity columns are connected in series during loading to increase resin binding capacity while a third column cycles through non wash, elution and cleaning steps [77–79]. A recent study evaluating PCC for affinity capture of AAV2 showed three-fold improvement in process productivity [72,77]. Overall, an SPTFF + PCC operation would be able to process a 2,000 L batch in <10 hours with 90% reduction in resin requirements.

Alternatively, novel approaches to affinity capture can be considered, such as IsoTag™ AAV [80] and continuous counter-current tangential chromatography (CTCC) [81]. In the former approach, a self-scaffolding recombinant protein reagent fusing an AAV-specific affinity ligand with a stimulus-responsive biopolymer is used that undergoes phase transition based on modification of salt concentration, enabling effective affinity capture of AAV with reported 25% reduction in COGs for a clinical AAV program [80]. In the CTCC approach, the chromatography setup is re-engineered such that resin beads recirculate across a flow path, enabling two- to four-fold decrease in resin requirements [82]. However, both these systems are not well-established in the industry and require novel operational equipment with limited scalability or robustness data.

Another approach to lowering COGs of affinity capture is enabling resin reuse, which would lower consumable costs as well as manpower needed for column packing and qualification. Recent data from the manufacturer on AVIPure AAV affinity resins suggest that these resins are caustic stable, enabling cycling up to 50 times with stable impurity clearance, potentially enabling significant reduction of the cost of affinity capture manufacturing processes. However, the current standard in the industry is to use a naïve affinity column for every run, and there is a paucity of literature data on cleaning efficiency of AAV affinity resins and on the stability of the ligands across cleaning cycles. A recent study purified five different AAV1 preparations with barcoded transgenes consecutively on a 1 mL POROS™ CaptureSelect™ AAVX column, with regeneration and wash steps with 6 M guanidine and 20% ethanol in each run [83]. The results showed that 99.93% of the genome in the elution pool of the fifth run contained the correct barcode, with negligible cross-contamination of product and <2% flow through loss across all runs. For larger columns, binding efficiency was observed to decline, but a more stringent cleaning protocol including 0.1 M phosphoric acid at pH 1.0 was found to restore efficient binding up to six cycles. In another study, affinity chromatography cycles were carried out with purified AAV9 model feeds as well as clarified lysate feeds on POROS CaptureSelect AAV9 and POROS CaptureSelect AAVX resins [84]. It was found that the presence of impurities in the latter feed led to increased aggregate, host cell DNA and histone protein content in the elution pools over multiple cycles, particularly for lower pH elutions. Overall, more resin characterization studies as well as advancements in resin engineering

to improve ligand stability and specificity are required before resin reuse becomes a norm in the industry.

Finally, as affinity capture resin is the major contributor to COGs/dose of this step, non-affinity methods to capture AAV can be considered. One approach is cation exchange (CEX) based capture, which was a standard method for AAV purification in the early stages of the industry, prior to the development of AAV-specific affinity capture resins [85]. CEX modalities specifically developed for AAV capture include CIM SO3 monoliths, which enable 3- to 30-fold higher flow rates than in resin-based systems along with ~100-fold lower costs [86]. Disadvantages of CEX include the possibility of less clearance of impurities compared to affinity capture resins which are the industry-standard workhorse for impurity clearance, as CEX processes have typically been used in conjunction with ultracentrifugation at lab-scale and thus residual impurities post-CEX capture in non-ultracentrifugation-based processes remains an open question. Other non-affinity capture methods are hydrophobic interaction chromatography (HIC) with ammonium sulfate treatment of cell culture lysate to enable separation of host cell proteins and DNA [87], and aqueous two-phase separation (ATPS) using polyethylene glycol to induce a two-phase system where AAV capsids are enriched in the interphase with reported 99% recovery [31,88]. Overall, though seemingly offering substantially lower COGs/dose than affinity capture, these methods require further study to ensure comparable AAV quality and process robustness.

Lowering COGs of full/empty separations

Separation of full and empty capsids is a key process step in AAV production due to the high proportion of empty capsids in the range of 50–90% that are produced in the upstream bioreactor [61]. The proportion of empty capsids produced is dependent on several factors including serotype, transgene properties, plasmid ratios during transfection, and bioreactor set-points during growth, transfection and production phases [89]. Genomic yields across the full/empty separation step can range from 30 to 70%, often impacted by the % full capsids produced in the upstream bioreactor but also dependent on process optimization and purity targets [25,90–92]. This three-fold difference in yield is a direct contributor to COGs/dose of the final AAV therapy.

Currently, there are two approaches for full/empty capsid separations in the industry: density gradient ultracentrifugation (UC) and anion exchange chromatography (AEX) [93]. UC is a well-established lab-scale approach for full/empty separations, leveraging the 1 MDa difference in weight between empty and full capsids to drive separations using ultracentrifugation with iodixanol- or cesium chloride-based density gradients [94]. However, scale-up of UC methods is well-known as an industry challenge because of limited volumetric capacity per ultracentrifuge run [32].

In contrast, AEX uses the differences in surface charge characteristics induced by the presence of encapsidated DNA to drive separations. The small pI difference between empty (pI ~5.8) and full (pI ~6.1) capsids makes the separation more challenging, especially due to the internalization of the DNA driving the pI change restricting the changes in surface charge one might expect from such a pI difference. Furthermore, the exact pI values, and more pertinently, the capsid surface charge, are further impacted by AAV serotype, divergent and stochastic capsid stoichiometry, and post-translational modifications as well as the nature and size of the therapeutic transgene [25,95–97]. In general, due to the low pI difference between empty and full species, extensive optimization is required to match the high yields and purities typically obtained in UC methods as well as to enable separation of partial capsids

potentially containing truncated genomes or other partial DNA fragments [92,98].

Ultracentrifugation based full/empty separations

Scaling out batch UC operations is challenging due to long run times of 8–12 hours with low loading volumes of <0.5 L of material per UC at the largest available equipment scale. Furthermore, batch UC at manufacturing scale was found to be an operationally unfeasible approach in COGs models developed for AAV processes in the literature [32]. An alternative approach to batch UC is continuous UC, a technique commonly used in vaccine manufacturing that is adaptable to AAV processes [99]. Advantages of continuous UC include the ability to reach high purity targets of >90% full capsids across a wide range of AAV serotypes without process optimization for each new product. Further advantages include sizing of the equipment based on process flow rate, enabling a scalable alternative to batch UC with similar yield and purity. However, continuous UC is not yet industrially established as a purification option for AAV, and creation of a stable operation at the large scale leads to operational challenges due to inconsistencies in pouring of density gradient media.

AEX-based full/empty separations

AEX methods significantly outperform both batch and continuous UC in terms of impact to COGs/dose, with AEX-based purification trains resulting in 50% lower downstream COGs/dose and 30% lower overall process COGs/dose than UC-based processes [32]. A variety of chromatographic units suitable to anion exchange are commercially available, including packed-bed resins such as POROS™ HQ and Capto™ Q, monoliths such as CIM QA and Prima T, and membranes such as Sartobind® Q and Mustang™ Q. Full/empty separations across these modalities have been described in the literature and are widely used in the industry [100]. In general, the highest resolution between full and empty capsids is obtained from monoliths or membranes due to elimination of pore diffusion limitations impacting separations of large AAV particles using conventional AEX resins of 20–50 µm size developed for separations of mAbs and smaller biologics [101]. Recent studies using isocratic elution blocks have been shown to enable excellent resolution between full and empty capsids at both the HPLC and preparative scales using packed-bed resins, though such processes can be quite sensitive to column loading, starting % full, and exact buffer preparation conditions at the preparative scale [91,92,102].

COGs for monolithic or membrane-based AEX processes can be lower than for packed-bed processes due to improved yields and productivities [103]. Firstly, higher resolution between full and empty capsids translates into wider pool collection criteria resulting in higher yields. Secondly, as monolithic separations rely on only convective rather than diffusive mass transfer, process flow rates typically run at 1–5 column volumes (CV)/min, compared to packed-bed processes which run at 0.3–0.5 CV/min resulting up to 15-fold higher productivities in terms of g produced per minute, and enabling rapid cycling of 4–8 L monoliths with <2 hour run times to process large volumes of material, rather than requiring scale up to >20 L column volumes with >20 hours of run time. Thus, switching to monolith-based separations can be key productivity enhancers for downstream AAV purification processes. However, a key issue with polymethacrylate monoliths is lack of sanitization stability with NaOH solutions which limits their reusability, as this can impact their binding capacity and cause elution profiles to shift across cycles. Lot-to-lot in variability in monolith structure has also been an issue faced in the industry and limited the transition to isocratic instead of linear gradient elutions, although the recent release of CIM QA HR 'high reproducibility' monoliths in 2023 may serve to mitigate this issue [104].

Lastly, with the continued titer improvements in upstream bioreactor processes, as well as the increase in AAV development for indications requiring high-dose systemic injections, the column scales required for AAV purifications are likely to exceed the current available commercial sizes. For example, a 2,000 L bioreactor producing 1×10^{15} cp/L requires a 4–8 L monolith to enable AEX operations with loading of $1–2 \times 10^{17}$ cp/L of monolith volume. These are already the largest monoliths commercially available, with costs in the range of US$50,000–100,000. Further increases in bioreactor titer may require a switch to very large packed-bed columns with long operational times, or an increase in the number of AEX cycles required per batch. Alternatively, approaches such as displacement chromatography have been shown to be particularly effective for high-loading full/empty capsid separations, enabling 10- to 50-fold higher loadings in the order of $1–5 \times 10^{18}$ cp/L of monolith volumes [105–107]. Displacement chromatography can enable both higher yields and purities than conventional bind-and-elute chromatography as it leverages competitive binding between capsid species. Overall, the >10-fold increase in binding capacity without impact on pool yield or purity can contribute to 10-fold reduced COGs of this step.

CONCLUSIONS

Current high COGs of AAV therapies in the range of US$0.5–1 million per dose are a critical contributor to the current pricing of these therapies in the range of US$1–3 million per dose [4]. This is a bottleneck limiting affordability and accessibility of potentially life-saving gene therapies to patient populations worldwide. The high costs also attract censure from both patients and potential investors, casting doubt on the feasibility of the technology over the long term.

In this article, we discussed key reasons for high COGs/dose, including low bioreactor production yields, significant product loss during purification, and limited process scalability. Approaches for process intensification include switching to plasmid-free production systems, improving bioreactor titers, and intensifying purification workflows. The need for expensive raw materials including plasmids, transfection reagents, endonuclease, and affinity resins throughout the manufacturing process were also discussed, along with a review of potential options to reduce or eliminate their use.

Finally, despite the currently high COGs, it is important to note that the AAV sector is in very early stages compared to other biologics such as mAbs and is likely to see continued rapid growth with significant improvements in production and purification methods. This will help to bring the costs of these therapies in line with costs associated with mAbs and other well-established biologics, enabling these curative therapies to become the most compelling option for patients and for the society.

REFERENCES

1. Shen W, Liu S, Ou L. rAAV immunogenicity, toxicity, and durability in 255 clinical trials: a meta-analysis. *Front. Immunol.* 2022; 13, 1001263.

2. Berger R. Cutting the Cost of Gene Therapy Manufacturing. *Roland Berger*. https://www.rolandberger.com/en/Insights/Publications/Cutting-the-cost-of-gene-therapy-manufacturing.html (accessed Feb 2024).

3. Naddaf M. $3.5-Million Hemophilia Gene Therapy Is World's Most Expensive Drug. *Scientific American.* https://www.scientificamerican.com/article/3–5-million-hemophilia-gene-therapy-is-worlds-most-expensive-drug/ (accessed Feb 2024).

4. Macdonald GJ. Vector Production Processes Play Key Role In High Gene Therapy Prices. *Genetic Engineering and Biotechnology News* 2022; Nov 22.

5. Grieger JC, Soltys SM, Samulski RJ. Production of recombinant adeno-associated virus vectors using suspension HEK293 cells and continuous harvest of vector from the culture media for GMP FIX and FLT1 clinical vector. *Mol. Ther.* 2016; 24(2), 287–297.

6. Chahal PS, Schulze E, Tran R, Montes J, Kamen AA. Production of adeno-associated virus (AAV) serotypes by transient transfection of HEK293 cell suspension cultures for gene delivery. *J. Virol. Methods* 2014; 196, 163–173.

7. The Center for Breakthrough Medicines. Manufacture the highest quality precision plasmids with a reliable proven platform process. *Cell & Gene*. https://www.cellandgene.com/doc/manufacture-the-highest-quality-precision-plasmids-with-a-reliable-proven-platform-process-0001 (accessed Feb 2024).

8. Challener CA, Alvarez D. Optimizing plasmid production to meet growing demand. *Pharma's Almanac* Apr 3, 2023. https://www.pharmasalmanac.com/articles/optimizing-plasmid-production-to-meet-growing-demand (accessed Feb 2024).

9. Borja GM, Meza Mora E, Barrón B, Gosset G, Ramírez OT, Lara AR. Engineering *Escherichia coli* to increase plasmid DNA production in high cell-density cultivations in batch mode. *Microb. Cell Fact*. 2012; 11, 132.

10. Smith J, Grieger J, Samulski J. Overcoming bottlenecks in AAV manufacturing for gene therapy. *Cell Gene Ther. Ins.* 2018; 4(8), 815–827.

11. Lara AR, Ramírez OT. Plasmid DNA production for therapeutic applications. *Methods Mol. Biol.* 2012; 824, 271–303.

12. Cranenburgh RM, Hanak JAJ, Williams SG, Sherratt DJ. *Escherichia coli* strains that allow antibiotic-free plasmid selection and maintenance by repressor titration. *Nucleic Acids Res.* 2001; 29(5), e26.

13. Mairhofer J, Cserjan-Puschmann M, Striedner G, Nöbauer K, Razzazi-Fazeli E, Grabherr R. Marker-free plasmids for gene therapeutic applications—lack of antibiotic resistance gene substantially improves the manufacturing process. *J. Biotechnol.* 2010; 146(3), 130–137.

14. Marie C, Vandermeulen G, Quiviger M, Richard M, Préat V, Scherman D. pFARs, Plasmids free of antibiotic resistance markers, display high-level transgene expression in muscle, skin and tumour cells. *J. Gene Med.* 2010; 12(4), 323–332.

15. Wood WN, Smith KD, Ream JA, Lewis LK. Enhancing yields of low and single copy number plasmid DNAs from *Escherichia coli* cells. *J. Microbiol. Methods* 2017;133, 46–51.

16. Rouches MV, Xu Y, Cortes LBG, Lambert G. A plasmid system with tunable copy number. *Nat. Commun*. 2022; 13, 1.

17. Shitik EM, Shalik IK, Yudkin DV. AAV-based vector improvements unrelated to capsid protein modification. *Front. Med.* 2023; 10.

18. Choi VW, McCarty DM, Samulski RJ. AAV Hybrid serotypes: improved vectors for gene delivery. *Curr. Gene Ther*. 2005; 5(3), 299–310.

19. Jain NK, Ogden PJ, Church GM. Comprehensive mutagenesis maps the effect of all single codon mutations in the AAV2 rep gene on AAV production. *eLife* 2024; 12.

20. Grimm D, Kern A, Rittner K, Kleinschmidt JA. Novel tools for production and purification of recombinant adenoassociated virus vectors. *Hum. Gene Ther*. 2008; 9(18).

21. van Lieshout LP, Rubin M, Katrina Costa-Grant K, *et al.* A novel dual-plasmid platform provides scalable transfection yielding improved productivity and packaging across multiple AAV serotypes and genomes. *Mol. Ther. Methods Clin. Dev.* 2023; 29, 426–436.

22. Ohba K, Sehara Y, Enoki T, Mineno J, Ozawa K, Mizukami H. Adeno-associated virus vector system controlling capsid expression improves viral quantity and quality. *iScience* 2023; 26(4), 106487.

23. AAVpro® Tet-One™ Inducible Expression System (AAV2).

24. Das AT, Tenenbaum L, Berkhout B. Tet-On systems for doxycycline-inducible gene expression. *Curr. Gene Ther*. 2016; 16(3), 156–167.

25. Ibreljic N, Draper BE, Lawton CW. Recombinant AAV genome size effect on viral vector production, purification, and thermostability. *Mol. Ther. Methods Clin. Dev*. 2024; 32(1), 101188.

26. Barreira M, Kerridge C, Jorda S, *et al.* Enzymatically amplified linear dbDNA™ as a rapid and scalable solution to industrial lentiviral vector manufacturing. *Gene Ther.* 2022; 30, 122–131.

27. What is doggybone DNA?—DNA vector and enzymatic manufacturing. *Touchlight*. https://www.touchlight.com/platform-technology/overview/ (accessed Feb 2024).

28. Bodicoat R. Touchlight signs patent license agreement with Pfizer for dbDNA. *Touchlight.* https://www.touchlight.com/news/pfizer-license/ (accessed Mar 2024).

29. Malm M, Rasool Saghaleyni R, Magnus Lundqvist M. Evolution from adherent to suspension: systems biology of HEK293 cell line development. *Sci. Rep.* 2020; 10, 18996.

30. Leinonen HM, Lepola S, Eevi M Lipponen EM, *et al.* Benchmarking of scale-X bioreactor system in lentiviral and adenoviral vector production. *Hum. Gene Ther*. 2020; 31(5–6), 376–384.

31. Kimura T, Ferran B, Tsukahara Y, *et al.* Production of adeno-associated virus vectors for *in vitro* and *in vivo* applications. *Sci. Rep*. 2019; 9, 1.

32. Lyle A, Stamatis C, Linke T, *et al.* Process economics evaluation and optimization of adeno-associated virus downstream processing. *Biotechnol. Bioeng.* 2023; 1–14.

33. Reducing Cost of Gene Therapy Commercialization. *Pall Corporation*. https://www.pall.com/en/biotech/posters-presentations/cost-modelling-comparison-static-suspension-fixed-bed-bioreactors-manufacture-commercial-gene-therapy-products.html (accessed Apr 2023).

34. Lennaertz A, Knowles S, Drugmand JC, Castillo J. Viral vector production in the integrity® iCELLis® single-use fixed-bed bioreactor, from bench-scale to industrial scale. *BMC Proc*. 2013; 7(Suppl. 6), 59.

35. iCELLis™ Single-Use Fixed-Bed Bioreactor Systems. *Cytiva*. https://www.cytivalifesciences.com/en/us/shop/cell-culture-and-fermentation/adherent-cell-single-use-bioreactors/icellis-single-use-fixed-bed-bioreactor-systems-p-36959 (accessed Feb 2024).

36. Cameau E, Pedregal A, Glover C. Cost modelling comparison of adherent multi-trays with suspension and fixed-bed bioreactors for the manufacturing of gene therapy products. *Cell Gene Ther. Insights* 2019; 5(11), 1663–1674.

37. Ladd B, Chotteau V, Gräslund T. An efficient microcarrier based adeno-associated virus production method. Poster presented at : *Advancing Manufacture of Cell and Gene Therapies VII*. Coronado, CA, USA. Feb 6–10, 2022.

38. Yang J, Guertin P, Jia G, Lv Z, Yang H, Ju D. Large-scale microcarrier culture of HEK293T cells and Vero cells in single-use bioreactors. *AMB Express* 2019; 9(1), 70.

39. Cytiva. AAV Production Using Microcarriers. *Cell and Gene* Jun 2, 2021. https://www.cellandgene.com/doc/aav-production-using-microcarriers-0001 (accessed Feb 2024).

40. Gallo-Ramírez LE, Nikolay A, Genzel Y, Reichl U. Bioreactor concepts for cell culture-based viral vaccine production. *Expert Rev. Vaccines* 2015; 14(9), 1181–1195.

41. NevoLine™ Platform. *Univercells Technologies*; https://www.univercellstech.com/nevoline-biomanufacturing-platform/ (accessed Apr 2023).

42. Oxford Biomedica. Achieving rAAV bioreactor titer Of >1E15 vg/L with novel plasmid transient transfection platform. *Bioprocess Online* Oct 12, 2022. https://www.bioprocessonline.com/doc/achieving-raav-bioreactor-titer-of-e-vg-l-with-novel-plasmid-transient-transfection-platform-0001 (accessed Apr 2023).

43. Zhao H, Lee KJ, Daris M, *et al*. Creation of a high-yield AAV vector production platform in suspension cells using a design-of-experiment approach. *Mol. Ther. Methods Clin. Dev.* 2020; 18, 312–320.

44. Hebben M, Nyamay'antu A. FectoVIR-AAV Transfection Reagent. *BioProcess International* Oct 29, 2021. https://bioprocessintl.com/manufacturing/cell-therapies/optimizing-the-aav-transfection-process-in-suspension-cells-with-fectovir-aav-transfection-reagent/ (accessed Apr 2023).

45. Coplan L, Zhang Z, Ragone N, *et al*. High-yield recombinant adeno-associated viral vector production by multivariate optimization of bioprocess and transfection conditions. *Biotechnol Prog.* 2024; Epub ahead of print.

46. Ladd B, Bowes K, Lundgren M, Gräslund T, Chotteau V. Proof-of-concept of continuous transfection for adeno-associated virus production in microcarrier-based culture. *Processes* 2022; 10(3), 515.

47. Shupe J, Zhang A, Odenwelder DC, Dobrowsky T. Gene therapy: challenges in cell culture scale-up. *Curr. Opin. Biotechnol.* 2022; 75, 102721.

48. Nitschke P. ELEVECTA®—The Next Level in Gene Therapy AAV Production. *BioPharm* Aug 10, 2020. https://www.biopharminternational.com/view/elevecta-next-level-gene-therapy-aav-production (accessed Mar 2024).

49. Harris E. Ultragenyx Explains Advancements In Manufacturing Scalability For Gene Therapy. *Cell & Gene* May 19, 2021. https://www.cellandgene.com/doc/ultragenyx-explains-advancements-in-manufacturing-scalability-for-gene-therapy-0001 (accessed Mar 2024).

50. Cevec launches ELEVECTA platform. *European Biotechnology* Apr 29, 2020. https://european-biotechnology.com/up-to-date/latest-news/news/cevec-launches-elevecta-platform.html (accessed Mar 2024).

51. LEK Consulting. Viral-Based Gene Therapy: Key Challenges and Bioprocessing Innovations, Dec 2022.

52. Jalšić L, Lytvyn V, Elahi SM, *et al*. Inducible HEK293 AAV packaging cell lines expressing Rep proteins. Mol. *Ther. Methods Clin. Dev.* 2023; 30, 259–275.

53. AAV production with HEK293 cell line and vector technologies. Lonza May 16, 2023. https://www.lonza.com/knowledge-center/cell-gene/brief/AAV-production-HEK293-cell-line (accessed Mar 2024).

54. Escandell JM, Pais DA, Carvalho SB, Vincent K, Gomes-Alves P, Alves PM. Leveraging rAAV bioprocess understanding and next generation bioanalytics development. *Curr. Opin. Biotechnol.* 2022; 74, 271–277.

55. Coronel J, Patil A, Wissing S. Efficient production of rAAV in a perfusion bioreactor using an ELEVECTA® stable producer cell line. *Genetic Engineering & Biotechnology News* 2021; 41(4), 1.

56. Dobrowsky T, Shupe JW. Seed culture process for AAV production. World Intellectual Property Organization Jul 30, 2020. https://patents.google.com/patent/WO2020154607A1/en (accessed Apr 2023).

57. Meet TESSA: a new standard in viral vector manufacturing *Nature Portfolio*. https://www.nature.com/articles/d42473–022–00030–3 (accessed Feb 2024).

58. Su W, Patrício MI, Duffy MR, Krakowiak JM, Seymour LW, Cawood R. Self-attenuating adenovirus enables production of recombinant adeno-associated virus for high manufacturing yield without contamination. *Nat. Commun.* 2022; 13(1), 1182.

59. Collins LT, Ponnazhagan S, Curiel DT. Synthetic biology design as a paradigm shift toward manufacturing affordable adeno-associated virus gene therapies. *ACS Synth Biol.* 2023; 12(1), 17–26.

60. The Future of AAV Gene Therapy Is Scalable. BioProcess International Aug 24, 2022. https://www.bioprocessintl.com/sponsored-content/the-future-of-aav-gene-therapy-is-scalable (accessed Mar 2024).

61. Adams B, Bak H, Tustian AD. Moving from the bench towards a large scale, industrial platform process for adeno-associated viral vector purification. *Biotechnol Bioeng.* 2020; 117(10), 3199–3211.

62. Qu G, Bahr-Davidson J, Prado J, *et al*. Separation of adeno-associated virus type 2 empty particles from genome containing vectors by anion-exchange column chromatography. *J. Virol. Methods* 2007; 140(1–2), 183–192.

63. Hebben M. Downstream bioprocessing of AAV vectors: Industrial challenges & regulatory requirements. *Cell Gene Ther. Insights* 2018; 4(2), 131–146.

64. Krishnan R, Bohonak D, Etnier E, Grobholz J, Stankiewicz N. Optimization Strategy and Process Economics of DNA Digestion in Viral Vector Production for Gene Therapy. *Sigma Aldrich* 2020.

65. Wright JF. Product-related impurities in clinical-grade recombinant AAV vectors: characterization and risk assessment. *Biomedicines* 2014; 2(1), 80–97.

66. Muralidharan N. Residual Host-Cell DNA Risk Assessment. *BioProcess International* Apr 20, 2023. https://www.bioprocessintl.com/cell-therapies/quantitative-risk-assessment-of-limits-for-residual-host-cell-dna-ensuring-patient-safety-for-in-vitro-gene-therapies-produced-using-human-derived-cell-lines (accessed Feb 2024).

67. Jokondo HZ, Voloshin A, Jonathan F. Anion-Exchange Chromatographic Clarification. *BioProcess International* Apr 9, 2024. https://bioprocessintl.com/downstream-processing/chromatography/anion-exchange-chromatographic-clarification-bringing-simplification-robustness-and-savings-to-mab-purification/ (accessed Sep 2023).

68. Almeida A, Chau D, Coolidge T, *et al.* Chromatographic capture of cells to achieve single stage clarification in recombinant protein purification. *Biotechnol. Prog.* 2022; 38(2), e3227.

69. O'Mara B, Singh NK, Menendez A, *et al.* Single-stage chromatographic clarification of Chinese Hamster Ovary cell harvest reduces cost of protein production. *Biotechnol. Prog.* 2023; 39(2), e3323.

70. Thakur G. Using Clarification Innovation to Eliminate Endonuclease from an AAV Production Process. Poster presented at: BPI East, Boston, MA, USA. Sep 21, 2023.

71. Mietzsch M, Smith JK, Yu JC, *et al.* Characterization of AAV-specific affinity ligands: consequences for vector purification and development strategies. *Mol. Ther. Methods Clin. Dev.* 2020; 19, 362–373.

72. Mendes JP, Bergman M, Solbrand A, Peixoto C, Carrondo MJT, Silva RJS. Continuous affinity purification of adeno-associated virus using periodic counter-current chromatography. *Pharmaceutics* 2022; 14(7), 1346.

73. Casey C, Gallos T, Alekseev Y, Ayturk E, Pearl S. Protein concentration with single-pass tangential flow filtration (SPTFF). *J. Memb. Sci.* 2011; 384, (1–2), 82–88.

74. Clutterbuck A, Beckett P, Lorenzi R, Sengler F, Bisschop T, Haas J. Single-Pass Tangential Flow Filtration (SPTFF) In Continuous Biomanufacturing. In: *Continuous Biomanufacturing: Innovative Technologies and Methods* (Editors: Clutterbuck A, Beckett P, Lorenzi R, Sengler F, Bisschop T, Haas J). 2017; 423–456, Wiley.

75. Young S, Welsh J, Hitchcock A, Mirmalek-Sani H. Purification of adeno-associated virus by single-pass tangential flow filtration (SPTFF). Poster presented at: American Chemical Society. Aug 22–26, 2021.

76. Yehl CJ, Jabra MG, Zydney AL. Hollow fiber countercurrent dialysis for continuous buffer exchange of high-value biotherapeutics. *Biotechnol. Prog.* 2019; 35(2), e2763.

77. Godawat R, Brower K, Jain S, Konstantinov K, Riske F, Warikoo V. Periodic counter-current chromatography—design and operational considerations for integrated and continuous purification of proteins. *Biotechnol. J.* 2012; 7(12), 1496–1508.

78. Mahajan E, George A, Wolk B. Improving affinity chromatography resin efficiency using semi-continuous chromatography. *J. Chromatogr.* A. 2012; 1227, 154–162.

79. Thakur G, Hebbi V, Rathore AS. An NIR-based PAT approach for real-time control of loading in Protein A chromatography in continuous manufacturing of monoclonal antibodies. *Biotechnol. Bioeng.* 2020; 117(3), 673–686.

80. Haley J, Jones JB, Petraki S, *et al.* IsoTag™AAV: an innovative, scalable & non-chromatographic method for streamlined AAV manufacturing. *Cell Gene Ther. Insights.* 2022; 8(10), 1287–1300.

81. Dutta AK, Fedorenko D, Tan J, *et al.* Continuous countercurrent tangential chromatography for mixed mode post-capture operations in monoclonal antibody purification. *J. Chromatogr. A.* 2017; 1511, 37–44.

82. Dutta AK, Tran T, Napadensky B, *et al.* Purification of monoclonal antibodies from clarified cell culture fluid using Protein A capture continuous countercurrent tangential chromatography. *J Biotechnol.* 2015; 213, 54–64.

83. Florea M, Nicolaou F, Pacouret S, *et al.* High-efficiency purification of divergent AAV serotypes using AAVX affinity chromatography. *Mol. Ther. Methods Clin. Dev.* 2022; 28, 146–159.

84. Soni H, Lako I, Placidi M, Cramer SM. Implications of AAV affinity column reuse and vector stability on product quality attributes. *Biotechnol. Bioeng.* 2023. Epub ahead of print.

85. Rieser R, Koch J, Faccioli G, *et al.* Comparison of different liquid chromatography-based purification strategies for adeno-associated virus vectors. *Pharmaceutics* 2021; 13(5),748.

86. Bažec K, Krašna M, Mihevc A, Leskovec M, Štrancar A, Tajnik Sbaizero M. Optimization of rAAV capture step purification using SO3 monolith chromatography. *Electrophoresis* 2023; 44(24), 1943–1952.

87. McNally DJ, Piras BA, Willis CM, Lockey TD, Meagher MM. Development and optimization of a hydrophobic interaction chromatography-based method of AAV harvest, capture, and recovery. *Mol. Ther. Methods Clin. Dev.* 2020; 19, 275–284.

88. Fu XQ, Leong HY, Wang GA *et al.* Enrichment and separation of recombinant adeno-associated virus particles by aqueous two-phase extraction. *Biochem. Eng. J.* 2023; 200, 109115.

89. Schofield M. Empty/full separation: gene therapy's hidden challenge. *Cell Gene Ther. Insights* 2021; 6(11), 1715–1722.

90. Segura MM, Kamen AA, Garnier A. Overview of current scalable methods for purification of viral vectors. *Methods Mol. Biol.* 2011; 737, 89–116.

91. Dickerson R, Argento C, Pieracci J, Bakhshayeshi M. Separating empty and full recombinant adeno-associated virus particles using isocratic anion exchange chromatography. *Biotechnol. J.* 2021; 16(1), e2000015.

92. Joshi PRH, Bernier A, Moço PD, Schrag J, Chahal PS, Kamen A. Development of a scalable and robust AEX method for enriched rAAV preparations in genome-containing VCs of serotypes 5, 6, 8, and 9. *Mol. Ther. Methods Clin. Dev.* 2021; 21, 341–356.

93. Joshi V, Kumar V, Rathore AS. Rapid analysis of charge variants of monoclonal antibodies using non-linear salt gradient in cation-exchange high performance liquid chromatography. *J. Chromatography A* 2015; 1406, 175–185.

94. Crosson SM, Dib P, Smith JK, Zolotukhin S. Helper-free production of laboratory grade aav and purification by iodixanol density gradient centrifugation: *Mol. Ther. Meth. Clin. Dev.* 2018; 10, 1–7.

95. Wörner T P, Bennett A, Habka S, *et al.* Adeno-associated virus capsid assembly is divergent and stochastic. *Nat. Commun.* 2021; 12, 1642.

96. Giles AR, Sims JJ, Turner KB, *et al.* Deamidation of amino acids on the surface of adeno-associated virus capsids leads to charge heterogeneity and altered vector function. *Mol. Ther.* 2018; 26(12), 2848–2862.

97. Wu J, Heger C. Establishment of a platform imaged capillary isoelectric focusing (icIEF) characterization method for adeno-associated virus (AAV) capsid proteins. *Green Analytical Chemistry* 2022; 3, 100027.

98. Wang C, Mulagapati SHR, Chen Z, *et al.* Developing an anion exchange chromatography assay for determining empty and full capsid contents in AAV6.2. *Mol. Ther. Methods Clin. Dev.* 2019; 15, 257–263.

99. Chen H, Marino S, Ho CY. 97. Large scale purification of AAV with continuous flow ultracentrifugation. *Molecular Therapy* 2016; 24, S42.

100. Khanal O, Kumar V, Jin M. Adeno-associated viral capsid stability on anion exchange chromatography column and its impact on empty and full capsid separation. *Mol. Ther. Meth. Clin. Dev.* 2024; 31, 101112.

101. Crowly L, Labisch JJ, Leskovec M, *et al.* Purifying Viral Vectors: A Review of Chromatography Solutions. In: *Bioprocess and Analytics Development for Virus-based Advanced Therapeutics and Medicinal Products (ATMPs)* (Editors: Gautam S, Chiramel AI, Pach R.) 2024; 171–202, SpringerLink.

102. Khatwani SL, Pavlova A, Pirot Z. Anion-exchange HPLC assay for separation and quantification of empty and full capsids in multiple adeno-associated virus serotypes. *Mol. Ther. Methods Clin. Dev.* 2021; 21, 548–558.

103. Adriaenssens EM, Lehman SM, Vandersteegen K, *et al.* CIM(®) monolithic anion-exchange chromatography as a useful alternative to CsCl gradient purification of bacteriophage particles. *Virology* 2012; 434(2), 265–270.

104. New CIM Monolith QA HR Line–Sartorius BIA Separations. *Sartorius.* https://www.biaseparations.com/en/featured/new-cim-monolith-qa-hr-line (accessed Mar 2024).

105. Davies S, Senaratne J. Use of anion exchange chromatography in weak partitioning mode to provide high empty AAV capsid removal and product yields. Poster presented at: Meria GTx London.

106. Di W, Koczera K, Zhang P, Chen DP, Warren JC, Huang C. Improved adeno-associated virus empty and full capsid separation using weak partitioning multi-column AEX chromatography. *Biotechnol. J.* 2024; 19(1), e2300245.

107. Kelley BD, Tobler SA, Brown P, *et al.* Weak partitioning chromatography for anion exchange purification of monoclonal antibodies. *Biotechnol. Bioeng.* 2008; 101(3), 553–566.

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CELL & GENE THERAPY INSIGHTS

VECTOR PROCESSING AND MATERIALS

SPOTLIGHT

INNOVATOR INSIGHT

Trends in innovation for downstream process intensification, product yield, single use closed systems, and gentle ATMP bioprocessing: a KOL panel discussing 2024 insights in gene therapy manufacturing

Rachel Legmann, Senior Director of Technology, Gene Therapy, Repligen, and **Andrew D Tustian**, Senior Director of Viral Vector Process Development Group, Regeneron, discuss downstream AAV processes while exploring the trends in outsourcing versus in-house manufacturing and evolving supply chain solutions.

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Q What are the challenges the market faces today in viral vector downstream processing and what trends to progress are available in this field?

AT: To discuss AAV downstream processes, it is best to walk through a typical process. Following cell culture and production, the downstream process usually begins with a lysis step to release any intracellular virus from the production cells. As AAV can exist both inside and outside the cells, achieving 100% yield requires cell lysis. The specifics of the lysis step depend on the AAV serotype but typically involve detergent lysis. Subsequently, solid-liquid separation is performed. However, due to the low concentration of the virus, some processes use a concentration step such as tangential flow filtration (TFF), to reduce the load time on the subsequent affinity capture step.

Affinity capture involves using a specific antibody-based capture ligand to capture the AAV. Following this, further separation via polishing chromatography is conducted, along with the removal of host cell proteins and residual DNA. One significant challenge in AAV manufacturing is empty-full separation. AAV contains a protein capsid around the DNA, and distinguishing between empty and full capsids is crucial where the empty capsids do not contain the desired DNA as opposed to not containing anything. The aim is to enrich the full capsids while removing the empty ones, as empty capsids lack the target gene and can contribute to immunogenicity and reduced potency. Afterward, a viral retentive filtration step is employed to control adventitious viruses that may have contaminated the cell line. Finally, a concentration and diafiltration (UFDF) step is performed to produce the desired drug substance.

The primary challenge in downstream purification currently lies in achieving full-empty separation as there is only a small difference in properties between the two types of capsids. For example, if chromatography is used to separate the empty and full capsids, this may not be expected to work due to the difference being in internal charge, however, a charge rearrangement may occur, leading to surface change and a conductivity difference on elution. Efforts to address this challenge have moved away from chromatography columns and toward monoliths and membranes to achieve a sharper separation, however, this has seen lower loading.

Additionally, improvements are being made in upstream processes achieving higher vector genome titer of 1×10^{12} vg/mL where TFF and concentration prior to affinity chromatography, can be eliminated. Those solutions can lead to increased productivity in cell culture and advancements in cost, time, and yield.

Further efforts are being made in lysis and clarification with novel filters, for example, chromatographic clarification devices that can remove part of the DNA. This allows for the reduction or elimination of endonuclease addition. During cell lysis, where free DNA is released, endonucleases are added in high amounts to remove this free host cell residual DNA to aid clarification and downstream processing. This can become quite costly and removing the free residual DNA with the novel filters becomes advantageous.

"Apart from the scalability and robustness challenges, in gene and cell therapy, rapid movement into clinical stages is crucial."

There are further opportunities to improve the affinity capture being explored, such as the use of new devices to increase flow rates. Although these may have reduced capacity it should be noted that capacity is not of great importance in the AAV process currently, but breakthroughs are happening that could potentially change the platform.

RL: I have a lot of experience with enveloped virus, a larger molecule that is far more fragile and sheer sensitive than the AAV. This has created another challenge, not only on the upstream but also on the downstream. The correct platform must be used to handle this with low-shear stress and a very low hold-up volume to increase the recovery yield of the viral vector, exosomes, and other fragile enveloped viruses.

Currently, there is a loss of almost 70% of the potent lentivirus during downstream processes. Despite the virus not requiring lysis since it buds out of the cell, implementing a perfusion system on the upstream side allows for better virus collection and removal from the cell environment. This results in a higher quality product and tighter yields downstream.

Instead of using an ion exchange resin, using affinity columns that run quickly with very low residence time can improve process efficiency by preventing hold time and particles aggregations. Apart from the scalability and robustness challenges, in gene and cell therapy, rapid movement into clinical stages is crucial. If scalability is not achieved in a linear manner of recovery yield and product consistency, the variability becomes too high for manufacturing.

In the expedited development step, the process must also be improved in terms of choosing redesigned systems with low shear stress and low non recoverable hold up volume to address these challenges and employing the right expertise for scalability. Another crucial aspect is reducing aggregation by significantly decreasing the hold-up time of the viral vector. While TFF is one method for concentration diafiltration to expedite downstream processing, other methods and strategies can also be explored.

Q How can customers release vector demand while maintaining reduced costs and without compromising product safety?

AT: Product demand with viral vectors is intriguing as, on one hand, several of the diseases that they treat tend to be genetic conditions and are quite rare. Unlike drugs such as Eylea, which treats thousands of patients, the drugs I work on typically target a smaller number—around 100–1,000 patients per year. While the demand may be lower, the cost per dose of viral vectors can be quite high due to their production in small amounts. This is

especially true for systemic therapies where high doses are needed, such as injecting AAV to treat Duchenne muscular dystrophy and hemophilia, for example.

When considering systemic therapies requiring high doses, the cost per batch can escalate to hundreds of thousands of dollars in cost of goods per dose, specifically for AAV. This significantly hinders access to treatment as the high cost per dose results in expensive single-dose treatments. The cost of goods for AAV and other viral vectors is thus a major limiting factor, along with ensuring a safe and efficacious manner of supply to market.

To address these challenges, considerable efforts have been made with robust practices to ensure enough material is being produced while maintaining a good and safe quality. These efforts have been made in the upstream side of the process where titers have significantly increased from around 1×10^{10} viral genomes per mL to routinely reaching 1×10^{12} vg/ml. This 100-fold increase in bioreactor production has been aided by new types of commercial cell lines, specifically in HEK293.

Plasmid optimization has helped to increase productivity and the number of full-to-empty capsids, having a knock-on effect on the purification yield. There have also been investigations into alternative systems such as Sf9 due to its increased scalability. Overall, there have been a lot of upstream changes that have decreased costs.

On the downstream end, there is a focus on analytics to better understand the characteristics of AAV and its effects on safety, efficacy, and immunogenicity. This has enabled the design of more robust processes and the use of quality by design principles. One aspect where the cost of downstream processes has been reduced is the industry's move away from ultra-centrifugation separation to chromatography separation. This is significantly more scalable therefore making the process cheaper.

A recent paper published by the National Institute for Innovation in Manufacturing Biopharmaceuticals (NIIMBL) and Alliance Regenerative Medicine (ARM) called Project A-Gene, outlines strategies for designing robust processes for a viral vector, providing valuable insights for those in the field.

RL: In terms of bioprocessing, I completely agree with intensifying the vector. From the biology side, the construct has been improved, resulting in higher yields. However, we must continue with the intensifying efforts in bioprocessing to generate even more doses per batch. In downstream processing, the focus is on developing a gentle and efficient process to maximize the recovery and yield generating more doses per batch.

AT: As mentioned, the mode of administration is a significant factor. It is always a relief when you have a process that does not put pressure on the cost of goods. For example, we have one drug in the clinic to treat otoferlin mutations in deafness that is administered in small doses into the ear. Programs like this alleviate the pressure on the cost of goods as fewer viruses are needed, allowing to produce more doses per batch.

Looking ahead, the hope is for more efficacious AAV designs to be achieved through various targeting approaches or evolution of the viruses, which could further reduce the required dosage. This is an area that we are actively working on.

Q Considering trends in single use for end-to-end processes and how companies are launching innovations to support manufacturers in meeting these evolving requirements and ensuring product quality and safety, what are the differences in addressing regulatory requirements in gene therapy manufacturing versus traditional biologics manufacturing?

RL: The complexity and the nature of the cell and gene therapy products—focusing on quality, safety, cost, and speed to market—are the main driving forces behind the trend toward single-use end-to-end bioprocessing. Open processing poses an increased risk of product contamination from the environment or operator, which may render patient material unrecoverable. Therefore, manufacturers are increasingly closing their processes to minimize the risk.

Closing the manufacturing process not only reduces contamination risk but also allows for the possibility of executing parallel batch manufacturing processes to meet the demand. Multiple closed-system processes handling different batches can operate simultaneously within the same manufacturing area, provided control measures are in place to prevent cross-contamination.

Unlike the standardized production of monoclonal antibodies, viral vector manufacturing remains variable across the industry. Gene and cell therapy products are large, complex living medicines involving viruses and human cells, making viral inactivation or removal steps impossible. Therefore, robust risk mitigation strategies, including the use of single-use systems, are essential for fragile enveloped vectors like lentivirus, VSV, and HSV.

Comprehensive analytics are crucial for both traditional biologics and cell and gene therapies. However, due to the difficulty in fully characterizing cell and gene therapy products using current analytical methods, there is a greater need for advanced analytics in this field.

Implementation of sterile single use closed systems and fluid management throughout the entire process is necessary to reduce the risk of batch failure, increase overall process recovery yield, obtaining real-time process data for robustness and consistency. For that, innovative online analytical techniques must be adopted and integrated into the single-use flow path during the process at all scales. For example, Repligen offers a TFF RPM system, a real-time process monitoring and control management system using an in-line flow VPX analytical tool that can be performed for plasmid DNA, mRNA, AAV, and other novel modalities.

Closed systems are also conducive to automation, reducing timelines, cost, and human error while ensuring consistent product quality. This can be achieved due to automation permitting higher process control and reproducibility. When selecting the vendor for single-use fluid management control systems and consumables, it is crucial to choose a solution designed to minimize shear force and non-recoverable volume, ensuring gentle handling of complex molecules throughout the process.

On the platform side, Repligen has developed a single-use KRM™ Chromatography System and RS TFF systems designed to increase process efficiency and yield. Over molding, seamless tubing connections, and compact valve designs minimize turbulence and maximize viral vector

recovery. Other examples include tangential single-use self-contained flat sheet cassettes and pre-packed OPUS® Columns for rapid setup, operational flexibility, and high recovery yields. AVIPure® affinity resins and other single-use solutions further enhance process efficiency and mitigate risk.

Overall, single-use technology enhances sterility assurance, mitigates contamination risk, ensures product purity and safety, and provides production flexibility for seamless adoption of various cell-line gene therapy approaches and product production scales. Accelerated research and developing efforts are essential to ensure that single-use solutions will be well-matched to the viral vector process needs rather than fitting the process to the selected or current equipment in manufacturing. It is also critical to engage partners with end-to-end single-use solutions to scale up for commercial manufacturing needs.

AT: I fully support the use of closed systems, especially with enveloped viruses, where activation steps or viral filtration are not feasible due to susceptibility to pH and detergent, and larger size. Having a closed system really is imperative for these viruses, and single-use technology can facilitate this effectively.

Considering shear force and the hold-up volume of the single-use systems, this is essential. Shear force can cause aggregation of viral vectors, and the hold-up volume can significantly impact batch yields, especially considering final pool volumes are often in the hundreds of milliliters while working with processes in the liter range. It is essential to consider hold-up volumes as processes are scaled.

Additionally, when considering a single-use system, ensuring the vendor has full extractable and leachables data, as recommended by the BioPhorum group, is crucial. This has become an industry standard and is essential to ensure product safety.

Regarding regulatory requirements, specifically in the biologics field, focus has moved away from the notion that the process is the product. There is now a better understanding of how quality attributes affect safety and efficacy, making it an easier journey when implementing changes to the process through analytical work. However, with AAV, regulators have released guidance on comparability, which is still a significant concern. It is crucial to be aware of this regulatory expectation when considering process changes, as demonstrating comparability may require extensive studies.

Q Considering outsourcing and addressing the unique needs of gene therapy CDMOs, what are the trends seen in outsourcing manufacturing versus in-house manufacturing for gene therapies?

AT: When I first became involved in gene therapy, one of the main drivers for the establishment of my group was the perceived capacity crunch in outsourcing CMOs or CDMOs. There was a bottleneck, and lining up a CMO could take a significant amount of time.

"The shift towards internal manufacturing may continue to be a prevalent trend in the outsourcing landscape of the industry."

However, this bottleneck has been somewhat alleviated recently. A contributing factor to these relief is the industry's recent reduced ability to secure financing, which has had a positive impact on the availability of the CDMOs. Despite this, there are many larger biotechnology companies, particularly those venturing into gene therapy, are now investing in building their own manufacturing plants and rely less on CMOs.

This appears to be a growing trend that will persist even as the timeline for accessing CMO services shortens, as it is often more efficient to work internally. While we have a good relationship with the CMOs we collaborate with, each step involves contractual negotiations, and these facilities are often shared with other clients.

Speed is crucial for gene therapies, and eliminating the need for contracting can significantly reduce timelines, allowing companies to focus on their top priorities without the need to negotiate with other customers. The shift towards internal manufacturing may continue to be a prevalent trend in the outsourcing landscape of the industry.

RL: It is worth noting that eight years ago, there were no gene therapy CDMOs in the USA, which caused a significant bottleneck. However, now there is sufficient capacity, experience, and expertise in the CDMO landscape in the cell and gene therapy field.

It should be emphasized that there are small startup developers who lack the capital expenditure and expertise to build their own manufacturing facilities. Additionally, they may not have the necessary talent to process their manufacturing and scalability operations. As a result, there is a trend among these small startups to seek out the right CDMO partners. Ensuring smooth tech transfer is crucial in the process, allowing them to expedite their entry into the clinical phase or potential acquisition by a larger company.

In the gene and cell therapy space, the trend among small startups is still to rely on CDMOs, similar to how it was with monoclonal antibodies initially before larger companies started building their own facilities.

Q In regard to supply chain, gene therapy manufacturers have unique and specific raw material requirements. How are suppliers evolving to meet the needs of this customer segment compared to advanced biologic manufacturers?

RL: Managing supply chain complexity is not a new challenge for the pharmaceutical industry. Every manufacturing process, including cell and gene therapy, requires starting materials, raw materials, and consumables to produce the required product samples.

However, cell and gene therapy add an additional complexity due to the immaturity of the process.

As mentioned, cell and gene therapy necessitate transient transfection, requiring critical raw materials such as plasmids and transfection reagents. These unique needs for biological raw material pose challenges, particularly in terms of shipping under strict temperature control and tight deadlines.

The cell and gene therapy industry faces specific supply chain challenges beyond those used in traditional pharmaceutical production. One key challenge is ensuring the quality and reliability of raw materials, including plasmids and viruses used in the process, lentivirus for CAR-T or HSV. Additionally, expensive media supplements and enzymes, like Benzonase®, must be sourced reliably to enable scalability and commercialization.

Variability in the quality and composition of the raw material can lead to inconsistent product quality, which is especially concerning given the high cost of gene and cell therapies. Thus, robust raw material sourcing and selection processes are essential, including developing dual sourcing and validating critical components early on. It is very important to consider the scalability of raw material suppliers as demands for the therapy increase when moving to Phase 3 and commercialization.

Establishing early-stage inventory management systems in the facility is crucial for business continuity, as missing a single item can stop the manufacturing process, causing delays in going to market. There are some key aspects that are needed to secure the material supply chain such as implementing facility supply chain management and ensuring sufficient forecasting for the raw material needed. The primary aspect is ensuring the supplier can consistently meet the raw material requirements through clinical trials and into commercialization, robust supply agreements, and transparent supply communication.

Transportation logistics are critical in cell and gene therapy, as environmental factors during transit can damage sensitive GMP-grade raw materials irreversibly. Further, navigating international regulations and policies remains a challenge, however, improvements are underway as regulatory frameworks evolve.

Talent acquisition is another critical aspect of supply chain management, as skilled professionals are needed to navigate the complexities of establishing and managing supply chain operations effectively.

By adopting an end-to-end approach that includes demand forecasting, stable formulation, and flexible, cost-effective raw material supplies, companies can optimize their supply chain and manufacturing capacity in the cell and gene therapy industry.

AT: It is worth noting that changing the process and ensuring comparability is particularly challenging with gene therapies due to the difficulty in establishing the necessary attributes for characterization. This complexity makes it much more challenging to make process changes after development.

This reality adds significant stress to the importance of getting the material selection right first time from the start. It is crucial to consider factors such as whether a material available in research grade can also be obtained in GMP grade, or if the cost will become prohibitive

when scaling up production. This consideration significantly heightens the importance of raw material selection when designing processes for gene therapy.

SUMMARY

In short, the interview highlights the intricate landscape of viral vector downstream processing and the evolving strategies to meet customer demands. From addressing challenges in achieving an industrial and scalable full-empty separation to adopting better fit innovations to complex new modalities such as single-use redesigned closed systems for enhanced sterility and recovery functional product yield, the industry remains committed to advancing processes with ensure product safety, efficacy, product intensification, and cost–effectiveness. It further emphasizes the importance of continuous improvement in both upstream and downstream processes, alongside careful consideration of raw material selection and supply chain management. As the gene therapy space continues to grow, these efforts pave the way for improved access to treatments and advancements in the field.

BIOGRAPHIES

RACHEL LEGMANN has more than 25 years of experience in the field of scalable biologics and gene therapy manufacturing of therapeutic products, viral vectors and proteins for gene therapy and biologics. She completed her PhD in Food Engineering and Biotechnology at the Technion-Israel Institute of Technology, Israel. Rachel joined Repligen in 2021 as a subject matter expert leading the global gene therapy organization helping customers achieve their technical and operational objectives in their manufacturing of vector-based therapeutics and vaccines with a focus on gene therapy processes including upstream, downstream, analytics, and scalability. In addition to supporting global customers and building high level networks, Rachel is supporting various internal cross-functional activities and external collaborations. Prior to joining Repligen, Rachel held several scientific and leadership roles at the Microbiology and Molecular Genetics department at Harvard Medical School, CRO SBH Sciences, Seahorse Biosciences part of Agilent, CDMO Goodwin Biotechnology, and Pall Corp part of Danaher.

ANDREW D TUSTIAN leads the viral vector process development group at Regeneron Pharmaceuticals, focused on developing bioprocesses for adeno-associated virus (AAV) based viral vectors. Andrew has worked at Regeneron since 2009. Prior to moving to gene therapy Andrew co-led the purification development group for protein therapeutics, working on Fc-fusion, monoclonal antibody, and bispecific processes. He has worked on the process development for the FDA-approved drugs Dupixent®, Libtayo®, Inmazeb™, Kevzara®, Evkeeza™, and Praluent®, and helped develop the bispecific antibody platform at Regeneron. Andrew received his doctorate in Biochemical Engineering from University College London and his undergraduate master's degree in Biochemistry from the University of Oxford.

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CELL & GENE THERAPY INSIGHTS

VECTOR PROCESSING AND MATERIALS

SPOTLIGHT

INNOVATOR INSIGHT

AAV capsids separation is critical for advancing gene therapies

Timotej Žvanut, Andreja Gramc Livk, and Aleš Štrancar

AAVs are widely used vectors in gene therapy. During the process of AAV viral vectors production, complex process and product related impurities are introduced. Anion exchange chromatography is one of the most widely used downstream purification processes for separation of empty and full AAV capsids. However, to achieve baseline separation between empty and full, as well as resolution of different AAV capsids, new approaches are required to meet increasingly stringent safety and efficacy criteria. Although anion exchange chromatography is a powerful tool in many rAAV manufacturing processes, it sometimes reaches its limits in separation of the target entity from other product-related impurities. Therefore, chromatographic methods that enable better separation between different AAV capsids are sought after. In this paper, an improved anion exchange analytical method is presented. It delivers enhanced separation of full AAV capsids from other common product-related impurities such as aggregates, and empty, partially filled, high density, or damaged capsids. The improved anion exchange analytical method has been shown to be applicable for different AAV serotypes. Importantly, it exhibits linear elution properties. Favorably, the improved anion exchange method can be used for both analytics and manufacturing of full AAV capsids.

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AAV CAPSIDS SEPARATION

AAVs are widely used vectors in gene therapy, primarily due to its safety profile and efficient transduction to various target tissues. Production of AAV viral vectors is a complex process and requires innovative approaches to meet safety and efficacy requirements, clinical and market demands. Various chromatographic approaches have already been implemented for separation of AAV capsids but each one with clear

limitations such as non-scalability, poor selectivity, and poor separation of different sample entities [1].

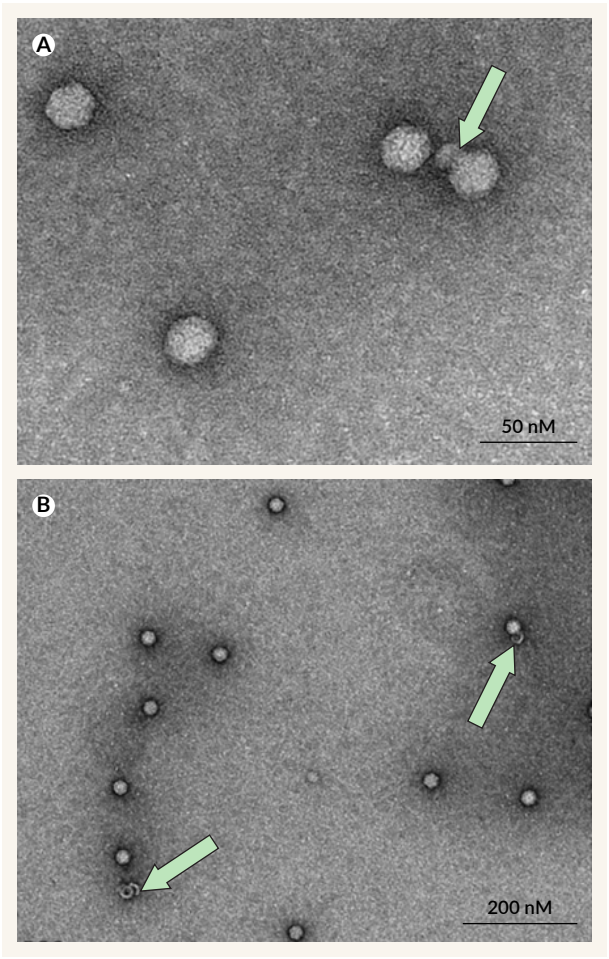
Removal of complex process and product related impurities from preparations of AAVs and to maximize the ratio between full and empty capsids is a particular goal of purification to address safety and regulatory recommendations for AAV-based gene therapy [2–4]. However, AAV capsids containing host cells and/or helper DNA and product related impurities may represent an immunological risk to patients [5]. Therefore, the development of robust, reproducible, scalable, and selective chromatographic methods for monitoring and optimization of AAV purification processes is required.

To separate empty and full AAV capsids, anion-exchange (AEX) chromatography is commonly used. However, there is just a small charge difference between empty (E), full (F), and partially filled (P) capsids. Full capsids have almost the same size as the empty capsids but have a slightly lower isoelectric point (pI) than empty rAAV capsids (a difference in the range of 0.4 pH units) [1,6]. Therefore, removing the unwanted contaminants and impurities by conventional methods often leads to a loss of yield of the target entity.

The aim of this paper is firstly to compare a conventional AEX method, which is also commonly used AEX elution mechanism with an improved AEX method for enhanced AAV8 capsids separation. Furthermore, the aim is to test the robustness of the improved AEX analytical method and to expand its application also to other AAV serotypes.

FIGURE 1

Model AAV8 sample analyzed by TEM.



Two different zoomed images, 50 nm and 200 nm, are presented (Figure 1A and 1B respectively). Green arrows indicate possible aggregates or surface impurities. TEM: transmission electron microscopy.

CHARACTERIZATION OF MODEL AAV SAMPLES BY ORTHOGONAL ANALYSES

It is important to characterize samples before any method optimization. In this study, the model AAV8 sample, which represents a pre-purified AAV8 fraction, obtained from the cation exchange (CEX) capture step, was characterized first by orthogonal analysis such as mass photometry (MP), density gradient ultracentrifugation and by transmission electron microscopy (TEM) as shown in Table 1 and Figure 1. The common characteristic for all three techniques is their scalability - none of the used methods can be applied to larger scale. Techniques such as AUC, TEM and MP require sample purification and concentration to accurately determine the empty/full rAAV ratio. Moreover, MP is relatively new and innovative experimental technique that needs better understanding and validation especially when it comes to different AAV capsids e.g., partially filled AAV.

The analyses of MP and density gradient ultracentrifugation show comparable results, where only empty (65% vs 70%) and full (35% vs 30%) AAV8 capsids were observed (Table 1). The model AAV8 sample was further characterized by transmission electron microscopy (TEM), where some heterogeneity is observed. In particular, TEM results (Figures 1A and 1B) implied possible aggregates and additional impurities with some viral particles expressing some amount of material on the surface, mainly on empty capsids. This could indicate the beginning of particle disintegration or impurity on the viral surface.

AEX METHOD FOR IMPROVED SEPARATION OF EMPTY AND FULL CAPSIDS

In initial experiments, a conventional AEX analytical method with commonly used increased linear elution of salt gradient (0–100%) was applied to separate full and empty AAV8 capsids [7].

Relatively poor empty and full resolution with considerable empty and full peak overlapping was obtained, shown in Figure 2. This initiated a deep investigation of the mechanism behind AAV8 capsids separation to develop a chromatographic method capable

TABLE 1

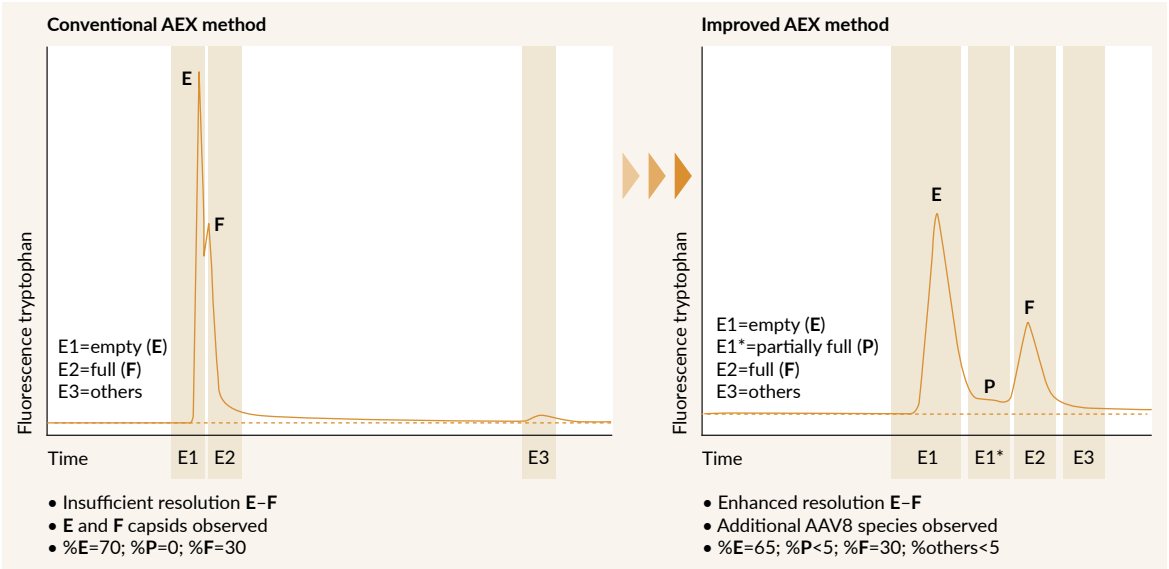
Characterization of model AAV8 sample by mass photometry and density gradient ultracentrifugation

Model AAV8 sample	MP	UC
% E	65	70
% P	0	0
% F	35	30
% others	0	0

MP: mass photometry; UC: ultracentrifugation; E: empty; P: partially filled; F: full; 'others' refers to the other AAV-related impurities, e.g., aggregates, heavy or overfilled AAV capsids, etc.

FIGURE 2

Model AAV8 sample analyzed by the conventional and the improved AEX analytical method.



AEX: anion exchange chromatography.

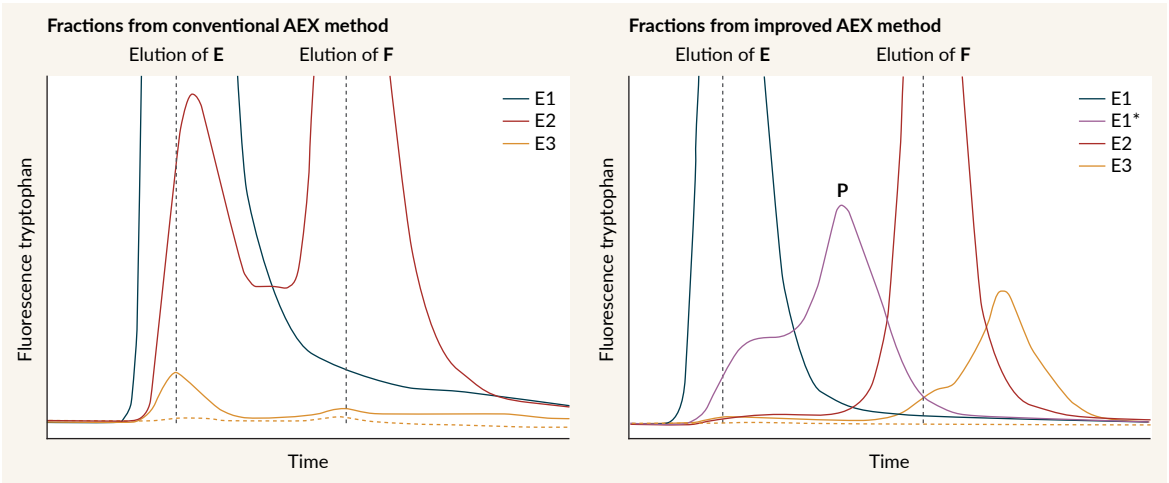
of separating not only empty and full but also other subpopulations of AAV8 capsids.

By following the elution mechanism of the conventional AEX method, new approach to achieve enhanced separation of AAV8 empty/full capsids, and multiple subpopulations was developed (Figure 2). Further information about the improved AEX method is presented in a webinar [8].

PATfix™ HPLC analytical system connected to UV-Vis, fluorescence, and light scattering detector was used for analysis of AAV species. Only fluorescence tryptophan results are shown. The separation between AAV species was achieved by the new line of CIMac quaternary amine high reproducibility (QA HR) monolithic column, used for the conventional and the improved AEX method approach. HR stands for high reproducibility between column batches and different scales. CIM monolith QA HR provides reproducible purity allowing for enrichment of any full AAV capsids and its chimeras, or surface modified capsids, regardless of the batch or

FIGURE 3

Chromatographic results of fractions collected, as depicted in Figure 2.



All the fractions were diluted 15-times with loading buffer to enable the same binding conditions. Fractions were re-analyzed by improved AEX method. AEX: anion exchange chromatography.

TABLE 2

Average percentage of AAV8 capsids present in collected fractions, analyzed by MP and improved AEX method, as depicted in Figure 2.

Conventional AEX method				Improved AEX method				
MP	E1	E2	E3	MP	E1	E1*	E2	E3
% E	95	30	N/A	% E	100	90	10	30
% P	0	0	N/A	% P	0	0	0	0
% F	5	70	N/A	% F	0	10	90	70
% others	0	0	N/A	% others	0	0	0	0
PATfix	E1	E2	E3	PATfix	E1	E1*	E2	E3
% E	95	20	N/A	% E	>95	25	0	0
% P	0	0	N/A	% P	0	70	0	0
% F	0	70	N/A	% F	0	0	95	15
% others	5	10	N/A	% others	<5	5	5	85

AEX: anion exchange chromatography; MP: mass photometry; E: empty; P: partially filled; F: full; others: e.g. overfilled or aggregates; N/A: value was under limit of detection. In the conventional AEX method; E1: empty; E2: full; E3: others; 'others' refers to the other AAV-related impurities, e.g., aggregates, heavy or overfilled AAV capsids, etc.

size of the column used. The new CIM QA HR line features the same QA ligand as the standard CIM QA columns but implements an additional specialized testing procedure with strict release criteria (detail information about the new CIM QA HR line is available on the Sartorius website.

A patent application for the improved methodology has been filed and is pending. Fluorescence tryptophan (FLD) profile was monitored. Note: axes for both methods are not unified.

Elution profiles of model AAV8 sample obtained by conventional and improved AEX methods, and their fraction collection are shown in Figure 2. Fractions E1–E3 were collected and further analyzed by orthogonal methods, mass photometry (MP) and AEX.

Although percentage of empty and full AAV8 capsids for the model AAV8 sample was comparable to both methods (Figure 2), the multiple AAV8 subpopulations were only observed with the improved AEX method.

The improved AEX method was used to assess purity of collected fractions and to check the presence of other capsid subpopulations that cannot be observed by the conventional method. Note: axes for both methods are not unified.

Compared to the conventional AEX analytical method, the improved AEX method delivers much purer full AAV capsids, see comparison of E2 fractions shown in Figure 3 and Table 2.

Partially filled AAV8 capsids (P) are expected to elute between empty and full AAV8 which was evidently observed with the improved AEX method shown in Figure 3.

Whereas aggregates, heavy or overfilled capsids (all noted as others) are expected to elute after full AAV8 elution (E3 fraction in Figure 3).

The heterogeneity of the model sample, which was indicated by TEM (Figure 1), was observed by the PATfix improved AEX method, where different AAV8 capsids can be detected.

According to Table 2 marked deviation between MP and PATfix orthogonal methods occurred in E1* fraction where 90% of empty capsids is determined by MP and only 25% empty by PATfix. Such deviation might be due to the low titer of this fraction. Limit of detection (LOD) for MP is considered to be at 500 counts which is approximate 5×10^{10} vp/mL. For the improved PATfix AEX method, the LOD is approximate 1×10^{8} vp/mL. Similar deviation was observed in E3 fraction, where most capsids were observed by MP as full (70%), whereas by the PATfix method, the majority of capsids (85%) were noted as others.

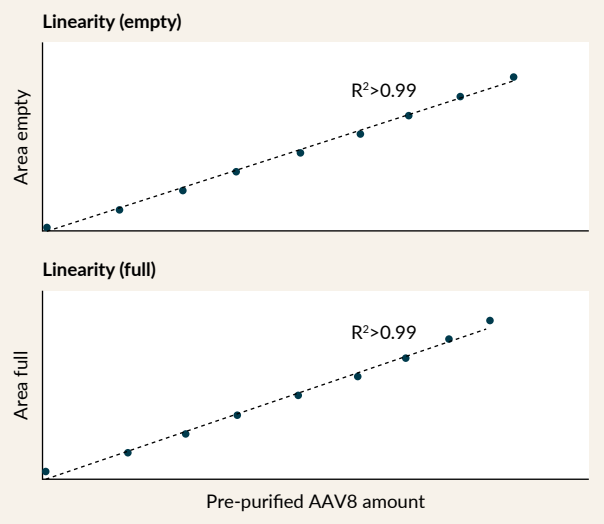
Linearity of the improved AEX method was confirmed for different amounts of model AAV8 sample (Figure 4).

SUCCESFUL SEPARATION OF DIFFERENT AAV SEROTYPES

The strategy for separating AAV8 capsids was implemented to other AAV8 samples and other serotypes (AAV5). Each serotype, as well as each sample of the same serotype, may behave differently in AEX chromatography. This may be due to sample heterogeneity

FIGURE 4

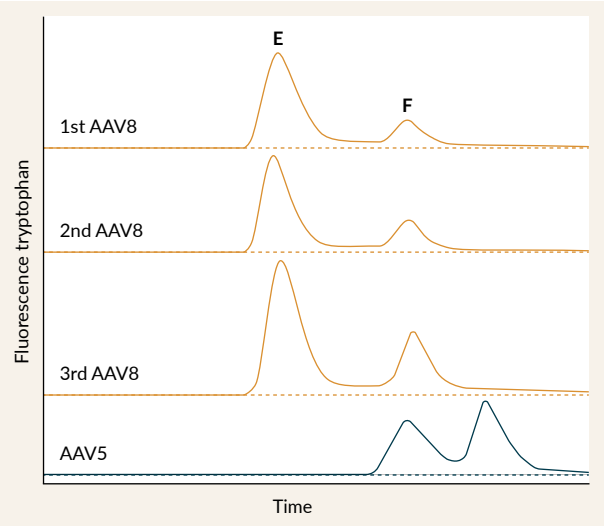
Linearity of the improved AEX method for 8 different loading amounts of model AAV8 sample, presented with linear trendline and R^2.



AEX: anion exchange chromatography.

FIGURE 5

Stacked chromatograms representing fluorescence signal versus time, trace for three different in-house AAV8 samples by improved PATfix AEX analytical method.



In blue, AAV5 in-house sample was analyzed by the same method. AEX: anion exchange chromatography.

(impurities) and overall differences in pI or amino acid composition, resulting in varying capsid surface charge profile and overall structural characteristics of different AAV serotypes [9]. To expand the improved AEX analytical method for a broader application and as a proof-of-concept this study, in-house produced AAV8 and AAV5 samples were used.

From the results obtained, the improved AEX analytical method was applicable also to other serotypes, specifically AAV5. In this case, only separation of empty and full of AAV5 capsids was observed as shown in Figure 5. Though it is necessary to optimize the conditions for each serotype separately to achieve enhanced AAV peaks separation, while using the same core principles and conditions of the original method.

A set of three different AAV8 samples, were evaluated as shown in Figure 5. Each sample showed two distinct peaks for empty and full capsids. The retention times of both empty and full peaks for all samples were consistent. This indicates that the method performance is robust and independent of different AAV8 samples.

SUPERIOR SEPARATION OF AAV CAPSIDS BY THE IMPROVED AEX METHOD

Chromatography has been proven to be a powerful tool in separation and of target entities in production process, also due to its scalability.

Most of the earlier conventional approaches had resulted in only partial separation of empty and full capsids, whereas the new method allows to achieve baseline separation between these two entities, detecting also some additional AAV8 capsids (e.g., partially filled, damaged, higher density AAV8 capsids and aggregates) indicating the heterogeneity of AAV samples.

The improved PATfix approach described in this paper utilizes only linear gradient elution, which is preferable gradient overstep gradient in liquid chromatography analytics. The use of isocratic holds or steps was omitted due to possible lower reproducibility or robustness of the method.

Compared to the conventional AEX analytical method, almost 4-times higher resolution was achieved by the improved AEX analytical method, consequently resulting also in lower peak overlapping and more accurate results of capsids. Compared to the conventional analytical method (Figure 2), the improved analytical method delivers higher purity of full AAV (Figure 2, Figure 3, and Table 2), which was confirmed with also the collected fractions and orthogonal analytics. In general, MP and AEX methods show comparable results. However, only with AEX analysis, is the separation of additional product related impurities (especially partially filled AAV8 capsids) achieved. Therefore, additional orthogonal methods are required to gather more information about these heterogenous impurities.

Performance of the improved method was found to be robust and largely unaffected by sample type changes (Figure 5). Additionally, the improved AEX method shows linearity (Figure 4).

The improved PATfix analytical method described above has shown to be suitable for its intended purpose. It can serve as a valuable and powerful tool to achieve in-depth

characterization of AAV capsids, and most importantly achieves better purity and safety of AAV final products.

The improved methodology outlined in this paper is patent pending and a forthcoming detailed scientific publication will be published and elaborated on the preparative scale-up process. This will be accompanied by various orthogonal methods, stress studies and controls to accurately distinguish and analyze all individual peaks separated by the improved AEX method.

SUMMARY

Compared to the conventional method, the improved AEX analytical method delivers enhanced separation of not only empty and full AAV capsids, but also other impurities, such as partially filled and heavy capsids, and aggregates.

Higher purity of full AAV8 capsids was obtained using the improved AEX method compared to the conventional method. The improved method offers a promising alternative for the development and production of safer AAV-based gene therapies.

The improved AEX analytical method was shown to be applicable for different AAV serotypes such as AAV8 and AAV5.

The improved chromatographic approach is scalable, an important advantage of liquid chromatography, which is of great benefit, especially when it comes to AAV purification.

REFERENCES

1. Wang C, Mulagapati SHR, Chen Z, *et al.* Developing an anion exchange chromatography assay for determining empty and full capsid contents in AAV6.2. *Mol. Ther. Methods Clin. Dev.* 2019; 15, 257–263.

2. Wright JF. AAV empty capsids: for better or for worse? *Mol Ther.* 2014; 22(1), 1–2.

3. Ling C, Wang Y, Lu Y, *et al.* The adeno-associated virus genome packaging puzzle. *J Mol. Genet. Med.* 2015; 9(3), 175.

4. Gao K, Li M, Zhong L, *et al.* Empty virions in AAV8 vector preparations reduce transduction efficiency and may cause total viral particle dose-limiting side-effects. *Mol. Ther. Methods Clin. Dev.* 2014; 1(9), 20139.

5. Gimpel AL, Katsikis G, Sha S, *et al.* Analytical methods for process and product characterization of recombinant adeno-associated virus-based gene therapies. *Mol. Ther. Methods Clin. Dev.* 2021; 20, 740–754.

6. Gagnon P, Goričar B, Prebil SD, Jug H, Leskovec M, Štrancar A. Separation of empty and full adeno-associated virus capsids from a weak anion exchanger by elution with an ascending ph gradient at low ionic strength. *BioProcess J.* 2021; 20.

7. Gagnon P, Goricar B, Mencin N, *et al.* Multiple-monitor HPLC assays for rapid process development, in-process monitoring, and validation of AAV production and purification. *Pharmaceutics* 2021; 13(1), 113.

8. Aleš Štrancar. Webinar: Novel Methods to Separate Empty, Partial, Full and Heavy AAV. *Cell & Gene Therapy Insights* Nov, 2023. https://www.biaseparations.com/en/library/seminars-webinars/1234/new-methods-to-separate-empty-partial-full-and-heavy-aav-capsids.

9. Khatwani SL, Pavlova A, Pirot Z. Anion-exchange HPLC assay for separation and quantification of empty and full capsids in multiple adeno-associated virus serotypes. *Mol. Ther. Methods Clin. Dev.* 2021; 21, 548–558.

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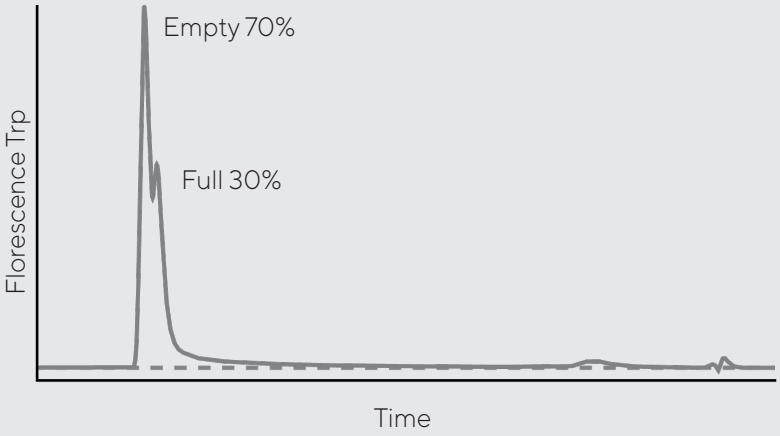
AAV Capsids Separation is Critical for Advancing Gene Therapies

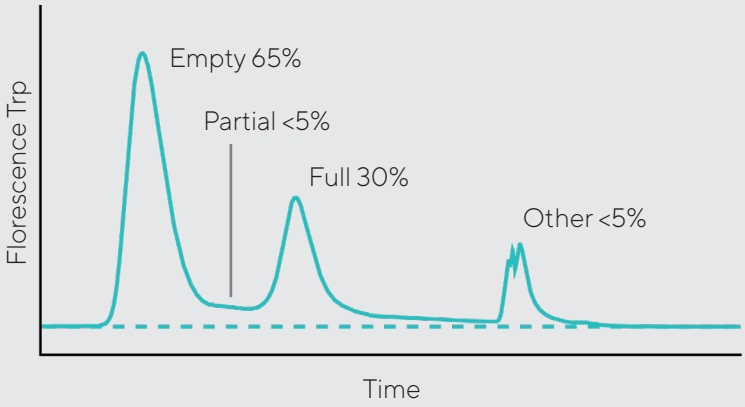
Separate AAV-related impurities and improve safety of the final product by using novel AEX method and line of CIM® QA HR monolithic columns, connected with the PATfix® analytical system.

Enhanced AAV Capsid Separation Using the Novel AEX Method

Conventional AEX Method



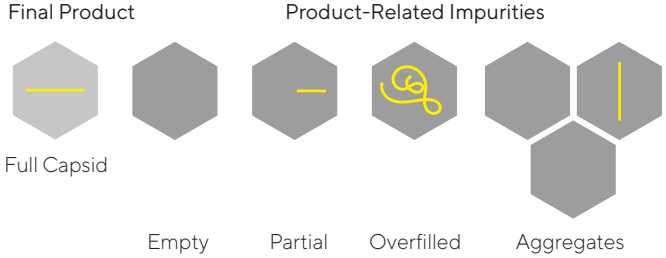
Novel AEX Method



Advantages of the Novel AEX Method:

- Higher purity of full AAV8
- Superior separation of AAV8 capsids
- 4x higher resolution between E and F
- Suitable for different AAV serotypes
- A promising tool for safer AAV-based gene therapies
- Scalability

What Are AAV-Related Impurities?



The Novel AEX method delivers enhanced separation of full (F), empty (E), partially filled (P) capsids and other AAV impurities.

CELL & GENE THERAPY INSIGHTS

VECTOR PROCESSING AND MATERIALS

SPOTLIGHT

INNOVATOR INSIGHT

Expediting development and manufacturing of advanced therapies: critical starting materials case studies

Andrew Frazer

With upwards of 30 FDA-approved cell and gene therapy products to date, plus many on the horizon, plasmid DNA (pDNA) continues to play a crucial function within advanced therapies medicinal product (ATMP) development. Plasmids are commonly used both as direct therapeutic products and as critical starting materials where they contribute directly to the quality and function of mRNA and viral vector-based drug products. This article discusses the role of pDNA as a critical starting material and highlights the evolving regulatory guidance to safeguard pDNA programs. Case study examples will demonstrate expedited development and manufacture of ATMPs and highlight manufacturing and supply scenarios seen for plasmid starting materials alongside the strategies implemented to support rapid delivery while maintaining focus on end product quality.

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PLASMIDS AS A CRITICAL STARTING MATERIAL

Plasmids are widely utilized for storage and transfer of genetic information within ATMPs and play a key role as a critical starting material for onward processing for a range of different advanced therapy applications. This includes viral vector-based cell and gene therapies, transient protein expression and

as templates for linearization and mRNA production.

Within their role as critical starting materials, plasmids can contain genetic information used for the generation of viral vector delivery vehicles but also therapeutic genes that are incorporated structurally into drug products. It is therefore critical to have high levels of quality control to deliver safe and effective treatments to patients however, from a manufacturing and quality perspective, it is important to differentiate between plasmids used directly as medicinal products and those used as critical starting materials.

Historically, regulatory guidance for plasmids has focused on use as a drug substance wherein common benchmark specifications, such as >80% supercoiled conformation and <1% residuals for host cell genomic DNA, RNA, and protein [1] have become commonplace for all types of plasmids produced within the industry. In 2018, the EMA determined plasmid as a starting material rather than raw material and added further guidance around ID testing, in particular sequencing of the therapeutic gene of interest (GOI) [2]. This was then taken further by the US FDA in 2020 where additional guidance was provided for the manufacturing and testing of plasmids used for gene therapy [3].

While the potential for the use of intermediate plasmid quality grades was introduced in 2005 [4], guidelines were left open to interpretation, with drug developers and plasmid suppliers adopting a range of quality standards related to processing, testing, and facility control making it challenging to clearly define exact specifications and quality attributes for plasmids used as critical starting materials. The 2021 EMA Q&A document on principles of GMP [5] helped to address this issue by providing some clear guidance on the expected quality standards for pDNA used for advanced therapies, advising that a product-specific, risk-based approach is utilized to identify appropriate GMP principles that must be implemented for the manufacturing and testing of plasmid critical starting materials. In addition, the US Pharmacopeia (USP) chapter <1040> aims to provide dedicated quality considerations for pDNA as a starting material and will help to further clarify and provide much needed alignment on pDNA manufacturing and quality standards [6].

PLASMID PRODUCTION AND EXPEDITED ADVANCED THERAPIES

With plasmids continuing to play a key role in many advanced therapy applications, their high demand necessitates that manufacturers and end-users review and clearly define quality requirements for specific applications, while also carefully considering key drivers like cost of goods and speed to market.

To address these challenges, Charles River launched the eXpDNA platform to deliver industry-leading timelines while maintaining a focus on process and product quality attributes. The eXpDNA platform is based on three core capabilities and over 20 years of experience in the manufacture of pDNA: an expanded plug-and-play screening toolbox for complex plasmids, a standardized platform manufacturing process to allow a streamlined supply chain and documentation, and 100% in-house analytics. Within the expDNA platform, Charles River offers three grades of pDNA (research, high quality [HQ], and GMP) to support all stages of development, clinical, and commercial supply.

In addition, a range of commonly used off-the-shelf (OTS) plasmids are now available as part of CRL's service offering to complement the eXpDNA platform and they offer developers immediate supply and the opportunity to standardize. The current portfolio of OTS plasmids include VSV-G, Gag-Pol, and Rev plasmids for third generation lentiviral vector production, an AAV Helper plasmid and new for 2024, AAV Rep/Cap plasmids for serotypes 2, 5, 6, 8, and 9. All OTS plasmids are manufactured from fully characterized master cell banks (MCBs) with purified plasmid products available in research, HQ or GMP

quality grades with associated testing and stability data.

To highlight some advantages of the expDNA platform and usage of off-the-shelf plasmids, two case study examples are presented below.

CASE STUDY 1: PHASE-APPROPRIATE SUPPLY

The first case study uses the example of an experienced product developer aiming to build out their product pipeline and progress manufacturing of multiple plasmid products while performing development work at their manufacturing site to fast-track their route to the clinic. This includes a priority focus on product quality and consistency, and the desire to establish a replicable platform approach for the rapid progression of future pipeline products.

The strategy used raises some interesting considerations for phase-appropriate supply solutions and how they can be leveraged to incorporate quality while accelerating timelines. In phase-appropriate supply, one generally considers quality standards to increase as a product moves through development and the various stages of the clinic. To ensure that the transition between quality grades is as simple as possible, the HQ plasmid offered by Charles River is closely matched to the GMP plasmid, particularly with regard to process unit operations, raw materials, and testing. This provides a solid option for fast and cost-effective supply with a smooth transition to GMP at later stages, particularly from a CMC perspective.

The supply approach is outlined in Figure 1, wherein initially, a screening evaluation and next-generation sequencing (NGS) is used to mitigate the risk of instability as the plasmids are all inverted terminal repeat (ITR)-containing AAV constructs that are inherently prone to recombination and deletion. MCB pre-banking is performed to ensure that the selected colonies from the initial transformation contain plasmids with intact ITR regions. In this example, generation of the pre-banks provides the option to manufacture HQ-grade plasmid to support the client's internal R&D requirements, and rapidly deliver multiple constructs without incurring the high costs associated with full GMP supply. At the point of candidate selection and progressing a construct to clinical supply, the pre-MCB is progressed to a full GMP master cell bank with full analytics and release testing and the resulting MCB is used to supply GMP-grade plasmid.

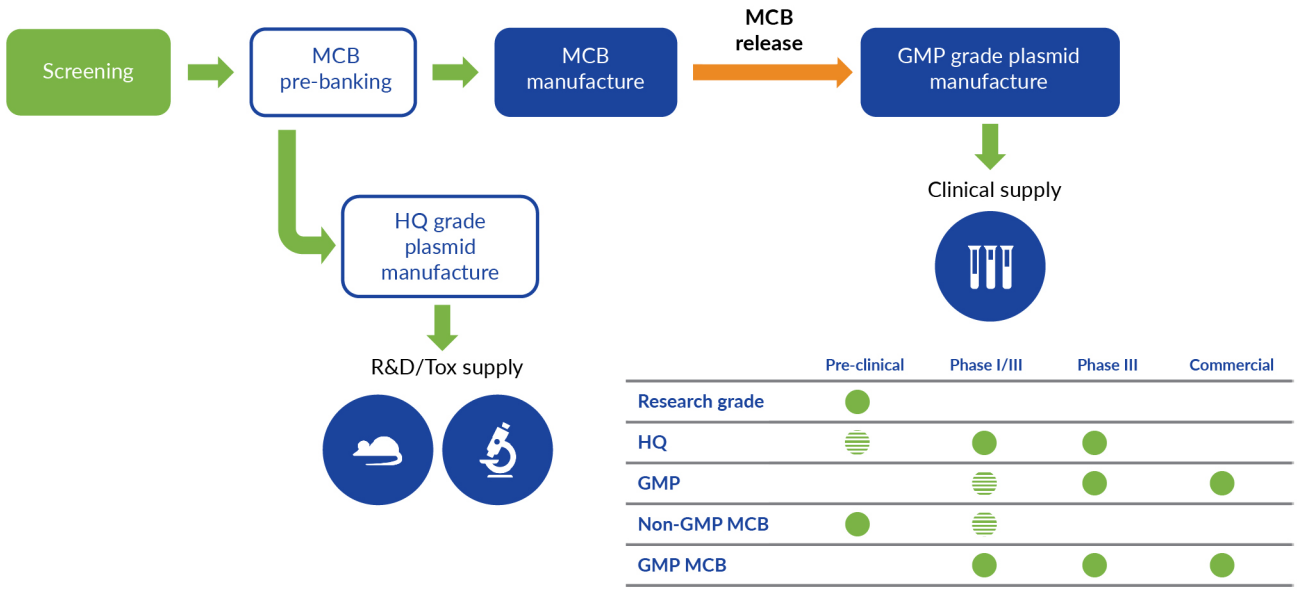
Key learnings from this case study include the importance of maintaining a focus on QC at an early stage, particularly for challenging plasmid types such as ITR-containing plasmids. This establishes a foundation for future production and helps avoid issues like the detection of low-level ITR deletions later on during manufacture that could result in significant setbacks. Parallel tracking of multiple constructs was critical to meet timelines and in this example, an estimated 75% reduction in overall project timelines could be realized versus sequential production. In order to run multiple products in parallel without incurring financial challenges, phase-appropriate quality grade supply with the HQ platform was important. Finally, long-term progress should always be part of decision-making. The immediate cost and time benefits of a phase-appropriate supply solution like HQ are soon negated if there is a need to perform extensive comparison studies at a later date when moving to GMP. For a developer considering their plasmid supply and looking at intermediate-grade plasmids, it is critical to plan in advance and understand the quality attributes of that supply and how to coordinate with longer term program aims.

CASE STUDY 2: OFF-THE-SHELF SUPPLY

This example once again focuses on pDNA supply as a critical starting material for AAV gene therapy. In this case, the focus is on a relatively new developer aiming to progress

FIGURE 1

Case study 1: phase-appropriate supply solutions.



their AAV product to clinic with an emphasis on rapid delivery to maintain timelines and a need to minimize cost. Manufacturing requirements for the project include MCBs and GMP-grade pDNA for the GOI, Helper, and Rep/Cap plasmids with the supply approach outlined in Figure 2. utilizing off-the-shelf supply for Helper and Rep/Cap plasmids and custom manufacture for the GOI plasmid.

The first step focuses once again on delivery of the GOI plasmid, which would be the most challenging to manufacture. In addition to the clone screening and QC, a host cell line evaluation was performed. This step only adds around 1 week to a manufacturing program and has demonstrated the ability to deliver stable plasmid and significant uplifts in productivity during production. At the same time, research-grade off-the-shelf Helper and Rep/Cap plasmids are supplied to the customer to allow evaluation within their chosen AAV platform while GOI MCB manufacturing progresses.

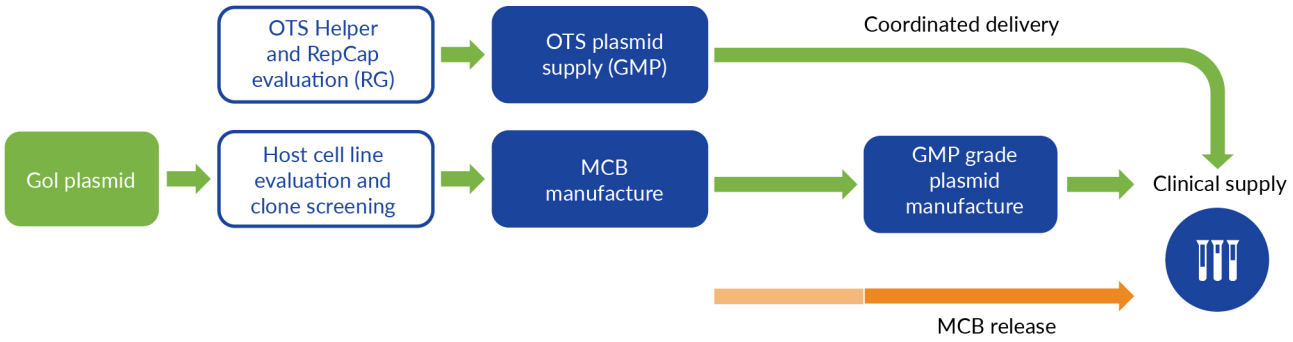
To support additional reduction in timelines, the GOI MCB is progressed directly to manufacture with prioritized testing prior to full release. This supports the expedited release of the GOI GMP plasmid in line with the full MCB release. Finally, the delivery of GMP grade off-the-shelf Rep/Cap and Helper plasmids is coordinated with the GOI supply to support AAV manufacture for clinic.

In this case study, there are clear time and cost savings associated with the use of OTS plasmids as, effectively only a single manufacture for the GOI plasmid was required versus three custom batches. Importantly, there are several additional advantages to the use of OTS products. Pre-production activities like slot allocation, supply of plasmid starting material, documentation review, and approvals were only required for a single plasmid versus three, which represents a significant reduction in workload.

Looking at immediate post-production benefits, the GOI plasmid is the only item on the critical path for this campaign and as a result, there is less risk of encountering manufacturing or testing issues. Additionally, as the Rep/Cap and Helper are manufactured in advance, the delivery of all three plasmids can be planned reliably and well in advance

FIGURE 2

Case study 2: off-the-shelf supply solutions.



of the AAV manufacturing slot with the required amounts of Rep/Cap and Helper tailored specifically to the AAV production process requirements to help avoid wastage. An added benefit of OTS products is the option of immediate resupply should the program encounter an issue at a later manufacturing or testing stage. A good approach for this type of program could be to establish a contingency stock for the GOI plasmid and order OTS Helper and Rep/Cap on an as-needed basis to help manage cost and mitigate the risk of supply issues.

This case study highlights that overall manufacturing effort should be considered in addition to live processing requirements which often receive the most attention. OTS products can provide immediate cost and time savings versus custom manufacturing but also offer extensive secondary benefits for wider advanced therapy programs.

SUMMARY

For plasmid critical starting materials, regulatory guidance is evolving and there is now dedicated guidance for managing starting materials versus drug substances. This will support a greater range of manufacturing options for developers and can ultimately help with costs and timelines for advanced therapies. To get the most out of what plasmid suppliers have to offer, it is important to understand the necessary quality attributes of the required plasmids and how they align with a specific application. The use of intermediate plasmid quality grades like HQ can provide a viable option to support reduced costs and shorten timelines with opportunities to parallel track development activities for larger programs. Additionally, the utilization of off-the-shelf plasmid products can be highly advantageous in certain circumstances and the use of platform screening and manufacturing with proven delivery can also help to embed quality at an early stage and avoid delays later in a program. Engaging early and working closely with your chosen plasmid supplier is advised, as is planning ahead to develop strategies that deliver the best options for both immediate needs and for the success of longer-term programs.

Q&A

Andrew Frazer

Q What has the biggest impact on timelines for plasmids?

AF: There are challenges across the CDMO industry regarding plasmid supply that can impact timelines, including long wait times for slots and material procurement difficulties. These barriers are related to the worldwide demand for plasmids. Charles River has put a lot of focus into developing the ability to fully segregate our production lines, using single-use materials, and holding on-site batch packed materials that are ready to go. This provides better flexibility within scheduling and helps avoid upfront delays on timelines.

In terms of process and release, the testing side of things has often had the biggest impact on timelines for plasmid supply. This is an area where we have seen significant improvements by bringing all our testing in-house within the Charles River network. We are looking to further reduce timelines by bringing more of the historically longer lead tests like GMP sequencing directly into our manufacturing sites.

Q Is it possible to provide small samples of off-the-shelf plasmids so they can be tested at a customer's lab?

AF: Yes, we have small volume research samples available for testing in external labs or production platforms. With our handling and use of the plasmids, we find that they perform favorably versus similar commercially available products.

For our customers to get the most out of them, we can provide technical support from our plasmid and vector subject matter experts to help optimize performance with offsite evaluations in our customer labs.

Q Do you have any advice for a risk-based approach to plasmid-critical starting materials?

AF: This is something that we routinely support for our customers. This is an important step in deciding which plasmid grade to use and how this will develop through the life cycle of a product. We have tried to make our platform as standardized as possible, making comparability assessments between quality grades as easy as possible.

For a risk-based approach to the use of plasmids as critical starting materials, the general rule is that the closer the plasmid is to the patient, then the higher the quality grade needs to be. There is good existing guidance in the form of the EMA Q&A document to provide support for product-specific risk-based approach and this is also expected to be covered in the upcoming USP chapter on plasmid critical starting materials.

Q What manufacturing production scale do you have and what is a typical batch size?

AF: At Charles River's Keele GMP manufacturing site, we offer plasmid manufacturing scales of up to 50 L GMP. Yields vary depending on plasmid type but we have process solutions to deliver batch sizes from milligrams through to multigram quantities. At Alderley Park, there are a range of different process options that we can implement depending on the plasmid amounts that are required, typically with a 15 L bioreactor for high-quality plasmids. Both GMP and HQ production use aligned testing, vendor-approved and fully traceable raw materials and single-use process equipment throughout.

REFERENCES

1. *FDA*. Guidance For Industry, Considerations For Plasmid DNA Vaccines For Infectious Disease Indications, 2007.
2. EMA. Guideline On The Quality, Non-Clinical And Clinical Aspects Of Gene Therapy Medicinal Products, 2018.
3. *FDA*. Chemistry, Manufacturing, and Control (CMC) Information For Human Gene Therapy Investigational New Drug Applications (INDs), 2020.
4. EMA. Guideline on Development And Manufacture Of Lentiviral Vectors, 2005.
5. EMA. Questions and Answers On The Principles of GMP For The Manufacturing Of Starting Materials Of Biological Origin Used To Transfer Genetic Material For The Manufacturing Of ATMPs, 2021.
6. United States Pharmacopeia. <1040> Quality Considerations of Plasmid DNA As A Starting Material For Cell And Gene Therapies (unpublished).

BIOGRAPHY

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COMMENTARY

Combining best practices and analytical reference materials to tackle challenges in AAV manufacturing

Mark Verdecia

The gene therapy industry is booming, as evidenced by the hundreds of AAV-based gene therapies in clinical trials and even more in development. This rapid increase creates a high demand for plasmids and other starting materials. However, manufacturing challenges and compressed development timelines, compared to traditional biologics, make scaling production difficult from early development stages. Despite these challenges, gene therapy products must still comply with existing regulations. Yet, the absence of standards and validated reference materials in this field complicates the development and implementation of methods to achieve compliance in a quality control environment. Standards-setting organizations, like the National Institute of Standards and Technology (NIST) and the United States Pharmacopeia (USP), are well-equipped to address these complex issues by developing tools to ensure the quality of AAV-based gene therapies. This article discusses the ongoing efforts by these organizations to create documentary standards and reference materials, facilitating consistent and predictable manufacturing processes for AAVs.

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INTRODUCTION

In the field of gene therapy, adeno-associated viral (AAV) vectors have emerged as the leading choice for delivering therapeutic genes into cells. They are favored for their low immunogenicity and ability to transduce both dividing and non-dividing cells. Currently, AAV-based gene therapies are being used to treat various genetic and acquired human diseases, including lipoprotein lipase deficiency, hemophilia type A and B, spinal muscular atrophy (SMA), and retinal dystrophy [1–3]. With numerous AAV-based gene therapies in clinical trials and many more in development, we can anticipate an increase in treatments based on these promising vectors [4–6].

To meet the expected future demand for AAV-based gene therapies, manufacturers will need to increase their production scale. However, there are still many challenges in purifying AAV vectors, which makes scaling up difficult [7]. For instance, lysates containing AAV are often contaminated with host-cell DNA and host-cell proteins, which can foul filtration media and limit the concentration of the drug substance [7]. These impurities also pose immunogenic risks to patients and can degrade the product if not removed to sufficiently low levels [8,9]. The current manufacturing process can also produce AAV vectors that lack the necessary genomic information [10,11].

In addition to manufacturing challenges, gene therapies face compressed development timelines. While traditional biologics can take up to a decade to develop, gene therapies have accelerated timelines of only 3 to 5 years from the start of clinical trials to regulatory approval. Therefore, considering manufacturing and scale from the early stages is critical, since attempting to fix a manufacturing process later in development can significantly impact submission timelines. Early planning should include implementing a wide range of process testing for every viral vector lot manufactured; and the analytical assays used should be rapid, accurate, and support validation, document control, and reproducibility [12].

Currently, gene therapy products must comply with applicable sections of the Code of Federal Regulations Title 21, Parts 211 and 610. However, the lack of standards hinders the development and implementation of methods to achieve compliance in a quality control environment. Manufacturers are forced to use multiple orthogonal methods to examine the same attribute, leading to a variety of analytical approaches without consensus on best practices for analysis. Addressing this lack of harmonization requires industry-wide collaborations that go beyond the scope or expertise of any single manufacturer. However, standards-setting organizations like the National Institute of Standards and Technology (NIST) and the United States Pharmacopeia (USP) are well-suited to tackle these complex issues and facilitate the development of tools for ensuring the quality of AAV-based gene therapies.

BUILDING STANDARDS THROUGH STAKEHOLDER ENGAGEMENT

Developing and implementing standards for AAV vectors presents its own set of challenges. Global manufacturers and regulators must first come to a common understanding of the most suitable standards. Then, after reaching a broad consensus, there remains the technical challenge of producing highly purified and validated materials. Overcoming these barriers requires collaboration between industry, academia, and regulatory bodies to establish and create globally accepted standards that not only ensure the safety and efficacy of AAV-based therapies but also drive innovation and excellence in the field.

To achieve these goals, NIST collaborates with the US Food and Drug Administration (FDA) to host events focused on developing global standards underpinned by a robust measurement infrastructure. For example,

in November 2023, NIST and the FDA jointly hosted a series of consecutive workshops for advanced therapy. The NIST-FDA Workshops on Measurements and Standards for Advanced Therapy brought together stakeholders from other federal agencies, industry, and academia to provide perspectives on identifying measurement challenges and innovative technology solutions, as well as standards to promote manufacturing innovation, improve supply chain resilience, support characterization and testing to facilitate regulatory approval, and explore opportunities for cross-consortia collaboration. The workshops validated the need for continuing efforts to develop standards and resulted in the launch of a new working group (WG) on gene delivery systems [13].

USP is also dedicated to working with all stakeholders and actively participates in facilitating discussions on these topics. In March 2023, USP co-hosted the AAV Analytical Characterization Workshop with the Alliance for Regenerative Medicine. This event provided a platform for manufacturers and regulators to discuss important issues in AAVs, such as evaluating process-specific impurities, measuring genome titer, assessing empty capsid titers, and developing best practices to meet regulatory expectations.

This kind of regular engagement enhances understanding of industry needs, informing the development of comprehensive standards that support analytical methods. While not all challenges can be solved through discussion alone, stakeholder dialogue helps establish consensus on best practices and identify opportunities for guidelines and analytical control standards for AAVs. This article describes some of the work that has resulted from these outreach efforts.

DEFINING MATERIALS AND BEST PRACTICES

Defining various aspects of a manufacturing process is crucial for understanding how to apply quality control and develop best practices. However, this may not always be straightforward when dealing with complex products like gene therapies. For example, determining what should be treated as a starting material can be challenging. Traditionally, starting materials are significant structural fragments incorporated into the drug substance. However, manufacturers use multiple plasmid constructs to make AAV vectors. The plasmids contain structural and helper genes, as well as a therapeutic transgene to be packaged in each particle. Each plasmid can significantly impact the final drug product's quality, but they are not part of the final AAV product. But given their impact on final product quality, recent regulatory guidance suggests treating plasmid DNA as starting materials [14].

Since plasmid DNA is considered a starting material, it should be produced using a phase appropriate GMP quality system and characterized using assays defined in a risk management strategy [14]. These assays should include tests for identity, sterility, endotoxins, and levels of host cell genomic DNA (gDNA) and protein. Regardless of whether the DNA plasmids are made by the sponsor or a contract manufacturer, all information on the manufacturing procedures and reagents used must be included in the Investigational New Drug (IND) submission. This is particularly important because many gene therapy developers outsource the production of plasmid DNA, and there is a lack of harmonization among suppliers in terms of manufacturing approaches and specifications.

To promote standardized practices for plasmids, USP has published <1040> Quality Considerations of Plasmid DNA as a Starting Material for Cell and Gene Therapies in Pharmacopeial Forum (PF) and General Chapter <1047> Gene Therapy Products in the USP-National Formulary (USP-NF). These non-compendial documentary standards aim to describe considerations for the manufacture and testing of various vectors used in gene therapy products, including AAVs. <1040> provides guidance on building

a control strategy for plasmid DNA, discussing sourcing and residual impurities testing, while General Chapter <1047> emphasizes the application of well-defined decision criteria for key in-process intermediates and the use of in-process controls to monitor production and specific activity.

Apart from starting materials, there are numerous ancillary materials that come into contact with product intermediates but are not intended to be present in the final product. Examples of ancillary materials include fetal bovine serum, enzymes (e.g., trypsin, collagenase, DNase/RNase, restriction endonucleases), growth factors, cytokines, monoclonal antibodies, antibody-coated beads, antibiotics, media, media components, and detergents. These reagents can impact the safety, potency, and purity of the final drug product by introducing adventitious agents or other impurities. Therefore, manufacturers need to consider how to validate them. For the qualification and testing of ancillary materials, USP has published General Chapter <1043> Ancillary Materials for Cell, Gene, and Tissue-Engineered Products to provide guidance on the development of appropriate material qualification programs.

ANALYTICAL REFERENCE MATERIALS (ARM)

According to guidelines from the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), it is crucial to develop and validate assay performance using high-quality, authenticated reference materials. This ensures reliable, reproducible, robust, and comparable results. A high-quality and validated analytical reference material (ARM) can be an invaluable tool for compliance with ICH regulations when combined with best practices.

Unlike reference standards, ARMs are not linked to compendial methods and can be used at various stages of the product lifecycle, including research and development, raw material testing, and system suitability. ARMs give manufacturers more flexibility, which is especially important for AAV developments where there are not many compendial methods yet.

Reference materials can serve an important role in the development and commercialization of viral vector-based gene therapies. ARMs enable comparability of different viral vector preparations for physical and infectious titer measurements. They also greatly enhance the reliability of results obtained from analytical procedures used to monitor AAV production, even across multiple locations, which is particularly important in today's manufacturing environment. This is especially relevant when production occurs at multiple sites. Finally, ARMs can help reduce barriers to entry for early-stage biotechnology companies. These companies may lack the necessary manufacturing experience to produce high-quality AAVs, and ARMs can provide them with the tools to establish their own production capabilities or support partnerships with contract manufacturers.

To develop ARMS, NIST is collaborating with the FDA and other industry partners to characterize the next generation of viral vector reference materials. NIST is currently working on multiple reference material projects, including developing physical titer methods to quantitatively measure loaded viral particle state and developing advanced measurements by imaging and flow cytometry of infectious titer to correlate with physical titer measurements [15]. USP is also developing several high-quality ARMs to assist manufacturers with AAV manufacturing. These include ARMs for host cell gDNA, genome titer, residual plasmid, and empty/full capsid ratio.

RESIDUAL GENOMIC DNA

One of the main concerns when using complex expression systems to produce AAV vectors is the potential presence of residual host cell gDNA in the final product. Contamination of the final product with host cell gDNA

can pose safety risks, such as immunogenicity and oncogenicity, and therefore it must be removed. Regulatory agencies such as the FDA, European Medicines Agency (EMA), and World Health Organization (WHO) have established criteria for the maximum allowable amount of residual host gDNA in products [16–18]. It is the responsibility of the manufacturer to demonstrate that they have effectively removed gDNA impurities using accurate and sensitive methods.

Many manufacturers use quantitative PCR (qPCR) to monitor residual host cell gDNA levels, which requires well-calibrated standard curves. Due to the high sensitivity of these tests, even small changes in kits, whether from different lots or vendors, can significantly impact the assay results. The method of quantifying the standard for value assignment can also affect the results. To ensure reliable and consistent results, it is important to validate and run the methods with high-quality controls that have been authenticated [19].

To address this need, the USP has collaborated with the American Type Culture Collection (ATCC) to provide high-quality, accurately quantitated gDNA controls for the detection of residual DNA in cell lines commonly used for manufacturing gene therapies, including HEK293. The USP-ATCC gDNA reference materials have been manufactured, evaluated, and precisely quantitated using robust processes. In addition, PCR-based protocols in USP-NF General Chapter <509> Residual DNA Testing support laboratories in accordance with current ICH guidelines.

GENOME TITER

Clinical trials have demonstrated that high doses of AAV vectors can induce antigen-specific memory $CD8^+$ T cells, antibodies, and interferon-γ production in certain patients [20,21]. Lower doses of AAV vectors may reduce these immune responses, but could potentially result in decreased efficacy [22]. Therefore, it is crucial to determine the minimal effective and maximum tolerable dose of a candidate vector before conducting clinical trials to ensure the success of gene therapy [23]. To minimize the risk of under- or over-dosing manufacturers typically focus on quantifying the genome present in the capsid, since this is effectively the active pharmaceutical ingredient (API) [24,25].

qPCR and digital PCR (dPCR) are commonly used to determine AAV genome titers [26]. While qPCR results can vary due to different protocols, reagents, and standards, dPCR offers absolute quantitation without relying on amplification efficiency, making it more precise and accurate than qPCR. However, manufacturers must still validate their dPCR methods and ensure consistency across production sites due to the potential variability of PCR-based assays across laboratories. Regardless of the analytical method used there can still be significant variability in titer measurements across laboratories, or even within the same site among different analysts, instruments, and methods. This highlights the need for standardization in quantification methods. Having a reliable reference material for genome titer measurements is critical to enable result standardization [27]. But establishing standards for AAV genome titer quantification is challenging due to variations in AAV vector constructs, host cell lines, and titer determination methods, making accurate inter-laboratory comparisons difficult [28].

One crucial consideration when developing a standard for AAV genome titer is the conformation of the DNA. DNA topology can hinder assay performance, leading to overestimation of absolute titers and increased result variability [29]. If the plasmid DNA used as a reference standard for AAV genomic titer exists in different conformations, it can affect the accessibility of PCR primers to the target sequence, resulting in variations in primer binding efficiency and target amplification [29]. This variability not only impacts the accuracy of AAV titer calculations but also prevents cross-comparison

of individual AAV samples. To address this problem, USP has developed a linearized plasmid DNA containing the inverted terminal repeat (ITR) regions found in all AAV genomes. This approach capitalizes on the fact that regardless of the AAV serotype, the viral genome encapsulated within the most commonly used recombinant AAV systems contains the same ITRs. Quantification is based on amplification of the ITRs using primers designed to avoid any secondary structural elements that could hinder primer annealing. This approach can be used to assess system suitability or assay performance regardless of the specific AAV drug substance.

In a complimentary effort, NIST is also developing techniques and protocols to evaluate the viral vector genome integrity. For instance, NIST is developing physical titer methods to quantitatively measure loaded viral particle state to provide manufacturers quality attributes on their viral vector preparation. These assays can potentially reduce the sample handling time and variability and improve the reproducibility of viral vector titration.

RESIDUAL PLASMIDS

The same plasmids used to produce AAVs can also contaminate the final product. Removing residual plasmid DNA impurities in AAV vectors is complicated because even with nuclease treatment during vector purification, fragments of DNA may remain packaged in the AAV vector, making them resistant to nuclease treatment [25]. This poses potential safety risks as residual plasmid DNA packaged into the capsid may contain bacterial sequences and antibiotic resistance genes, which could be harmful to patients. Additionally, residual plasmids may trigger an immune response in the patient, neutralizing the AAV vectors and reducing their effectiveness.

Measuring residual plasmid DNA is crucial to minimize adverse effects and ensure consistency between batches. Therefore, manufacturers must demonstrate control over residual plasmid DNA levels in their products to obtain regulatory approval for clinical trials or commercialization. PCR methods such as qPCR and dPCR are commonly used to detect and quantify residual plasmid DNA in the final product. Given the sensitivity of these assays, manufactures need to develop them carefully. For example, the selection of representative target amplicons is an important consideration. Furthermore, optimizing and validating PCR workflows against reference materials with known residual DNA profiles is important for standardizing purity tests across laboratories and AAV products.

To address this need, USP has developed an ARM for residual plasmid that contains the kanamycin resistance gene (KanR), commonly found in plasmids used for AAV production. PCR primers specific to this gene can be used to assess the level of residual plasmid in the drug substance. The USP residual plasmid reference material is useful for creating a standard curve for qPCR, validating system suitability, training personnel, and piloting transitions in manufacturing scale; making it useful for tackling some of the challenges faced in commercial production of AAV-based gene therapies [30].

FULL AND EMPTY CAPSID STANDARDS

The objective of any AAV manufacturing process is to maximize the production of full AAV capsids in the final product. This is accomplished by separating particles containing the desired construct from empty vectors that lack the therapeutic gene. Unwanted capsids can be completely empty or contain small fragments of DNA, such as AAV inverted tandem repeats (ITRs), host cell DNA, or plasmid DNA. These defective particles do not contribute to potency and increase the risk of immunogenicity by delivering antigenic DNA fragments to patients. Therefore, it is necessary to characterize and monitor empty capsids, as they have the potential to elicit an undesired immune

response without providing the intended therapeutic benefit [31].

To address this issue, NIST is collaborating with the FDA and industry partners to develop and characterize an adenovirus serotype 5 reference material. The original source for this material is a plaque isolate from a serially plaqued sample of adenovirus, type 5 (VR-5, ATCC). The amplified plaque isolate was used to manufacture a virus bank [32]. The adenovirus was cultured on microcarriers seeded with HEK 293 cells from a certified Working Cell Bank. The reference material was further developed under the guidance of the Adenovirus Reference Material Working Group (ARMWG) and the FDA [33,34]. NIST has recently performed final stability testing and is coordinating interlaboratory testing for physical and infectious titer to bridge measurements between the original material and this new reference material.

In addition to acquiring validated reference materials, there is also the challenge of determining which analytical technologies are best suited to provide the most information about AAV capsids. There are several possible approaches, including UV spectroscopy which uses the A260 to A280 ratio to determine the ratio of empty to full capsid. This method is simple but vulnerable to interference from sample components that absorb light in the UV spectrum. Another approach involves using a combination of qPCR and ELISA to quantify the viral genome and viral capsid proteins. However, this method may lack precision and requires a well-calibrated standard curve to accurately calculate the DNA component.

Analytical ultracentrifugation (AUC) is widely regarded as the gold standard method for determining the empty/full AAV capsid ratio [35]. It offers high-resolution separation, allowing for quantitation of empty, partial, and full capsids, and is independent of serotype. However, it requires a larger sample volume for routine analysis compared to other technologies. Anion exchange chromatography (AEX) is another commonly used method for determining the empty/full capsid ratio. AEX relies on differences in surface charge to distinguish between the different capsids. The empty/full ratio is obtained by integrating the peaks representing empty and full capsids and comparing their areas. AEX offers lower resolution compared to AUC but is suitable for GMP testing and requires a relatively small sample volume. However, this approach is serotype dependent, necessitating method optimization for each of the 13 AAV serotypes.

To establish best practices for these methods, USP, NIST, and the National Institute for Innovation in Manufacturing Biopharmaceuticals (NIIMBL) have partnered in an effort to develop tools that will allow better assessment of AAVs. NIST's long-standing experience with measurement sciences and USP's established role in the application of measurement to the development of methods and associated reference standards is a great combination to advance the field of testing biopharmaceuticals [36]. Also, NIST and USP both have extensive experience conducting multi-laboratory studies, and NIIMBL has a diverse membership across industry, academia, and government. Together, they are well-equipped to carry out an interlaboratory study aimed at improving the measurement of AAV quality attributes, with a particular focus on the empty-full capsid ratio [37,38]. The methods currently being investigated by this collaboration include charge detection mass spectrometry (CDMS), capillary electrophoresis (CE) immunoassays, and isoelectric focusing (IEF). Each of these techniques require minimal amounts of material and can provide information on the mass and charge of capsid populations, as well as a relative quantification of capsid content.

CONCLUSION

Many organizations, including NIST and USP, are committed to addressing industry-wide concerns regarding the purity of

AAV vectors and are taking a collaborative approach by bringing together stakeholders from academia, industry, and regulatory bodies to establish consensus on the critical quality attributes for gene therapies. This collective wisdom can then be translated into universally applicable best practices and reference materials for assessing AAV vector quality that play a crucial role in improving the manufacturing and quality control of AAV vectors. The use of documentary standards along with ARMs can facilitate consistent and predictable manufacturing processes and product testing throughout the product lifecycle. With these tools, manufacturers can reduce the burden of in-house method development and validation, gain valuable knowledge on vector production, phase-appropriate comparability, and meet regulatory expectations.

REFERENCES

1. Keeler AM, Flotte TR. Recombinant adeno-associated virus gene therapy in light of Luxturna (and Zolgensma and Glybera): where are we, and how did we get here? *Annu. Rev. Virol.* 2019; 6, 601–21.

2. Ozelo MC, Mahlangu J, Pasi KJ *et al.*; GENEr8-1 Trial Group. Valoctocogene roxaparvovec gene therapy for hemophilia A. *N. Engl. J. Med.* 2022; 386(11), 1013–25.

3. Prado DA, Acosta-Acero M, Maldonado RS. Gene therapy beyond luxturna: a new horizon of the treatment for inherited retinal disease. *Curr. Opin. Ophthalmol.* 2020; 31(3), 147–54.

4. ClinicalTrials.gov.

5. Kuzmin DA, Shutova MV, Johnston NR *et al.* The clinical landscape for AAV gene therapies. *Nat. Rev. Drug Discov.* 2021; 20(3), 173–4.

6. Issa S, Shaimardanova A, Solovyeva V, Rizvanov A. Various AAV serotypes and their applications in gene therapy: an overview. *Cells* 2023; 12(785), 1–41.

7. Gagon P, Leskovec M, Goričar B, Štrancar A. Streamlining industrial purification of adeno-associated virus. *BioProcess Int.* 2020; 18(11–12), 14–20.

8. Jones M, Palackal N, Wang F *et al.*. "High-risk" host cell proteins (HCPs): a multi-company collaborative view. *Biotechnol. Bioeng.* 2021; 118(8), 2870–85.

9. Jawa V, Joubert MK, Zhang Q *et al.* Evaluating immunogenicity risk due to host cell protein impurities in antibody-based biotherapeutics. *AAPS J.* 2016; 18(6), 1439–52.

10. Wright JF. AAV empty capsids: for better or for worse? *Mol. Ther.* 2014; 22, 1–2.

11. Hinderer C, Katz N, Buza EL *et al.* Severe toxicity in nonhuman primates and piglets following high-dose intravenous administration of an adeno-associated virus vector expressing human SMN. *Hum. Gene Ther.* 2018; 29, 285–98.

12. *International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH).* Q5B Analysis of the Expression Constructs in Cells Used for Production of r-DNA Derived Protein Products. November 1995.

13. Wang L, Maragh S, Kwee E *et al.* Measurement solutions and standards for advanced therapy. *Mol. Ther. Methods Clin. Dev.* 2024; 32, 1–4.

14. *European Agency for the Evaluation of Medicinal Products.* Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products 2018.

15. Gene Delivery Systems. NIST 2024.

16. *US Food & Drug Administration*. Guidance for industry: characterization and qualification of cell substrates and other biological materials used in the production of viral vaccines for infectious disease indications 2010.

17. *European Agency for the Evaluation of Medicinal Products*. Position statement on the use of tumourigenic cells of human origin for the production of biological and biotechnological medicinal products 2021.

18. Knezevic I, Stacey G, Petricciani J. Sheets R. WHO Study Group on Cell Substrates. Evaluation of cell substrates for the production of biologicals: revision of WHO recommendations. Report of the WHO study group on cell substrates for the production of biologicals. *Biologicals* 2010; 38(1), 162–9.

19. American Type Culture Collection. Application of a HEK-293 gDNA control in the detection of residual host cell DNA in biopharmaceuticals 2023.

20. Khabou H, Cordeau C, Pacot L, Fisson S, Dalkara D. Dosage thresholds and influence of transgene cassette in adeno-associated virus-related toxicity. *Hum. Gene Ther.* 2018; 29, 1235–1241.

21. Yang TY, Braun M, Lembke W *et al.* Immunogenicity assessment of AAV-based gene therapies: an IQ consortium industry white paper. *Mol. Ther. Methods Clin. Dev.* 2022; 26, 471–94.

22. Wang D, Tai PWL, Gao G. Adeno-associated virus vector as a platform for gene therapy delivery. *Nat. Rev. Drug Discov.* 2019; 18(5), 358–78.

23. Martinez-Fernandez de la Camara C, McClements ME, MacLaren RE. Accurate quantification of AAV vector genomes by quantitative PCR. *Genes (Basel)* 2021; 12(4), 601.

24. D'Costa S, Blouin V, Broucque F *et al.* Practical utilization of recombinant AAV vector reference standards: focus on vector genomes titration by free ITR qPCR. *Mol. Ther. Methods Clin. Dev.* 2016; 5, 16019.

25. Sanmiguel J, Gao G, Vandenberghe LH. Quantitative and digital droplet-based AAV genome titration. *Methods Mol. Biol.* 2019; 1950, 51–83.

26. Clarner, P, Mansfield, KM, PCR-based methodologies to characterize AAV gene therapy vectors. *American Pharmaceutical Review* 2022; 1–11.

27. Shmidt AA, Egorova TV. PCR-based analytical methods for quantification and quality control of recombinant adeno-associated viral vector preparations. *Pharmaceuticals* 2022, 15, 23.

28. Meierrieks F, Kour A, Pätz M, Pflanz K, Wolff MW, Pickl A. Unveiling the secrets of adeno-associated virus: novel high-throughput approaches for the quantification of multiple serotypes. *Mol. Ther. Methods Clin. Dev.* 2023; 31, 101118.

29. Hou Y, Zhang H, Miranda L, Lin S. Serious overestimation in quantitative PCR by circular (supercoiled) plasmid standard: Microalgal pcna as the model gene. *PLoS ONE* 2010; 5, e9545.

30. Hitchcock T. Manufacturing plasmid DNA. *BioProcess Int.* 2016; 14(9), 12–9.

31. Rumachik NG, Malaker SA, Poweleit N *et al.* Methods matter: standard production platforms for recombinant AAV produce chemically and functionally distinct vectors. *Mol. Ther. Methods Clin. Dev*. 2020; 18, 98–118.

32. Human adenovirus 5 VR-1516, ATCC, 2010.

33. Hutchins B. Development of a reference material for characterizing adenovirus vectors. *BioProcess J.* 2002; 1(1), 25–9.

34. Hutchins B, Sajjadi N, Seaver S *et al.* Working toward an adenoviral vector testing standard. *Mol. Ther*. 2000; 2(6), 532–4.

35. Wada M, Uchida N, Posadas-Herrera G *et al.* Large-scale purification of functional AAV particles packaging the full genome using short-term ultracentrifugation with a zonal rotor. *Gene Ther.* 2023; 30(7–8), 641–8.

36. Eglovitch JS. USP, NIST, and NIIMBL collaborate to boost viral vector quality standards. Regulatory Focus 2021.

37. Welch AR. Can USP, NIST, and NIIMBL conquer the biggest AAV quality challenge? Cell and Gene Collaborative 2022.

38. Shmidt AA, Egorova TV. PCR-based analytical methods for quantification and quality control of recombinant adeno-associated viral vector preparations. *Pharmaceuticals (Basel)* 2021; 15(1), 23.

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Accelerate time to clinic for advanced therapies with a platform approach

John Lee, Global Head of Cell and Gene Therapy, **Cédrick Rousseaux**, Innovation, Analytical, and Process Development Director, and **Dana Cipriano**, Global Head of Testing and Analytical Services, SK pharmteco

As the advanced therapies sector advances, the importance of adaptable and scalable platform solutions to reduce expenses and make patient access more widespread grows significantly. Covering areas from viral vector manufacturing to cell therapy processing, as well as plasmid production and associated analytics, many hurdles to establishing resilient platforms for cell and gene therapies must be addressed. This FastFacts poster outlines crucial insights for utilizing state-of-the-art technologies and streamlining unit operations, aiming to accelerate the advancement and production of advanced therapies.

LENTISURE™ AND AAVELOCITY™: SCALABLE AND HIGHLY PRODUCTIVE MANUFACTURING

LentiSure* for lentivirus and AAVelocity* for AAV manufacturing are two platform approaches developed to address current challenges in the cell and gene therapy space. Both are plug-and-play, robust, and scalable and can release a clinical batch within 12 months of receiving plasmids.

These platforms consistently achieve high titers, regardless of batch size, and productivity remains steady at cell culture harvest, whether at 250 mL or 200 L as seen in **Figure 1**. With LentiSure, titer yield after harvest reaches up to 5.9×10^7 IG/mL, and with AAVelocity, up to 2×10^{11} VG/mL.

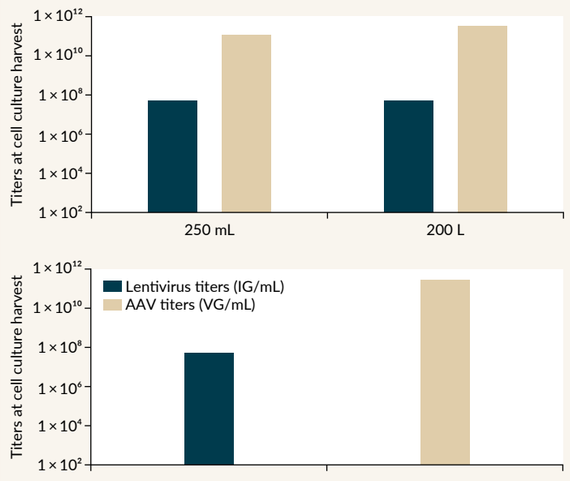
AAVelocity can achieve up to 80% full particles, making the manufacturing process highly productive. Additionally, total yields range between 30% and 50%, with the possibility of reaching a final titer yield after purification of 1×10^{14} VG/mL, and a proven scalability of up to 1,000 L.

Both platforms have a strong track record, with over **100** batches produced each. For LentiSure, this includes **40** cGMP batches and **14** different transgenes, while for AAVelocity, it is **55** and **5**, respectively.

AAV AND LENTIVIRUS PLATFORM ANALYTICAL CAPABILITIES AND ASSAY DEVELOPMENT

Accurate characterization and testing of cell and gene therapies is crucial for minimizing regulatory delays. The AAVelocity and LentiSure platforms are are equipped with analytical procedures suitable for testing the quality attributes of various products without significant changes to their operational conditions, system suitability, and reporting structure. They enable rapid and specific testing across five key domains throughout the manufacturing process and product lifecycle: potency, purity, identity, safety, and stability. The AAV method platform package supports CDMO clients and external testing, reducing GMP batch usage and release times, particularly for safety assays like replication-competent virus and adventitious agents testing. Similarly, for lentivirus, many required assays are provided as a platform service. As the technology expands for lentivirus, safety testing for cell-based replication-competent lentivirus GMP will be introduced in 2024.

Figure 1. Titers at cell culture harvest for lentivirus and AAV titers using LentiSure and AAVelocity platforms at 250 mL and 200 L.



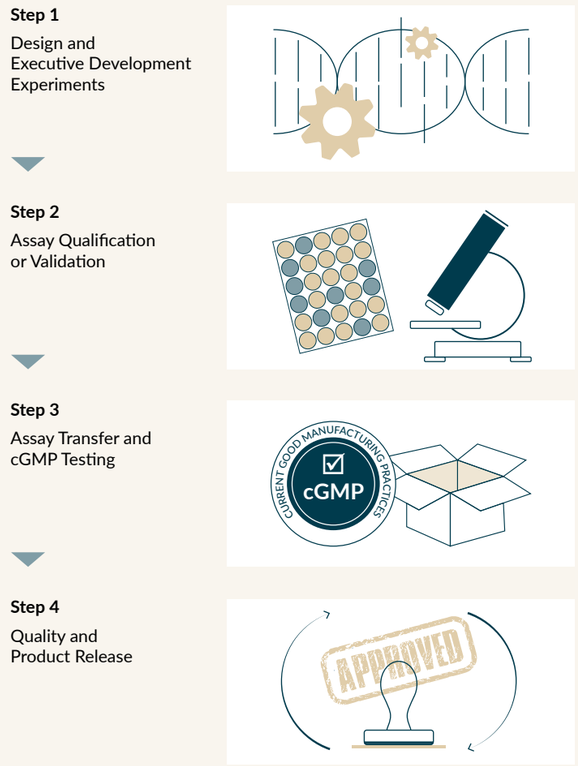
The Assay Development and Testing approach revolves around creating and implementing complex assays to support various modalities of cell and gene therapy. The four-step approach to assay development and analytical testing of platform assays is outlined in **Figure 2**.

SUMMARY

LentiSure and AAVelocity are proven and robust platform approaches that tackle the challenges currently faced by the cell and gene therapy space. With the integration of their key features, they provide production efficiency for viral vector manufacturing and release analytics.

*Proprietary information of Yposkesi SAS, an SK pharmteco company.

Figure 2. The four-step approach to assay development and analytical testing of platform assays.



CELL & GENE THERAPY INSIGHTS

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In partnership with: SK pharmteco

CELL & GENE THERAPY INSIGHTS

VECTOR PROCESSING AND MATERIALS

SPOTLIGHT

INNOVATOR INSIGHT

Accelerating AAV process development with a PAT-driven TFF system

Teva Smith

Ultrafiltration/diafiltration represents a crucial step in bioprocessing, but traditional methods can pose a range of challenges. Some of these issue stem from current mass-dependent methods used in tangential flow filtration (TFF) systems. This article describes streamlined process development with a system uniquely controlled by real-time titer measurement acquired through in-line variable pathlength spectroscopy. AAV case study data from real users demonstrate automated process control, exhibiting the system's efficacy across various final endpoints.

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CURRENT TFF CHALLENGES

In gene therapy manufacturing, traditional methods of ultrafiltration/diafiltration (UF/DF) often pose challenges related to variability and lack of real-time monitoring. Some key issues stem from reliance on mass balance calculations, which have potential to introduce and propagate errors throughout the TFF process. Errors may also arise during volume hold-up calculations or any of the steps requiring human interaction with the system, creating multiple opportunities to compromise accuracy before, during, and after the process (Figure 1).

Addressing these challenges are an industry-wide priority. In a survey of 207 qualified biopharmaceutical professionals [1],

real-time, in-line product testing, automation instrumentation, and continuous bioprocessing were identified as key new product development areas of interest to address process analytical technology (PAT) needs.

COMBINING TECHNOLOGY SOLUTIONS TO ACHIEVE A PAT-DRIVEN TFF PROCESS

The KrosFlo® KR2i Real-time Process Management (RPM™) is first in the Repligen PAT-driven TFF System. Repligen is addressing the needs of the industry by integrating the KrosFlo® KR2i TFF System with the CTech™ FlowVPX® in-line variable pathlength UV-Vis technology. The integrated system provides UF/DF process monitoring and control through real-time concentration measurements and set points.The KR2i system is an automated lab-scale TFF system used in downstream applications. It is a turnkey, benchtop TFF system that enables walkaway automation. There are 13 automated process control modes, mainly comprised of combinations of concentration and diafiltration. With robust processing and highly configurable flow paths and setups, processing volumes as low as 10 milliliters or as high as 10 liters are possible.

The FlowVPX System is the only in-line UV-Vis spectrophotometer that utilizes variable pathlength technology to allow for real-time, continuous concentration measurement throughout the UF/DF process. Variable pathlength technology yields a broad dynamic range, as the system adjusts to the right pathlength range to achieve accurate measurements regardless of the molecule of interest.

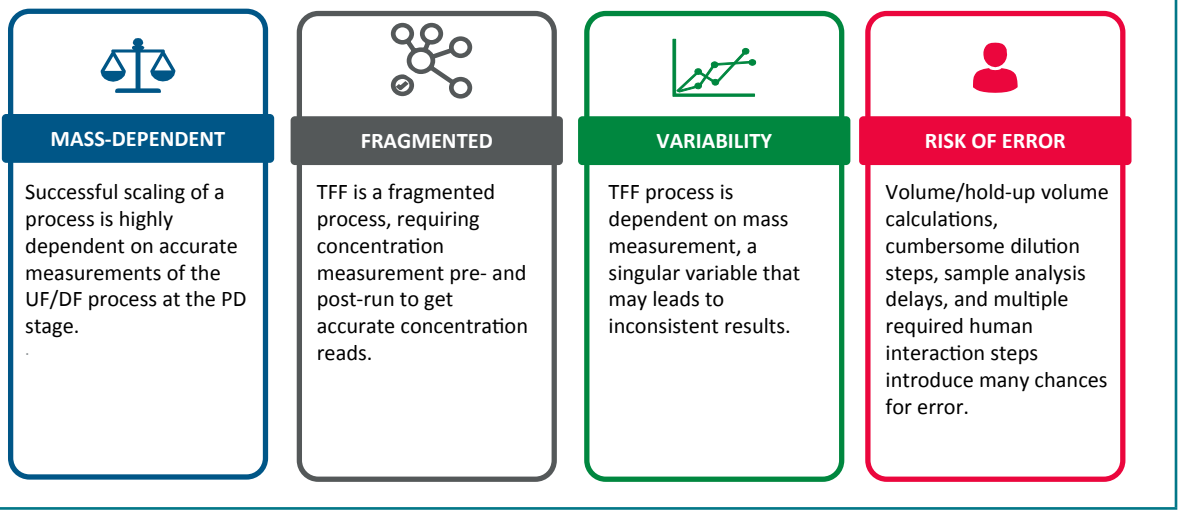
These two products are unified with a common software platform, allowing the user to utilize the FlowVPX not just as a monitoring system, but as a control and management system, by using the data that is created and captured by the FlowVPX System. This enables all of the familiar TFF automation control, but now based on in-line concentration. This integrated system gives real-time data at every step of the filtration process, and automatically generates graphs, charts, and trends, providing all of the inputs and outputs that are crucial to understand for optimising a process. It is also a flexible plug-and-play technology that can be set up as needed for specific applications.

TRADITIONAL UV-VIS VERSUS VARIABLE PATHLENGTH SPECTROSCOPY

UV-Vis spectroscopy is based on the Beer-Lambert law where absorbance (A) is directly

FIGURE 1

Traditional TFF system challenges.



proportional to the concentration (c), pathlength (l), and extinction coefficient (ε) (Figure 2). In traditional UV-Vis, the pathlength is a fixed value; sample dilution is therefore required to ensure the absorbance falls within the linear range of the spectrophotometer. In variable pathlength spectroscopy, the system rapidly measures absorbance at multiple pathlengths to produce a linear regression in accordance with the Beer-Lambert law. Concentration can then be collected neat, as the need for sample dilution is eliminated.

KROSFLO RPM SYSTEM SETUP

The RPM system setup (Figure 3) is similar to that of a traditional TFF system, and various configurations are possible depending on the concentration and diafiltration requirements of the process. What distinguishes the RPM system is the integration of the FlowVPX instrument, strategically positioned in-line immediately after the feed tank. This placement is pivotal, as it provides the best representation of the material concentration inside the feed vessel. In contrast, the material in the retentate line would appear more concentrated, as it most recently passed through the filter; however, this concentration would not reflect the entire batch, as it has not yet mixed with the material in the feed tank.

AAV CASE STUDY DATA

Case study 1: viral titer determination—AAV2

In the first case study, the objective was to evaluate a variable pathlength spectroscopy method for AAV viral titer monitoring. The assessment utilized the FlowVPX system to monitor the concentration of AAV2 during a UF/DF run (Figure 4). Each stage in the UF/DF was compared to ddPCR, ddPCR GOI, and ELISA, as shown in Table 1.

The results illustrate that this approach is able to accurately capture real-time titer and is comparable to offline methods. The run met expected values and the final UF2 target, and the FlowVPX System results were successfully acquired in real time. In contrast, off-line methods were generated by two different users in the analytical group with a 2-week turnaround time. The final product testing is highlighted in the data table. The average variation was found to be >15% for capsid titer and >7% for genome titer. At the final UF2 stage, this was within 5% of other methods with a high linearity, demonstrating

FIGURE 2

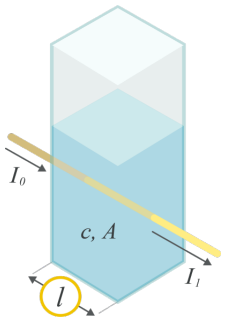
Traditional UV-Vis versus variable pathlength spectroscopy.

Traditional UV-Vis Spectroscopy

Single absorbance measurement

Pathlength (*l*)
is a fixed value

Concentration (*c*)
is variable; requires sample dilution to ensure concentration falls within the linear range of the spectrophotometer



Variable Pathlength Spectroscopy

Multiple absorbance measurements for slope-based concentration results

Pathlength (*l*)
is a variable value, capturing multiple absorbance measurements in less than 1 minute

Concentration (*c*)
is kept fixed and does not require sample dilution

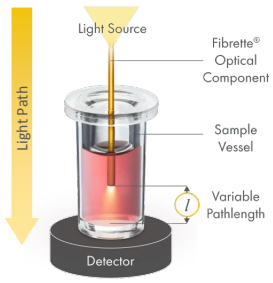
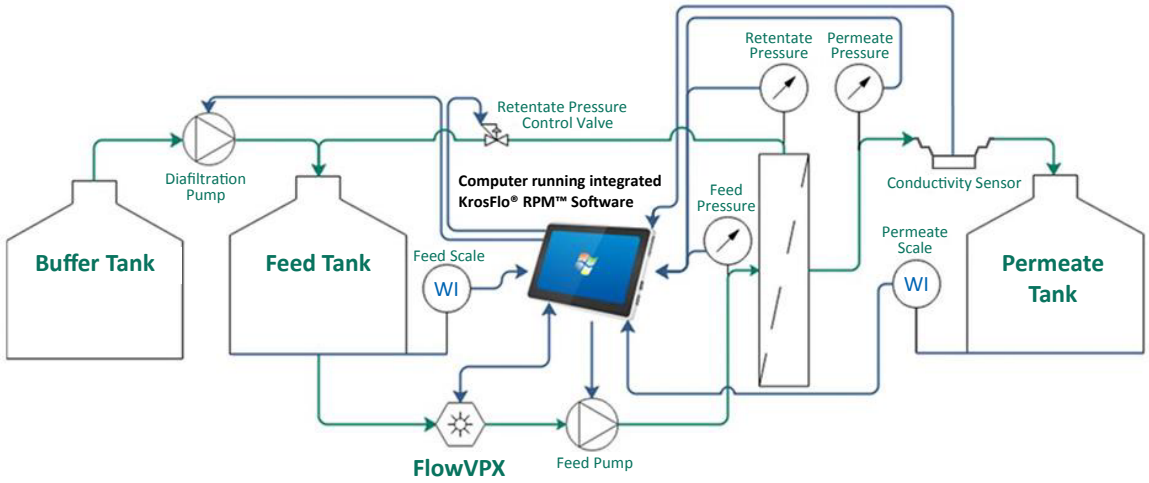


FIGURE 3

Example KrosFlo RPM system diagram.



Green arrow: Flow path; blue arrow: Communication line.

FIGURE 4

UF/DF AAV titer process monitoring using in-line VPT technology: AAV2 TFF run.

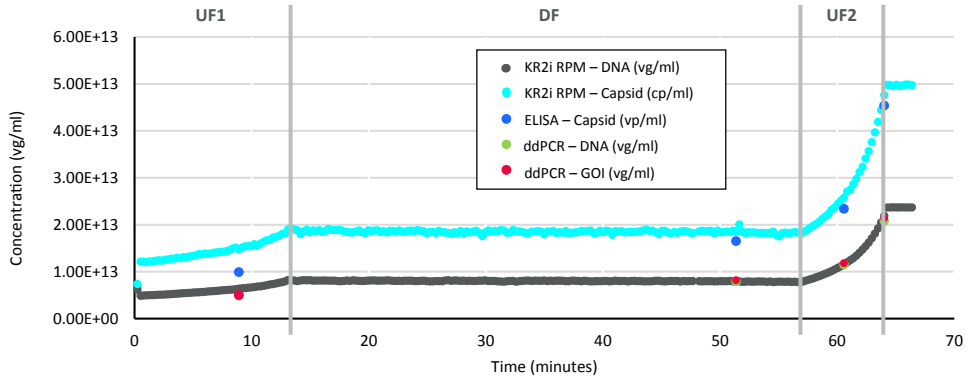


TABLE 1

UF/DF AAV2 titer process monitoring—KR2i RPM system results versus ELISA and ddPCR.

Step	KR2i RPM: DNA (vg/ml)	KR2i RPM: capsid (cp/ml)	ELISA: capsid (vp/mL)	ddPCR: DNA (vg/ml)	ddPCR: GOI (vg/mL)	% Difference capsid	% Difference ddPCR	% Difference ddPCR GOI
UF1	6.39×10^{12}	1.48×10^{13}	9.91×10^{12}	4.94×10^{12}	4.99×10^{12}	-32.97	-22.75	-21.97
DF	7.93×10^{12}	1.85×10^{13}	1.65×10^{13}	7.94×10^{12}	8.17×10^{12}	-11.05	0.09	2.99
Mid-UF2	1.16×10^{13}	2.58×10^{13}	2.34×10^{13}	1.15×10^{13}	1.18×10^{13}	-9.19	-0.66	1.93
End-UF2	2.19×10^{13}	4.77×10^{13}	4.54×10^{13}	2.08×10^{13}	2.14×10^{13}	-4.73	-5.11	-2.38

consistency during the process of collection with the FlowVPX.

Case study 2: viral titer determination—AAV5

A second study was performed using AAV5 to assess robustness of the FlowVPX System for in-line titer measurement of another standard serotype. This assessment utilized the FlowVPX System to monitor the concentration of AAV5 during a UF/DF run (Figure 5). Each stage in the UF/DF was compared to ddPCR, ddPCR GOI, and ELISA (Table 2).

As with the first case study, FlowVPX System results were successfully acquired in real time. Off-line methods were generated by two different users in the analytical group with a 2-week turnaround time. In this study a higher percent difference was seen between methods. However, this remained consistent and linear with the data it was capturing throughout the entire process. This shows trackability and comparability to other methods.

Case study 3: comparing different off-line methods

The third example used AAV2 and compared different offline methods: qPCR, Octet, and Repligen's own at-line SoloVPE method. As shown in Figure 6 and Table 3, the FlowVPX and the SoloVPE both collected AAV titer successfully and the real-time process insight allowed for the run to be modified. In the diafiltration stage, the variable pathlength instruments detected a decrease in concentration that was not registered by the other analytical methods. This suggested a gel layer was forming during the diafiltration stage.

The solution to this issue was to reduce the flux, i.e., lowering the transmembrane pressure to allow for better mixing throughout the process. However, this issue could only be detected using real-time, in-line analytics.

FIGURE 5

UF/DF AAV titer process monitoring using in-line VPT technology: AAV5 TFF run.

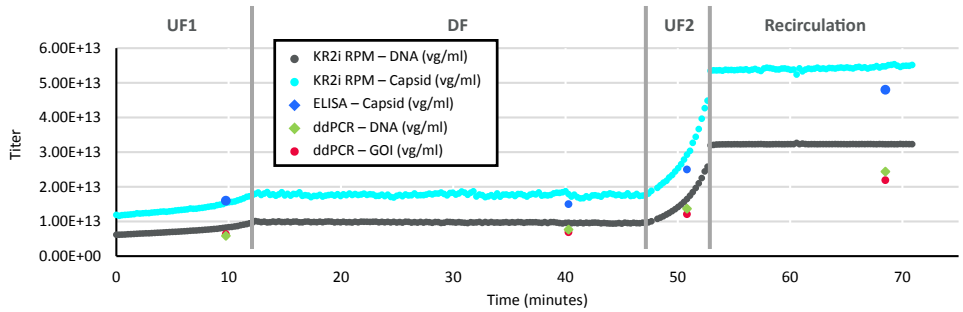


TABLE 2

UF/DF AAV5 titer process monitoring—KR2i RPM system results versus ELISA and ddPCR.

Step	KR2i RPM: DNA (vg/ml)	KR2i RPM: capsid (cp/ml)	ELISA: capsid (vp/mL)	ddPCR: DNA (vg/ml)	ddPCR: GOI (vg/mL)	% Difference capsid	% Difference ddPCR	% Difference ddPCR GOI
UF1	8.24×10^{12}	1.54×10^{13}	1.60×10^{13}	6.35×10^{12}	5.86×10^{12}	-4.03	-22.92	-28.86
DF	9.66×10^{12}	1.79×10^{13}	1.50×10^{13}	6.88×10^{12}	7.68×10^{12}	19.19	-28.78	-20.50
Mid-UF2	1.65×10^{13}	2.93×10^{13}	2.50×10^{13}	1.21×10^{13}	1.37×10^{13}	17.09	-26.56	-16.84
End-UF2	3.23×10^{13}	5.48×10^{13}	4.80×10^{13}	2.19×10^{13}	2.44×10^{13}	14.08	-32.15	-24.40

FIGURE 6

UF/DF AAV titer process monitoring using in-line VPT technology.

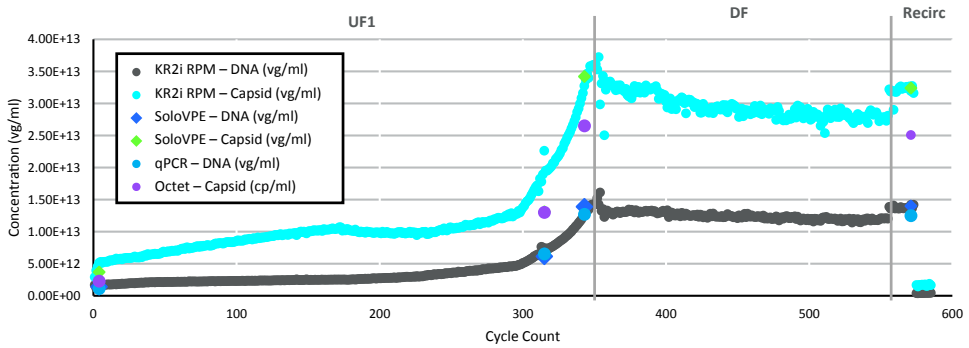


TABLE 3

UF/DF AAV titer process monitoring—KR2i RPM system results versus qPCR, Octet and SoloVPE.

Step	KR2i RPM: DNA (vg/ml)	KR2i RPM: capsid (cp/ml)	ddPCR: DNA (vg/ml)	Octet: capsid (cp/ml)	DNA % difference VPX vs qPCR	Capsid % difference VPX vs octet	DNA % difference VPE vs VPX	Capsid % difference VPE vs VPX
UF1	1.58×10^{12}	5.12×10^{12}	1.11×10^{12}	2.3×10^{12}	42.67	124.59	12.32	38.72
6x	6.94×10^{12}	2.26×10^{13}	6.52×10^{12}	1.3×10^{13}	6.51	73.75	13.10	72.60
12x	1.26×10^{13}	3.27×10^{13}	1.27×10^{13}	2.7×10^{13}	-0.45	23.45	-9.04	-4.28
DF	1.42×10^{13}	3.19×10^{13}	1.25×10^{13}	2.5×10^{13}	13.39	27.40	1.24	-1.43

SUMMARY

The KR2i RPM system seamlessly integrates the in-line FlowVPX spectrophotometer with the automated KR2i TFF System, establishing a first-of-its-kind PAT-driven system control by real-time titer measurement. This integration strengthens process control and ensures high-quality, reproducible results. Real-time data collection enhances process efficiency by reducing cycling time, while in-line measurement minimizes process risk by eliminating dependence on error-prone calculations.

Repligen is paving the way towards PAT-driven TFF systems and plans to expand the RPM product line with larger-scale systems in the near future.

Q&A

Teva Smith

Q How does the in-line titer value from the FlowVPX measured during TFF differ from the traditional offline titer measured after product recovery?

TS: We are using the UV-Vis with the relationship between capsid and genome titers. The output is going to be comparable to your ELISA, ddPCR or qPCR. The only difference here between the offline and the on-line is that we're able to provide a much more efficient output so you can have this information instantly, instead of taking a week or 2 to actually collect those offline data points. This reduces how many data points you have to collect, whether it's having to collect for DF for your TF1 and then your TF2. Here, you can just take your final sample and test it offline with your AD group or QC group.

Q Is there a single-use version of FlowVPX?

TS: Yes, the single-use flow cells are available now and they can come X-ray irradiated. This can be implemented into your own setup or within our custom flow paths to optimize your process.

Q Is the addition of a DF buffer to be used manually, or is it an automated calculation based on concentration input/output?

TS: We have two KR2i 'minis' as we call them, and they are the automated pumps that will pump in the diafiltration buffers so you can run two DFs if that's your process. I have some AAV clients that go with this approach, and it will be done automatically and based on

volume. We will capture the titer for your TF1 and TF2, but use volume exchange for the diafiltration.

Q Does this UV concentration utilize one or multiple wavelengths? Additionally, is multiwavelength measurement possible, and how does that impact the measurement time?

TS: This equation requires multiple wavelengths, so it's going to capture 260 and 280. We use four extinction coefficients, and this is based on a paper with an equation on UV-Vis with AAV [2]. We use those inputs to essentially generate automatic extinction coefficients for your capsids. If you have a custom capsid, you'd have to put that extinction coefficient in for 260 and 280, but we use the ratio within that paper to generate both capsid and genome titer output. So it does do multiwavelength and the range is 190 to 1,100 nanometers. If you had other modalities that you are interested in and you wanted to input this VPX technology to record a different process as well as your AAV process, you can do that.

Q Can you elaborate more on how the FlowVPX can quantify total capsids and viral genomes just by absorbance?

TS: How it does this is not just by absorbance—it's the ratio between the absorbances at 260 and 280, so it would actually give you an output of empty/full. It doesn't capture just the raw overall titer of your process or overall amount of capsid. It gives you the in-line titer. Your in-line titer would be how you'd be able to process your amount of mLs that you have in that solution. When we talk about the in-line mass collection, that's more regarding chromatography where everything is flowing through at once and you can capture that. This is going to be a recirculation, so it'll give you the titer, but not overall capsid number.

Q What is your minimum process volume using both VPX and KrosFlo RPM systems?

TS: For our AAV clients, we have been able to optimize even custom flow paths down to 4 mL, using L14-size tubing and a hollow fiber from Repligen. The output is really based on what our clients need. We have varying sizes based on the quantity that you're starting with.

Q Can you control concentration to specific ranges with a specified minimum and maximum?

TS: For titers, you won't control for range. The beauty of having an in-line PAT is that you can actually target your target—you can set it to your ideal number, instead of having to overshoot it like in traditional mass balance and then having to re-dilute it after.

Q Does the VPX use disposable fibrettes, and have you seen issues with protein absorption onto the probe during processing? Could this be recovered by clean in place (CIP)?

TS: Yes, this does not use the flow cell other than the single-use model, which would be just the disposable whole flow cell. The stainless steel model is CIP, and you would do 0.5 NaOH for 30 minutes followed by a water flush. We've found this to be below the 500 ppb, which is in the pharmacopeia standards.

Q In the TFF, what are the limits of sample in terms of viscosity, solid content, and protein titer?

TS: In your traditional process, viscosity doesn't affect the FlowVPX reading, besides micro-bubbling. If it does have a known micro-bubbling effect, that can impact UV-Vis. But traditionally, as long as the filter can support the level of viscosity you are working with and doesn't gel up, you would be able to get accurate readings in-line.

Q What are the limits of operation of the TFF regarding sample viscosity and protein titer?

TS: Regarding viscosity for the titer for AAV clients, one of them can be gel layer formation on your hollow fibers. AAV is notorious for forming a gel layer. You'll see this drop in titer during the diafiltration that actually jumps back up after the diafiltration once you have recirculation going and pulling things off of that filter. The viscosity itself isn't as much of an issue—it's more so if you are having an even distribution with mixing in your process. Definitely having a stir bar and making sure that your flux is optimized in order to get the best output. Using this PAT also allows you to optimize these other aspects of your process.

Q How robust is the UV measurement with regard to precipitants which can occur during TFF?

TS: Precipitants can affect the system. Where you would see this impact would be through a failing R-squared value. The VPX and the SoloVPE both have this indicator, R-squared, that lets you know that the collection is not collecting linearly, which can happen if a precipitant comes through the solution that's not in the next collection. So you would have an indicator to let you know something is precipitating in your solution.

Q If you have a continuous manufacturing process, would it be better to place FlowVPX in the feed or retentate line?

TS: If you're working at that scale it is better to place it on the retentate. Your target of interest for the titer or the concentration reading is where you'd want the VPX. The reason that we have it on the feed side is because the target in this RPM system, the smaller scale, is your feed vessel. If it's passing on to another spot, you move it to where your target is. This is similar to chromatography, where you're going to have it post-column to collect the information that's going into your next stages.

Q Is there any flow rate limit range for optimized measurement?

TS: No—we do have a maximum flow rate, but this can collect even at zero flow rate as well. It is really based on what is required to optimize your mixing in your setup. There is no optimized flow rate, it's based on your actual molecule. A flow rate that prevents any settling, any precipitants or any gel layer from fully forming are how we would work with that. That is something we would provide to the client, and work with them to help optimize what their process is and get the best in-line readings possible.

REFERENCES

1. 20th Annual Report and Survey of Biopharmaceutical Manufacturing Capacity and Production. A Study of Biotherapeutic Developers and Contract Manufacturing Organizations. 2023; BioPlan Associates.
2. Sommer JM, Smith PH, Parthasarathy S, *et al.* Quantification of adeno-associated virus particles and empty capsids by optical density measurement. *Mol. Ther.* 2003; 7(1), 122–8.

BIOGRAPHY

TEVA SMITH is an accomplished researcher with a BSc in Biology from the University of North Carolina at Chapel Hill, now specializing in bioprocessing. Currently serving as a Field Application Scientist at Repligen Corporation, Teva has amassed over 2 years and 7 months of experience in delivering onsite client demonstrations on Process Analytics Technology, with a primary focus on optimizing UF/DF, chromatography, and fill-to-finish processes. Teva plays a crucial role in post-sale customer support, overseeing implementation, process validation, SOP creation, and method transfers. Notably, Teva collaborates with the engineering division, actively participating in alpha and beta testing of new devices and providing valuable customer feedback for research and development projects. This commitment extends to contributing to research papers and posters on innovative device applications. In Teva's previous role as a Product Development Research Associate at AstraZeneca, he demonstrated expertise in simulated use test inhalation trials and cGMP data collection and recording. Teva's proficiency in incorporating R statistical analysis streamlined data review and manipulation, contributing to efficient research design troubleshooting and coordination of multi-team trials. Teva's earlier experiences at the University of North Carolina at Chapel Hill and the US Environmental Protection Agency (EPA) involved conducting research on neuronal cellular adhesion molecules and independently preparing high-throughput

toxicology assays, showcasing a diverse skill set in PCR, SDS-PAGE, Western Blotting, and high-throughput research methodologies. Teva Smith emerges as a driven scientific professional, blending comprehensive expertise in bioprocessing with a commitment to advancing research and development in the field.

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Field Applications Scientist,
Repligen

AUTHORSHIP & CONFLICT OF INTEREST

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TAKE CONTROL OF YOUR UF/DF PROCESS

The new PAT-driven KrosFlo® KR2i RPM™ System combines the power of automation with real-time concentration data, so you can take control of your UF/DF process. Using variable pathlength technology, the system continuously measures concentration and controls the process to deliver the desired concentration end points, every time.

- **Strengthen your process control** with high accuracy and reproducible results
- **Increase process efficiency** by reducing cycling time and increasing yield throughput
- **Reduce process risk** by ensuring accurate concentration throughout the TFF process

Take Control

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CELL & GENE THERAPY INSIGHTS

VECTOR PROCESSING AND MATERIALS

SPOTLIGHT

Best practices and considerations for outsourcing with CDMOs

Joseph W Graskemper
Biogen, Inc.

"...the question of how companies should approach CDMO selection is more important than ever."

VIEWPOINT

Cell & Gene Therapy Insights 2024; 10(3), 291–295

DOI: 10.18609/cgti.2024.043

On March 4, 2024, **David McCall**, Senior Editor, *Cell & Gene Therapy Insights*, spoke to **Joseph W Graskemper**, Director of External Network Strategy at Biogen, about recent trends in the gene therapy CDMO sector and what these mean for the industry as a whole. This article is based upon that conversation. The views expressed in this article belong exclusively to the author and not to Biogen, Inc.

PAST TO PRESENT: THE CURRENT STATE OF THE GENE THERAPY CDMO INDUSTRY

Before outlining the current state of the field, it's important to firstly acknowledge some key events in our industry throughout the past several years.

The COVID-19 pandemic triggered a significant shift in the industry, creating a 'perfect storm' of factors that resulted in a substantial increase in demand for raw materials, consumables, and drug manufacturing, along with the corresponding capacity thereof. This had a huge impact on cell and gene therapy (CGT) supply chains. Companies without strong dual-sourcing strategies and robust business continuity plans were in a tough position as previously readily available materials became hard to find. At the same time, there was also great excitement building around advanced therapy technologies, with multiple recent gene therapy approvals and mRNA beginning to show promise in the first COVID-19 vaccines. This exacerbated the difficulties already facing gene therapy supply chains. As skyrocketing investment and fundraising efforts funneled into CGT, those working in the area battled challenges including severe shortages of single-use consumables and critical raw materials that were being diverted to vaccines manufacture, an insufficient talent pool due to the novelty of the field, and practically non-existent manufacturing capacity.

In early 2021, there was a reactionary phase that saw contract development and manufacturing organizations (CDMOs) building out capacity as quickly as possible to meet demand for advanced therapies. And at the same time, sponsors built or were considering $100+ million facilities of their own, preferring to keep their processes internal to retain control and safeguard IP.

Fast forward from this surge in activity and investment to today, and we see that the market did not hold up. The last 2 years have been characterized by a challenging environment for CGT biotech fundraising, especially for early-phase companies that built their own facilities. Those investments in 'build' versus 'buy' led to negative consequences for some, where the overhead of maintaining an advanced therapy manufacturing facility may not have been fully considered. If early-phase programs did not pan out as hoped, companies became stuck with expensive, empty facilities. The March 2022 deal between Oxford Biomedica and Homology Medicines to form Oxford Biomedica Solutions is just one example of a CDMO coming in and purchasing a facility from an innovator company.

The extra capacity that was being built out in 2020–2021 has now come online—however, the tough financial climate means that the demand is not what it once was, resulting in unfilled capacity at CDMOs. For example, some CGT developers are no longer moving multiple assets forward in parallel and are rather focusing on a smaller, re-prioritized portfolio to progress. We are just now seeing this capacity starting to be taken up and utilized based on the investment environment improving.

In addition, the introduction of manufacturing platforms has brought about a profound shift in the field. For example, in 2020, AAV vector production platforms were not established or widely being offered by CDMOs. Just a few years later, new platforms covering several different AAV serotypes are coming out that developers can plug into. With the industry having largely completed its move towards the suspension culture-transient transfection model and away from adherent cell culture, the field is now feeling cautious optimism that industrialization may soon be a reality.

HOW TO FIND THE RIGHT CDMO PARTNER

With these recent trends having strengthened both the hand of the CDMO sector through the offering of manufacturing platforms, and the hand of the CGT sponsor by

way of increased availability of manufacturing capacity, the question of how companies should approach CDMO selection is more important than ever. There are myriad pitfalls to avoid. But where to start?

For example, as a company works to advance an asset from the preclinical to clinical stage, a common misstep is not starting the search for a CDMO early enough. Once there is a particular asset in R&D with a nomination in sight and the beginnings of a target product profile, it is vital to discuss internally the external manufacturing strategy, and the costs, dosages, and amount of material required in a variety of scenarios. By the time the point of nomination is reached, assumptions must have been pressure-tested internally. So, start early, go through scenario planning, and determine what you require in a partner before formally beginning your CDMO search. You may miss the mark if you do not align internally on your target product profile, dose, potential volumes, and prospective multi-year plan. It is vitally important to go in knowing what you need in terms of high-level capabilities.

The process to find the right CDMO starts with an initial landscaping assessment, and there could be many CDMOs that meet all your high-level criteria. At that point, you will send out a Request for Information (RFI) to help you figure out a given CDMO's technical capabilities, quality capabilities, and site history on a deeper level. That RFI can then be escalated to a request for proposal (RFP) once the range of target companies has been narrowed down. An RFP shares with the CDMO exactly what you want them to do for you. This is typically sent to a maximum of three to five CDMOs.

As part of this process and of your due diligence, you must do a site visit of any CDMO that you are seriously considering. Walking the floor, seeing the facility, and meeting the site leadership are all critical. Importantly, you also need to present and sell your company to that CDMO. If you look at this relationship on a transactional level, you will lose. To establish a fruitful partnership, you need to look at the relationship with the CDMO as being an extension of your team, and vice versa. On site visits, you can gauge the CDMOs interest in working with you. Through face-to-face discussions, you can tell if they share the same excitement as you, and use such subjective cues to potentially walk away from that relationship, if needed.

When you find a CDMO that wants to partner with you, the next step is to establish a strong foundation for a business relationship that will get you where you ultimately need to go. In other words, you must consider what this relationship and asset lifecycle will look like from clinical through to commercial. Ask yourself: is this CDMO a potential one-stop shop all the way through to commercial manufacturing, or is it more specialized on the clinical side? There is no perfect answer or preference; it is dependent on the holistic manufacturing strategy for the innovator company.

Having selected your preferred CDMO and awarded it your business, you may encounter difficulties when you begin negotiating the Master Services Agreement (MSA). At that stage, you may realize that you are not aligned with the CDMO on aspects such as IP or batch failure. Suddenly, there is the need for a monstrous negotiation, which isn't in the interests of either party. It is therefore critical that during the RFP process, you either obtain a copy of the CDMO's MSA or share your company's template MSA to facilitate an initial discussion to understand the terms and conditions that will need to be negotiated. Terms and conditions and contracts with the CDMOs should be a key component of your selection process.

As discussed previously, a further important consideration in defining the strategic direction of a modern CGT development company is that manufacturing platforms now exist. Today, not every company has to build its own homegrown production process with the required internal resourcing thereof.

There are processes out there that can be tapped into at CDMOs, meaning you only need a relative few subject matter experts in each department for a single asset. It is possible to be a virtual company through the early-phase delivery of clinical trial material. CGT companies should carefully evaluate just how much they need to build in the way of internal assets and capabilities, especially at a time when capital is still relatively difficult to raise. More and more biotech companies are now taking this virtual approach and relying on CDMO platforms.

However, this method does come with its own risks. There are a few items to bear in mind with CDMO platforms, such as IP and transferability. This will be a different situation with each individual CDMO, so it is important to look at those terms and conditions in detail. Understand how locked in you could potentially be and what alternative options are available.

FUTURE DIRECTIONS

We are starting to see more partnerships between CDMOs and innovator companies. They represent a great way to take the pressure off raising capital in the current environment. This can also provide a win-win for both biotech companies and CDMOs, as the biotech companies can try out CDMO platforms and by doing so, help to refine and improve them.

The industry landscape is rapidly changing. Recently, there have been two major occurrences that could have a considerable impact on the CGT industry. Firstly, Novo Holdings acquired Catalent, the largest gene therapy CDMO in the world, for US$16.5 billion—an unprecedented deal driven by the monumental demand for GLP-1 receptor agonist drugs, including Ozempic® and Wegovy®. Novo Holdings has taken the decision to acquire Catalent in order to secure capacity and capability, which is something that nobody predicted. Consequently, from a risk management perspective, gene therapy companies should be thinking differently about the business continuity of their supply chains and trying to understand if this is a one-off deal, or part of a larger trend. Considering this, CGT biotech companies may wish to build a greater degree of flexibility into their contracts with CDMOs moving forward and/or modify manufacturing strategies.

Secondly, the BIOSECURE Act, which was introduced in late January 2024, is another key story to watch, as it may restrict the ability to work with certain named companies in China. This carries a wide range of implications that may affect the overall sourcing of raw materials, intermediates, and products from China. Here, current geopolitical tensions are creating an environment that poses further difficulties to those who are outsourcing to other regions because of the pandemic. This is a serious consideration that needs to be accounted for in manufacturing strategy and business continuity.

It is also important to acknowledge that there is tremendous pressure on pricing for CGT products in general. This is a long-standing talking point in the industry, with key figures like Dr Peter Marks of the US FDA saying that we need to do better. Currently, gene therapies are the most expensive medicines in the world, potentially costing millions of dollars per patient. We need process intensification (and policy negotiation) to get these prices down. This is where advances in platforms can potentially come in to help lower the cost of goods.

A final point to consider around pricing is the Inflation Reduction Act—a result of the tremendous pressure on the pricing of these products. The biopharma industry is still figuring out exactly what this means, but while it is not likely to have as severe an impact as it may on the small molecule drug space, it is likely to make strategy and planning somewhat more difficult for CGT developers, especially those working in non-orphan indications.

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CELL & GENE THERAPY INSIGHTS

VECTOR PROCESSING AND MATERIALS

SPOTLIGHT

INTERVIEW

Harnessing producer cell lines and process intensification to address the AAV process productivity conundrum

In the battle to drive AAV process productivity to the levels required to ensure a successful commercial future, gene therapy developers are increasingly exploring non-traditional tools and methodologies. **David McCall**, Senior Editor, *Cell & Gene Therapy Insights*, talks to **Ying Cai**, Executive Director, Manufacturing Sciences and Technology, Ultragenyx, about the growing impact that producer cell lines and perfusion processes are having on manufacturing yields and consistency.

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Q What are you working on right now?

YC: My team and I are currently working on process validation activities for several late-phase AAV programs. One of our programs is called UX111 (also referred to as ABO-102). It is an AAV9 gene therapy used to treat patients with a rare genetic disorder—Sanfilippo syndrome type A. Ultragenyx announced data in February 2024 that treatment with UX111 resulted in rapid and sustained reduction of heparan sulfate in cerebrospinal fluid, which correlated with improved long-term cognitive development. Our main focus right now is to

partner with Andelyn (CDMO) to execute a process performance qualification (PPQ) campaign and prepare for biologics license applications and marketing authorization applications for commercial launch.

Ultragenyx has a robust pipeline of AAV gene therapies at different clinical phases, and has relied solely on CDMOs to produce clinical materials previously. However, it became clear that internal manufacturing was necessary to enable our long-term success. We finished construction of our 110,000-square-foot Gene Therapy Manufacturing Facility (GTMF) in Bedford, MA last year. This facility is capable of manufacturing AAV with multiple platforms. We have already produced clinical materials using our proprietary Pinnacle producer cell line (PCL™) platform. Several early products with transient transfection process will also be made at GTMF. This GMP facility is well positioned to deliver both clinical and commercial materials, with an ultimate goal of providing low-cost and high-quality gene therapies to patients.

Q Where have improvements been made recently in terms of boosting yields in viral vector manufacturing, and how far do we still have to go in this regard?

YC: It has been a keen industry focus to boost viral vector titer responding to increased clinical demand and the steep cost of approved therapies. Recent approval of Elevidys for the treatment of Duchenne muscular dystrophy also emphasized this need. My company is addressing this issue from three different angles.

First of all, scaling out or scaling up is the most straightforward approach with less burden on process development. We applied this approach to different AAV platforms, and strongly encourage other companies to consider it. For example, pooling 4 × 30 HYPERStacks® (HS) into one downstream process is a lot more efficient than individually processing 30 × HS into drug substance (DS), as in-process sampling and DS testing consumes a large portion of drug product (DP) that could otherwise be destined for patients. Scaling up the suspension culture from 200 L bioreactor to multiple 500 L bioreactors might require scale-up optimization and facility planning. Typically, a manufacturing suite may only have two production bioreactors, but the clarified harvest pool can be concentrated with tangential flow filtration and held frozen before pooled into the downstream process. For new products using the PCL process, we started at the commercial scale (2,000 L) for Phase 1/2 clinical manufacturing and will keep the same process for commercial supply (thus reducing the burden of comparability testing). In short, scaling the upstream scale out or up should be the first consideration to improve overall productivity.

Second, cell line engineering has been our unique strength, particularly on the PCL. Through genome modifications and iterative clone screening, the latest PCL clones showed 50- to 100-fold titer increase compared to the earliest clones. There is also enhanced product quality such as increased full particles and decreased impurities. This has been crucial for enabling Ultragenyx's pathway towards commercial success.

Lastly, process development teams made great strides in applying process intensification techniques to AAV. We have always used perfusion for N-1 stage before the AAV production, but we are now taking it one step further to continue pushing for cell growth post-infection and to optimize/extend AAV production. We demonstrated a >10-fold increase of volumetric titer not only in process development, but also at the pilot scale, and plan to tech transfer to GMP manufacturing soon.

"...the PCL approach demands more effort up front on cell line engineering and offers less flexibility for the candidate screening phase. But it offers major reward to a product with commercial viability."

Ultragenyx is working diligently on all these fronts, leveraging advancements in cell line engineering, process innovation, and different tools to lead and drive improvements in viral vector manufacturing. We believe process intensification coupled with Pinnacle PCL will provide a means to realize truly affordable AAV gene therapies in the near future.

Q Can you tell us more about Ultragenyx's PCL?

YC: The Pinnacle PCL platform was initially derived from the HeLa S3 cell line sourced from the American Type Culture Collection. This initial iteration was a polyclonal packaging cell line with truncated inverted terminal repeats (ITRs) and a truncated P5 promoter. We constructed a single plasmid with all the components—rep, cap, and transgene—and put that into the cell line.

We enhanced that iteration with the next-generation Pinnacle PCL Platform. This version underwent significant genome modifications, incorporating full-length ITRs and transitioning to a biotype P5 promoter for improved stability and performance. Through rigorous screening and genetic optimization, we advanced to a monoclonal cell line, ensuring greater genetic stability across multiple programs.

More recently, we have updated the platform with several tools that enable transient or stable knockdown/knockout of multiple genes. Knocking out these nonessential genes further enhanced AAV production capacity. We have applied the Pinnacle PCL platform to several programs requiring high-dose applications targeting diseases like Duchenne muscular dystrophy.

Q How does the PCL compare with transient transfection- and helper virus-based approaches, particularly in terms of improving productivity? What are the relative pros and cons of the different platforms as they currently stand?

YC: Transient transfection provides flexibility and speed, being a lead choice for fundamental and exploratory research applications. Therapeutic transgenes flanked by AAV ITRs, AAV rep and cap genes, and helper function genes are delivered via plasmids to transfect human cell lines during the production phase. Cells can also be optimized to insert requisite helper genes in the genome, for example, current HEK-293 genome contains stable E1A and E1B genes because it was derived from cell transfection with sheered adenovirus type 5 genes [1]. Major disadvantages of transient transfection process are low titer and high impurities. It lacks the intricacies of a natural viral system, to maintain proper balance of helper gene expression, capsid protein expression, and transgene replication. It often leads to a high percentage of

"We are still at the early stage of understanding and characterizing the AAV product and process, due to several challenges including a lack of industry reference standards, complex analytical methods, and limited knowledge sharing in the CMC field."

defective viral capsids, adding burden to downstream purification or leading to higher capsid dose (to achieve desired genome dose) in clinical studies.

In contrast, the PCL approach demands more effort up front on cell line engineering and offers less flexibility for the candidate screening phase. But it offers major reward to a product with commercial viability. With the genetic makeup of the PCL already tailored to include necessary elements such as rep/cap genes and the transgene flanked with ITRs, there is no need to supply plasmids during production. Although a helper virus bank is needed, it can self-replicate, so only a small volume is required; it is also one time only and generic to all AAV, therefore more favorable in cost than plasmids. Most importantly, AAV production via Ad5 infection resembles natural viral infection, which is the outcome of billions of years of evolution. I think it is still far more efficient than newly developed systems, at least for now, as we don't truly understand all the viral replication and packaging details at the molecular level. We have compared the same AAV product made with transient transfection and PCL processes—the latter showed much higher productivity and full AAV capsid percentage.

Transient transfection and the PCL platform each offer distinct advantages and limitations, and it is unlikely one will replace another in the near future. Understanding the tradeoffs of each platform is crucial to selecting the right process for a given product, ultimately advancing therapeutic developments for various medical conditions.

Q Where do we stand as an industrializing sector in terms of enabling process intensification? What are the key remaining issues and challenges here?

YC: Despite rapid clinical progress, advancements in AAV bioprocessing are limited, and clinical supply remains a bottleneck. Regarding all of the four manufacturing platforms (baculovirus, HSV, transient transfection, and Ad5 helper), the baculovirus platform reported the greatest improvement in scaling up and high-yield production, in addition to optimizing Bac-vector system at the molecular design level [2]. Ultragenyx focused on the advancement of PCL/Ad5 system and started to apply process intensification techniques to PCL platform.

While process intensification was extensively studied with traditional biologics, adapting to AAV production is not easy. There is a key difference of AAV production compared to antibodies or enzymes that are secreted to extracellular environment: viral infection triggers cell autophagy and autophagy-triggered caspase activity leads to adenovirus-mediated cell lysis [3]. Cell death is just the beginning—build-up of cell debris and increased culture viscosity (from host cell DNA) will quickly compromise oxygen transfer and decrease AAV productivity of remaining viable cells.

Ultragenyx has studied different phases of PCL process and put effort into optimizing growth conditions pre-infection and for a short duration after cell infection. We used perfusion at multiple stages and achieved 10-fold increase of volumetric titer. There is ongoing effort to optimize media components and feeding strategy. Downstream also has opportunities in terms of overall yield, automation, and smaller scale. Charged membranes and monolith columns can replace packed columns for efficiency gain. Even recently, simulated moving bed is being considered for viral purification. We also explored continuous chromatography for anion exchange chromatography: with a small column and optimized loading, we achieved a significant yield increase and improved full/empty separation.

Process intensification is a holistic approach to maximize overall cell productivity. As an industry of AAV manufacturing, I think we are just at the beginning of the journey. Merten *et al.* reported 10^6 vg/cell for HeLa + Ad system, with a cell density of 10^7 VCD/mL. We could achieve a potential of 10^{13} vg/ml [4]. Realizing such productivity necessitates a paradigm shift and a concerted effort across the industry. A more ambitious goal is to transform a batch process to a continuous process, which I will talk about later.

Q Regarding the reduction of batch-to-batch variability, what are the key processing steps to take to improve repeatability and robustness in AAV manufacture? How is Ultragenyx addressing this particular issue?

YC: My current role in MSAT is developing PPQ strategies for late-phase AAV programs. As such, I am a strong advocate for understanding and reducing batch-to-batch variability at the manufacturing scale.

As described in the FDA guidance [5], a successful validation program requires the manufacturer to understand the sources of variation, build tools to detect the presence and degree of variation, understand the impact, and finally apply controls commensurate to the risk level. It is a lot more challenging to apply this guidance to AAV than traditional well-characterized antibodies or biosimilars. We are still at the early stage of understanding and characterizing the AAV product and process, due to several challenges including a lack of industry reference standards, complex analytical methods, and limited knowledge sharing in the CMC field. Companies are independently investing in process development, but it often competes with company goals (speed to market and R&D cost reduction). I hope to see more knowledge sharing and industry collaborations in this area, as well as more understanding and flexibility from health authorities (especially for rare disease programs).

Regarding variation, I think it is important to firstly distinguish special cause variation from normal process variation. The former is associated with deviations and can be removed when root causes are identified. The latter is the outcome of many manufacturing variables including production environment, equipment, raw materials, analytics, personnel, and manufacturing procedures. We often focus on controlling critical process parameters, but the other variables should also be considered as well.

Regardless of the complexity of manufacturing variations, there are a few common approaches to consider during process design stage for a more robust process. Firstly, cell growth rate and AAV production rate differ from batch to batch, so it would be beneficial to use growth indicators (such as viable cell density and cell viability) as controls instead of a fixed harvest time. Secondly, upstream titer may vary by several-fold (more for transfection than PCL), so

"One area I would like to highlight is the continued optimization of cell lines. Whilst most focus in the AAV space has been on capsid engineering for tissue tropism, we could apply these approaches to improve AAV productivity."

downstream design could be tailored to the lowest titer and enable multiple cycles for higher titer harvest stream. More specifically, if column loading impacts yield and purity, we could design a smaller column, keep a tight optimal loading range, and vary cycle number by load. Having multiple sizes of tangential flow filtration is another practical approach to better control DS concentration and increase step yield. These approaches can be applied to any AAV manufacturing platform.

It is more difficult to investigate equipment, analytical methods, and raw materials for their contributions to variability. For instance, variations in analytical methods can sometimes be mistaken for process variability. Rigorous method validation and continuous monitoring are essential to ensure the reliability of analytical data. Raw materials also introduce significant variability into the manufacturing process. Any change in the grade of a raw material can have profound implications, as evidenced by our own experience of having to reject multiple GMP batches due to a change in the grade of transfection reagent.

To summarize, Ultragenyx employs a multifaceted approach to reduce the sources of variation in AAV manufacturing. We have invested significant effort in process characterization studies to define critical process parameters and critical raw materials, and implemented extensive sampling and rigorous process monitoring while preparing for a PPQ. We are committed to high quality standards from raw material release to personnel training/qualification in the facility. By implementing all these strategies, we can have a high assurance of delivering consistent and reliable gene therapies to patients.

Q What are the key questions and considerations around adding perfusion to suspension-based production of viral vectors?

YC: We have been actively exploring the integration of perfusion into suspension-based production processes, which is a crucial element of process intensification.

The primary consideration revolves around how to overcome the 'cell density effect'. That is, in the mAb space, process intensification leads to increased cell numbers as well as a higher productivity. But enriching cells or infecting cells at high cell densities could result in a lower AAV yield [6]. Therefore, there are more variables to study to truly increase viral vector titer. Our current approach involves using alternating tangential flow systems for perfusion, although we are closely monitoring advancements in this area, such as the innovative perfusion devices being developed by Repligen [7].

I also think there is potential in leveraging this technique throughout the entire production process, especially the vector production phase, by re-engineering traditional alternating tangential flow devices and by further advancing and refining perfusion-intensified AAV production. With proper membrane pore size, it would allow for continuous extraction of viral vectors from the permeate while simultaneously removing cell debris. One step further, a

feedback loop to supplement fresh cells from N-1 into the production bioreactor would maintain a healthy cell population and allow continuously removing spent cells. This system could operate indefinitely, thus creating a likely continuous cell culture process for AAV. It will be an intricate system to maintain a steady state, but the reward is paramount in terms of efficiency and facility usage.

While this vision may seem ambitious, I think it represents the future of viral vector manufacturing—one product is made from a small-scale single-use bioreactor to supply all patients in the world, and one GMP facility can make hundreds of products at the same time with a small footprint. By pushing the boundaries of perfusion technology and exploring novel approaches of process integration, we can unlock new levels of efficiency and scalability in AAV production.

Q What will be some future directions for technological innovation for both upstream and downstream processing?

YC: One area I would like to highlight is the continued optimization of cell lines. Whilst most focus in the AAV space has been on capsid engineering for tissue tropism, we could apply these approaches to improve AAV productivity. Researchers like David Schaffer from UC Berkeley have demonstrated the effectiveness of directed evolution in improving clinical efficacy [8,9], and it is possible to apply similar methodology to optimize rep/cap genes and other components affecting the production process. It has been reported that modifying the rep gene and optimizing the transgene cassette and ITRs can benefit AAV production. Modifying certain codons in these genes could lead to improvements by modulating mRNA transcription and balancing AAV and adenovirus production.

I have already discussed the potential of continuous AAV production. It has been successfully applied to therapeutic proteins leading to a commercial product nearly ten years ago [10]. If it is realized for AAV, downstream processing may become the new bottleneck. Therefore, we need to place a focus on downstream innovations. Continuous chromatography, membrane-based techniques, and new separation methods tailored to subtle differences of AAV species will transform downstream processing. By seamlessly integrating upstream and downstream operations and leveraging continuous processing strategies, we can streamline manufacturing workflows and reduce variability, ultimately improving product consistency and yield.

Q Lastly, can you sum up one or two key goals and priorities that you have for your work over the foreseeable future?

YC: My team and I have two important goals. Firstly, Ultragenyx research and development teams have spent years improving our AAV analytical methods and manufacturing processes. My team is dedicated to advancing our programs towards commercialization. In the next 2–3 years, we are looking to bring 3–4 programs to the finish line for both biologics license applications and marketing authorization applications submissions, which is something we are really excited about. My priority is to ensure that we successfully execute the PPQ campaigns and ultimately, to help bring these important therapies to market. It is incredibly

meaningful to see years of research and collaboration culminate in therapies that can make a real difference in the lives of patients.

Secondly, I am committed to addressing the issue of high drug prices in the cell and gene therapy industry. We recognize the challenges associated with the high cost of manufacturing these therapies, and my goal is to contribute to efforts aimed at reducing CMC-related expense. We are actively working on incorporating innovations such as process intensification, automation, and second-generation processes to drive down the manufacturing cost. For example, I led CMC development for an AAV9 gene therapy for Wilson Disease. We have successfully reduced clinical cost per patient by ten-fold compared to traditional transient transfection processes. This accomplishment brings us one step closer to making AAV gene therapies more accessible and affordable for patients who need them.

REFERENCES

1. Graham FL, Smiley J, Russell WC, Nairn R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* 1977; 36(1), 59–74.
2. Joshi PRH, Venereo-Sanchez A, Chahal PS, Kamen AA. Advancements in molecular design and bioprocessing of recombinant adeno-associated virus gene delivery vectors using the insect-cell baculovirus expression platform. *Biotechnol. J.* 2021; 16(4), e2000021.
3. Jiang H, White EJ, Ríos-Vicil CI, Xu J, Gomez-Manzano C, Fueyo J. Human adenovirus type 5 induces cell lysis through autophagy and autophagy-triggered caspase activity. *J. Virol.* 2011; 85(10), 4720–4729.
4. Merten OW, Gény-Fiamma C, Douar AM. Current issues in adeno-associated viral vector production. *Gene Ther.* 2005; 12, S51–S61.
5. *US FDA.* Process Validation: General Principles and Practices 2021.
6. Lavado-García J, Pérez-Rubio P, Cervera L, Gòdia F. The cell density effect in animal cell-based bioprocessing: questions, insights and perspectives. *Biotechnol. Adv.* 2022; 60, 108017.
7. Mendes JP, Fernandes B, Pineda E, *et al.* AAV process intensification by perfusion bioreaction and integrated clarification. *Front. Bioeng. Biotechnol.* 2022; 10, 1020174.
8. Jang JH, Lim KI, Schaffer DV. Library selection and directed evolution approaches to engineering targeted viral vectors. *Biotechnol. Bioeng.* 2007; 98(3), 515–524.
9. Byrne LC, Day TP, Visel M, *et al. In vivo*-directed evolution of adeno-associated virus in the primate retina. *JCI Insight.* 2020; 5(10), e135112.
10. Warikoo V, Godawat R, Brower K, *et al.* Integrated continuous production of recombinant therapeutic proteins. *Biotechnol. Bioeng.* 2012; 109(12), 3018–3029.

BIOGRAPHY

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CELL & GENE THERAPY INSIGHTS

INTERVIEW

Challenges and benefits in harnessing platform processes for viral vector manufacture



As the demand for AAV and lentiviral vector-driven gene therapies continues to grow, the manufacturing space must overcome manufacturing challenges including issues of cost, scalability, and consistency. **David McCall**, Senior Editor, BioInsights, spoke with Dark Horse Consulting Group's **Scott Cross**, **Christina Fuentes**, and **Jacob Staudhammer** (pictured left to right) about the current utility and future potential of platform technologies and processes for viral vector manufacturing.

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Q What are you each working on right now?

CF: As part of our consulting practice, I support a range of product types in the cell and gene therapy space with particular emphasis on viral vector and gene editing based technologies. I also support companies across various stages of development, with more recent focus on early-stage companies, providing embedded support ranging from vector design, regulatory writing, and process development.

JS: My role involves providing support to AAV and LVV gene therapy companies across various stages of development, spanning early preclinical development through to late-phase and Biologic Licensing Application (BLA) submissions.

Much of what I have been working on lately involves the middle and late stages of clinical development, where companies are characterizing their processes and planning for BLA submission, and require support with process validation, commercial planning, and comparability assessments.

SC: My main focus so far this year has been running diligence for companies and assets, ensuring companies have robust CMC strategies, and supporting my team.

Q Can you give us your high-level commentary on the key current issues and trends across both AAV and lentiviral vector manufacturing, including those being driven by regulatory guidance evolution?

CF: One of the key challenges across the viral vector manufacturing industry is the complex biological systems involved. Compared to monoclonal antibodies, viral vectors are much larger and more complex modalities, consisting of multiple proteins, lipid bilayers, and nucleic acids; unlike mRNA therapeutics, these systems do not simply involve easily scalable enzymatic reactions. The products we work with are complex and require deep process knowledge and good characterization. This poses challenges in terms of costs and timelines.

However, thanks to evolving analytical methods, our understanding of these products continues to grow. For example, we recently submitted a proposed draft guidance to the US FDA on the measurement and reduction of empty capsids in AAV products. Empty capsids are increasingly becoming a part of the conversation. The recognition of potential safety impacts of these impurities, particularly in high doses and large indications, underscores the importance of refining analytical methods and enhancing product quality control.

Scalability is another crucial consideration in the space at the moment. It is essential to design processes that are not only effective in small-scale production but also scalable for large-scale manufacturing to meet the needs for commercialization as well as of larger patient

"We are frequently asked to provide support when processes are moving into later-stage trials, and the processes are not scaling appropriately."

populations. Investing upfront in scalable processes can yield long-term benefits as the demand for these therapies grows.

Q How do you gauge progress in terms of ongoing efforts to develop platform technologies and processes for viral vector manufacturing?

JS: The answer to this question involves assessing the evolution of gene therapy CDMOs' capabilities, and the alignment between CDMOs and sponsors in supporting the development of safe and effective products.

Currently, many CDMOs have developed, or are developing, platform processes for AAV and lentiviral vectors, which helps streamline sponsors' speed to the clinic and drives down early-stage manufacturing costs. However, these platform processes still require customization to fit each new product, such as particular transgenes or serotypes. Therefore, custom development work is still necessary to tailor the platform processes to the unique needs of each product. Some groups are attempting to develop a one-size-fits-all platform process that requires less individual development, or removes it all-together, but such endeavors have not yet come to fruition. Additionally, sponsors should consider the impact of using a platform process to their commercial COGs—it's important to negotiate commercial and technology transfer terms into your manufacturing agreements early on, ensuring future royalties and milestone payments are clearly agreed upon before clinical manufacturing begins.

As these platform processes continue to become more common, it is even more important to have robust process analytical technology and automation to ensure that nuances within products can be appropriately tailored to each platform in real-time.

Q What are the pros and cons of building these platforms? Firstly, what are some of the key risks, challenges, and considerations in undertaking this work?

SC: The platform process is meant for speed, which often comes at the cost of optimization. However, one size does not always fit all, so spending some time and money early on in the optimization process can pay big dividends later. We are frequently asked to provide support when processes are moving into later-stage trials, and the processes are not scaling

"Platforms provide a starting point rather than a one-size-fits-all solution, but they do serve as a structured foundation for process development, guiding the way forward."

appropriately. This problem requires reoptimization or performing the optimization that was skipped early on, along with potential comparability exercises. All of these extra steps incur additional expenses and additional time, delaying clinical trials.

Q And what are the key benefits of taking a platform approach?

CF: It is essential to understand that your process is *not* your product. Platforms provide a starting point rather than a one-size-fits-all solution, but they do serve as a structured foundation for process development, guiding the way forward.

This approach is particularly advantageous for programs targeting rare indications, where resources, patient populations, and timelines may be limited. While these programs still require an investment of time early on in the process for optimization purposes, having a starting point is hugely beneficial.

Furthermore, platforms leverage prior knowledge from other product types, accelerating development and providing insights into scalability. When leveraged appropriately, this could lead to cost savings and faster timelines, especially in the early stages of development.

Q Reducing batch-to-batch variability is a key point of focus for the field. Moving forward, what tools and strategies offer the potential for advancement in this area?

JS: There are a few important aspects to consider in terms of controlling batch-to-batch variability, but before we can control it, we must firstly understand it and measure it accurately. A highly-characterized reference standard that could be used across multiple platforms and products would be hugely beneficial, but has proven difficult due to the physical differences in serotypes and transgenes. It is also crucial to have reliable and consistent analytics to take those measurements, ensuring consistent repeatability.

Once you can accurately measure batch-to-batch variability, the focus shifts to reducing it. Having a robust and highly automated process that takes out as much variability as possible is important. Utilizing process analytical technology also allows for real-time control and adjustment of the process, allowing more consistent product quality.

"...we still have a long way to go before we have a truly reproducible and automated viral vector manufacturing process."

While we have been seeing gradual advances in this area, with both CDMOs and sponsors making progress, we still have a long way to go before we have a truly reproducible and automated viral vector manufacturing process.

Q Lastly, can you each pick out one or two key goals and priorities that you have for your work over the coming 12–24 months?

CF: I am looking forward to continuing to support clients across multiple programs, providing expertise in areas where we have seen repeat challenges or barriers to success. I am particularly interested in supporting clients in the gene modification field, whether that is with knock-out, knock-in, upregulation, or downregulation. There is a lot of exciting work coming out of that space, and I am eager to see that work advance.

JS: We have seen a lot of advancements in the AAV analytics and manufacturing technologies over the past few years, including the analysis of various types of product-related impurities and heterogeneity in AAV. I have supported a lot of clients in these types of requests, and I am excited to continue to support these programs and help advance the field.

At Dark Horse, we are expanding our service offerings to provide clients with additional support and capabilities for analyzing their AAV products and interpreting the results. I am looking forward to continuing to support clients right from the early stages of their programs and help carry them through commercialization.

SC: Our primary goal every year is to advance the field by progressing these products through clinical stages and increasing access for patients. To support this, my team will continue to expand our offerings and expertise in the viral and non-viral gene therapy space.

BIOGRAPHIES

SCOTT CROSS is a Senior Principal and the Head of Gene Therapy CMC at Dark Horse Consulting Group. Scott is a molecular biologist by training, earning a BS degree from Grand Valley State University and an MS from Western Michigan University. Scott's career has focused on the development, manufacturing, and commercialization of gene and cell therapies and has previously spent time at Merck & Co, Indiana University School of Medicine, Cincinnati Children's Hospital, University of Florida/Florida Biologix, and Orchard Therapeutics.

CHRISTINA FUENTES is a Senior Consultant with DHC and a bioengineer by training, earning a BS degree from Northwestern University and a PhD from UC Berkeley. She has extensive experience in molecular cloning, viral production and purification, CRISPR/Cas9 design and application, and polymer synthesis.

JACOB STAUDHAMMER is a Principal with DHC, holding a BS in Bioengineering from Oregon State University. His expertise includes process development, manufacturing, and analytics for AAV and Lentiviral vectors. Jacob previously worked at Adverum Biotechnologies, Orchard Therapeutics, and CODA Biotherapeutics.

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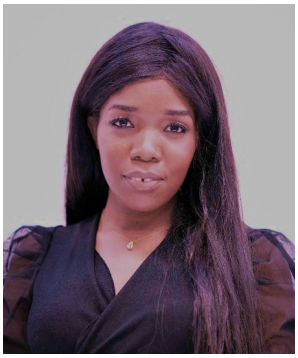
CELL & GENE THERAPY INSIGHTS

VECTOR PROCESSING AND MATERIALS

SPOTLIGHT

INTERVIEW

Delivering on a vision of a digitally enabled future for viral vector manufacturing



Consultants **Sönke Brunswieck** and **Tania Chilima** share their thoughts on the need for a holistic approach to product and process development in the gene therapy space—an objective that can only be achieved by correctly harnessing the power of digital tools.

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Q What are you working on right now?

TC: Currently, my focus lies in providing strategic consulting services to companies across various domains. I specialize in enhancing product and portfolio value propositions, identifying opportunities for cost optimization in goods and services. In addition to my consulting work, I am collaborating with Sönke on a groundbreaking project. We are spearheading the establishment of a new company (Newco) aiming at transforming the decision-making processes within the bioprocessing industry. By empowering bioprocessing companies with

data-driven insights and predictive models, ultimately enhancing their operational efficiency, productivity, and overall success.

This endeavor aligns perfectly with my passion for innovation and my expertise in leveraging technology to drive transformative change. I'm excited about the potential impact our project could have on the bioprocessing sector and beyond.

SB: About 2 years ago, I founded 7AlpsBio, a company with a focus on strategic, operational, and capex project consulting especially aimed at companies and organizations in the field of cell and gene therapy. In this short time, my new environment has enabled me to get to grips with new developments at breathtaking speed. And there were and are more than enough of them in cell and gene therapy spanning various areas, whether it is facility engineering and related strategies, process technologies and manufacturing platforms, or the development of new and optimized cell and gene therapy products. These three pillars are still almost always only considered or developed in isolation from each other, but we take a holistic approach to them through Newco. In addition, we also focus on the networking of all information and knowledge within a company right down to the patient level. This represents a quantum leap, so to speak, from decision-making based on faith. In other words, moving to quantifiable analyses and predictable decisions based on facts and knowledge rather than possibilities and feelings. With the help of this approach, it is possible to map reality and scenarios in advance and plan optimal strategies.

The breadth of benefits from this approach is probably unforeseeable at the moment, but it will hopefully help to successfully translate the immense potential of these highly innovative therapies into products that reach the market, thus helping the many patients who need them to finally get a chance to actively participate in life again.

Q How would you describe the recent history and evolution of viral vector bioprocessing tools, particularly in the digital realm—what have been the key developments, for you?

TC: The recent history of viral vector bioprocessing tools has witnessed remarkable advances across all domains, from upstream to downstream processing and analytics, with a notable emphasis on digitalization. In upstream processing, there has been a surge in the development of improved cell lines boasting >ten-fold increases in titer, alongside efforts to enhance stable producer cell lines, particularly in the lentivirus sector. Companies still relying on transient transfection have seen progress in the utilization of multi-gene plasmids to address issues related to process robustness and cost efficiency. Moreover, novel transfection reagents and additives have emerged, promising increased transfection efficiencies and higher yields and better product quality. Moreover, the shift towards suspension cell-based processes has gained momentum to augment capacity and process understanding.

Advances in downstream processing have centered around boosting yields and refining full/empty capsid separation techniques. Analytical tools for characterizing viral vectors have

"By leveraging digitalization, bioprocessing companies can achieve greater efficiency, agility, and precision in developing and scaling up viral vector production."

evolved to meet industry needs, with a steadfast focus on identity, strength/potency, purity, safety, and stability. Techniques such as PCR, genome sequencing, and mass spectrometry have become indispensable for confirming genetic and protein identities, ensuring product consistency and safety. Moreover, Advanced methods like droplet digital (dd)PCR are gaining traction for their enhanced sensitivity and reproducibility in quantifying viral genomes.

In recent years, the implementation of digital tools has been pivotal across various axes of bioprocess operations, extending from personalized medicine management to mechanistic modeling aimed at elucidating and optimizing critical process steps such as transfection. Digital twins, in particular, have emerged as powerful predictive models, facilitating simulation and optimization of bioprocessing workflows. By leveraging digitalization, bioprocessing companies can achieve greater efficiency, agility, and precision in developing and scaling up viral vector production.

Q Can you characterize the current state of implementation in the gene therapy space, as you see it?

TC: The current state of implementation underscores the critical importance of digitalization tools. There is widespread recognition within the gene therapy industry that digitalization is not just advantageous but essential to its growth and success. However, a significant challenge lies in accessing and utilizing data effectively. Many companies face hurdles in data sharing due to intellectual property protection concerns, necessitating the generation of data in-house. This process can be both costly and time-consuming. Particularly in the context of gene therapies where high-throughput scale-down models and inline measurement tools for data collection and analysis are lacking. As a result, technology gaps persist in this area, presenting challenges for viral vector developers who are often constrained by both financial resources and time. Balancing immediate priorities with the investment required for advancing digitalization efforts remains a formidable task in the gene therapy space.

SB: If you look at the current status of the sector, which is about further optimizing processes, increasing the safety and effectiveness of gene therapies, and harnessing automation and robotics to reduce costs and increase quality, you can't help but realize that all of this together can only be achieved with the help of digitalization. Actually, there is much deep-dive expertise and many platforms coming from the biologicals industries, but relatively little of this meets the specific development requirements of personalized medicines, such as the circumstances that must be dealt with when working with living cells of our own immune

"Artificial intelligence and democratizing data-driven decision-making will be critical to success."

system and cell signaling cascades. Furthermore, we have to consider that cell and gene therapies, or any other personalized medicines, will need to combine an understanding and deep knowledge not only of medicine, but also of physics, chemistry, and biology. Artificial intelligence and democratizing data-driven decision-making will be critical to success.

Without new approaches such as ours, my fear would be that we would lose many years, many investments, and many opportunities. In all likelihood, we would even end up in despair if we had to recognize that we are not sufficiently able to process all the information efficiently and use it successfully to provide the best possible therapies.

Q What are the key learnings that you would draw to help carry the industry forward towards harnessing the full potential of these tools?

TC: Investing early in high-throughput, representative scale-down models and developing inline analytical methods are crucial steps to harnessing the full potential of digitalization tools in the gene therapy industry. Firstly, these investments allow for a deeper understanding of bioprocessing dynamics, enabling companies to identify critical process parameters and potential bottlenecks more effectively. This understanding is essential for optimizing processes early in development, ultimately leading to improved efficiency and productivity. Additionally, inline analytical methods facilitate real-time monitoring of key variables, enabling process control and ensuring process consistency and quality. By prioritizing the development of such tools, the industry can overcome current challenges and pave the way for more streamlined and cost-effective gene therapy manufacturing processes.

Q What is your vision for a future where viral vector manufacture makes full use of available and forthcoming vector bioprocess technology, and what will be the key steps towards harnessing this power?

TC: The key lies in harnessing the power of advanced digital tools and fostering collaboration across the industry. My vision entails the creation of anonymized data libraries encompassing diverse variables present in the gene therapy landscape, including patient characteristics, capsids, vectors, cargo, and more. These data libraries would serve as invaluable resources for

rapid product development and optimization, facilitating informed decision-making and reducing the need for costly experimentation.

Advanced digital tools, such as real-time monitoring systems and mechanistic modeling platforms, will play a central role in this future scenario. These tools enable precise control of critical process parameters, optimize workflows, and empower predictive simulation of process behavior, ultimately accelerating development and scale-up. Additionally, seamless data integration and collaboration facilitated by digital platforms foster transparency and innovation across cross-functional teams, enabling knowledge sharing and accelerating collective progress.

Furthermore, advanced analytics and decision support tools, powered by machine learning algorithms, will drive continuous improvement by optimizing processes and enhancing yields. Automation and robotics will streamline workflows, enhancing efficiency and reproducibility whilst minimizing human error. By embracing digitalization and fostering collaboration, the gene therapy industry can unlock unprecedented levels of efficiency, agility, and productivity, ultimately expediting the development and delivery of life-saving therapies to patients worldwide.

SB: I share Tania's vision that the future of viral vector manufacture will be one where we can guarantee the highest levels of safety and efficacy, combined with the highest level of quality and the ability to limit cost of goods at any scale and manufacturing site. Considering the need for scalability and consistency from laboratory-scale onwards while maintaining the quality of the product will have to be a routine pathway for all from early in development, independent of the size and funding of the R&D project. As Tania pointed out, the creation of anonymized data libraries is crucial to enabling that, and overall, the implementation of digital tools will be the central key that unlocks all doors for gene therapy manufacturing.

BIOGRAPHIES

SÖNKE BRUNSWIECK has close to 25 years of experience in the biopharmaceutical industry in the areas of pharmaceuticals, diagnostics, and cell and gene therapies. Sönke is Founder and CEO of 7AlpsBio GmbH based in Switzerland. Before 2022, Sönke was Vice President of Cell and Gene Therapies at Celonic AG in Switzerland and a member of the management team. Prior to Celonic, Sönke worked at PharmaCell BV from 2013 to 2018 as Director of Business Development and a member of the management team. Sönke started his work in cell and gene therapy back in 2002 at CellGenix GmbH in Germany, where he was Director of Global Marketing and Sales, and a member of the management team for more than 10 years. Sönke began his professional career in the pharmaceutical and diagnostics industries, working in business and marketing for Pharmacia Upjohn, Glaxo Wellcome, SmithKline Beecham and Innogenetics Group. He earned his doctorate in biology at the Klinikum Rechts der Isar and the Technical University of Munich and holds master's degrees in biology from the Max Planck Institute for Psychiatry in Munich and the Technical University of Munich, as well as a business trainee from the University of Applied Sciences in Munich.

TANIA CHILIMA is a biochemical engineer, inventor, and entrepreneur. She currently works as an independent consultant focusing on providing strategic consulting services to companies across various domains with emphasis on enhancing product and portfolio value

propositions and identifying opportunities for cost optimization in goods and services. Previously, she held the position of Chief Technology Officer at Univercells Technologies. Tania's post-doctorate research at UCL and the Bill & Melinda Gates Foundation focused on building advanced decisional tools to facilitate cost-effective regional vaccine manufacture in low- and middle-income countries. Her Engineering Doctorate from University College London and Pall Life Sciences delved into financial and operational perspectives of cell therapy bioprocessing, addressing critical questions and optimizing strategies for cost-effective production. Tania's educational background includes a BSc in biochemical engineering from University College London, where she still continues to teach as a guest lecturer on commercialization, regulation and ethics of advanced therapies.

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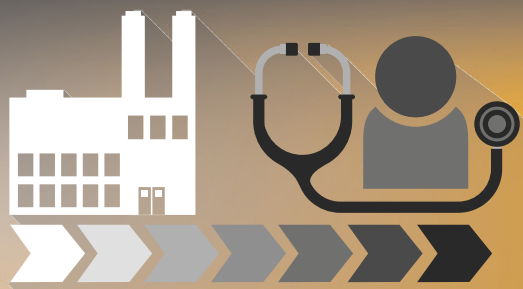
CELL & GENE THERAPY INSIGHTS

SUPPLY CHAIN CHANNEL EDITION

Scaling the supply chain



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CELL & GENE THERAPY INSIGHTS

SCALING THE SUPPLY CHAIN

CHANNEL CONTENT

EXPERT INSIGHT

Innovation in hematopoietic stem cell cryopreservation and cold chain management

Michele Prisciandaro, Michele Santodirocco, Giuseppe Fania, and Michele Vacca

Over the last decade, hematopoietic cell transplantation (HCT) and the demand for cell therapies have expanded significantly due to their potential to treat various hematological and non-hematological malignancies [1–4]. The successful implementation and standardization of cryopreservation and cold chain management will be crucial in this context. What is the importance of cryopreservation in the era of new cellular therapies? What are the critical factors to manage?

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Cryopreservation is the primary method for long-term cell preservation, significantly extending cell viability and providing ample time for quality control and regulatory testing, thereby facilitating clinical application of the product. This procedure must ensure that the cells maintain their viability and functionality after thawing and facilitate rapid and durable engraftment in the days following infusion, leading to an effective and safe transplant procedure. Several cryopreservation protocols are employed worldwide. Cells products should be processed and stored in accordance with the relevant Medical Council, responsible local, overarching authorities, as well as scientific society guidelines [5] and cell therapy companies.

Despite the widespread adoption of regulatory standards, quality control measures, and testing protocols for frozen products

across all processing laboratories, several challenges persist, including cell viability loss following thawing, adverse events induced by cryoprotectant toxicity, and the standardization of cold chain management. These factors are of significant importance for ensuring reproducibility, patient safety, and optimizing cost–effectiveness. Additionally, the commercial or academic development of cell therapy products, such as CAR-T cells, presents further challenges. These products can serve as both starting material (intermediate product) and post-production drug. In either case, suboptimal cryopreservation, transport, and storage can have a detrimental impact on the quality and efficacy of these specialized and costly products.

The complexity of current and future cold chain that support the transport of live cells and engineered tissues, organs has been highlighted by the worldwide SARS-Cov-2 pandemic experience [6]. For more than 2 years, the COVID-19 pandemic has had a significant impact on HCT. During this period, transplant centers have implemented notable alterations to their procedures, including the adoption of alternative donor types and the unprecedented utilization of cryopreservation for allogeneic hematopoietic progenitor cell grafts [7].

Despite documented adverse reactions associated with its use which can range from mild to life-threatening [8–13], dimethyl sulfoxide (DMSO) remains the gold standard for cell cryopreservation and is the most widely utilized cryoprotectant for hematopoietic stem cells (HSCs) and it is universally used as a cryoprotectant in clinical adoptive cell-therapy settings to treat hematological malignancies and solid tumors [14]. What are the current strategies and actions to mitigate cryoprotectant-associated adverse events?

Cryopreservation can damage cells through cryoinjuries caused by osmotic shock, dehydration, solution effects, and mechanical damage from ice crystal formation during freezing and thawing. However, two key strategies can mitigate these injuries: cryoprotectants (CPAs) and controlled-rate cooling.

At present, the primary cryopreservation strategies are freezing and vitrification. They are typically classified based on whether ice crystal formation occurs during sample cooling or is effectively suppressed, respectively [15]. The preservation of cells through freezing necessitates a delicate equilibrium of protective measures to counterbalance the detrimental effects of ice formation and temperature fluctuations. This balance must also safeguard the efficient recovery and viability of cells, as well as minimize adverse reactions in patients induced by cryoprotectants.

In most cell therapy processing facilities, technologists constitute freezing media or solutions using various reagents and concentrations per institutional and cell product policy. There is no current 'standard' or uniform solution used across the cell processing field for cryopreservation [16–18].

DMSO is a commonly used cryoprotectant, but it can cause dose-dependent adverse events during and after transplantation. These adverse events can stem from the DMSO itself, DMSO-induced histamine release, or cell debris and lysis products present in the thawed HPC grafts [19,20]. Studies have shown a link between the amount of DMSO in the HPC graft and/or the granulocyte content, and the occurrence and severity of these adverse reactions [19–24].

Several techniques can be employed to alleviate adverse events associated with DMSO:

1. Pre-freezing methods:

- Decreasing the initial concentration of DMSO used in the cryopreservation solution
- Adding a second cryoprotectant to the solution to dilute the concentration of DMSO
- Changing ice pattern formation
- Using alternatives to DMSO

2. Post-thawing methods:

- DMSO removal
- Extended infusion time of the HPC graft [25]
- Hydration after reinfusion of HPC [26]

PRE-FREEZING METHODS

Decreasing the initial concentration of DMSO used in the cryopreservation solution

Cryopreservation of cells is generally performed using a solution containing a final concentration about 10% DMSO. This technique was established in the 1950s [27] and is now commonly used in cell banks. The European Society for Blood and Marrow Transplantation (EBMT) handbook reports that the final product contains 5–10% dimethyl sulfoxide (DMSO) as a cryoprotectant and 0.05–0.25 mL of acid-citrate dextrose (ACD-A) stabilizer solution per milliliter of transplant. Freezing at a controlled rate of 1–2 °C per minute is recommended. Cells need to be stored in vapor or liquid phase nitrogen at a temperature of ≤–140 °C [5]. Recently, ultra-low temperature freezers are replacing liquid nitrogen tanks [28].

Several studies have investigated the impact of DMSO concentration on HSC cryopreservation [29–35]. A clinical study showed faster white blood cell recovery for patients receiving autologous transplants with PBSC cryopreserved in 7.5% DMSO compared to 10%, but the frequency of adverse events (AEs) was unchanged among two groups [30]. A similar neutrophil and platelet engraftment was also observed for PBSC cryopreserved with 5% and 10% DMSO [33,35]. Instead, Fry *et al.*'s research on cord blood (CB) cryopreservation found that using a 5% DMSO concentration resulted in cell loss compared to samples frozen with 10% DMSO. Their findings suggest a 7.5–10% DMSO range as optimal for CB cryopreservation, with a maximum exposure time of less than 1 hour before freezing and 30 minutes after thawing [36].

A prospective randomized study of Mitrus *et al.* showed reduction in DMSO concentration had no impact on engraftment, but adverse reactions were lowest in patients who received cells had been frozen in 5% DMSO [31]. A recent review of preclinical and clinical trials suggests that reducing the concentration of DMSO to 5% could become the new standard for hematopoietic stem cell cryopreservation [30,31,37].

Adding a second cryoprotectant to the solution to dilute the concentration of DMSO

Complementation of DMSO with sugars or alternative CPA can now be used to reduce his concentrations and minimize the DMSO side effects for patients and cytotoxicities.

The inclusion of macromolecules, such as polymers, proteins and/or polysaccharides, in cryopreservation solutions is widespread [38]. For example, hydroxyethyl starch (HES) [39–41], autologous plasma and human serum albumin [42], dextran, PEG and trehalose [43] (amongst others) are added into cryoprotectant solutions.

In vitro studies of Stiff *et al.* [41] and McCullough *et al.* [39] explored the use of HES mixed by DMSO to enhance HSCs cryopreservation. Both studies showed that *in vitro* measurements indicate that HSCs can be successfully frozen and stored using a combination of DMSO and HES providing smaller amounts of DMSO. Rowley *et al.* [40] performed a single-blinded, randomized study comparing these cryoprotectant solutions for patients undergoing autologous peripheral blood stem cell transplantation. A total of 294 patients were evaluable; 148 received cells frozen with 10% DMSO and 146 received cells frozen in 5% DMSO/6% HES. In this study they found that patients who received cells frozen with this combination recovered

their white blood cell count faster (median of 10 days) compared to those who received cells frozen with the standard method (DMSO alone). This difference was statistically significant (p-value=0.04). The benefit was even greater for patients who received a higher number of stem cells. However, there was no significant difference in platelet recovery time between the two groups. Additionally, the number of red blood cell and platelet transfusions needed were similar for both groups.

Smagur *et al.* [42] investigated whether autologous plasma (AP) could replace human albumin (HSA) in cryopreservation solutions containing 7.5% DMSO prediluted with 5% HSA or AP. Their findings showed that median recovery of nucleated cells and the number of colony-forming units did not differ between tested cryoprotective mixture, and clinical part of the study found no significant difference between the two groups in terms of WBC and platelet engraftment.

In another study, Chen *et al.* [43] compared the effects of different cryoprotectants on stem cells from umbilical cord blood. The cryoprotectants tested were: 10% ethylene glycol and 2.0% dimethyl sulfoxide (DMSO) (v/v), 10% DMSO and 2.0% dextran-40, 2.5% DMSO (v/v) + 30 mmol/L trehalose, and a group with no cryoprotectant. The data showed that solutions containing trehalose exhibited higher cell viability and CFUs and a lower apoptosis rate after thawing than either group.

These findings suggest that it is possible to reduce DMSO concentration by adding macromolecules to the CPA solution. There is growing interest in discovering new synthetic and natural polymers that can replace or reduce the amount of organic solvents and increase post-thaw yields, including those that do not have specific ice binding or ice recrystallization inhibitory activity [38].

Using alternatives to DMSO

Trehalose and sucrose emerged as the most prevalent alternatives to DMSO in the context of HSC cryopreservation. Trehalose is a small disaccharide of glucose that does not permeate mammalian cell membrane, it is considered an osmolyte that can stabilize proteins and cell structures during freezing and it protects cells by stabilizing membranes and proteins by direct interactions through the water replacement hypothesis [44,45]. Trehalose has been approved for human consumption and has demonstrated a good safety profile, with no adverse effects reported up to a maximum oral dose of 50 g [46]. It has been explored in cryopreservation protocols for various human cell types [47–51]. Notably, research suggests its potential use in alternative cryopreservation methods for clinical applications involving stem cells derived from CB, bone marrow, mobilized peripheral blood (PB), and non-mobilized PB [52–55].

Mantri *et al.* study's results revealed 0.5 M trehalose and DMSO 5% showed the highest viability of 91.8±2.8% of HSCs; 5% DMSO inclusion to trehalose (0.5 M) ameliorated hematopoietic colonies such as erythroid and myeloid colonies with no significant difference from that of 10% DMSO [56].

Studies have demonstrated their ability to directly interact with cellular membranes during the freezing process. This direct interaction, along with their capacity to influence ice crystal formation patterns, has rendered them promising additives or standalone cryoprotective agents [55,57].

Other DMSO alternatives: development of new commercial solutions in the likes of CryoSoFree™ (MilliporeSigma), CryoNovo™ (Akron Biotech), StemcellKeep™ (Diagnocine), CryoProtect Pure™ (AdInfinitum Cell Preservation Technologies—Spectacular Diagnostics) and Pentaisomaltose® (PIM) has been investigated in the context of hematopoietic stem cell grafts [58–61].

Moreover, amino acids and derivatives such as poly-L-lysine , glycine and isoleucine have been studied in different type of cells [62–66]. The carboxylated ε-poly-L-lysine exhibited remarkable cryoprotective properties, enabling the recovery of viable mouse fibroblast cells in

the absence of DMSO [42] and demonstrating potential for preserving human mesenchymal stem cells [62], and natural killer cells for cancer immunotherapy [67]. Rami *et al.* demonstrated the preservation of human cytokine-activated natural killer (NK) cell viability and function following cryopreservation using a cocktail of biocompatible, bioinspired cryoprotectants (i.e., dextran and carboxylated ε-poly-L-lysine). Results showed that the recovered NK cells after cryopreservation and rewarming maintained their viability immediately after thawing at a level comparable to controls (dimethyl sulfoxide-based cryopreservation). However, their viability dropped in the first day in culture compared to controls. Nevertheless, the cells grew back to a level comparable to controls after 1 week in culture [67].

Changing ice pattern formation

Changing ice pattern formation can influence the freezing response. Wu *et al.* reported that the supplementation of the cryoprotectant solution with mono- and di-saccharides possessing the ability to inhibit ice recrystallization improved the post-thaw viability of $CD34^+$ cells from cryopreserved UCB [68]. A category of small molecules, ice recrystallization inhibitors (IRIs), has been identified that can control ice growth or recrystallization. These molecules have been shown to be effective cryoprotectant additives for HSCs and UCB [68,69].

IRIs, which are carbon-linked antifreeze glycoprotein analogs, block ice recrystallization during freezing and thawing. This reduces the average ice crystal size in frozen samples, ultimately protecting cells from cryoinjury [38,69,70].

POST-THAWING METHODS

DMSO removal

Previous washing for purposes of DMSO depletion is not routinely performed, as the loss and damage of HSCs are regarded as too high. According to a survey among transplantation centers by the Cellular Therapy and Immunobiology Working Party of the European Society for Blood and Marrow Transplantation (EBMT) and, only a minority of responding institutions remove DMSO before infusion [71]. Several methods for removal of DMSO before infusion have been developed, ranging from centrifugation to dialysis-like filtration, and diffusion-based approaches [72–74].

Centrifugation methods can be either manual or automated. Manual centrifugation methods were found to reduce the occurrence of AEs, but also to reduce post-wash $CD34^+$ counts, to increase time to platelet (PLT) engraftment in some series, and to raise overall cost [75].

Automated washing methods include commercially available washing devices, such as the CytoMate™, Sepax™, and Lovo™, which have been developed to automate the washing process. Studies have demonstrated high recovery of viable $CD34^+$ cells with good engraftment potential after automated washing using these devices [76–78]. These devices need a medium. Recent reports [79] described the validation of the use of a colloid solution of succinilgelatine (Gelfusine™) as an alternative to 10% dextran for washing thawed HSCs products with removal of DMSO.

Several new methods of DMSO removal without the need for centrifugation are currently being investigated. These methods include filtration using spinning membranes, DMSO extraction through diffusion in microfluidic channels and dilution through hollow-fiber membranes. They present several limitations, including the processing of large volumes of products, cell loss, and high costs [37].

Ensuring accurate representation of the overall quality and viability of the product using the vials associated with the product is a critical issue that is often highlighted during regulatory inspections. What are the main perspectives on this issue?

Consensus on the optimal methods for assessing post-cryopreservation viability of HPC products remains unclear [80]. While culture-based clonogenic assays, flow cytometry, and image-based approaches offer viability insights, each method presents limitations. Culture-based assays are time-consuming and subjective [81], flow cytometry techniques lack standardization [82], and image-based approaches risk misinterpretations. Furthermore, no established viability or cell recovery threshold exists to predict successful engraftment, hindering clinical decision-making based on these values. Despite the requirements of voluntary accreditation organizations, such as the Association for the Advancement of Blood and Biotherapies (AABB) and the Foundation for the Accreditation of Cellular Therapy (FACT), for a written HPC stability program for cryopreserved products [83,84], a lack of a consensus test to predict HPC engraftment contribute to the challenges of establishing such a program [85].

The EBMT recommended that a reference sample (vials) must be cryopreserved under identical conditions as the cell product and assessed for $CD34^+/CD45^+$ cell viability prior to product release. Moreover, during the pandemic, the WMDA strongly recommended that conditioning not be commenced until the viability of the cryopreserved HPC product is established using an attached segment or retention/pilot vial [86]. This would enable the compromised products to be identified in a timely manner and a second graft to be collected from the donor. However, this precautionary measure has not been widely implemented in the past [82], and accordingly, there is limited data on how well it correlates with product viability [87].

Although a standard test for baseline product viability hasn't been established, the CFU assay offers insight into the function of specific stem cells and progenitor cells. However, current methods based on the morphology of colonies and cells for evaluation have limitations. Despite the importance of this test, the subjectivity of colony identification due to user variability and differences in inter-laboratory formation may hinder reproducibility [88–91]. The EBMT recommended that performing a clonogenic assay (e.g., colony-forming assay) of the vials is not considered a release criterion and should only be conducted during process validation procedures or in the event of extended cryostorage (>2–5 years) [5].

The scientific literature has not yet reached a consensus on the precise correspondence between cryopreserved viability of the product and its reference samples. Some studies report that pilot vials exhibit lower colony formation after cryopreservation and thawing compared to the corresponding product [82,92–94]. This discrepancy between viability and poor colony formation could be attributed to the way the vials are prepared at the end of the product preparation process, the stratification of the content in a small container compared to the bag, which would impact the penetration of DMSO inside the cells, the type of pilot vial or attached sample (vial, segment, microsac) and their exposure to faster freezing rates due to their smaller size (1–2 mL).

As is known, a major cause of cellular injury during cryopreservation is attributed to the growth of ice crystals during the thawing process in a phenomenon known as ice recrystallization. Ice recrystallization leads to an increase in the mean ice crystal size which can cause mechanical damage to cell membranes [68].

Thawed HSC cells are particularly vulnerable and require careful handling. Slow thawing can lead to mechanical damage. This occurs when intra- or extra-cellular ice crystals, instead of melting, grow larger during the process (recrystallization). This enlargement can rupture cell membranes, causing irreversible damage and cell death. To prevent this, rapid thawing is crucial to minimize ice recrystallization. Studies have shown that thawing HSC cells at around 100 °C/min using a 37 °C water bath yields the best results in terms of post-thaw recovery and viability [95].

Another way to prevent the discrepancy could be improving the handling of pilot vials intended for functional evaluation could contribute to greater reliability. For example, storing pilot vials in racks and frequently removing them from the freezer to store samples could expose them to repeated reheating cycles, compromising their viability. The use of dedicated vial freezing containers could mitigate this issue by slowing the rate of vial freezing [96]. In addition, it is necessary to periodically (at least annually) perform correlation studies between vials and their respective products by comparing the viability of cells in the vials and the cells in the infused product. However, some studies support the correspondence between vials and product viability [97–100], and other suggest that neither the type of container (bag or vial) nor the freezing temperature has a major impact on how well HPCs survive cryopreservation [97].

Some studies have suggested a negative effect on the quality of the biological material caused by an increase of cell concentration [101,102]. These authors suggested a relationship between the quantity of nucleated cells in the graft and delayed engraftment. What are the detrimental factors to the quality and functionality of the cryopreserved product?

According to the literature and EBMT guidelines, adherence to stringent collection parameters and intermediate storage conditions prior to cryopreservation is critical for successful cryopreservation outcomes [74].

The collection goals are: concentration of nucleated cells (NC): $\leq 2 \times 10^8$ NC/ml, with a maximum of 5×10^8 NC/ml; volume: 100–400 ml; hematocrit ≤5%, with a maximum of 10%. Intermediate storage conditions are: storage temperature: +2 °C to +6 °C, NC concentration: $\leq 2 \times 10^8$ NC/ml, time between collection and cryopreservation ≤48 hours (strong recommendation), with a maximum of ≤72 hours [5,103]. Any deviation from these goals can have detrimental effects on the product's functionality and, consequently, on the engraftment [104].

Cell concentration is an important parameter that requires careful consideration before freezing, as a low cell concentration is associated with more DMSO usage, higher cost, and greater patient toxicity [37]. Despite these factors, the influence of cell concentration on cryopreservation outcome is not well studied [105].

As suggested by two study a cell concentration of 2×10^8 cells/mL still yielded high recovery of viable cells and excellent engraftment after autologous PBSC transplantation [105,106].

A significant correlation was found between delayed engraftment and the number of granulocytes in the product greater than 2×10^8 NC/ml. The authors suggest that this negative effect is due to the damage to the homing of stem cells caused by the pro-inflammatory cytokines and metalloproteases produced by granulocytes. *In vivo* persistence of metalloproteases has a negative feedback effect on stromal-derived factor-1 (SDF-1), which plays a key role in homing via the CXCR4 receptor. The increase in cytokines (IL-8, IL1β, IL6) and metalloproteases (MMP-9) would therefore lead to a delay in engraftment [102].

Even an earlier study indicated a cell concentration of $3.7 \pm 1.9 \times 10^8$ for cryopreservation of PBSCs did not result in loss of engraftment potential [107].

However, it is important to note that high cell concentration in cryopreserved products can be detrimental, potentially leading to cell loss, clumping after thawing, or even seizures during cell infusion. Therefore, special handling is crucial for products with high cell concentrations [108,109].

Cold chain compliance for cellular products is a series of meticulously managed steps to ensure temperature compliance within specific parameters throughout the entire supply chain. What are the main challenges in maintaining an efficient cold chain?

Cold chain compliance for cellular therapy products is essential to preserve the biological characteristics, viability, and functionality of

the product throughout all phases of manufacturing, cryopreservation, storage, transportation, and distribution.

Key elements of an efficiently, safely, and tightly monitored cold chain include [110–113]:

1. Understanding product stability under processing, storage, preservation, and distribution conditions: it is important to generate data to support the stability of the product within the temperature range it will be exposed to during distribution. Typical temperature ranges for cellular therapy products are: i) controlled ambient temperature (15–25 °C) or refrigerated (2–8 °C), both of which require just-in-time delivery of cells to patients after manufacture of the cellular product; or ii) cryogenic temperature (vapor phase of liquid nitrogen [LN2]) for cells cryopreserved or for transportation via dry shipper;

2. Another critical factor is the freezing rate. Two main approaches exist: controlled-rate freezing and uncontrolled-rate freezing in a mechanical freezer at −80 °C or −135 °C [39,114–116]. The chosen program significantly impacts cell viability. Excessively rapid cooling can lead to intracellular ice crystal formation, potentially rupturing cell membranes. Conversely, overly slow cooling promotes extracellular ice formation, causing cellular dehydration through osmosis [117,118]. Regardless of the freezing method, storage of HPCs typically occurs between −80 °C and −196 °C;

3. The effect of storage in vapor-phase nitrogen versus liquid nitrogen was studied and no differences in total WBC recovery were found between storage in vapor phase or liquid nitrogen [116, 119];

4. Courier qualification: couriers must be qualified and trained to ensure that transport temperatures are maintained throughout the journey. They must also ensure the safety and traceability of the product, such as by avoiding exposing the product to X-rays;

5. Container qualification: containers must be qualified through validation testing and must ensure the maintenance of transport temperatures and the integrity of the product contained therein. This point includes qualification of shipping containers for both temperature maintenance and physical protection (dry shipper). It also encompasses post-shipping or post-thaw container/closure integrity testing to guarantee sterility and maintain production quality;

6. Product monitoring during shipment: it is important to monitor all shipments using portable temperature recorders to confirm the quality conditions of the shipment.

The main challenges in maintaining an efficient cold chain for cellular products include:

1. Temperature excursions and Interruptions in the cold chain: frozen cell products are not stable when exposed to ambient temperature despite their frozen appearance. Transient warming events (TWEs) are defined as brief exposures of a cryopreserved product to temperatures above a recommended critical storage temperature [60]. TWEs can result in the growth of small ice crystals into larger ice crystals (re-crystallization) which can irreversibly damage cells. TWE can occur during routine operational activities such as the retrieval of vials for quality testing or during retrieval of a HPC from the liquid nitrogen (LN2) dewar storage tank for shipping and distribution. Poor cryopreservation procedures or TWEs have been reported to reduce the viability and potency of lymphocyte [120], PBMC [121], and MSC [122,123] products;

2. Human error: human error, such as mishandling of products or incorrect temperature monitoring, can also contribute to cold chain failures.

To mitigate these challenges, it is important to implement a comprehensive cold chain management program that includes:

1. Thorough training of all personnel involved in the cold chain;
2. Use of validated processes and equipment;
3. Regular monitoring of temperature compliance;
4. By taking these steps, healthcare organizations can help to ensure that cellular products are delivered to patients in a safe and effective manner.

Processing and Cryopreservation Facility, part of a comprehensive transplantation program, is subject to regular inspections to ensure adherence to established guidelines, certifications, and national regulations. In particular, manipulations and cryopreservation laboratories must maintain and present comprehensive documentation to demonstrate compliance with these requirements.

What specific documentation does a manipulation and cryopreservation laboratory need to maintain and provide to regulatory inspectors to demonstrate compliance with applicable regulations and guidelines?

Within the Processing and Cryopreservation Facility, comprehensive documentation of all processing and preparation steps, from receipt from the collection facility to release to the clinical unit, must be maintained in either paper or electronic format and readily accessible for review [84]. A comprehensive product dossier must include, at a minimum, the following documentation:

1. Receipt and eligibility: consent for manipulation, eligibility documents, release documents from the collection facility;
2. Characterization and qualification: virological qualification exams, collection yield report, storage location records, treatment documentation, traceability records, and evidence of manipulations;
3. Release and transport: suitability for clinical use, release and transport documents, and receipt confirmation by the clinical unit;
4. Outcome documentation: granulocyte and platelet engraftment data.

REFERENCES

1. Passweg JR, Baldomero H, Chabannon C, *et al.* Hematopoietic cell transplantation and cellular therapy survey of the EBMT: monitoring of activities and trends over 30 years. *Bone Marrow Transplant.* 2021; 56(7), 1651–64.
2. Schett G, Mackensen A, Mougiakakos D. CAR T-cell therapy in autoimmune diseases. *Lancet* 2023; 402(10416), 2034–44.
3. Rotz SJ, Bhatt NS, Hamilton BK, *et al.* International recommendations for screening and preventative practices for long-term survivors of transplantation and cellular therapy: a 2023 update. *Bone Marrow Transplant.* 2024.
4. Niederwieser D, Baldomero H, Bazuaye N, *et al.* One and a half million hematopoietic stem cell transplants: continuous and differential improvement in worldwide access with the use of non-identical family donors. *Haematologica* 2022; 107(5), 1045–53.

5. Carreras E, Dufour C, Mohty M, Kröger N. *The EBMT Handbook: Hematopoietic Stem Cell Transplantation and Cellular Therapies.* 2019; Springer.

6. Criswell T, Swart C, Stoudemire J, *et al.* Shipping and logistics considerations for regenerative medicine therapies. *Stem Cells Transl. Med.* 2022; 11(2), 107–13.

7. Worel N, Ljungman P, Verheggen ICM, *et al.* Fresh or frozen grafts for allogeneic stem cell transplantation: conceptual considerations and a survey on the practice during the COVID-19 pandemic from the EBMT Infectious Diseases Working Party (IDWP) and Cellular Therapy and Immunobiology Working Party (CTIWP). *Bone Marrow Transplant*. 2023; 58(12), 1348–56.

8. Cordoba R, Arrieta R, Kerguelen A, Hernandez-Navarro F. The occurrence of adverse events during the infusion of autologous peripheral blood stem cells is related to the number of granulocytes in the leukapheresis product. *Bone Marrow Transplant.* 2007; 40(11), 1063–7.

9. Donmez A, Tombuloglu M, Gungor A, Soyer N, Saydam G, Cagirgan S. Clinical side effects during peripheral blood progenitor cell infusion. *Transfus. Apher. Sci.* 2007; 36(1), 95–101.

10. Zenhäusern R, Tobler A, Leoncini L, Hess OM, Ferrari P. Fatal cardiac arrhythmia after infusion of dimethyl sulfoxide-cryopreserved hematopoietic stem cells in a patient with severe primary cardiac amyloidosis and end-stage renal failure. *Ann. Hematol.* 2000; 79(9), 523–6.

11. Cox MA, Kastrup J, Hrubiško M. Historical perspectives and the future of adverse reactions associated with haemopoietic stem cells cryopreserved with dimethyl sulfoxide. *Cell Tissue Bank.* 2012; 13(2), 203–15.

12. Ikeda K, Ohto H, Okuyama Y, *et al.* Adverse events associated with infusion of hematopoietic stem cell products: a prospective and multicenter surveillance study. *Transfus. Med. Rev.* 2018; S0887–7963.

13. Kollerup Madsen B, Hilscher M, Zetner D, Rosenberg J. Adverse reactions of dimethyl sulfoxide in humans: a systematic review. *F1000Res* 2018; 7, 1746.

14. Yao X, Matosevic S. Cryopreservation of NK and T cells without DMSO for adoptive cell-based immunotherapy. *BioDrugs* 2021; 35(5), 529–45.

15. Bojic S, Murray A, Bentley BL, *et al.* Winter is coming: the future of cryopreservation. *BMC Biol.* 2021; 19(1), 56.

16. Zinno F, Landi F, Scerpa MC, *et al.* Processing of hematopoietic stem cells from peripheral blood before cryopreservation: use of a closed automated system. *Transfusion* 2011; 51(12), 2656–63.

17. Berz D, McCormack EM, Winer ES, Colvin GA, Quesenberry PJ. Cryopreservation of hematopoietic stem cells. *Am. J. Hematol.* 2007; 82(6), 463–72.

18. Jansen J, Nolan PL, Reeves MI, *et al.* Transportation of peripheral blood progenitor cell products: effects of time, temperature and cell concentration. *Cytotherapy* 2009; 11(1), 79–85.

19. Calmels B, Lemarié C, Esterni B, *et al.* Occurrence and severity of adverse events after autologous hematopoietic progenitor cell infusion are related to the amount of granulocytes in the apheresis product. *Transfusion* 2007; 47(7), 1268–75.

20. Martín-Henao GA, Resano PM, Villegas JMS, *et al.* Adverse reactions during transfusion of thawed haematopoietic progenitor cells from apheresis are closely related to the number of granulocyte cells in the leukapheresis product. *Vox Sang.* 2010; 99(3), 267–73.

21. Morris C, de Wreede L, Scholten M, *et al.* Should the standard dimethyl sulfoxide concentration be reduced? Results of a European Group for Blood and Marrow Transplantation prospective noninterventional study on usage and side effects of dimethyl sulfoxide. *Transfusion* 2014; 54(10), 2514–22.

22. Windrum P, Morris TC, Drake MB, Niederwieser D, Ruutu T. Variation in dimethyl sulfoxide use in stem cell transplantation: a survey of EBMT centres. *Bone Marrow Transplant.* 2005; 36(7), 601–3.

23. Akkök CA, Liseth K, Melve GK, Ersvaer E, Hervig T, Bruserud Ø. Is there a scientific basis for a recommended standardization of collection and cryopreservation of peripheral blood stem cell grafts? *Cytotherapy* 2011; 13(8), 1013–24.

24. Foïs E, Desmartin M, Benhamida S, *et al.* Recovery, viability and clinical toxicity of thawed and washed haematopoietic progenitor cells: analysis of 952 autologous peripheral blood stem cell transplantations. *Bone Marrow Transplant.* 2007; 40(9), 831–5.

25. Khera N, Jinneman J, Storer BE, *et al.* Limiting the daily total nucleated cell dose of cryopreserved peripheral blood stem cell products for autologous transplantation improves infusion-related safety with no adverse impact on hematopoietic engraftment. *Biol. Blood Marrow Transplant.* 2012; 18(2), 220–8.

26. Kortleve JP, Kisch AM, Piepenbroek B, *et al.* Variation in hydration use after reinfusion of autologous stem cells in dimethyl sulfoxide (DMSO): a survey of EBMT centers on behalf of the EBMT Nurses Group. *Bone Marrow Transplant.* 2023; 58, 1062–4.

27. Lovelock JE, Bishop MWH. Prevention of freezing damage to living cells by dimethyl sulphoxide. *Nature* 1959; 183(4672), 1394–5.

28. Powell S, Molinolo A, Masmila E, Kaushal S. Real-time temperature mapping in ultra-low freezers as a standard quality assessment. *Biopreserv. Biobank.* 2019; 17(2), 139–42.

29. Smagur A, Mitrus I, Giebel S, *et al.* Impact of different dimethyl sulphoxide concentrations on cell recovery, viability and clonogenic potential of cryopreserved peripheral blood hematopoietic stem and progenitor cells. *Vox Sang.* 2013; 104(3), 240–7.

30. Mitrus I, Smagur A, Giebel S, *et al.* A faster reconstitution of hematopoiesis after autologous transplantation of hematopoietic cells cryopreserved in 7.5% dimethyl sulfoxide if compared to 10% dimethyl sulfoxide containing medium. *Cryobiology* 2013; 67(3), 327–31.

31. Mitrus I, Smagur A, Fidyk W, *et al.* Reduction of DMSO concentration in cryopreservation mixture from 10% to 7.5% and 5% has no impact on engraftment after autologous peripheral blood stem cell transplantation: results of a prospective, randomized study. *Bone Marrow Transplant.* 2018; 53(3), 274–80.

32. Hayakawa J, Joyal EG, Gildner JF, *et al.* 5% dimethyl sulfoxide (DMSO) and pentastarch improves cryopreservation of cord blood cells over 10% DMSO. *Transfusion* 2010; 50(10), 2158–66.

33. Akkök CA, Liseth K, Nesthus I, *et al.* Autologous peripheral blood progenitor cells cryopreserved with 5 and 10 percent dimethyl sulfoxide alone give comparable hematopoietic reconstitution after transplantation. *Transfusion* 2008; 48(5), 877–83.

34. Veeraputhiran M, Theus JW, Pesek G, Barlogie B, Cottler-Fox M. Viability and engraftment of hematopoietic progenitor cells after long-term cryopreservation: effect of diagnosis and percentage dimethyl sulfoxide concentration. *Cytotherapy* 2010; 12(6), 764–6.

35. Trummer T, Fox R, Koç JR, de Lima M, Otegbeye F. Cryopreservation of hematopoietic cells using a pre-constituted, protein-free cryopreservative solution with 5% dimethyl sulfoxide. *Cytotherapy* 2020; 22(11), 613–6.

36. Fry LJ, Querol S, Gomez SG, McArdle S, Rees R, Madrigal JA. Assessing the toxic effects of DMSO on cord blood to determine exposure time limits and the optimum concentration for cryopreservation. *Vox Sang.* 2015; 109(2), 181–90.

37. Hornberger K, Yu G, McKenna D, Hubel A. Cryopreservation of hematopoietic stem cells: emerging assays, cryoprotectant agents, and technology to improve outcomes. *Transfus. Med. Hemother*. 2019; 46(3), 188–96.

38. Murray KA, Gibson MI. Chemical approaches to cryopreservation. *Nat. Rev. Chem.* 2022; 6(8), 579–93.

39. McCullough J, Haley R, Clay M, Hubel A, Lindgren B, Moroff G. Long-term storage of peripheral blood stem cells frozen and stored with a conventional liquid nitrogen technique compared with cells frozen and stored in a mechanical freezer. *Transfusion* 2010; 50(4), 808–19.

40. Rowley SD, Feng Z, Chen L, *et al.* A randomized phase III clinical trial of autologous blood stem cell transplantation comparing cryopreservation using dimethylsulfoxide vs dimethylsulfoxide with hydroxyethylstarch. *Bone Marrow Transplant.* 2003; 31(11), 1043–51.

41. Stiff PJ, Murgo AJ, Zaroulis CG, DeRisi MF, Clarkson BD. Unfractionated human marrow cell cryopreservation using dimethylsulfoxide and hydroxyethyl starch. *Cryobiology* 1983; 20(1), 17–24.

42. Smagur A, Mitrus I, Ciomber A, *et al.* Comparison of the cryoprotective solutions based on human albumin vs. autologous plasma: its effect on cell recovery, clonogenic potential of peripheral blood hematopoietic progenitor cells and engraftment after autologous transplantation. *Vox Sang*. 2015; 108(4), 417–24.

43. Chen G, Yue A, Ruan Z, *et al.* Comparison of the effects of different cryoprotectants on stem cells from umbilical cord Blood. *Stem Cells Int*. 2016; 2016, 1396783.

44. Yu G, Li R, Hubel A. Interfacial interactions of sucrose during cryopreservation detected by Raman spectroscopy. *Langmuir* 2018; 35(23), 7388–95.

45. Crowe JH, Crowe LM, Carpenter JF, *et al.* Interactions of sugars with membranes. *Biochimica Biophys.* Acta 1988; 947(2), 367–84.

46. Richards AB, Krakowka S, Dexter LB, *et al.* Trehalose: a review of properties, history of use and human tolerance, and results of multiple safety studies. *Food Chem. Toxicol.* 2002; 40(7), 871–98.

47. Wolkers WF, Walker NJ, Tablin F, Crowe JH. Human platelets loaded with trehalose survive freeze-drying. *Cryobiology* 2001; 42(2), 79–87.

48. Pellerin-Mendes C, Million L, Marchand-Arvier M, Labrude P, Vigneron C. *In vitro* study of the protective effect of trehalose and dextran during freezing of human red blood cells in liquid nitrogen. *Cryobiology* 1997; 35(2), 173–86.

49. Eroglu A, Toner M, Toth TL. Beneficial effect of microinjected trehalose on the cryosurvival of human oocytes. *Fertil. Steril.* 2002; 77(1), 152–8.

50. Beattie GM, Crowe JH, Lopez AD, Cirulli V, Ricordi C, Hayek A. Trehalose: a cryoprotectant that enhances recovery and preserves function of human pancreatic islets after long-term storage. *Diabetes* 1997; 46(3), 519–23.

51. Erdag G, Eroglu A, Morgan JR, Toner M. Cryopreservation of fetal skin is improved by extracellular trehalose. *Cryobiology* 2002; 44(3), 218–28.

52. Motta JPR, Paraguassú-Braga FH, Bouzas LF, Porto LC. Evaluation of intracellular and extracellular trehalose as a cryoprotectant of stem cells obtained from umbilical cord blood. *Cryobiology* 2014; 68(3), 343–8.

53. Zhang XB, Li K, Yau KH, *et al.* Trehalose ameliorates the cryopreservation of cord blood in a preclinical system and increases the recovery of CFUs, long-term culture-initiating cells, and nonobese diabetic-SCID repopulating cells. *Transfusion* 2003; 43(2), 265–72.

54. Scheinkönig C, Kappicht S, Kolb HJ, Schleuning M. Adoption of long-term cultures to evaluate the cryoprotective potential of trehalose for freezing hematopoietic stem cells. *Bone Marrow Transplant*. 2004; 34(6), 531–6.

55. Martinetti D, Colarossi C, Buccheri S, Denti G, Memeo L, Vicari L. Effect of trehalose on cryopreservation of pure peripheral blood stem cells. *Biomed. Rep.* 2017; 6(3), 314–8.

56. Mantri S, Satpathy AK, Mohapatra PC. Trehalose: effect on cryopreservation of umbilical cord blood-derived hematopoietic stem cells. *Acta Haematologica Polonica* 2021; 52(2), 121–6.

57. Rodrigues JP, Paraguassú-Braga FH, Carvalho L, Abdelhay E, Bouzas LF, Porto LC. Evaluation of trehalose and sucrose as cryoprotectants for hematopoietic stem cells of umbilical cord blood. *Cryobiology* 2008; 56(2), 144–51.

58. Fujita Y, Nishimura M, Wada T, Komori N, Otoi T. Dimethyl sulfoxide-free cryopreservation solution containing trehalose, dextran 40, and propylene glycol for therapy with human adipose tissue-derived mesenchymal stromal cells. *Cytotechnology* 2022; 74(5), 515–29.

59. Svalgaard JD, Talkhoncheh MS, Haastrup EK, *et al*. Pentaisomaltose, an alternative to DMSO. engraftment of cryopreserved human CD34(+) cells in immunodeficient NSG mice. *Cell Transplant.* 2018; 27(9), 1407–12.

60. Jahan S, Kaushal R, Pasha R, Pineault N. Current and future perspectives for the cryopreservation of cord blood stem cells. *Transfus. Med. Rev.* 2021; 35(2), 95–102.

61. Kaushal R, Jahan S, McGregor C, Pineault N. Dimethyl sulfoxide-free cryopreservation solutions for hematopoietic stem cell grafts. *Cytotherapy* 2022; 24(3), 272–81.

62. Matsumura K, Hayashi F, Nagashima T, Hyon SH. Long-term cryopreservation of human mesenchymal stem cells using carboxylated poly-l-lysine without the addition of proteins or dimethyl sulfoxide. *J. Biomater. Sci. Polym. Ed.* 2013; 24(12), 1484–97.

63. Seong Jin O, Ho Lee J, Cheol Shin Y, *et al.* Cryoprotection of fibroblasts by carboxylated poly-L-lysine upon repeated freeze/thaw cycles. *CryoLetters* 2013; 34(4), 396-403.

64. Li Y, Si W, Zhang X, Dinnyes A, Ji W. Effect of amino acids on cryopreservation of cynomolgus monkey (*Macaca fascicularis*) sperm. *Am. J. Primatol*. 2003; 59(4), 159–65.

65. He S, Woods Iii LC. Effects of glycine and alanine on short-term storage and cryopreservation of striped bass (*Morone saxatilis*) spermatozoa. *Cryobiology* 2003; 46(1), 17-25.

66. Pollock K, Samsonraj RM, Dudakovic A, *et al.* Improved post-thaw function and epigenetic changes in mesenchymal stromal cells cryopreserved using multicomponent osmolyte solutions. *Stem Cells Dev.* 2017; 26(11), 828–42.

67. El Assal R, Abou-Elkacem L, Tocchio A, *et al.* Bioinspired preservation of natural killer cells for cancer immunotherapy. *Adv. Sci. (Weinh.)* 2019; 6(6), 1802045.

68. Wu LK, Tokarew JM, Chaytor JL, *et al.* Carbohydrate-mediated inhibition of ice recrystallization in cryopreserved human umbilical cord blood. *Carbohydr. Res.* 2011; 346(1), 86–93.

69. Briard JG, Jahan S, Chandran P, Allan D, Pineault N, Ben RN. Small-molecule ice recrystallization inhibitors improve the post-thaw function of hematopoietic stem and progenitor cells. *ACS Omega* 2016; 1(5), 1010–8.

70. Jahan S, Adam MK, Manesia JK, Doxtator E, Ben RN, Pineault N. Inhibition of ice recrystallization during cryopreservation of cord blood grafts improves platelet engraftment. *Transfusion* 2020; 60(4), 769–78.

71. Holbro A, Baldomero H, Lanza F, *et al.* Handling, processing and disposal of stem cell products in Europe: a survey by the cellular therapy and immunobiology working party of the European Society for Blood and Marrow Transplantation. *Cytotherapy* 2018; 20(3), 453–60.

72. Akkök ÇA, Holte MR, Tangen JM, Østenstad B, Bruserud Ø. Hematopoietic engraftment of dimethyl sulfoxide–depleted autologous peripheral blood progenitor cells. Transfusion 2009; 49(2), 354–61.

73. Syme R, Bewick M, Stewart D, Porter K, Chadderton T, Glück S. The role of depletion of dimethyl sulfoxide before autografting: on hematologic recovery, side effects, and toxicity. *Biol. Blood Marrow Transplant.* 2004; 10(2), 135–41.

74. Lemarie C, Calmels B, Malenfant C, *et al.* Clinical experience with the delivery of thawed and washed autologous blood cells, with an automated closed fluid management device: CytoMate. *Transfusion* 2005; 45(5), 737–42.

75. Rohner N, Passweg JR, Tsakiris DA, *et al.* The value of the post-thaw $CD34^+$ count with and without DMSO removal in the setting of autologous stem cell transplantation. *Transfusion* 2019; 59(3), 1052–60.

76. Calmels B, Drezet A, Huynh C, *et al.* Automated washing of autologous hematopoietic stem cell grafts after thawing does not impair engraftment. *Bone Marrow Transplant*. 2014; 49, 1127–8.

77. Mfarrej B, Bouchet G, Couquiaud J, *et al.* Pre-clinical assessment of the Lovo device for dimethyl sulfoxide removal and cell concentration in thawed hematopoietic progenitor cell grafts. *Cytotherapy* 2017; 19(12), 1501–8.

78. Sánchez-Salinas A, Cabañas-Perianes V, Blanquer M, *et al.* An automatic wash method for dimethyl sulfoxide removal in autologous hematopoietic stem cell transplantation decreases the adverse effects related to infusion. *Transfusion* 2012; 52(11), 2382–6.

79. Nappo SEM, Maisto G, Casalino R, *et al.* Use of Gelofusine for washing hematopoietic stem cells for transplant. *Abstract Book Sidem 21° Congresso Nazionale SIDEM*. 2023.

80. Reich-Slotky R, Vasovic LV, Land KJ, *et al.* Cryopreserved hematopoietic stem/progenitor cells stability program-development, current status and recommendations: a brief report from the AABB-ISCT joint working group cellular therapy product stability project team. *Cytotherapy* 2022; 24(5), 473–81.

81. Pamphilon D, Selogie E, McKenna D, *et al.* Current practices and prospects for standardization of the hematopoietic colony-forming unit assay: a report by the cellular therapy team of the Biomedical Excellence for Safer Transfusion (BEST) Collaborative. *Cytotherapy* 2013; 15(3), 255–62.

82. Rosskopf K, Ragg SJ, Worel N, *et al.* Quality controls of cryopreserved haematopoietic progenitor cells (peripheral blood, cord blood, bone marrow). *Vox Sang.* 2011; 101(3), 255–75.

83. Association for the Advancement of Blood and Biotherapies. *Standards for Cellular Therapy Services.* 2021; AABB.

84. FACT-JACIE International Standards for Hematopoietic Cellular Therapy. Product Collection, Processing, and Administration. Eighth Edition, Version 8.12021, 2021.

85. Reich-Slotky R, Vasovic LV, Land KJ, *et al.* Cryopreserved hematopoietic stem/progenitor cells stability program-development, current status and recommendations: a brief report from the AABB-ISCT joint working group cellular therapy product stability project team. *Transfusion* 2022; 62(3), 651–62.

86. World Marrow Donor Association. WMDA Rapid Alert, May 2020.

87. Marinelli Busilacchi E, Costantini A, Mancini G, *et al.* A novel method to evaluate prethawing viability of cryopreserved CD34$^+$ hematopoietic stem cells for autologous transplantation. *Transfusion* 2020; 60(7), 1529–35.

88. Thompson EN, Carlino MJ, Scanlon VM, Grimes HL, Krause DS. Assay optimization for the objective quantification of human multilineage colony-forming units. *Exp. Hematol.* 2023; 124, 36-44.e3.

89. Lumley MA, Burgess R, Billingham LJ, McDonald DF, Milligan DW (Editors). *Colony Counting Is A Major Source Of Variation In CFU-GM Results Between Centres*. 1996; Blackwell Science.

90. Lamana M, Albella B, Rodriguez F, Regidor C, Bueren JA. Conclusions of a national multicenter intercomparative study of *in vitro* cultures of human hematopoietic progenitors. *Bone Marrow Transplant.* 1999; 23(4), 373–80.

91. Moroff G, Eichler H, Brand A, *et al.* Multiple-laboratory comparison of *in vitro* assays utilized to characterize hematopoietic cells in cord blood. *Transfusion* 2006; 46(4), 507–15.

92. Castelhano MV, Reis-Alves SC, Vigorito AC, *et al.* Quantifying loss of CD34$^+$ cells collected by apheresis after processing for freezing and post-thaw. *Transfus. Apher. Sci.* 2013; 48(2), 241–6.

93. Solomon M, Wofford J, Johnson C, Regan D, Creer MH. Factors influencing cord blood viability assessment before cryopreservation. *Transfusion* 2010; 50(4), 820–30.

94. Fu SQ, Abboud CN, Brennan JK, Ifthikharuddin JJ, Nichols D, Liesveld JL. Impact of mobilized blood progenitor cell quality determined by the CFU-GM/CD34$^+$ ratio on rapid engraftment after blood stem cell transplantation. *Blood Cells Mol. Dis.* 2002; 28(3), 315–21.

95. Woods EJ, Liu J, Pollok K, *et al.* A theoretically optimized method for cord blood stem cell cryopreservation. *J. Hematother. Stem Cell Res.* 2003; 12(3), 341–50.

96. Watts MJ, Linch DC. Optimisation and quality control of cell processing for autologous stem cell transplantation. *Br. J. Haematol.* 2016; 175(5), 771–83.

97. Majado MJ, Salgado-Cecilia G, Blanquer M, *et al.* Cryopreservation impact on blood progenitor cells: influence of diagnoses, mobilization treatments, and cell concentration. *Transfusion* 2011; 51(4), 799–807.

98. Fietz T, Reufi B, Mücke C, Thiel E, Knauf WU. Flow cytometric CD34$^+$ determination in stem cell transplantation: before or after cryopreservation of grafts? *J. Hematother. Stem Cell Res.* 2002; 11(2), 429–35.

99. Lee HR, Shin S, Yoon JH, *et al.* Attached segment has higher CD34$^+$ cells and CFU-GM than the main bag after thawing. *Cell Transplant*. 2015; 24(2), 305–10.

100. Bai L, Peters L, Xia W, *et al*. Assessing pilot vial material as a surrogate for functional and phenotypic stem cell markers in cryopreserved haematopoietic stem cell product. *Bone Marrow Transplant*. 2016; 51(12), 1631–2.

101. Araújo AB, Salton GD, Angeli MH, Furlan JM, Schmalfuss T, Röhsig LM. Effects of cell concentration, time of fresh storage, and cryopreservation on peripheral blood stem cells: PBSC fresh storage and cryopreservation. *Transfus. Apher. Sci.* 2022; 61(1), 103298.

102. Trébéden-Negre H, Rosenzwajg M, Tanguy ML, *et al*. Delayed recovery after autologous peripheral hematopoietic cell transplantation: potential effect of a high number of total nucleated cells in the graft. *Transfusion* 2010; 50(12), 2649–59.

103. Klein G, Wuchter P. *Stem Cell Mobilization*. (Methods in Molecular Biology, 2017). Humana; 2019.

104. Pavel P, Laier S. A freezing protocol for hematopoietic stem cells. *Methods Mol Biol.* 2019; 2017, 177–92.

105. de Oliveira Félix OMW, Tunes G, Ginani VC, *et al*. The influence of cell concentration at cryopreservation on neutrophil engraftment after autologous peripheral blood stem cell transplantation. *Hematol. Transfus. Cell Ther.* 2018; 40(3), 233–9.

106. Alencar S, Garnica M, Luiz RR, *et al*. Cryopreservation of peripheral blood stem cell: the influence of cell concentration on cellular and hematopoietic recovery. *Transfusion* 2010; 50(11), 2402–12.

107. Rowley SD, Bensinger WI, Gooley TA, Buckner CD. Effect of cell concentration on bone marrow and peripheral blood stem cell cryopreservation. *Blood* 1994; 83(9), 2731–6.

108. Bachier C, Potter J, Potter G, *et al*. High white blood cell concentration in the peripheral blood stem cell product can induce seizures during infusion of autologous peripheral blood stem cells. *Biol. Blood Marrow Transplant*. 2012; 18(7), 1055–60.

109. Villalon L, Odriozola J, Ramos P, Ramos ML, Herrera P, de Oteyza JP. Cryopreserving with increased cellular concentrations of peripheral blood progenitor cells: clinical results. *Haematologica* 2002; 87(2), ELT06-ELT.

110. Meacle F, Salkin J, Rice M, Harris I. Key considerations of cell and gene therapy cold chain logistics. *Cell Gene Ther Insights.* 2016; 2, 223–36.

111. Myles L, Church TD. An industry survey of implementation strategies for clinical supply chain management of cell and gene therapies. *Cytotherapy* 2022; 24(3), 344–55.

112. Meneghel J, Kilbride P, Morris GJ. Cryopreservation as a key element in the successful delivery of cell-based therapies—a review. *Front. Med.* 2020; 7, 592242.

113. Wilde AD. The Fifth Edition of the ISBER Best Practices: user feedback supports broad applicability. *Biopreserv. Biobank*. 2023; 21(6), 635–6.

114. Sputtek A, Jetter S, Hummel K, Kühnl P. Cryopreservation of peripheral blood progenitor cells: characteristics of suitable techniques. *Contrib. Infus. Ther. Transf. Med.* 1997; 34, 79–83.

115. Tijssen MR, Woelders H, De Vries-van Rossen A, Van Der Schoot CE, Voermans C, Lagerberg JWM. Improved postthaw viability and *in vitro* functionality of peripheral blood hematopoietic progenitor cells after cryopreservation with a theoretically optimized freezing curve. *Transfusion* 2008; 48(5), 893–901.

116. Valeri CR, Pivacek LE. Effects of the temperature, the duration of frozen storage, and the freezing container on *in vitro* measurements in human peripheral blood mononuclear cells. *Transfusion* 1996; 36(4), 303–8.

117. Mazur P. The role of intracellular freezing in the death of cells cooled at supraoptimal rates. *Cryobiology* 1977; 14(3), 251–72.

118. Rowley SD. Hematopoietic stem cell processing and cryopreservation. *J. Clin. Apheresis* 1992; 7(3), 132–4.

119. Dijkstra-Tiekstra MJ, Setroikromo AC, Kraan M, Gkoumassi E, de Wildt-Eggen J. Optimization of the freezing process for hematopoietic progenitor cells: effect of precooling, initial dimethyl sulfoxide concentration, freezing program, and storage in vapor-phase or liquid nitrogen on in vitro white blood cell quality. *Transfusion* 2014; 54(12), 3155–63.

120. Germann A, Oh Y-J, Schmidt T, Schön U, Zimmermann H, von Briesen H. Temperature fluctuations during deep temperature cryopreservation reduce PBMC recovery, viability and T-cell function. *Cryobiology* 2013; 67(2), 193–200.

121. Cosentino LM, Corwin W, Baust JM, *et al*. Preliminary report: evaluation of storage conditions and cryococktails during peripheral blood mononuclear cell cryopreservation. *Cell Preserv. Technol*. 2007; 5(4), 189–204.

122. Chabot D, Tremblay T, Paré I, Bazin R, Loubaki L. Transient warming events occurring after freezing impairs umbilical cord–derived mesenchymal stromal cells functionality. *Cytotherapy* 2017; 19(8), 978–89.

123. Hawkins BJ, Abazari A, Lock LT, *et al*. The effect of cryomedia selection and transient warming events on post-cryopreservation human MSC function. *Cytotherapy* 2018; 20(5), S40.

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CELL & GENE THERAPY INSIGHTS

INTERVIEW

Achieving scalability: strategic insights into avoiding the pitfalls of the cell and gene therapy supply chain

Abi Pinchbeck, Commissioning Editor, *Cell & Gene Therapy Insights*, speaks to **Donna Rill**, Chief Technology Officer, Triumvira Immunologics. The discussion delves into the current state of the art in scaling the cell and gene therapy supply chain, emphasizing the importance of securing critical reagents, ensuring scalability for custom supply, and achieving the required scales for cGMP vector production.

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Q What are you working on right now?

DR: Triumvira is currently focused on our ongoing clinical trial targeting Claudin 18.2 and the final optimization of our automated manufacturing process.

Q What is the current state of the art in scaling the cell and gene therapy (CGT) supply chain?

DR: Several factors contribute to the scaling of the CGT supply chain. To successfully secure a consistent source of critical reagents and consumables with a standing lot reservation order, you must be able to understand and reasonably predict what your needs will be over the next year. This facilitates vendors in planning to meet the expectations of the field. Recently, there have been many back orders that have occurred for various reasons. Providing the vendors with a reasonable expectation of what you will need over the next year lets them manage their manufacturing process to not over or underestimate.

It is important to build out your material specifications with defined critical quality attributes. The field has often been faced with only having one source of critical materials. It is important to understand what aspect of a component, or a reagent, makes your manufacturing process successful. Once understood, the process involves qualifying backup sources of those critical materials. There are many more vendors in the field than in previous times, but switching does not happen immediately. It requires qualification and ensuring that any backup source of critical material is as effective and does not compromise the quality of your product.

With respect to custom supply, for example a viral vector, it is important to work with a reliable vendor with a slot reservation program and scalability. In the early stages before proof of concept, you do not want a 500 L vat of virus because you do not yet know whether that construct will work well going forward. It is key to have a vendor that can do both the small and large scales, from 20 L to 50 L to 100 L to 500 L. Then, when rolling into Phase 3 and a potential commercial launch, you will know that you are working with a company with a path that can take you to that commercial stage.

It is key to ensure that a vendor has that breadth of capabilities in addition to a slot reservation system so that you as a company can make predictions. If you are seeing real success in a Phase 1 clinical trial, you can plan ahead for Phase 2/Phase 3, and likewise going forward. You can reserve the slot to have another batch made in advance to be ready for that next stage clinical trial. Equally important is to develop a manufacturing process that incorporates cohesive, interconnected automation and is scalable to meet current and future manufacturing needs.

Q Specifically, how can the required scales for cGMP vector production be achieved?

DR: From the perspective of a small biotech company, the same considerations as previously mentioned are still true. Having a reliable path forward to take you to commercialization is key. There are many more viral vector companies now than there used to be in the field, and many companies in the early stages produce small amounts of GMP plasmids for vector production without yet having a scale-out plan for larger production. Timing is critical

"Small companies that start with their own vector production must be especially cognizant of scalability."

to ensure that while you may not need commercial launch-type volumes of vector for several years, knowing that you can have a consistent, reliable, quality source of vector that can take you to that point without switching vendors is hugely important. It is critical to be aware of the ultimate scalability of a viral vector production company.

A company producing its own vectors is a different matter entirely. Small companies that start with their own vector production must be especially cognizant of scalability. Most would never be able to do a full-scale commercial launch of viral vectors initially. They should consider having the means and awareness to transition to a viral vector company if needed to move forward with larger productions.

Q Where do the key challenges remain in ensuring the scalability of varying CGT supply chains? Where are the greatest pressure points right now?

DR: The greatest pressure points now are the continual back orders of critical materials, as well as logistical shipping issues. Staying ahead of your demands and having a consistent influx of material becomes important. It is critical to have the ability to set up standing lot reservations with key vendors. This requires a company to understand their workflow, especially from R&D to process development and optimization to clinical production. A company must make a reasonable projection of how much material they need on hand, in addition to the absolute minimal stock and the maximum that can be stored. Then, set up those standing orders so that stock is coming in on a consistent basis, minimizing the possibility of reaching a crisis point. There will be times when there are major back orders or recalls of a critical reagent. That is where it is important to build out backup sources of critical reagents in the event you cannot get your preferred reagent or consumable.

Q What are some issues you have faced in terms of leukapheresis shipping specifically?

DR: Throughout my years involved in CGT, there have been manufacturing successes and failures. It is important to understand the root cause of those failures. Often, the failures are rooted in the actual leukapheresis material from the subject. Learning what the problems are to better optimize the seed material coming into your facility as well as how to best process

"If you start out using GMP reagents and optimize your processes for those, it can be very helpful."

that material before manufacturing is hugely important. Maintaining conditions that support the immunological function of T cells is important for Triumvira, thus utilizing controlled room temperature shipping provides the best scenario. We have performed many stability studies to learn that functionality starts falling off after 36 hours. To ensure that we get the best quality material for our manufacturing, we ship at controlled room temperature and all materials are delivered within 24 hours.

Pre-COVID, logistics were much easier as flight logistics were more stable. The large delivery companies, such as FedEx, World Courier, DHS, and UPS, were consistent with their ability to turn around 24-hour deliveries. Post-COVID, it has become increasingly difficult for these companies to meet overnight demands. Triumvira currently works with a company that utilizes retired flight attendants and pilots who hand-carry leukapheresis. They pick it up from the apheresis center, take it to the airport, and can manage if a flight is canceled to get on the next available flight. They hand deliver it to the cell processing center of the manufacturing facility. Since we have been using this company, we have had no issues with delivery within 24 hours.

Q With your three decades of extensive CGT clinical and research laboratory experience, do you have any key pieces of advice for those starting out in CGT lab development, and those seeking to scale their supply chains robustly?

DR: Across the board, most smaller companies are coming out of academic technologies and are familiar with the supplies they need in their early stages of development. Issues arise at the stage of scaling up, which can be avoided by understanding sources for GMP products for your manufacturing process. Even in the early R&D stages, using reagents and consumables that you can translate straight into a manufacturing GMP environment saves a lot of time. Too frequently, a research use-only reagent does not translate to an equivalent GMP reagent, even within the same company. If you start out using GMP reagents and optimize your processes for those, it can be very helpful.

Also, it is important to understand the lot-to-lot variability in reagents and consumables. Establishing a program where you can ensure that as you transition from one lot to another the quality attributes are maintained is essential. One should establish critical quality attributes early in development and build an internal process control seed bank of cells for testing each lot. This can be done easily by establishing a prior preserved bank of cells that can be taken into an assay or manufacturing scenario with two lots to show equivalency. This way, you have

established expectations with your current lot or reagent and can check that you get an equivalent output with a new lot of that same reagent. Many smaller companies often are not engaged in these kinds of programs. As you establish those internal process controls, you get a running history of what a specific reagent and/or a lot of that reagent does. Once you feel comfortable that a company has good internal controls and lot-to-lot consistency after working with them for several lots, these programs can be relaxed. Instead of having to run in parallel, you can then bring in that new lot and simply run it and use data to show that this new lot is equivalent without having to duplicate, which is a cost-saving measure.

Q How do you imagine the CGT supply chain will differ from now in a decade's time?

DR: There are many newer vendors with reagents that work as well as some of the reagents provided by more established companies. The durability of those companies over time must be considered as the market becomes more competitive. Part of what has plagued the field for a while is only having a single source for many critical reagents. Now, it has become a field with a few more supply chain options for vendors, but the question of how durable they are remains.

One hugely important aspect is being able to manufacture critical reagents in a manner that is more compatible with full automation. As an example, most of our reagents are in valves and are manually drawn out with a syringe and injected into our automated system. To fully automate in the future, being able to have critical reagents in a manner that can be aseptically docked into a system will become much more important. This is one way the field should move to minimize hands-on time to further reduce costs and be able to turn out products at a commercial cell in a more feasible, cost-effective manner. Much of the expense of CGT manufacturing comes from labor, and the less necessary physical activities there are, especially those that require a biological safety cabinet, the more efficient the workflow. Seeking out sources that provide materials that can be built directly into a workflow path for manufacturing with minimal human intervention.

Q What are your key goals and priorities in your work and for Triumvira over the next 1 to 2 years?

DR: I hope to see the field of CGT move forward more effectively to efficient successful commercial launches. There are still many hurdles to tackle in the field, which I want to prioritize and spend more time on. For Triumvira, our main objective over the next 12 to 24 months is to have successful proof of concept clinical trials and to be able to move forward into pivotal studies.

BIOGRAPHY

DONNA RILL currently serves as the Chief Technical Officer of Triumvira Immunologics, USA, Inc. She has extensive manufacturing, clinical, and translational research laboratory experience in CGT, monoclonal antibody production, viral vector production, and protein production. She has setup and managed core development laboratories covering a large range of testing services to facilitate research, core drug development activities, as well as manufacturing, quality systems and quality control laboratories. Her experience covers a broad range of activities inclusive of laboratory construction, project management, development and operations, cGMP, cGTP, and GLP regulatory compliance, quality control/assurance system development, database development, and clinical standards of practice. She has designed and qualified/validated cGMP CGT laboratories, cGMP vector production facilities, core service laboratories, and translational research labs. Rill has previously held the positions of: Vice President of Manufacturing for Cell Medica, Chief Development Officer for Opexa Therapeutics, Laboratory Director of Cell and Gene Therapy, Translational Research Laboratories for Cell and Gene Therapy, Baylor College of Medicine; Associate Scientist/Lab Manager of the Bone Marrow Transplant Research Laboratory, and the GMP CGT laboratories, St Jude Children's Research Hospital; Education Coordinator and Clinical Instructor, Department of Clinical Laboratory, LeBonheur Children's Medical Center, and University of Tennessee Center for the Health Sciences.

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CELL & GENE THERAPY INSIGHTS

LATEST ARTICLES

CELL & GENE THERAPY INSIGHTS

INTERVIEW

Insights into advanced therapies in the context of evolving funding and M&A environments

David McCall, Senior Editor, BioInsights, spoke to **Lee Brown**, Healthcare Sector Global Team Leader, Third Bridge Group, on Tuesday, February 6, 2024 to gain his insights into the fast-moving market landscape of advanced therapies. The discussion outlines key recent news and strategic moves from prominent pharma and biotech companies in the field, as well as the prospects for specific technologies such as CRISPR-based genome editing and next-generation CAR-T therapies.

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Q What are you working on right now?

LB: As always, the news flow in healthcare is fast-moving. The industry is currently advancing at a breakneck speed, especially on the heels of the recent JP Morgan Healthcare Conference that kicked off the new year, and which was bookended by a spate of announced strategic mergers and acquisitions (M&As). That is a good sign as we discuss the thawing of the biotech funding market.

I am fortunate to lead a talented global healthcare team at Third Bridge with analysts in Shanghai, London, and Manhattan. My team covers healthcare across the entire capital structure, including public equities as well as the credit and private markets, and I'm excited to

discuss where we are focusing our time in terms of our strategic coverage of the healthcare industry.

Q How would you summarize the current state of affairs in biotech funding for the sector?

LB: After a tricky 2023 for advanced therapies, and the fallout seen in the CRO, CDMO, and life sciences tools markets, there are reasons to be optimistic as we enter 2024. This is largely due to the recent M&A activity as well as several recent successful IPOs.

We saw Bristol Myers Squibb acquire Karuna Therapeutics for $14 billion, a strategically savvy deal that strengthens the big pharma company's central nervous system franchise. We also saw AbbVie acquire Cerevel Therapeutics in an $8.7 billion deal. Cerevel's lead asset is emraclidine, a potential best-in-class next-generation antipsychotic to treat schizophrenia and Alzheimer's disease psychosis. With two huge deals in the space, we are seeing an evolving competitive landscape in schizophrenia, as well as in Parkinson's disease and Alzheimer's disease psychosis.

Late last year, we also saw AstraZeneca announce the acquisition of Gracell Biotechnologies for $1.2 billion, adding GC012F to its cell therapy clinical pipeline. This is a FasTCAR-enabled BCMA and CD19 dual-targeting autologous CAR-T therapy that is being evaluated as a potential new treatment in multiple myeloma and other hematologic malignancies and autoimmune diseases. The deal highlights the significant interest in next-generation CAR-T therapies.

Turning to the reopening of the IPO window, CG Oncology recently brought in $380 million in the first biotech IPO of the year. The stock was priced at $19 per share, above the forecast $16 to 18 range. Today, CG Oncology stands at $39 per share with a market cap of $2.5 billion, speaking to the current enthusiasm in the space. CG Oncology's lead candidate, cretostimogene grenadenorepvec, is a targeted oncolytic intravesical immunotherapy agent currently in two Phase 3 trials for the treatment of bladder cancer.

Q What other recent company news have you seen in the advanced therapies field that stands out for you?

LB: Just this morning, Kyverna Therapeutics announced that it increased its IPO deal size by offering 14.5 million shares in the range of $20 to 21 per share, around a 50% increase on the previous filing to offer just 11.1 million shares in the range of $17 to 19. The IPO is expected to close this week with a valuation of around $850 million. This Gilead- and Bain Capital Life Sciences-backed company is focused on developing cell therapies for autoimmune diseases. Its lead program is KYV-101, an autologous CD19 CAR-T cell therapy

"Companies with interesting technology that can offer new approaches and accelerate pipeline candidates could be of interest to the large-cap biotech and pharmas."

being evaluated in rheumatology and neurology. The initial rheumatology development is for lupus nephritis (LN), which will be followed by systemic sclerosis (SSC). The company plans to conduct two trials of KYV-101 in patients with LN and has received IND clearance for a Phase 1/2 study in SSC. In neurology, Kyverna has received IND clearance for Phase 2 studies in both myasthenia gravis and multiple sclerosis.

Metagenomi is a testing point for the market's appetite for preclinical companies. This is an interesting company that has many industry eyes on it at the moment—the large-cap biotechs and a few mega-cap pharmaceutical companies are increasingly interested in bolt-on deals. They are battling exclusivity challenges and Inflation Reduction Act pricing issues with some blockbuster drugs that have enjoyed market dominance, so they are looking for opportunities to bridge the gap. Companies with interesting technology that can offer new approaches and accelerate pipeline candidates could be of interest to the large-cap biotech and pharmas.

Tome Bioscience emerged with around $210 million in funding between Series A and B rounds in mid-December. The firm has licensed a CRISPR-based genome editing technology that was developed by its co-founders, formerly of Massachusetts Institute of Technology's McGovern Institute. They are looking at monogenetic liver diseases and cell therapies for auto-immune diseases. The CEO recently made a bold statement that we will be able to finally reprogram the human genome with an elegance and efficiency previously unimagined. That gets those following healthcare, especially cell and gene therapy, excited. For patients with rare monogenic diseases, Tome could offer potentially curative treatments with a single drug per disease approach, regardless of genetic heterogeneity. For patients with more common disorders, this is an exciting time.

Beam Therapeutics uses CRISPR technology to change single bases in the genome with a technique called base editing. This breaks only one strand of DNA and therefore, may result in a higher cell survival rate. Its lead programs include sickle cell disease, T cell cancers, and glycogen storage disease Type 1A. In September 2023, Beam reported positive preclinical data from its BEAM-302 program, which was designed to treat both lung and liver manifestations of α-1 antitrypsin deficiency.

Caribou Biosciences is a clinical-stage biotech company looking at gene-edited allogeneic cell therapies to treat cancer. In July 2023, Caribou reported positive early-stage trial data from its most advanced program, CB-010, for treating relapsed or refractory B-cell non-Hodgkin's lymphoma. Of its other programs, CB-011 is aimed at treating patients with relapsed or refractory multiple myeloma, while CB-012 targets relapsed or refractory acute myeloid leukemia. Caribou has a differentiated approach to gene editing and cell therapy production, with innovation around manufacturing. Often, analysts' focus is on preclinical and clinical development and innovation, but it can be important to focus on manufacturing to drive

"In terms of the already approved and commercialized CAR-Ts, we have seen a market embrace that was well below early expectations."

down costs, including reducing batch-to-batch variation in cell therapy manufacturing. This will enable companies to meet the needs of larger markets efficiently with scalable manufacturing and without a lot of waste, which is a common issue in earlier-stage manufacturing.

In December, Vertex Pharmaceuticals and CRISPR Therapeutics announced the FDA approval of Casgevy™, a CRISPR/Cas9 genome-edited cell therapy for the treatment of sickle cell disease in patients 12 years and older with recurrent vaso-occlusive crises. Casgevy offers the real potential of a one-time transformative therapy for eligible patients. This requires specialized stem cell transplantation, so Vertex is engaging with experienced hospitals and establishing networks of independently operated authorized treatment centers throughout the USA. In terms of the already approved and commercialized CAR-Ts, we have seen a market embrace that was well below early expectations. However, the development of authorized treatment centers is building momentum and will help drive much better performance, not only in newly launched cell and gene therapies but also in already-approved ones. With Casgevy, the FDA surprised us and approved the treatment of transfusion-dependent β-thalassemia (TDT). TDT is a very serious life-threatening genetic disease. It requires frequent blood transfusions and iron chelation—a therapy that is required throughout a person's life—so this is big news.

I also want to highlight bluebird bio's gene replacement therapy, Lyfgenia™, to treat sickle-cell disease. This has a black box warning, noting that in rare cases, the therapy could cause certain blood cancers. The US FDA added the warning after two patients who had received Lyfgenia in a clinical trial died from a form of leukemia. The FDA stated that it is unclear whether Lyfgenia itself or another part of the treatment process, such as chemotherapy, caused the cancer. However, the black box warning along with a higher wholesale acquisition cost of $3.1 million, which is 40% higher than the $2.2 million list price for Casgevy, tempers our view of Lyfgenia.

I recently had the honor of hosting an interview on BioMarin, focused on its gene therapy, Roctavian™, to treat adults with severe hemophilia. The expectations for Roctavian's uptake should be conservative despite a sizable market opportunity of approximately $7+ billion in the USA alone. The market is fantastic, but Roctavian's experience in Europe did not get off to a good start due to its high cost and the need for patient education. It is predicted that Roctavian will experience a similarly slow start in the USA, with a metered uptake. The Pfizer-Sangamo hemophilia A therapy may be more selective than Roctavian, with a potentially more efficient serotype that could require a lower dose.

In January 2023, Sarepta Therapeutics announced positive data from Part B of its MOMENTUM study—a global Phase 2 multi-ascending dose clinical trial of SRP-5051, a next-generation peptide phosphorodiamidate morpholino oligomer (PMO) treatment for patients with Duchenne muscular dystrophy (DMD) amenable to skipping Exondys 51™ (vesleteplirsen). However, the top-line data from Sarepta's Phase 3 EMBARK confirmatory study of Elevidys failed to meet its

primary endpoint. When the data was announced on October 30, 2023, Sarepta's stock plunged by over 40%. On December 22, 2023, the company filed an efficacy supplement to expand the Elevidys label to include all DMD without age or ambulatory status restriction. Sarepta has also submitted the EMBARK post-marketing requirement that the FDA sought in converting the Elevidys accelerated approval to a traditional approval. Sarepta released preliminary Q4 results on January 8 and Elevidys generated Q4 sales of around $131 million as the first approved gene therapy for treating DMD.

Regarding AbbVie and Regenxbio's ABBV-RGX-314, positive interim data from the Phase 2 AAVIATE trial was recently announced. This is for wet age-related macular degeneration, which is a huge and growing market given our aging populations. Eylea is the market leader right now, which requires regular injections. ABBV-RGX-314A would be hugely advantageous as a one-time cure.

Q You mentioned that the approved CAR-T's performance to date has been underwhelming compared to initial predictions. How do you see that space developing further?

LB: Bristol-Myers Squibb's Abecma® and Johnson & Johnson's Carvykti® are both approved for relapsed or refractory multiple myeloma. It will be interesting to see how this large market develops. Bristol Myers's Breyanzi® and Gilead's Yescarta® are for large B-cell lymphoma including diffuse large B-cell lymphoma. Meanwhile, Novartis's CAR-T, Kymriah®, is for treatment of relapsed or refractory follicular lymphoma, as well as acute lymphoblastic leukemia and mantle cell lymphoma.

These commercialized products have perhaps not received the market embrace some might have hoped for, but I am excited to learn about innovations in next-generation CAR-Ts. The whole idea is to improve efficacy while reducing toxicity. Another important aspect, as we get smarter about manufacturing, is how we can drive down the cost at large-scale.

Q What are the key takeaways from this recent news as we move further into 2024?

LB: Personally, I would like to spend more time looking at longevity studies. There are differences in the current approaches to curative therapies. For example, Recursion Pharmaceuticals approaches curative therapies from the cellular level and works backward (although this can create challenges in terms of defining the mechanism of action with the FDA). There needs to be an evolving paradigm shift in terms of how the FDA looks at this because mechanistically, we will become stuck in a single approach if we do not expand our view, especially as we better understand how things are functioning at the cellular level.

I think the most important takeaway is that healthcare innovation is alive and well. We are fortunate to have the human capital that is driving this. We are accelerating at an exponential rate in terms of our understanding. We are seeing a thawing of the IPO window as well as increased M&A. We have many smart people working on things at both preclinical and clinical stages. All in all, it is one of the most exciting times to be a part of cell and gene therapy right now. The future is now.

BIOGRAPHY

LEE BROWN joined Third Bridge in 2021 as the Global Healthcare Team Leader where he manages a team of twenty analysts and associates across offices in Manhattan, London, and Shanghai. His team conducts primary due diligence and produces over 1,600 interviews annually with respected industry key opinion leaders and C-suite executives covering every sector within the healthcare industry. Before joining Third Bridge, Lee was the Founder and Chief Investment Officer of Reef Knot Capital from 2016 to 2021 where he executed a market neutral healthcare strategy that consistently generated superior risk-adjusted returns. Prior to launching Reef Knot, Lee was a Portfolio Manager at Visium Asset Management where he worked from 2009 to 2016 and managed a market neutral healthcare portfolio. Before joining Visium, Lee was Senior Analyst at Highland Capital Management from 2007 to 2009 where he helped to manage a multi-asset healthcare portfolio during the financial crisis. Prior to joining the buy-side, Lee was a Vice President at Merrill Lynch on a consistently top ranked II team where he worked as a lead, publishing sell-side equity analyst covering Medical Technology from 2002 to 2007. Before joining Wall Street, Lee served as an officer in the US Marine Corps for 5 years and earned the rank of Captain. Lee is a distinguished graduate from the United States Naval Academy where he earned a BS. Lee also earned a MBA with second-year honors from Harvard Business School.

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CELL & GENE THERAPY INSIGHTS

2024: the year of the genome editing technologies?

Harriet Edwards
Associate Director of Regulatory Affairs,
Boyds

"The coming years will define a new era of medicine and consequently, regulation..."

VIEWPOINT

Cell & Gene Therapy Insights 2024; 10(3), 231–235

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Over the last decade, the development of innovative medicines has experienced exponential growth, with the move towards more personalized medicines and the introduction of scalable and tailored approaches to cell and gene therapies. In more recent years, the potential benefits of AI in drug development have provided a new challenge for developers and regulators alike and as newer, more complex medicines begin to emerge, there is an undeniable shift towards incorporating these medicines as the new standards of care. Genome editing technologies are an example of a new, complex niche of gene therapies that are now realizing their potential to cure previously untreatable diseases.

Genome editing (GE) incorporates many different techniques and technologies, from the earlier introduced methods such as zinc finger nucleases (ZFN), which are less specific in their approach to introducing changes to the DNA sequence, to clustered regularly interspaced short palindromic repeat (CRISPR)-associated (Cas) nucleases, which are targeted to a specific site of interest. The diversity of GE technologies in addition to the mechanism of action (i.e., nuclease-dependent or independent) further adds to the complexity and difficulty in creating regulation to support development of this sub-class of gene therapies.

Due to the inherent nature of how GE technologies work, through the ability to definitively treat the root causes of life-threatening and life-altering diseases, they provide the opportunity to move beyond the capabilities of more traditional gene therapy methodologies. However, with great potential comes great risk and where GE offers the ability to fundamentally alter a patient's genetic makeup for the better, there is also the possibility that these irreversible genetic changes could extend to unknown off-target effects of an unpredictable magnitude. It is therefore understandable that GE development, at least in medical research, has taken years to get to the point of commercialization. However, with the rapid advancement of innovative technologies and scientific approaches, developing GE technologies to a scalable and reproducible standard has been demonstrated on multiple occasions by academic researchers. Furthermore, now that the theoretical possibilities of GE have been proven—for example, with CRISPR winning the Nobel Prize for Chemistry in 2020—there has been significant interest and investment from the pharmaceutical sector to further build on the work of academics and take GE technologies from bench to bedside.

Whilst the physical development of these technologies is increasing at an unprecedented rate, there are still many hurdles to navigate before successfully reaching the intended patient population, not least the ability to demonstrate safety and efficacy to the regulators. Due to the inherent complexity of GE products, there are also still many limitations in the production of GE products, including the ability to manufacture to cGMP standards in a reproducible and scalable manner [1]. The validation of methods used to manufacture and test GE products also presents significant challenges due to the complexity and relative novelty of the methods required.

Due to the lack of existing case studies in clinical and real-world settings, in addition to the challenges with manufacture, there is little precedent for regulators to draw upon in order to develop appropriately insightful and supportive guidance for development of GE products and eventual licensure. As such, there is much discordance between regulators on what constitutes the definition of a GE product—for example, the US FDA defines GE as "a process by which DNA sequences are added, deleted, altered, or replaced at specified location(s) in the genome of human somatic cells, *ex vivo* or *in vivo*, using nuclease-dependent or nuclease-independent GE technologies," whereas the European Medicines Agency (EMA) currently incorporates GE under the umbrella definition of a 'gene therapy' product. Furthermore, some regions have not yet defined GE products as a separate class of medicine, let alone come to a harmonized agreement on recommendations for the most suitable course of development. However, with the increasing number of GE products in development, there is a broad recognition from the major global regulatory agencies that the development of guidelines and legislation specifically for GE technologies is required.

Improvements have been made by some regulators to support developers of GE technologies through the introduction of draft guidance documents and concept papers, in addition to raising awareness through webinars and the creation of dedicated working groups. However, it is important to note that this is very much a first step towards the development of a regulatory framework, rather than a definitive output. Additionally,

when a dedicated regulatory framework is developed for GE products, it will most likely be national or at best, regional, rather than harmonized global frameworks.

Despite the current lack of specific regulations, or harmonized approaches, 2023 was a landmark year for the development of GE therapies. The first ever CRISPR-based technology was approved by a regulator—the UK MHRA, in November, just 3 years after the technology won the Nobel Prize in Chemistry. Casgevy™ (exagamglogene autotemcel), a CRISPR-Cas9 GE tool developed through a collaboration between Vertex Pharmaceuticals and CRISPR Therapeutics, is designed to treat β-thalassaemia and sickle-cell disease, two rare, inherited genetic diseases caused by mutations in the gene (*BCL11A*) that encodes hemoglobin [2]. Other regulatory agencies followed suit shortly afterwards with both the EMA and US FDA also approving the same product in subsequent months (December for both the EMA and FDA approving Casgevy for the treatment of sickle-cell disease, but January 2024 for the approval of Casgevy to treat β-thalassaemia).

There are, however, many caveats to using this 'n=1' as a benchmark for other GE developers; most notably the precedent is limited to a positive benefit:risk profile for patients with a rare disease associated with a poor prognosis. Furthermore, as the number of GE products in the clinic increases and knowledge of long-term side effects becomes more apparent, there may be a change in approach to regulation, so what may have been acceptable for the approval of the first GE products may not be applicable as scientific and technical knowledge of these products increases. Similarly, as experience with these products grows within each regulatory agency, new guidance and legislation specific to GE technologies will become available and potentially, even new regulatory pathways and/or initiatives may appear to support GE developers.

Although there is currently only one approved GE product on the market, there are many more in clinical development and with the momentum of such a monumental approval, there is much excitement and interest from the industry in what 2024 may bring. The GE industry has a predicted worth varying between $10.8 billion in 2028 [3] to $19.9 billion in 2030 [4] which, given the current $2.2 million price tag of Casgevy and an estimated annual revenue of $2.6 billion [5], provides an attractive prospect to potential investors. As of March 2024, there are 97 publicly listed active clinical trials involving GE products, with seven in Phase 3 development and many more in the earlier phase of clinical development [6]. The development of GE products is spanning multiple indications, with >50 disease types currently being studied in clinical trials, although more than a quarter of these are devoted to blood disorders and just less than 20% are focused on oncology. This breadth and diversity reflect a healthy pipeline that is possibly capable of supporting the estimated market worth in just a few years.

2024 promises to bring new highlights to the GE space, with the first prime editing therapy (a new type of CRISPR technique) expected to reach the clinic just 5 years after the technology was first published [7–9]. At the other end of the development spectrum, Intellia Therapeutics was granted access to the EMA's PRIME scheme for its CRISPR-Cas9 technology at the end of 2023, which is currently in Phase 3 clinical trials for the treatment of transthyretin amyloidosis [10].

Interest, investment, and opportunities in the GE space are set to continue their rapid growth throughout 2024. However, the true potential of these products may only be fully realised if the regulatory framework is there to support it. Therefore, alongside the increasing efforts of GE developers, there is also a need for regulators to focus on the process for review and approval of GE products.

Aside from the obvious need to demonstrate safety and efficacy of any new therapeutic, additional critical areas for GE regulation include determining the potential

for off-target effects and the magnitude of these. The true impact of altering a genome may only be realised with long-term follow up studies and these take time to complete. Furthermore, regulators need time to understand how these technologies work in order to regulate them effectively, further highlighting the importance of collaboration and information sharing between industry and regulators.

Despite the regulatory challenges that exist for GE products, there has been an increased output in support from regulators. The start of 2024 has already marked the introduction of a final guidance from FDA incorporating specific information on genome editing [11] and this has been followed by a dedicated webinar to provide further context to the guidance and support GE developers. There has also been strong communication from the US regulator that all applications for GE products should be placed under accelerated approval pathways, demonstrating the importance and prioritisation of these products. Although there is not yet anything similar from EMA or MHRA, with the increased clinical pipeline for GE products, it is highly likely that they will follow suit and start implementing their own versions of similar guidelines and initiatives to support GE developers to accelerate approvals.

Undoubtedly, GE technologies have experienced significant progress into the clinic over a remarkably short period of time; however, there is still much work to be done to bring these innovative therapies to market. Regulators must play their part in ensuring that GE products have access to accelerated routes of approval in addition to early and frequent collaboration with developers to understand the risks of such products. Balancing patient safety with the provision of curative, one-time therapies is understandably challenging for regulators, but is critical in ensuring patients have access to these life-saving treatments. The coming years will define a new era of medicine and consequently, regulation, and whilst 2023 marked the start of this new era, it is 2024 that holds the greatest promise in building on the momentum of change.

REFERENCES

1. Doudna JA. The promise and challenge of therapeutic genome editing. *Nature* 2020; 578(7794), 229–36.
2. Wong C. UK first to approve CRISPR treatment for diseases: what you need to know. *Nature* 2023; 623(7988), 676–7.
3. Gene Editing Market Global Forecast to 2028.
4. Gene Editing Market Size Forecast 2022–2030.
5. Parish, M. The FDA just approved the first CRISPR drug. Here's what's next in gene editing. PharmaVoice, 2023.
6. ClinicalTrials.gov Search for: Other terms: gene editing, Phase: 2.
7. Zhang S, Wang Y, Mao D *et al.* Current trends of clinical trials involving CRISPR/Cas systems. *Front. Med. (Lausanne)* 2023; 10, 1292452.
8. Philippidis A. First prime editing clinical trial expected in 2024. *Hum. Gene Ther.* 2023; 21–22, 1077–80.
9. Prime Medicine reports full year 2023 financial results and provides business updates.
10. Intellia Therapeutics receives priority medicines (PRIME) designation from the European Medicines Agency for NTLA-2002, an investigational *in vivo* CRISPR genome editing treatment for hereditary angioedema.
11. *US FDA.* Guidance: human gene therapy products incorporating human genome editing.

BIOGRAPHY

HARRIET EDWARDS is Associate Director of Regulatory Affairs at Boyds. With over 10 years of experience in leading and supporting regulatory projects and business critical activities, Harriet is an award-winning regulatory professional and chartered scientist. Harriet provides strategic and operational regulatory advice to help guide clients' drug development journeys across a range of therapeutic applications and regions including the USA, EU, and APAC. She has also worked across development phases from early clinical development through to lifecycle management.

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CELL & GENE THERAPY INSIGHTS

VIEWPOINT

Human capital comes with a memory

Helena Strigård
CEO, Haeger & Carlsson Executive Search and Interim

"Being respectful will take you far. You are dealing with people, not digits!"

VIEWPOINT

Cell & Gene Therapy Insights 2024; 10(3), 211–214
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As a biotech company, accessing the right human capital and financial capital in a timely manner determines your success. Both entities are key to building your company. They also depend on each other in a way that creates a bit of a 'Catch 22' for any growing company in need of venture funding. Without the right team, your struggle to raise financial capital will be even more brutal. And without the latter, you might not be able to enroll the sharpest minds. For companies in new therapeutical areas such as cell and gene therapy that are already perceived as high-risk, you might be faced with an even tougher challenge to convince investors that you and your co-workers have what it takes.

As part of their due diligence, investors will look into your management team, your board of directors, and your ability to attract key talent over the growth years ahead.
Over the last few years of ups and downs in the industry, the intertwined relationship of human and financial capital has become even more impactful on companies' prospects of success.

The point of this article is to share some hands-on advice on how to cruise through the waves of access to capital through a clever, yet highly ethical way of dealing with human capital (with an emphasis on the 'human' part of this somewhat unempathetic term). As a Nordic-based recruiter specialized in life sciences, this is close to my heart.

THE CHALLENGE MANY OF YOU ARE FACING, OR HAVE JUST FACED

You staffed up in 2022 based on the ways things were during the pandemic years. Access to capital in 2021 was extraordinarily high and while this was not entirely unexpected, few were genuinely prepared for the sudden drop that came in 2022 when capital costs increased. For service providers, always lagging one or two steps behind the capital-eating R&D companies that they depend upon, there were less of an excuse to not hit the brakes in time. They should have seen it coming, but many continued staffing up well into 2022 with continuous growth in mind. However, by then, financial capital had become relatively costly and scarce, and the only way to stay in the game until the next inflection point and keep your investors happy was to cut down on human capital. This strategy was pursued with a sense of urgency that might not have permitted you to think twice about how it would affect your company, once (and if) the wheels started spinning again.

This is a scenario that will be all too familiar to many in the biotech space.

In some countries, cutting down on staff is a costly matter in the short run. Yet it is nothing compared to the cost of brain drain—of losing the key competences that you really needed to reach the long-term goals of the company. Having to let people go is awful, I know. It always is when we are affecting people´s lives in a way that is likely to be upsetting at the very least, and sometimes even devastating, and many people in this business have a loyalty with the company and its mission that goes beyond what could be expected. You most likely did not have a choice at the time. But how you did it mattered, and still does.

If your company is among those that are still standing, you might have to start thinking about bringing in key competence again sooner than you expected. Maybe your investors have already grasped that their whole investment is at stake unless the team is adequately staffed. However, your ability to attract talent moving forward will depend on how your company handled the recent downsizing. This is because human capital comes with a memory.

I believe this holds true regardless of whether you are operating in a hire-and-fire labor market such as the US, or a more regulated 'flexicurity' market such as that in the Nordic countries. It is also about ethics, of course. The following is advice for the various situations you might be facing when dealing with either downsizing or attracting human capital.

WHEN YOUR TEAM HAS TO BE DIMINISHED...

Provide transparency as far as possible. Explaining the underlying reasoning of the cut in human capital and what will have to be stripped in terms of activity builds trust.

Ensure a clear process. Set a timeline and stick to it. If you deviate from the process, explain why. Do not make promises you cannot keep.

Communicate! It is human nature to shy away from communicating in tough situations as people feel they do not have something constructive to say, or simply do not know how. Therefore, seek professional

help if needed to be clear on your messages. Communicate even when there is no definitive news to impart. This is the ultimate stress test of your internal information cascade. Staff members who are kept well informed, and who trust that they will continue to be, don´t spend as much time and energy guessing.

Avoiding discrepancies in access to information is key. However, there will be substantial discrepancies in both the detail of information that various parts of your team access, and the timing with which they receive critical information. This means that processing bad news occurs at different speeds in different parts of the company. Although this is somewhat inevitable, it is possible to avoid unwanted loss of competencies by staying close to the information reality of your co-workers. Using emotional quotient will sharpen your senses and has nothing to do with being soft (or maybe soft is being sharp?)

It might seem contradictory to work with employer branding during a downsizing, but that is exactly when you should stay true to your ethics and policies. Walk the talk.

WHEN YOUR TEAM IS GROWING...

When wheels are spinning fast in the other direction, you might tend to focus on the final candidates of your recruitment process and forget about the rest. How you deal with all candidates, or how your recruitment firm deals with them, will impact on your employer branding. And before you know it, a candidate who was turned down in your most recent recruitment drive might pop up again as the ideal one in the next, or at a client/partner company. It is important to remember that human capital does not only come with a memory—it has a voice as well.

Make use of your investors' experience in building teams. If you don´t have the full team already, share what competences you need and ask your investors to help through their vast networks. Additionally, the buy-in generated through this approach might prove helpful down the line.

Make every head count! Many start-ups are led by a CEO who is also the C-everything, tasked with taking on an international market while reporting to a Board of Directors of approximately seven members. Those men and hopefully, some women, should be strategic players on this mission, too.

That leads us to diversity of perspectives. What is the use in having seven pairs of cloned eyes, all with the same experience, background, and reasoning when facing challenges? You could make do with just one set of those particular eyes and cut down on cost significantly. Although this is most likely something that is not within your control, keep in mind that the more diverse your board is, the more likely you are to find the strategic advice needed on the complex, ever-changing journey of building a biotech company.

AND AT ALL TIMES...

Being respectful will take you far. You are dealing with people, not digits!

Consider whether those of your key competences that normally come at a high cost could be made available through part-time positions or interim consultants. In the Nordic life science ecosystem, the use of shared C-level staff and specialists is growing popular. For instance, a CFO of a start-up normally carries out a crucial role for operations and will matter when investors carry out their due diligence. You want your books in order and processes in place. So, how about hiring half a senior CFO, supported by junior staff, and splitting the cost with another company? For smaller ecosystems such as the Nordic one, this also opens up the possibility for knowledge-sharing and enables them to punch above their weight.

BIOGRAPHY

HELENA STRIGÅRD holds a MSc in Molecular Biotechnology Engineering and a BA in Management. She is the CEO and partner of Haeger & Carlsson Executive Search and Interim, a premier league recruitment company operating in the Nordic life science space. Prior to joining Haeger & Carlsson, Strigård held leading positions in the industry, including as CEO of Swedish-based CDMO NorthX Biologics and Director General of SwedenBIO, the Swedish life science industry organization. In her current undertaking, Strigård is building on her previous experience in the public sector, including as Head of Section at the Finance Ministry of Sweden between 2012–2017, as well as her roles in industry, to create strong teams, thus supporting life science companies from all over the Nordics and beyond on their international growth journey.

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CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Evaluation and performance of an AAV affinity resin: a CDMO case study

Spyridon Gerontas and Buzz Lobbezoo

Often referred to as the gold standard for *in vivo* gene therapy, the adeno-associated virus (AAV) has seen a huge increase in its use in clinical trials over the last few years, with various serotypes used depending on the target tissues and cells. There are now six AAV-based gene therapy products on the market, with many more in the R&D pipeline. This has led to an increased demand for the development of efficient and robust downstream purification processes to ensure the production of high-quality AAV vectors for clinical applications. The performance of the affinity capture step of AAV as a scalable unit operation is of particular importance to deliver high purity and recovery.

This article introduces POROS™ CaptureSelect™ AAVX affinity resins for the scalable downstream purification of a range of AAV serotypes for gene therapy applications. A case study from Pharmaron, a leading CRO/CDMO, provides an analysis of the performance of the resin under varying conditions at both small and large scales, from early development runs to assess the resin capabilities to the affinity capture of 22 L of AAV harvest material.

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INTRODUCING POROS™ CAPTURESELECT™ AFFINITY RESINS

Designed for improved process performance and productivity for a wide range of biomolecules, POROS CaptureSelect affinity resins are highly rigid and both chemically and mechanically stable, allowing for linear pressure drop versus flow curves up to very high pressures and flow rates. The POROS backbone has large through pores and a large internal pore volume for improved mass transfer, in addition to a 50 µm bead size for improved separation whilst retaining high flow rates. The pore structure of the POROS bead enables efficient purification of large molecules such

as plasmid DNA, viruses, viral vectors, and virus-like particles.

The CaptureSelect technology platform has a structure derived from the heavy chain antibodies found in *Camelidae*. The VHH domain, a small (12–15 kDa) fragment, is reverse-engineered into an animal origin-free nanobody, which is produced in yeast and is highly selective to its target—in this case, adeno-associated virus (AAV) serotypes. POROS CaptureSelect AAVX resin has an affinity to a wide range of serotypes, including both natural and chimeric vectors, with high binding capacity, purity, and recoveries.

The performance of POROS CaptureSelect AAVX resin is serotype-specific, so process development is critical to obtain optimal process performance. Despite commonalities between different serotypes, each step in the process should be optimized to improve performance, including loading, washes, elution, and clean-in-place.

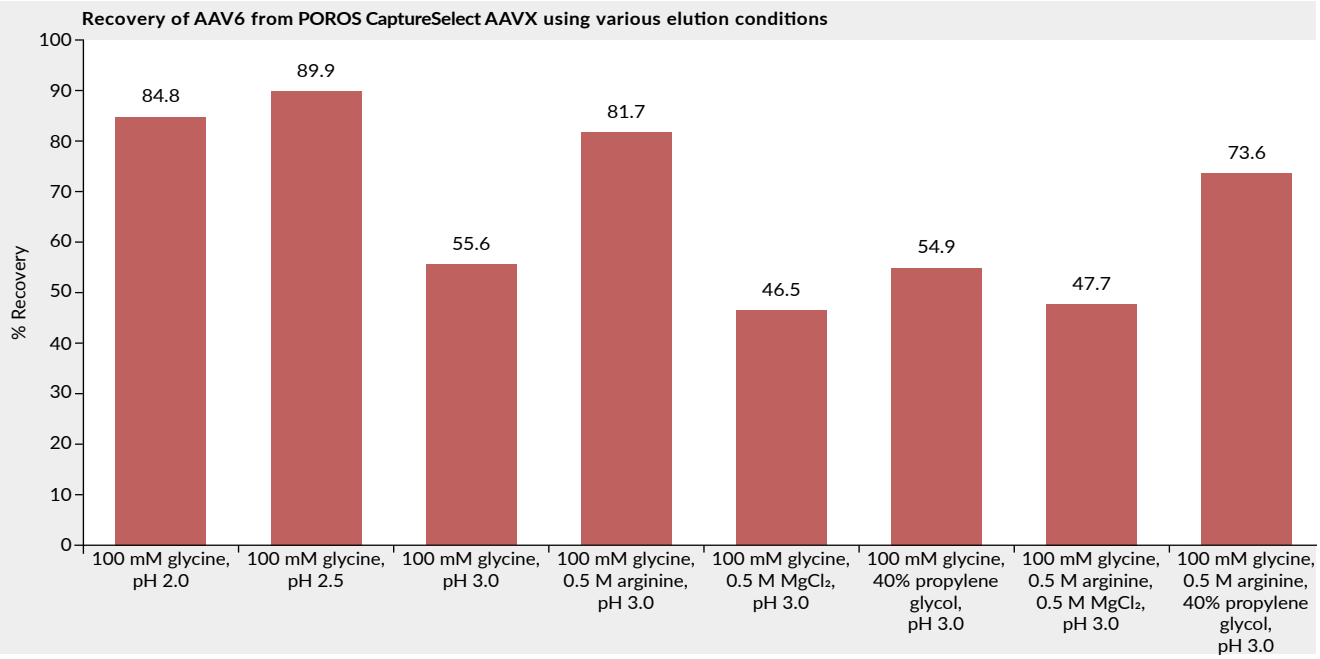
Intermediate wash optimization conducted by Thermo Fisher Scientific has been shown to improve the clearance of process-related impurities. A wash study was performed looking to remove additional non-specific DNA and host cell protein (HCP) binding employing low and high concentrations of NaCl washes with no salt and 1.5 M salt, and an increased pH wash at pH 9.0. With the washes, the levels of residual DNA and HCP were four times lower in the elution.

Optimization of elution conditions is also required to maximize AAV recovery. A study conducted by Thermo Fisher Scientific demonstrated the recovery of AAV6 was optimal at pH 2.5 yielding a recovery of around 90%. Figure 1 shows the recovery of AAV6 from POROS CaptureSelect AAVX resin using various elution conditions. This study shows that additives can be used in the elution buffer to improve recoveries at higher pH conditions.

Analytics also play an important role in ensuring accurate results. One important feature of POROS CaptureSelect AAVX is its high binding capacity for multiple serotypes at short residence times. As demonstrated in Figure 2, capacities have been shown to

FIGURE 1

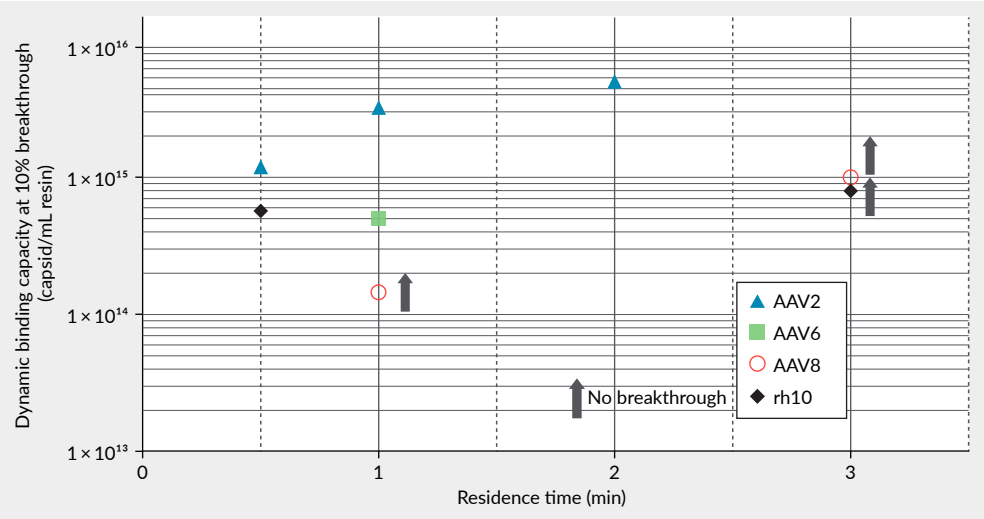
POROS CaptureSelect AAVX elution optimization study.



Data generated by Thermo Fisher Scientific.

FIGURE 2

Breakthrough as a function of capsids loaded per mL of resin.



Data generated by Thermo Fisher Scientific.

exceed 1×10^{15} viral capsids (vp)/mL of resin, achievable with no breakthrough in the case of AAV8 [1]. POROS CaptureSelect AAVX has also been shown to give consistent chromatographic performance and yield over 35 reuse cycles [2].

PHARMARON'S MULTI-SEROTYPE AAV PLATFORM PROCESS

Pharmaron is a leading fully integrated pharmaceutical R&D services platform with global operations. It has a well-established team of over 20,000 employees working in 21 different sites worldwide. Pharmaron's mission is to support their partners in discovery, development, and commercialization of innovative medicines with the vision to become the world-leading life science R&D service company.

Pharmaron Gene Therapy, Liverpool, focuses on viral vector development and clinical manufacture, delivered through our 80,000 sq ft MHRA-licensed cGMP facility. Notably, Pharmaron is embarking on an ambitious plan for the expansion of the Liverpool facility with a £151 million investment in the project, supported by a grant from the UK Government's Life Sciences Innovation Manufacturing Fund (LSIMF). This expansion will lead to a significant increase of Pharmaron' s gene therapy operations to 400,000 sq ft, facilitating the accommodation of viral vector, DNA, and RNA drug substances, along with drug product formulation.

In terms of AAV development and manufacturing, Pharmaron has established an AAV platform and purification toolbox to ensure the production of multiple AAVs, alongside a secure supply of the critical starting materials for these products. Pharmaron's upstream processing consists of a seed train and a production bioreactor, in which the AAV product is expressed following triple transfection of the human embryonic kidney 293 (HEK293) cells. During the seed train, the cells are expanded to inoculate the production single-use bioreactor. Then, they proliferate in a controlled environment to a target concentration, in preparation for the triple transfection, a step which has been optimized to maximize AAV yields and quality. Following harvest, the Pharmaron downstream processing (DSP) team uses its downstream purification toolbox to purify

the viral vector to formulated drug substance. The downstream processing is adapted to the serotype. In general, the clarified product is captured by affinity chromatography. The purification process may require an intermediate chromatography step to further reduce the impurity levels. The product is then loaded onto the polishing chromatography step to separate the genome containing or full capsids from the genome-free or empty capsids. Using ultrafiltration and diafiltration, the purified product becomes drug substance.

PHARMARON'S CASE STUDY ON EVALUATING THE POROS CAPTURESELECT AAVX AFFINITY RESIN

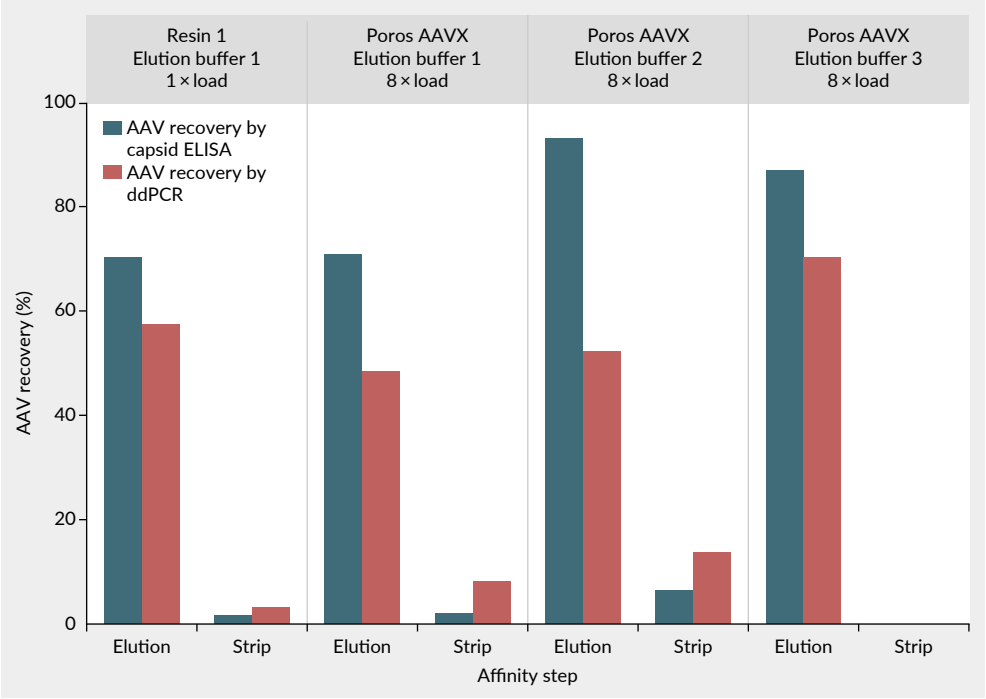
This case study focused on the capture step of the AAV platform and Pharmaron's work on evaluating the POROS CaptureSelect AAVX affinity resin. Pharmaron's assessment of the capabilities of the POROS CaptureSelect AAVX resin began through a series of milliliter-scale experiments. Subsequently, high-throughput (HTP) robotics were integrated with a design of experiments (DoE) approach to screen capture conditions using POROS™ CaptureSelect™ AAVX RoboColumns™. The focus then shifted to estimating the POROS CaptureSelect AAVX resin's dynamic binding capacity (DBC) and determining how much the resin could handle in terms of processing volume. Finally, findings were verified at larger scale by using manually packed columns filled with POROS CaptureSelect AAVX resin to capture AAV from a 22 L cell harvest.

FEASIBILITY RUNS TO ASSESS POROS CAPTURESELECT AAVX

An exploration into the capabilities of the POROS CaptureSelect AAVX resin

FIGURE 3

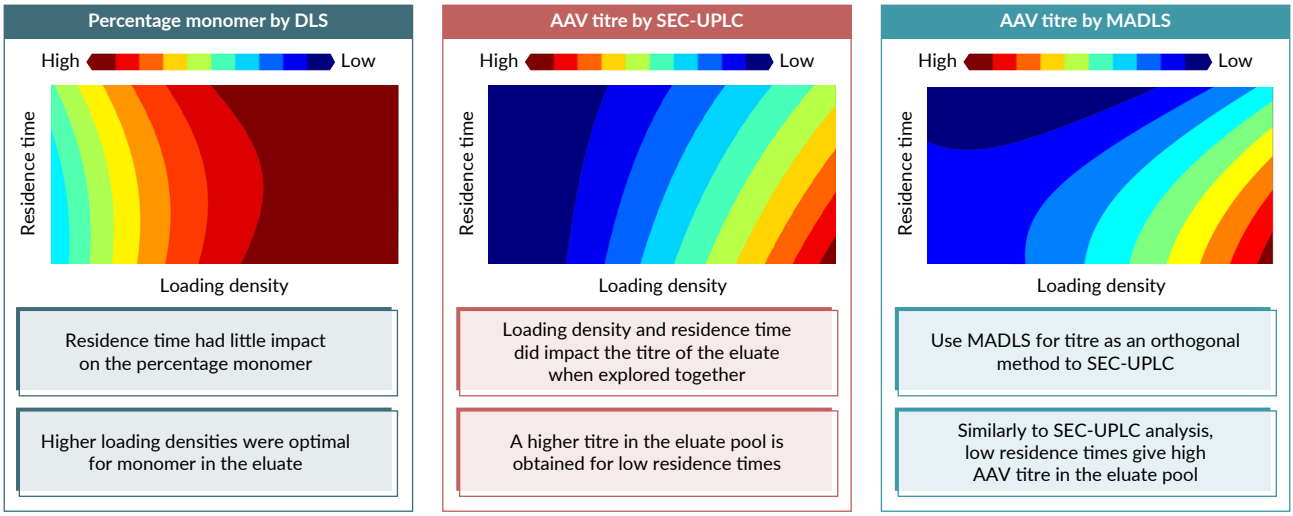
AAV recovery by capsid ELISA and ddPCR of feasibility runs to assess POROS CaptureSelect AAVX capabilities.



The chromatography runs and data analysis were performed by Pharmaron Gene Therapy.

FIGURE 4

HTP robotics/DoE contour plots showing the effect of POROS CaptureSelect AAVX process parameters on AAV monomer content and AAV elution titer.



The chromatography runs and data analysis were performed by Pharmaron Gene Therapy.

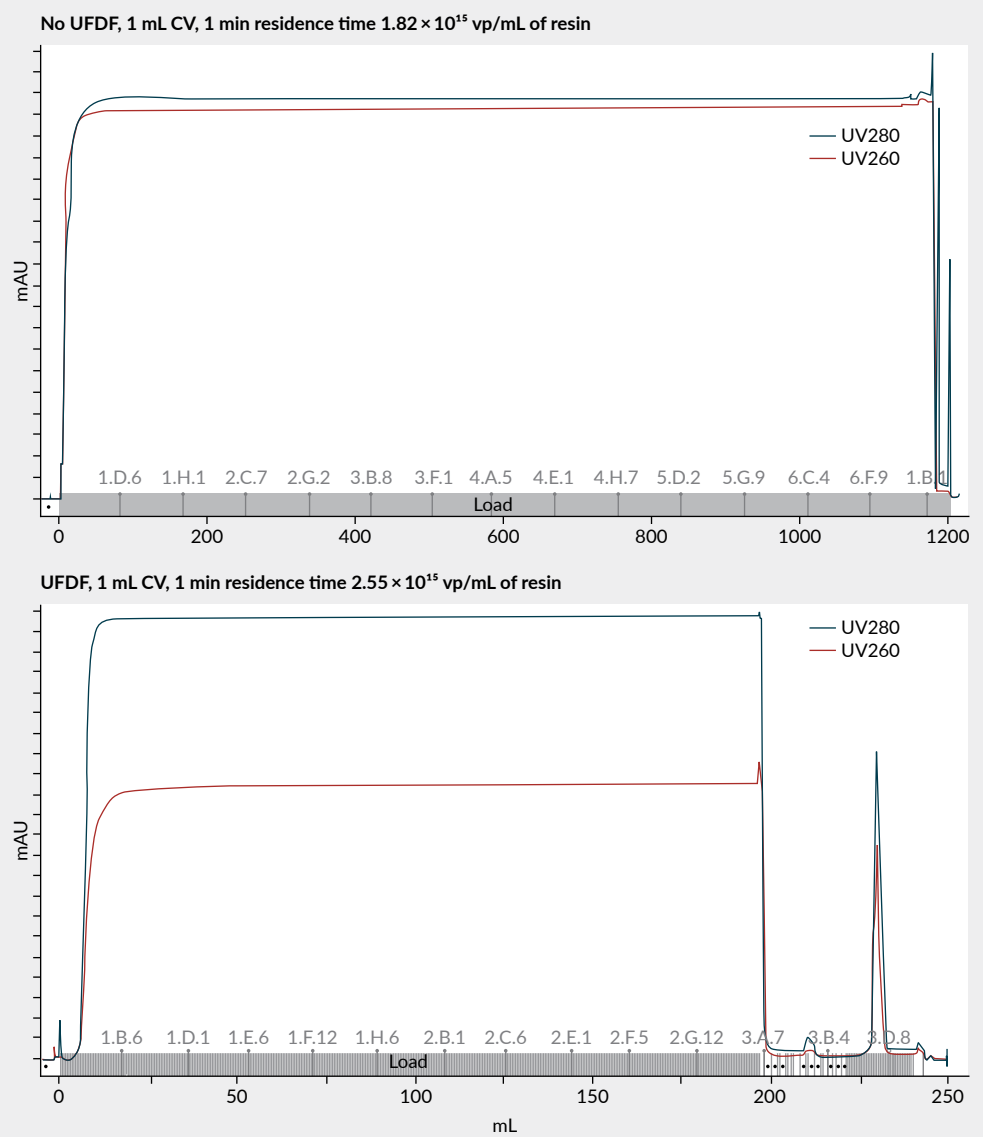
was initiated by conducting experiments on a small scale using 1 mL pre-packed columns connected to a Cytiva ÄKTA™ avant 25. The performance of the POROS CaptureSelect AAVX resin was compared in terms of recovery against a control affinity resin. Both resins were loaded at their recommended linear velocity for the same duration. The POROS CaptureSelect AAVX resin was shown to handle significantly higher linear velocities resulting in an 8-fold increase in loading volume. To ensure consistency, identical buffers were used for capturing material using both the control and POROS CaptureSelect AAVX resin. The effect of buffer composition on AAV recovery was investigated by testing two different elution buffer compositions. AAV recovery values, measured by capsid enzyme-linked immunosorbent assay (capsid ELISA) and droplet digital polymerase chain reaction (ddPCR), are shown in Figure 3. POROS CaptureSelect AAVX resin demonstrates similar recoveries with an 8-fold increase in load compared to the control resin. To enhance efficiency, the wash and elution buffers were fine-tuned to reduce the quantity of AAV in the strip.

UTILIZING HIGH-THROUGHPUT ROBOTICS AND DESIGN OF EXPERIMENTS TO OPTIMIZE POROS CAPTURESELECT AAVX FOR AAV CAPTURE

Following the feasibility runs, the use of HTP robotics and DoE was explored in the optimization of AAV capture parameters. Automation facilitated by HTP and DoE significantly streamlines product development processes. The HTP studies were conducted using POROS CaptureSelect AAVX 200 µL robocolumns in combination with a Beckman Biomek® i7 automated workstation. An experimental plan, based on the central composite design, was implemented using JMP® software to guide experiments. The focus was on optimizing titer and monomer content by exploring loading pH, loading density, and residence time. The success criteria for the HTP experiments comprised achieving a DBC>1×10^{14} vp/mL of resin, a

FIGURE 5

Loading profiles obtained by capturing AAV from post clarified material using 1 mL prepacked POROS CaptureSelect columns with and without UF/DF utilization before the affinity step.



Process parameters	No UFDF	UFDF
UV260/UV280 in loading	0.98	0.62
Loading time (min)	1180	200

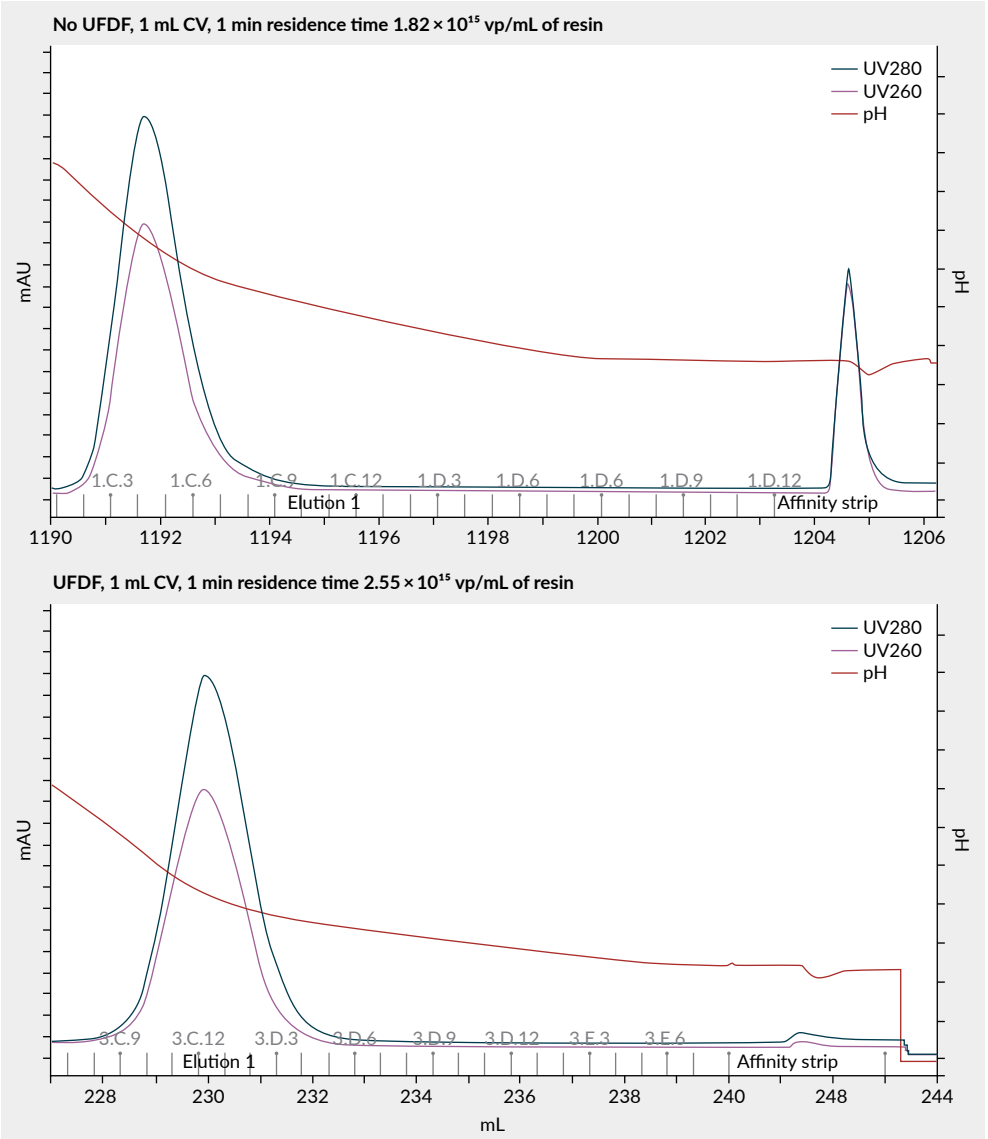
The chromatography runs and data analysis were performed by Pharmaron Gene Therapy.
UF/DF: ultrafiltration/diafiltration.

residence time <3 min, an AAV recovery rate >70%, and aggregate content <5%.

Analysis was conducted on the data produced from HTP robotics/DoE using contour plots (Figure 4). Residence time was shown to have little impact on the percentage monomer whilst higher loading densities were optimal for monomer in the eluate (left plot of Figure 4. Loading density and residence time were found to impact the titer of the

FIGURE 6

Elution profiles obtained by capturing AAV from post clarified material using 1 mL prepacked POROS CaptureSelect columns with and without UF/DF utilization before the affinity step.



POROS CS AAVX attributes	No UFDF	UFDF
Recovery by capsid ELISA (%)	107	121

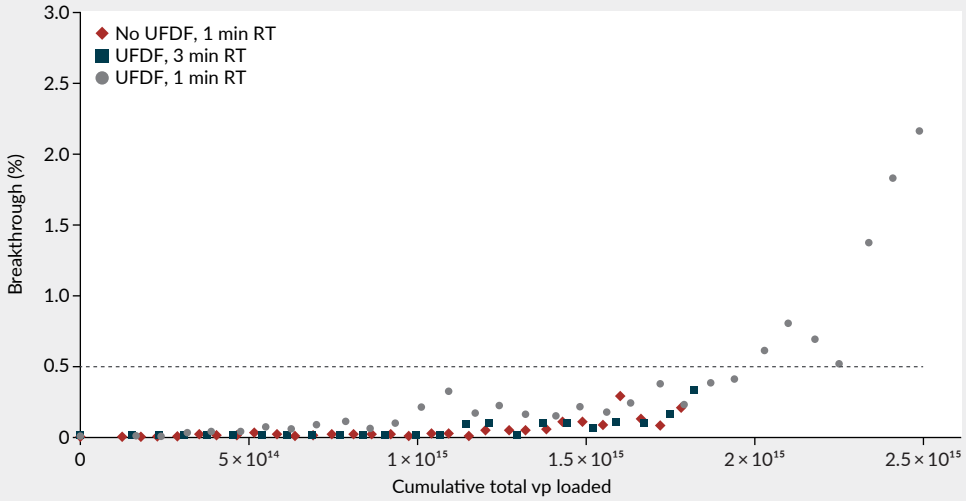
The chromatography runs and data analysis were performed by Pharmaron Gene Therapy.
UF/DF: ultrafiltration/diafiltration.

eluate when explored together, and a higher titer in the eluate pool was obtained for low residence times (center plot of Figure 4).

The AAV titer was measured by multi-angle dynamic light scattering using a Malvern Panalytical Zetasizer® Nano ZSP system in relation to loading density and residence time (right plot of Figure 4). This analytical method serves as an orthogonal method to size exclusion ultra-performance liquid chromatography (SEC-UPLC) for quantifying AAV using a Waters SEC column affixed to a Thermo Fisher Scientific Vanquish™ Horizon UHPLC. Some differences between

FIGURE 7

POROS CaptureSelect AAVX dynamic binding capacity estimation with and without UF/DF utilization before the affinity step.



POROS CS AAVX attributes	No UFDF, 1 min RT	UFDF, 3 min RT	UFDF, 1 min RT
Linear velocity (cm/h)	300	100	300
Loading time (min)	1180	100	300
Loading density (vp/mL of resin)	1.82×10^{15}	1.79×10^{15}	2.55×10^{15}
Recovery by capsid ELISA	107	99	121
HCP (ng/1 × 10^{13} vp)	<5.85	<5.43	<7.88
hcDNA (ng/1 × 10^{13} vp)	3.17	2.62	2.41

The chromatography runs and data analysis were performed by Pharmaron Gene Therapy.

the two analytical methods emerge at low loading densities, although these variations did not affect the conclusions drawn from the contour plots. As with SEC-UPLC analysis, low residence times yield higher AAV titer in the eluate pool.

To summarize the HTP results, the loading density has the greatest impact on generating monomeric AAV, and low residence times are essential in achieving high recoveries.

ESTIMATION OF POROS CAPTURESELECT AAVX DYNAMIC BINDING CAPACITY

To assess the dynamic binding capacity of the POROS CaptureSelect AAVX resin, 1 mL POROS CaptureSelect AAVX columns were loaded at a concentration of 2×10^{15} vp/mL of resin with a 1 min residence time using harvest material. Conditions in which the harvest material underwent buffer exchange and concentration using ultrafiltration/diafiltration (UF/DF) were explored to reduce loading time from 1180 to 200 min and to avoid exposing the harvest material to extended periods at ambient temperature before loading onto the resin (Figure 5). The ratio of UV260 to UV280 was also reduced indicating the removal of DNA-related impurities during the UF/DF step (Figure 5).

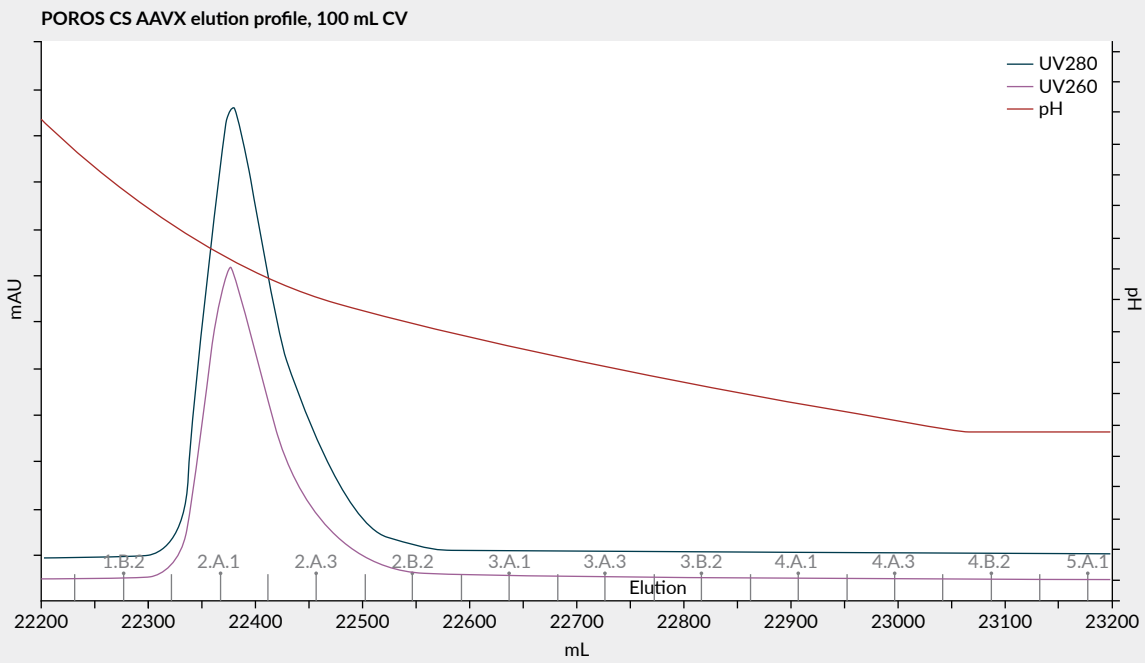
The POROS CaptureSelect AAVX elution profiles with and without UF/DF utilization before the affinity step were also analyzed.

In both cases, symmetrical AAV peaks were observed without any pre- or post-peaks, and minimal AAV loss was detected via capsid ELISA (Figure 6). Notably, in the absence of UF/DF, a peak was evident in the strip, indicating the presence of impurities during affinity load. These impurities were potentially non-specifically absorbed by the POROS CaptureSelect AAVX resin.

The percentage breakthrough of loaded capsids is shown in Figure 7, against the total viral particles loaded onto a 1 mL column for three distinct loading conditions. In the initial condition, the percentage breakthrough was assessed without using UF/DF to concentrate the material before loading, maintaining a residence time of 1 min. Even when loading POROS CaptureSelect AAVX at 1.82×10^{15} vp/mL of resin, no breakthrough was observed. Subsequently, the impact of residence time on the condition in which the cell harvest was concentrated using UF/DF was investigated. At a residence time of 1 min, the UF/DF condition showed a 0.5% breakthrough at 2×10^{15} vp/mL of resin (Figure 7). The residual host cell DNA

FIGURE 8

POROS CaptureSelect AAVX elution profile from 22 L clarified material.



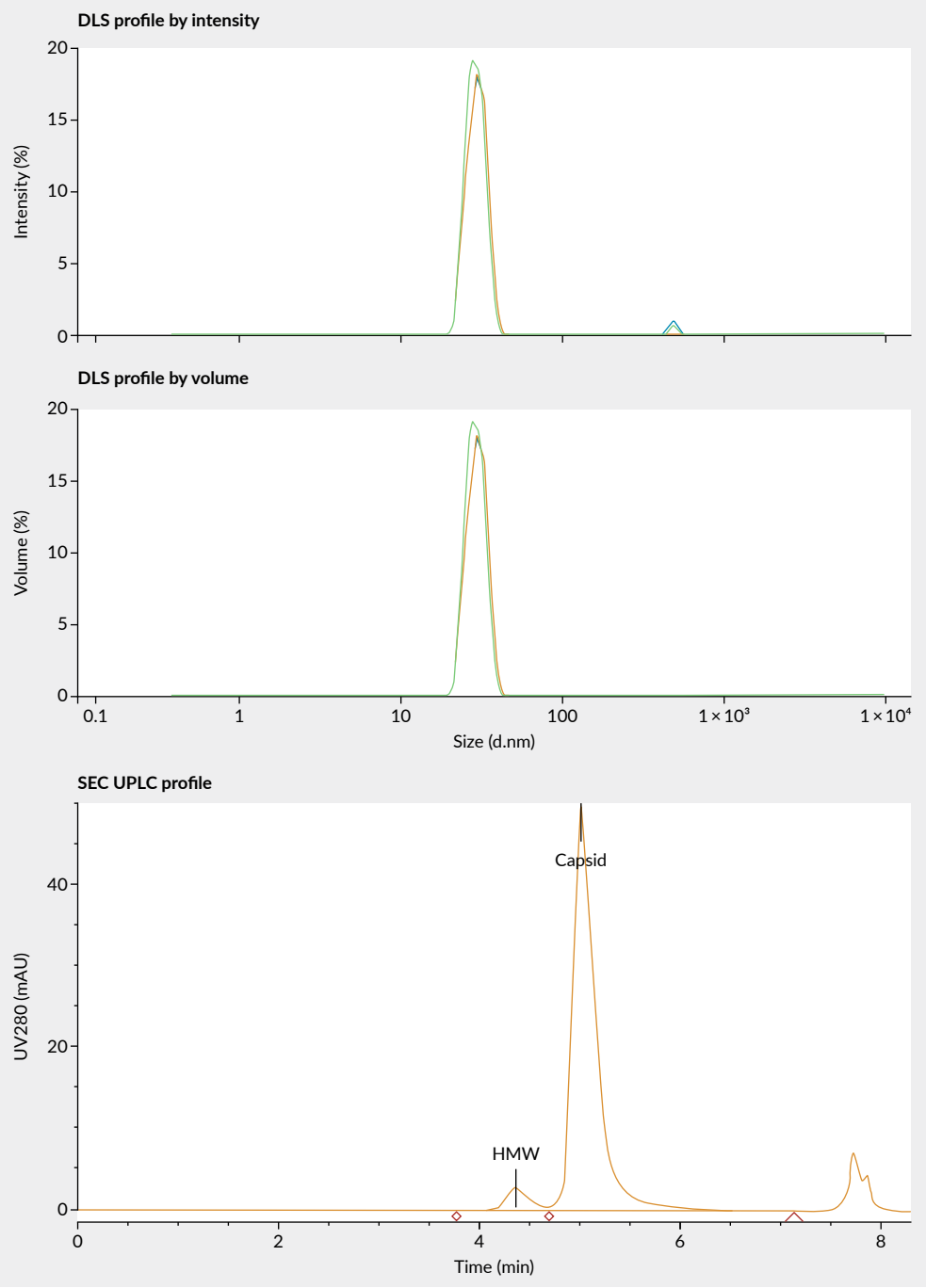
POROS CS AAVX running parameters and attributes
Processed volume=22.5 L of clarified material
Poros CS AAVX volume/height=96 mL/18.1 cm
Loading=3.90×10^{14} vp/mL
Residence time/linear velocity=~2 min; 450 cm/h
Recovery (by capsid ELISA/ddPCR)=93.4%/67.7%

The chromatography runs and data analysis were performed by Pharmaron Gene Therapy.

and HCP levels were <4 ng/1 × 10^{13} vp and <8 ng/1 × 10^{13} vp respectively for all runs (Figure 7). These experiments underscored the capability of POROS CaptureSelect AAVX resin to capture AAV at high loading densities and low residence times.

FIGURE 9

Monomer content of POROS CaptureSelect AAVX eluate from loading 22 L clarified material.



The chromatography runs and data analysis were performed by Pharmaron Gene Therapy.

AFFINITY CAPTURE OF 22 L OF AAV HARVEST MATERIAL

After small-scale runs, an evaluation of the performance of the POROS CaptureSelect AAVX resin was performed at a larger scale. The large-scale run began with manual packing of the AAVX resin following the Thermo Fisher Scientific Instructions [3].

Two columns were packed (2.6 cm diameter Cytiva HiScale™ 10/40 and 1 cm Cytiva AxiChrom™ 50) with POROS CaptureSelect AAVX resin and asymmetry results and the number of theoretical plates per meter met requirements [4].

The harvest material (22 L) was processed using a Cytiva ÄKTA avant 150 with a 2 min residence time, running at the linear velocity of 450 cm/h. Figure 8 shows the operating conditions and elution profile. the recovery rates validated via capsid ELISA and ddPCR were high. The UV elution profile showcased a single, sharp peak devoid of pre- or post-peaks, which supports the high recovery result. This result confirmed the optimized host and elution buffering conditions derived from the feasibility and DoE runs.

Additionally, analytical assessment was conducted to characterize the product quality. The findings are shown in Figure 9. The monomer content of POROS CaptureSelect AAVX eluate was estimated by dynamic light scattering (DLS) and SEC-UPLC. The DLS analysis was performed on a Malvern Panalytical Zetasizer Nano ZSP and provided an estimate of the large-size aggregates (typically, those >0.2 µm). The SEC-UPLC analysis estimated the small-size aggregates (typically AAV dimers, trimers, and tetramers) and it was performed using a Waters SEC column connected to a Thermo Fisher Scientific Vanquish Horizon UHPLC. A monomer content of 99% was measured by DLS and 93% by SEC-UPLC. The residual host cell DNA and HCP levels were <4 ng/1×10^{13} vp and <2 ng/1×10^{13} vp respectively. These large-scale run results confirmed the findings of the development work at small scale.

SUMMARY OF THE POROS CAPTURESELECT AAVX ASSESSMENT

The POROS CaptureSelect AAVX affinity resin is designed to address the high selectivity and capacity requirements for the large-scale downstream purification of a wide range of both natural and chimeric AAV serotypes. The use of Pharmaron's HTP robotics and DoE expertise in combination with POROS CaptureSelect AAVX RoboColumns delivered a rapid screening of AAV capture conditions. This helped Pharmaron to develop AAV capture conditions that enable low processing times and increased processing volumes, resulting in shorter development times for large-scale batch production. The POROS CaptureSelect AAVX rigid matrix facilitates column packing, ensuring alignment with specifications for both asymmetry factors and plates/meter, enabling Pharmaron to perform robust and repeatable large-scale affinity runs, achieving high AAV recovery and impurity clearance.

Q&A


Buzz Lobbezoo and Spyridon Gerontas

Q What is the binding capacity of the AAVX resin? Can this resin be reused?

BL: The binding capacity for the AAVX resin is variable and dependent on serotype. We have used a multitude of different serotypes and have achieved binding capacities in the region of 1×10^{15} vp. There are a few serotypes where slightly lower capacities are seen, so we recommend the completion of DBC evaluation work or breakthrough studies.

The resin can be reused. The number of times it can be reused depends on how you treat the feedstock and the resin. When the resin is treated with the right amount of cleaning material and regenerated with low pH strips, as well as a denaturant like urea guanidine, we have been able to reuse the resin for over 35 cycles.

Q What is the best approach to improving elution recovery on POROS CaptureSelect AAVX?

BL: The short answer is low pH. The best approach is to perform an optimization study looking at a range of pHs and additives. We also recommend the addition of pluronic in the elution. It is important to be confident in your analytics, so you can look at different orthogonal steps and assays to ensure that you are achieving accurate values.

Q Why does the lower residence time give a higher purification yield?

SG: AAVs can aggregate until they are loaded onto the resin. Low residence times may result in higher purification yields of AAV monomers, as they translate into shorter material hold times at ambient temperature, consequently minimizing the drop in purification yield

due to AAV aggregation. Furthermore, optimizing the buffer composition of the AAV feed solution can prolong the time AAVs remain in the monomeric state at ambient temperature, thereby achieving high purification yields of AAV monomers. Developing formulation recipes for in-process steps can be performed in-house or in collaboration with a CDMO.

BL: In our studies, we have seen that in some instances, reducing the residence time can be beneficial both from a stability standpoint and to reduce the amount of entrapment. When running at much lower flow rates, you can run the risk that some of the particles becoming trapped, leading to a reduction in recovery. It is worth investigating and optimizing residence times.

Q How does the low pH affect the infectivity of the virions?

BL: We have seen that lowering the pH, especially down to pH 2.0, can increase aggregation. This effect can be serotype-dependent. We also recommend performing the elution promptly to neutralize rapidly.

Q Can we apply cell lysate on POROS CaptureSelect AAVX resin directly without UF/DF buffer replacement?

SG: Yes, this can be done. It depends on the approach to retrieve AAVs from cell culture material. If the AAVs are retrieved through cell lysis, there can be more impurities, so a UF/DF step may be needed. The Cost of Goods (CoGs) must also be considered here, as the addition of a UF/DF step may increase DSP costs. The development team should perform a CoGs analysis to estimate whether it is better to load the AAV material directly onto the affinity resin without the UF/DF step, even though the capacities will be slightly lower.

Q What were the main challenges experienced in Pharmaron's platform process development?

SG: The main challenges were linked to the complexity of AAV vectors. The process must be looked at holistically; it is not as simple as optimizing the upstream process, and then passing the material to the downstream processing team for purification. It is crucial that the upstream and downstream processing teams work together to transform the material into a drug substance with low impurities, and to ensure a highly productive process. Achieving this goal required support from the Pharmaron analytics team, to provide high-sensitivity, low-volume analytical methods.

Q What is, in your experience, the best analytical method to detect the empty, partially full, and incomplete AAV capsids from the full capsids?

SG: The gold standard is analytical ultracentrifugation (AUC), which we use routinely at Pharmaron. We also employ mass photometry, another accurate method, to achieve higher throughput analysis with lower sample volumes for development purposes. Other methods include anion exchange-high performance liquid chromatography (AEX-HPLC) and ddPCR/capsid ELISA.

Q Why do the UF/DF versus non-UF/DF methods have different DBCs?

SG: This is due to the feed. It is key that the development team optimizes the feed composition. The preceding step always plays a huge role in what will happen, not only to the affinity step, but to the following downstream processing steps. When a team optimizes a chromatography step, for instance, they need to consider all preceding steps

BL: Different setups have different binding capacities due to how you have lysed cells and what is present in your feedstock. A high level of impurities can lead to a steric hindrance, an effect on the binding capacity, or some non-specific interaction. Washes can help with this. Potentially dirty feeds can reduce your DBC and the ability to reuse the resin. How you treat the feeders is key.

Q When evaluating purification yields, is it correct that both capsid ELISA and ddPCR methods are used, but only ddPCR measured the filled capsids?

SG: Yes, ddPCR measures the filled capsids. In the affinity step, we do not expect to have different recoveries by capsid ELISA and ddPCR, because we do not have separation of the full from empty capsids. However, ddPCR is used as an orthogonal method in this case to be sure that any buffers used do not cause rupture of the capsid surface and therefore ejection of the transgene.

REFERENCES

1. Ravault V, Laroudie N. Platform optimization for efficient AAV purification: insights from a CDMO. *Cell Gene Ther. Insights* 2022; 8(1), 145–158.
2. Laroudie N, Bazot Q. Advancing gene therapy development with a multi-serotype AAV affinity resin. *Cell Gene Ther. Insights* 2024; 10(2), 237–253.

3. User's manual for POROS CaptureSelect AAV8, AAV9 and AAVX affinity resins. Thermo Fisher Scientific, Aug 2017.

4. POROS™ GoPure™ Pre-Packed Chromatography Columns Pub. Thermo Fisher Scientific, Aug 2020.

BIOGRAPHIES

BUZZ LOBBEZOO is a Senior Field Applications Scientist at Thermo Fisher Scientific supporting the CaptureSelect and POROS resin for Bioproduction group in EMEA North. He holds a Bsc (Hons) from the University of Kent in Canterbury, UK, and has over 25 years of experience in downstream processing, working for various small and medium sized biotech companies as well as the University of Cambridge. Throughout his career, he has worked on every aspect of downstream processing, working with a full gamut of techniques for the purification of a range of biomolecules.

SPYROS GERONTAS is a Senior Technical Specialist in Process Sciences at Pharmaron Gene Therapy, Liverpool. He has over 15 years of experience in developing downstream processing (DSP) platforms and performing process modelling/economic analysis for biopharmaceuticals. In his role, he leads the bioprocessing development of gene therapy products in Pharmaron's cutting-edge facilities. Furthermore, he promotes the implementation of innovative technologies in gene therapy downstream processing through strategic technology partnerships in order that Pharmaron gains/maintains a competitive edge. Moreover, he leads the scale-up and technology transfer of DSP platforms for viral vectors and recombinant proteins to Pharmaron's strategic partners. Spyros holds a PhD in Biochemical Engineering from University College London.

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AUTHORSHIP & CONFLICT OF INTEREST

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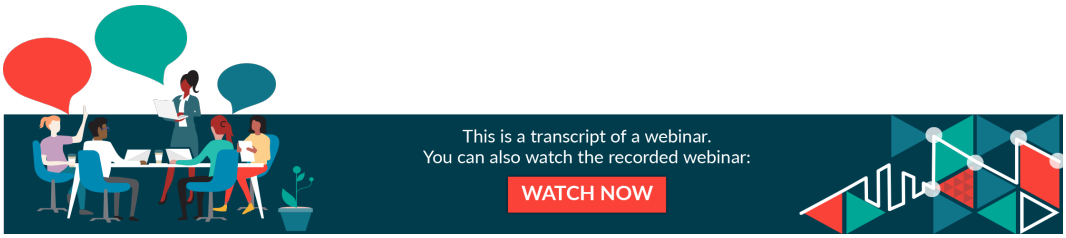

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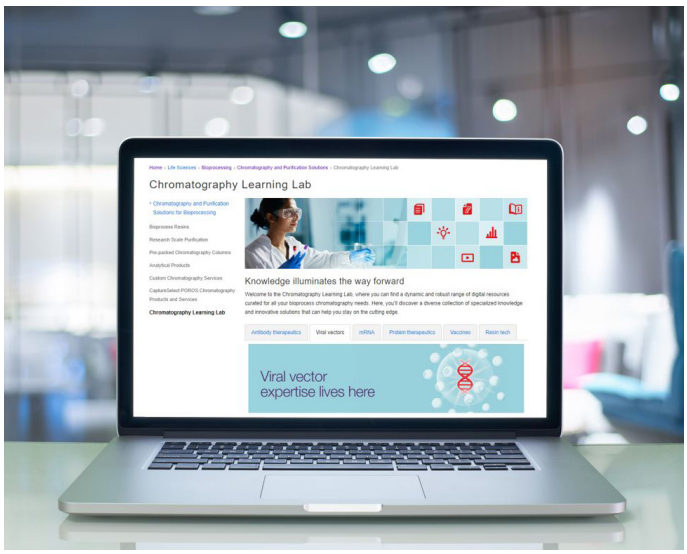

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