OCTOBER 2024 Volume 10, Issue 9

CELL & GENE THERAPY INSIGHTS

SPOTLIGHT ON
Gene editing

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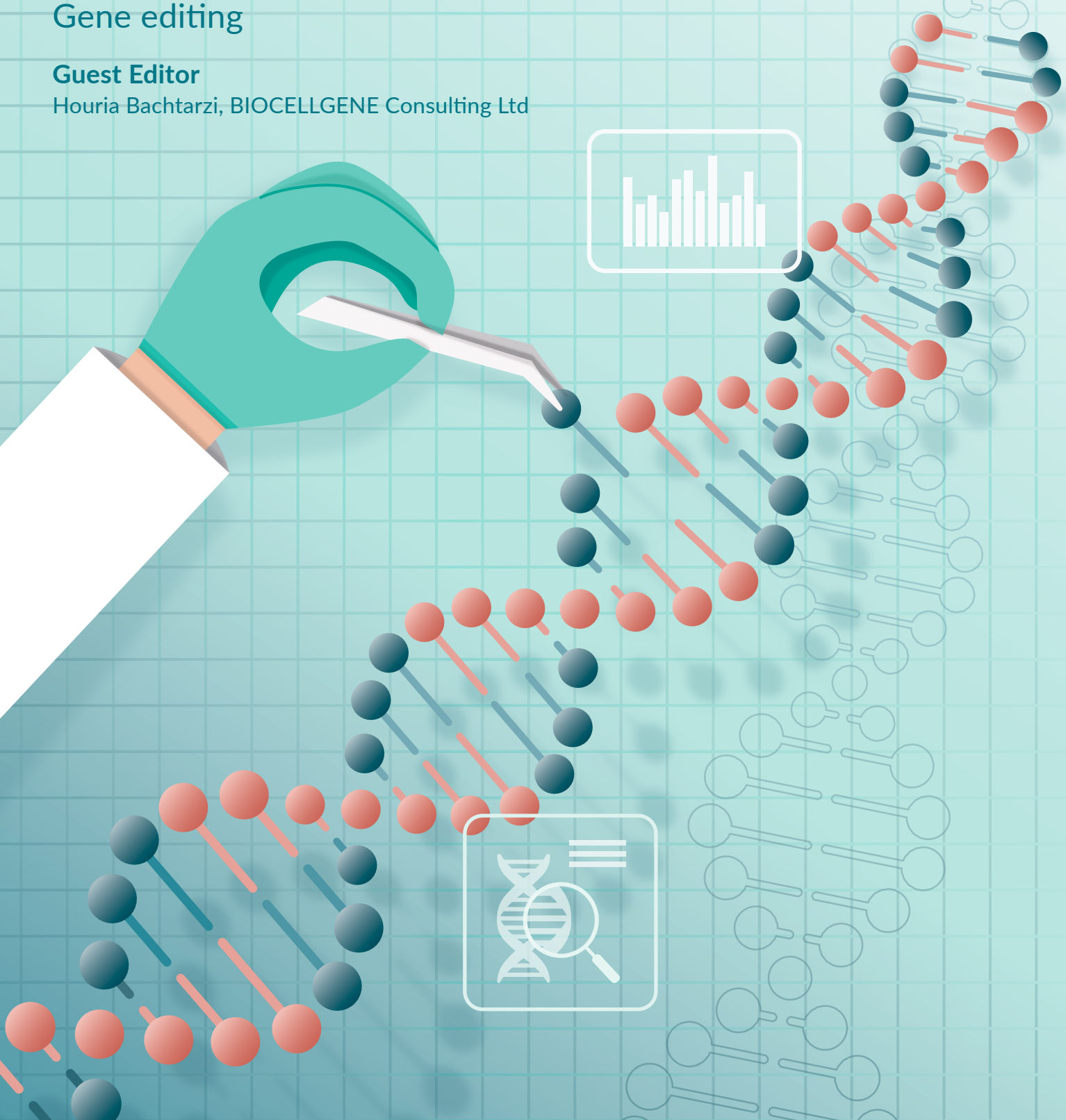

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CELL & GENE THERAPY INSIGHTS

GENE EDITING

SPOTLIGHT

EXPERT INSIGHT

Therapeutic epigenome editing: safety and quality considerations of a new class of gene-targeted medicines

Houria Bachtarzi and Tim Farries

This focused insight article sheds light into the safety and quality-related aspects of an emerging class of gene-targeted epigenetic medicines based on target sequence specific genome-directed approaches. These combine with mechanisms using protein activators and suppressors of transcription and those affecting DNA methylation and histone modifications. Therapeutic modalities based on post-transcriptional mechanisms of gene regulation (such as siRNA) or non-specific epigenetic controls (such as histone deacetylase (HDAC) inhibitors) are outside the remit of this article. The 'pros' and 'cons' of targeted epigenetic therapies/epigenome editing relative to 'standard' gene therapy and genome editing approaches are discussed; and their prospects including the various therapeutic opportunities and current commercial development activities are illustrated. Emphasis on the additional safety considerations, which can be potentially unique to this therapeutic class, is also discussed. These considerations aim to de-risk the development of these novel modalities, and hence facilitate their transition into the clinic and beyond.

Cell & Gene Therapy Insights 2024; 10(9), 1257–1272

DOI: 10.18609/cgti.2024.144

Advancement in molecular techniques notably high-throughput sequencing have shed light into our understanding of chromatin conformation and its interplay with epigenetics and functional genomics, giving rise to a new therapeutic class of gene-targeted medicines that can potentially change a 'disease' phenotype without changing genotype. Of these, epigenetic therapies are emerging as a unique class of targeted precision medicines that are specifically designed to modulate the 'epigenetic machinery' of cells through silencing or activation of a gene of interest, or in some cases through regulation of multiple genes simultaneously; hence controlling gene(s) of interest activity without causing any changes to the DNA sequence. Mechanistically, this can be achieved via a direct modification of the epigenetic marks on DNA (e.g., addition of a methyl group on cytosine nucleotides) or on histone proteins (e.g., addition of an acetyl group or a methyl group on lysine residues) using chromatin-modifying enzymes ('epi-effector' enzymes) [1–4]. Epigenetic editing-mediated transcriptional regulation can also be achieved indirectly using activators and repressors [5–7]. Broader reviews of the technologies used for targeted epigenome editing can be found in Feehley, *et al.* [4]; and Kungulovski and Jeltsch [5].

Leveraging the use of clinically validated delivery vectors (including adeno-associated viral vectors and lipid nanoparticles), and employing DNA-binding tools similar to those used in standard gene editing approaches (notably zinc finger proteins (ZFPs), transcription activator-like effector (TALE) proteins or guide RNA (gRNA)-directed Cas protein with ablated enzymatic activity), this technology is promising to open up alternative gene expression-tuning strategies in gene therapy; as well as potentially synergistic activities to enhance the safety and efficacy of cell-based therapies.

Table 1 shows some of the key components that may be used in epigenetic therapeutics, and their characteristics, which are further discussed later in this article.

This article relates to therapeutic epigenome editing, meaning therapy that acts on the epigenome and for which the term 'epigenetic therapy' is also used. This is therefore not the nomenclature equivalent 'gene therapy', the essence of which is therapy by means of genes rather than necessarily therapy on genes. As such epigenetic therapy and gene therapy are not alternatives and epigenetic therapy may be achieved through gene therapy.

MOVING FROM 'STANDARD' GENE THERAPY AND GENOME EDITING APPROACHES TO EPIGENETIC EDITING

The 'pros' and 'cons' of a new class of precision medicines

The main principle of sequence-specific epigenetic therapy is that it can specifically modify genome functions, and particularly up- or down-regulate the transcription of targeted endogenous genes. Targeted epigenetic treatments could find therapeutic applications where such regulation could be beneficial to a disease condition and if, and where, this approach has advantages over alternative therapeutic strategies, including genome editing and other gene therapies. In this section we consider what those advantages could be, and what conditions may therefore be treatable with an epigenetic approach.

At this stage, this evaluation is highly speculative and it remains to be proven if such approaches can be translated into practical therapeutic solutions. Regulation of gene expression involves multiple interacting mechanisms, and harnessing for therapeutic application will require understanding of which mechanisms are primary controls, which are not the primary signals but can sustain the effects of other stimuli on gene expression, and which are consequences that cannot themselves be modified to impact the gene expression. Furthermore, changes in expression of one gene can result in changes

TABLE 1

Examples of the components that could be used for targeted epigenetic therapeutics.

Component of epigenetic therapeutic (with examples)	Characteristics
Sequence-specific targeting element	
RNA (gRNA + dCas9) DNA-binding proteins (ZFP, TALE)	All can be tuned to high sequence specificity; all have some sensitivity to chromatin structure; DNA-binding proteins are more likely to be sensitive to DNA tertiary structure and methylation status
Effector domains (note that the epigenetic effects below may also indirectly promote or suppress other epigenetic mechanisms)	
Transcriptional activators (VP16, P65) Transcriptional repressors (KOX1)	Potential for rapidly reversible effects
DNA methylation (DNA methyltransferases, TET enzymes)	Effect can be sustained after removal of stimulus and through cell division
Histone modifications (histone acetyltransferases, histone deacetylases)	Typically, acetylation increases gene expression, and can affect neighboring genes; Effect can be sustained after removal of stimulus and through cell division
Delivery systems	
mRNA and LNP Viral vectors (e.g. AAV, AdV)	*In vivo* or *ex vivo* applications; May be modified for tissue specificity
RNP + electroporation	*Ex vivo* applications

AdV: Adenovirus; AAV: Adeno-associated virus; dCas9: Enzymatically inactive Cas9; gRNA: Guide RNA; KOX1: Krüppel-associated box (KRAB) repressor domain; LNP: Lipid nanoparticle; P65: Activation domain from the p65 subunit of NF-kB; RNP, ribonucleoprotein complex; TALE: Transcription activator-like effector; TET: Ten-eleven translocation methylcytosine dioxygenases; VP16: Herpes simplex virus protein vmw65; ZFP: Zinc finger protein.

in expression of other genes, some of which may be beneficial and others deleterious. Selection of the optimal target for regulation will therefore depend on: (i) the ability to therapeutically regulate that target; and (ii) the benefit and risks of other effects arising from that regulation.

We must also note the limitations of epigenetic approaches. Conventional gene therapy can do things that epigenetic therapy cannot do, such as introduce new genes or make coding changes to existing genes. Epigenetic therapy is primarily limited to changing the expression profile of existing genes. There are also some ways to do that with other gene therapy approaches, for example by editing promoters or by expressing RNA or protein factors that inhibit or enhance expression. It must further be noted that only a subset of genes may be amenable to regulation of expression by epigenetic mechanisms that can be retained through cell division, including those involving DNA methylation. Transient effects, for example by association of DNA-binding elements with protein repressors or activators, will likely be applicable to a much wider range of potentially therapeutically beneficial regulations of gene expression. Furthermore, these limitations do not ignore the possibility that the epigenetic mechanisms described herein could be combined with other forms of gene therapy. For example, by inclusion of the required target sequences in the transgene, it may thereby be possible to introduce epigenetic regulation of expression of those genes (as gene switches to control safety or efficacy).

Potential advantages of using epigenetic mechanisms to regulate gene expression therapeutically include:

1. Assurance that the gene being regulated remains in its correct genomic location, as is likely to be important for maintenance of appropriate tissue-specific levels of expression, and regulation by other cellular signals. In contrast, exogenous genes provided from a viral vector, whether genomically inserted (by a retroviral vector) or remaining episomal

(as from AAV or adenoviral vectors) will typically depend for expression on control elements provided with the vector, which may not be able to reproduce all the control properties of the correct endogenous genome location. However, genome editing technologies such as CRISPR can be used to precisely insert a new sequence at an endogenous locus;

2. Avoidance of immunological issues for transgene products for which there are protein variations between individuals and for which expression of a different variant might therefore generate an immune response;

3. Avoidance of genotoxicity, meaning in this case DNA rearrangements and other unwanted changes to the genome that can be associated with gene insertions and editing and are considered to be risks for oncogenesis (as discussed further below);

4. Differences from genome editing in the profile of off-target effects, even if the same targeting moiety (such as a guide RNA) is used, on account of factors such as the impacts of pre-existing epigenetic modifications and differential sensitivity of different parts of the genome to epigenetic modification. This in turn could alter the safety profile, and hypothetically this could be favorable if off-target effects are thereby more restricted. A refinement of the specificity of the technology could be to make the targeting and/or activity sensitive to the epigenetic state (notably the DNA methylation state) as well as to the sequence of the target. However, the potential for variations in epigenetic status between individual patients would need to be taken into account in designing a viable therapeutic;

5. The possibility for regulation of expression of genes that are too large to incorporate into conventional vectors;

6. The regulatory advantage that handling and applications of cells (allogeneic or autologous) manipulated ex vivo by purely epigenetic modifications would not be subject to the additional regulatory approvals that are currently required in many jurisdictions for genome-modified cells under regulations for use of genetically modified organisms (GMOs) [8]. However, any viral vectors used to deliver the epigenetic machinery would still be classified as GMOs;

7. Other advantages and disadvantages of epigenetic interventions are strongly related to the durabilities and reversibilities of the effects, with the following possibilities:

   - Transient effects that revert when the intervention is no longer present. When applicable such effects would be expected to avoid risks of long-term transformation of cells into undesirable phenotypes, such as cancerous (or pre-cancerous) states;

   - Sustained effects that persist after the removal of the initial stimulus. The treatment agent may be transient, but the effect is long-lasting (so called 'hit-and-run' treatments). Some of these effects may be retained through cell division, which is a characteristic of epigenetic changes associated with cell differentiation;

   - Imprinted effects: whilst most epigenetic genomic profiles are reset in germ cells and/or in early embryogenesis, at least some epigenetic effects can be retained by ('imprinted' on) the next generation. While this is only applicable to effects that may impact the germ cells, any risk of this happening would be an important safety consideration to address.

Whilst more sustained effects may be necessary for therapeutic benefit, these would also carry more potential safety risks, especially in effects that are transmitted through cell division.

Opportunities for therapeutic epigenetic intervention

Like gene therapies, epigenetic editing-based therapies could be envisaged to be applied either *in vivo*, subject to the availability of delivery systems suitable for the target tissue, or *ex vivo* to autologous or allogeneic cells that can be harvested, treated, and then redelivered as needed. These various scenarios are considered further below.

Transient increases or decreases in expression of specific genes

Since the earliest sequence-targeting technology with engineered zinc finger proteins, it has been envisaged that there could be therapeutic applications (and other applications) of using this property to regulate the expression of specified genomic genes by coupling the DNA-targeting elements to activators or repressors of transcription. It was found that such agents can regulate the expression of endogenous genes, albeit subject to influences of the chromatin structure [9]. However, since then other means of regulating gene expression have come to the fore. To be practical, therapeutic repression of gene expression by the epigenetic mechanisms described would therefore need to have advantages over alternative post-transcriptional means including use of inhibitory RNAs (such as siRNA), which would generally be easier to manufacture and deliver than the machinery required for chromosomal epigenetic regulation. Such advantages might come from a more favorable duration of action, especially if repeated administration is problematic. However, for longer term effect the repeat administration of the biologics required to achieve this using an epigenetic-based therapy, will generally be more costly and challenging than for a smaller molecule. The other option would be to use a system for long-term expression of the epigenetic effector, such as an AAV or lentiviral vector, although that negates the main safety advantages over other gene therapy approaches (as discussed under 'product safety considerations'). For applications requiring up-regulation of a target gene expression, post-transcriptional mechanisms are less applicable and the genome-based alternatives to the epigenetic mechanisms would be through new gene insertion or editing of regulatory sequences, with the consequence issues of irreversibility and potential for genotoxicity.

The early study by Zhang *et al.* demonstrated the possibility to upregulate expression of human erythropoietin [9]. Other examples of applications of using epigenetic approaches to elicit transient changes in gene expression, which are readily reversible by removal of the therapeutic, could include enhancing local production of endogenous growth factors, such as VEGF-A [10], to improve blood supply during tissue regeneration, or of cytokines to stimulate an immune response to a vaccine or against a tumor target. A study with zinc finger units linked to a KRAB repressor domain suggested the possibility of such a therapeutic for suppressing transcription and hence replication of integrated HIV-1 [11]. Alternatively, for an allogeneic cell therapy, there could be an application for transient suppression of HLA molecules that would trigger immune rejection of the therapeutic. In that case the transience of the suppression might, in comparison to a genetic knock-out, reduce the risk that the cells could transform into an oncogenic phenotype that avoids immune rejection, even mitigating a genotoxicity risk that is increased if those cells are also genomically modified (for example CAR-T cells).

The ability to control the expression of specific genes could also be harnessed to control expressions of genomically-integrated transgenes, to create 'gene switches' that can

either upregulate expression when and where needed, or down-regulate expression if safety issues occur. The principle is to include in the gene therapy, machinery for epigenetic control targeted to sequences either in the transgene insert, or near its sites of integration (the latter strategy limiting the control function to specific on-target gene insertions). The epigenetic control may be linked to respond to either endogenous signals, such as a tumor environment, or to exogenous molecules that could be administered to regulate the response. It is noted that such gene switches can alternatively utilize post-transcriptional mechanisms of gene regulation (e.g., siRNA) which are not covered in this article.

Stable increases or decreases in expression of specific genes

In some cases, changes in gene expression mediated by epigenetic intervention may be maintained after the removal of the triggering agent. This is most likely if the epigenetic intervention mediates a change in DNA methylation and/or histone modification and hence the effector domain could include enzymes with such activity. The principle is that the transient presence of an epigenetic therapeutic (perhaps administered as an mRNA or from another transiently-expressing vector system) would direct the chromosomal modification at the gene of interest such that there would be a change in expression of the target gene that would be maintained after the intervention ceases to be present. This 'hit and run' strategy could be a major advantage for an epigenetic mechanism of action as it minimizes the safety issues of prolonged exposure to the therapeutic, including the potentials for immunogenicity of treated cells or for accumulation of off-target effects over time. The extended duration of effect may also avoid the challenges of repeat administration. Such a 'hit and run' strategy should also have the advantage over genome edits of avoiding genotoxicity risks associated with DNA breaks. However, it may be that only some genes are amenable to such sustained epigenetic control.

For example, Cappelluti, *et al.* [12] demonstrated in mice the potential for downregulating the Pcsk9 gene in hepatocytes, which is involved in cholesterol homeostasis. They used a zinc finger protein targeting the murine Pcsk9 gene promoter fused to engineered DNA methylating repressor proteins. A single administration mRNAs for expression of the fusion protein, delivered in an LNP formulation, achieved an almost 50% reduction in the circulating levels of PCSK9 protein sustained for nearly a year. There could be an application for epigenetic suppression of activation of endogenous retroviruses (as suggested by the study of Reynolds, *et al.* [11] in situations of immunosuppression, such as transplantation [used to treat either the patient or the donor tissue]). For example, Tune Therapeutics have a program for epigenetic repression of viral activation in chronic hepatitis B infection [13].

Changes in DNA methylation profiles are observationally associated with long-term aspects of neuronal functions, including learning and memory [14]. However, the mechanisms and drivers of these effects are not yet sufficiently understood to be able to confidently design a therapeutic epigenetic intervention. In addition, neuronal cells in the brain represent particularly difficult target cells to reach efficiently and safely with the biotherapeutics that would be required.

Epigenetic changes in DNA methylation and histone structure, which are maintained through cell division, are thought to be critical to the mechanisms of cell differentiation. A potential *ex vivo* application would then be to create epigenetic tools to precisely control cell differentiation, perhaps making more efficient and controlled customized tools that could replace some of the complex protocols, including empirically-derived combinations of growth factors, inhibitors and selection conditions, that are currently used to derive different cell types from pluripotent stem cells. For direct therapeutic application (not

just as a tool for manufacture), it may also be asked if it could be possible to use the same technologies to reprogram cell phenotypes *in vivo*. As evidence in favor of this principle, scientists from Tenaya Therapeutics have published a study in rats that showed that a cocktail of factors, including microRNAs, delivered by a viral vector can reprogram cardiac fibroblasts into cardiomyocytes to repair heart muscle damaged by ischemic injury [15]. Therefore, it seems feasible that similar effects might be similarly achievable by specific epigenetic mechanisms, although it is also not yet apparent if that could provide advantages in efficacy or safety.

Stable correction of pathological disorders associated with aberrant epigenetic signals

There are diseases that are associated with apparently aberrant epigenetic profiles that may offer potential for epigenetic therapeutic intervention. However, the mechanistic association between the epigenetic signal and the pathologies are generally unclear, so it remains to be demonstrated if an epigenetic normalization will improve the condition for the patient.

Abnormal DNA methylation is a characteristic of tumor cells. However, as with so many epigenetic phenomena, it is not yet clear if the signal seen is in any way causative of, or required for, the pathology or just an effect thereof. The fact that tumors represent an aberrantly differentiated (or de-differentiated) state of cells that is maintained through cell replication suggests that epigenetic mechanisms may at least be required to sustain that state. This in turn suggests that it is worth exploring if epigenetic treatments can reverse any of the abnormality and, if so, could they be therapeutically beneficial. The activation of some oncogenes is associated with DNA hypomethylation at CpG islands, and so the impact of specifically remethylating the affected regions can be explored. Targeting specific regulatory elements within the insulated genomic domains (IGDs) repress MYC expression in hepatocellular carcinoma and other types of solid tumors is also being investigated using LNP-based delivery of mRNA for expression of modular proteins consisting of a DNA-binding domain and an epigenetic effector [16]. Epigenetic modification can control the use of alternative promoters of a gene [17]. One study found that differential DNA methylation affected the use of alternative promoters of the oncosuppressor gene *PRDM1* leading to increased expression of the alternative form that interferes with its normal functioning [18]. The authors duly suggested that epigenetic drugs targeting the *PRDM1* promoters could have therapeutic potential in multiple myeloma. Another study reported that tumor cells typically become hypomethylated, and disproportionately in CpG poor regions which then opens up the chromatin for use of alternative promoters that then lead to misregulation of the cellular transcriptomes. Again, this suggests that epigenetic remethylation of effected genomic sequences could help to restore the normal cellular phenotype. However, such mechanistic considerations may still not translate into a feasible therapeutic strategy for oncogenic indications, particularly because of the presumed need for a delivery that reaches a very high proportion of the pathogenic cell population of a tumor.

Another therapeutic opportunity for regulation of expression from different promoters is evidenced by the alternative expressions of fetal and adult hemoglobin. Casgevy [19] is an approved autologous cell therapy for sickle cell anemia and β-thalassemia that uses CRISPR-mediated genome editing to prevent expression from the defective adult hemoglobin gene and thereby allow beneficial expression of the fetal hemoglobin. It could be envisaged that the same effect could potentially be achieved by epigenetic means (reversing the epigenetically mediated switch from fetal to adult gene expression), perhaps with safety advantages over using genome-modified cells

and potentially performed *in vivo* instead of *ex vivo* [20,21].

As the technology now allows detailed mapping of DNA methylation, many other diseases are being found to be associated with epigenetic changes. Alterations in epigenetic profiles have been described in the settings as diverse as aging, exercise, and B cell differentiation. However, our mechanistic understanding is currently too limited to predict that intervention to normalize the profile could be beneficial, or even what the target genes for that intervention would be. A causative association of aberrant epigenetic cell profiles with pathology is probably more evident from the rare cases of diseases associated with genetic imprinting effects that are transmitted to subsequent generations. This includes cases of the developmental disorders of Angelman and Prader–Willi syndromes in which a functional gene from one parent is epigenetically silenced by differential DNA methylation failing to correct the impact of a defective gene from the other parent [22]. An epigenetic therapeutic strategy to 'unsilence' the imprinted gene could be envisaged if it could be administered early enough for the developmental benefit.

With all these strategies that may be envisaged for epigenetic therapy, a key challenge will be how to achieve effective delivery to the target tissues. The same issue exists for all gene therapy, but for the epigenetic mechanism, that difficulty may be amplified by the size and complexity of the functional elements that need to be included.

Active commercial development activities

Active commercial development activities of epigenetic therapies are currently ongoing using various approaches targeting different indications. The majority of these are in early-stage development, and it is expected that such a progress will continue as the technology gains traction. Table 2 captures some examples of current ongoing commercial development activities using different epigenetic modalities that target various pathologies including neurological conditions, neuro-muscular disorders, solid tumors, infection, and liver-targeted diseases (such as: heterozygous familial hypercholesterolemia and alpha-1 antitrypsin deficiency).

Sangamo Therapeutics are pioneers of therapeutic uses of engineered sequence-specific zinc finger proteins, including for epigenetic therapies and for genome editing applications. As shown in the table below, they are still active in the field with a number of therapeutic programs, although none of these are yet at the clinical phase. Omega Therapeutics are one of the first companies to take a therapeutic with such a mechanism into clinical studies, with a treatment for hepatocellular carcinoma that is designed to epigenetically down-regulate MYC expression using two fusion proteins that combine ZFP units that target the *MYC* gene and units that induce methylation of DNA and histones [23]. The company reports that the Phase 1/2 study is currently recruiting patients in the USA and Asia. These factors are delivered as mRNAs in liver-targeting lipid nanoparticles. Other companies with pre-clinical programs in the field include Tune Therapeutics, Chroma Medicine, Epicrispr biotechnologies, and Modalis Therapeutics. It may be expected that many other start-up companies will emerge as the technology gains traction.

DE-RISKING THE DEVELOPMENT OF TARGETED EPIGENETIC THERAPIES

As with any novel therapeutic technology, developers and regulators are often faced with an unchartered territory for which there is no prior or very limited product and/or relevant platform-related safety experience. Progress may be further hampered by limitations in technology-enabling analytics, pre-clinical models that are relevant to the efficacy and safety of the novel paradigm as well as absence of precedence or regulatory guidelines.

TABLE 2

Examples of commercial development activities using targeted epigenetic therapy.

Technology	Condition(s)	Clinical Phase	Company
Zinc finger proteins linked to transcriptional repressors	Chronic neuropathic pain, amyotrophic lateral sclerosis (ALS), frontotemporal lobar degeneration (FTLD), Huntingdon's disease, prion disease	Pre-clinical	Sangamo Therapeutics
Epigenomic programming	Hepatocellular carcinoma and other types of solid tumors	Phase 1/2	Omega Therapeutics
Various combinations of targeting and effector units	Hepatitis B	Pre-clinical	Tune Therapeutics
Targeting units combined with CHARM effector units that recruit DNA methyltransferases to silence target genes	Prion disease	Pre-clinical	Chroma Medicine
Gene expression modulation system combining DNA-binding proteins, guide RNAs and a wide range of modulators (activators/repressors)	Facioscapulohumeral muscular dystrophy, heterozygous familial hypercholesterolemia, alpha-1 antitrypsin deficiency, retinitis pigmentosa 4, retinitis pigmentosa 11, blood cancers	Pre-clinical	Epicrispr Biotechnologies
Guide RNA, enzymatically inactive Cas9 linked to a modulator of expression (activators/repressors), delivered by an AAV vector	Various	Pre-clinical	Modalis Therapeutics

Experience with other ground-breaking gene and cell therapy modalities have taught us the importance of a scientifically driven tailored risk-based approach to product development. Such approach is proving to be beneficial, where there are gaps and challenges that cannot be resolved within the current regulatory and industry guidance, hence shaping and tailoring the specific data requirements and providing some flexibility, when it comes to the data package to support first-in-human studies and subsequent marketing authorization applications.

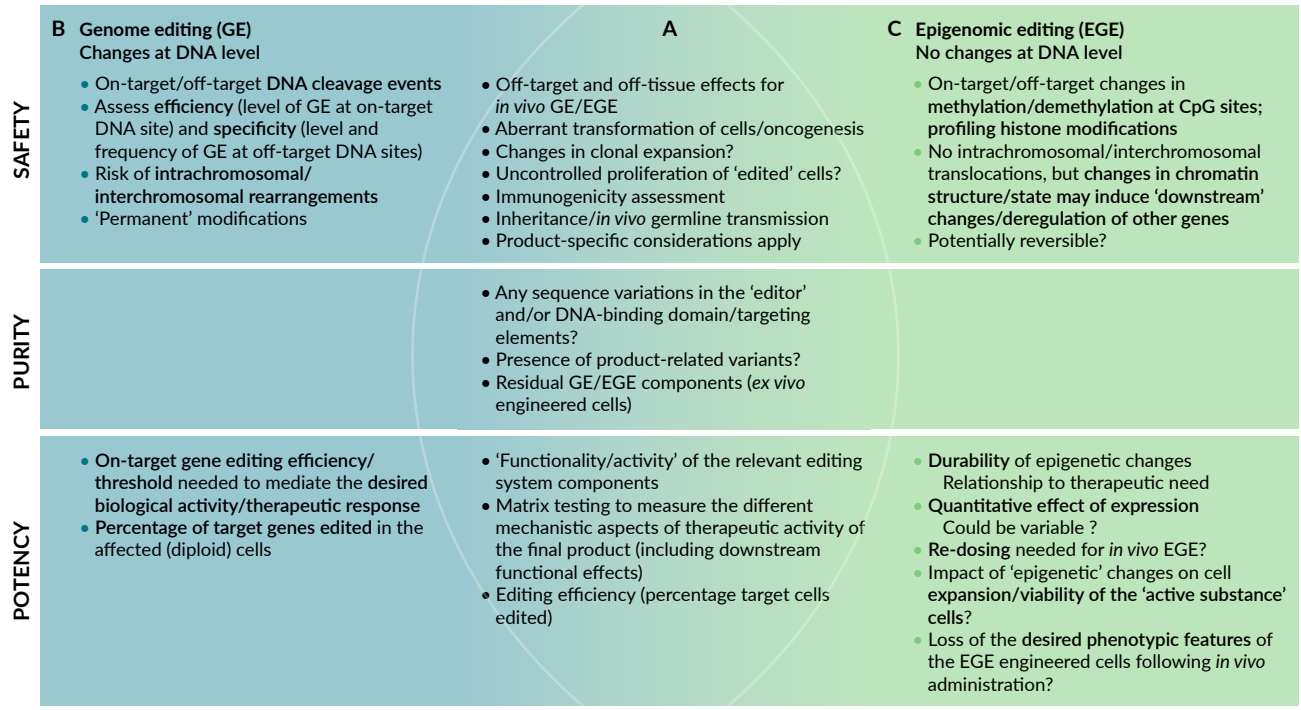
For epigenetic therapy medicinal products, developers can still leverage the substantial amount of regulatory guidance and recommendations previously published for gene therapy (including genetically modified cells) and genome editing products [24–28] . While this latter category is distinct from therapeutic epigenome editing, some fundamental safety and quality-related commonalities still apply when it comes to establishing safety, purity, and potency of the final therapeutic product (Figure 1).

Characterization of epigenetic activity

It will be critically important to demonstrate that the designed therapeutic has the planned effect through the envisioned mechanism of action. Initial characterization should demonstrate that expression of the target gene is up- or down-regulated as intended. Such studies would initially be performed *in vitro* on a relevant cell type(s). Studies incorporating testing for safety (see below) of *in vivo* treatments would need to be conducted on a range of cells representing the various cell tissues that could be affected. Ideally the tests could also show a cellular functional response related to the intended mechanism of action. Such activities will likely also lead to development of an *in vitro* potency assay for the product, as will be required for clinical development. This work should also guide design of studies for *in vivo* proof-of-concept, though noting that these may be challenged by species-specificity of the genome target site and/or the epigenetic response.

FIGURE 1

Key similarities and differences between epigenome editing and genome editing in terms of safety, purity, and potency.



The key commonalities between epigenome editing (EGE) and genome editing (GE) are categorised into safety, purity, and potency. **(A)** Common product characterisation, safety, and quality-related features; **(B)** Specific features and elements that apply to GE modalities; **(C)** Specific features and areas of considerations that apply to EGE modalities.

For *ex vivo* cellular therapies with one or multiple epigenetic modifications (multiplex epigenetic engineering), the stability of the induced epigenetic changes to maintain the desired biological activity of the final cell product; and any potential risk/impact of reversibility or loss of the desired phenotypic features *in vivo*, following administration/transplantation into the right microenvironment, should be understood. For instance, measuring the efficiency and durability of silencing or induced expression of gene(s) of interest, in the engineered 'active substance' cells, can potentially be assessed via re-stimulation/repeated re-stimulation or repression, respectively, over a justified defined period of time, taking into account the mechanism of action/the proposed biological activity of the final cell product and its intended use.

Secondary effects of the epigenetic treatment on cells may also be relevant. It would be appropriate to ensure that the treatment does not interfere with critical cellular functions including viability, ability to replicate and cell-type specific functional activities.

Product safety considerations

When it comes to safety, one must take into account the potential outcome of the therapeutic epigenetic intervention employed, leading to either transient or sustained effects. As such, some of the relevant safety considerations can potentially be categorized and de-risked on a product-specific case-by-case basis.

Basic characterization should include testing the effects of the epigenetic therapeutic on targeted gene expression, and the specificity of that effect with respect to other genes. As with genome editing technologies, unwanted effects on the expression of other

genes could arise from off-target binding of the DNA sequence-targeting element. These may be rare events, depending on the specificity of the sequence targeting. However, effects on gene expression could also occur with high frequency to genes neighboring the target in the genome as a result of the local modifications in chromatin structure or to genomically unconnected genes as secondary effects of control networks connected to the products of the targeted gene. For targeted epigenetic-based modalities with transient effects that are not expected to persist after removal of the therapeutic agent, changes in gene expression that could be linked with unexpected abnormalities/toxic signals should be assessed using transcriptomic/proteomic-based analysis and/or other relevant bioanalytical assays in representative human cells. Observed changes, wanted or unwanted, should be investigated and demonstrated to revert back to 'pre-treatment' state, when the agent is removed.

Where these targeted epigenetic interventions are used in combination with other pharmacological agents, the impact of this concomitant treatment on the safety profile of the epigenetic therapy product would need to be evaluated, and again transcriptomic/proteomic-based analyses would be appropriate tools to incorporate.

For targeted epigenetic-based modalities intended to provide sustained effects, as with genome editing products, the potential risk of transformation of cells and acquisition of an oncogenic phenotype, which could predispose to cancer, will need to be assessed. Whilst mutations and other genotoxic events are recognized as important factors in oncogenesis, not all tumorigens are mutagens and it appears that genotoxicity is not a prerequisite for dysregulation of oncogenes leading to oncogenic transformation [29]. Also, in the absence of evidence to the contrary, it cannot be ruled out that even a transient disturbance in the epigenome machinery could potentially lead to an irreversible switch to an abnormal phenotype. It is also worth highlighting that such a switch may only require a small fraction of cells to become problematic, and as such the depth of analysis and the use of additional complementary orthogonal testing systems beyond the standard *in vivo* and *in vitro* tumorigenicity testing approaches, are warranted.

Testing for potentially pathogenic effects of epigenetic therapeutics should also account for the less frequent off-target events, even if these are occurring at a low frequency. Similar to gene editing interventions, when it comes to epigenetic editing, *in silico* analysis to predict potential off-target sites of the DNA-binding elements, would still be relevant, looking at the closest transcription start site and the closest methylated region for each potential off-target site; and reviewing the data for the presence of off-targets of concern, particularly those that can activate oncogenes or repress tumor suppressor genes. However, current bioinformatics technologies are not expected to be able to predict potential for off-target effects that may occur to chromosomal segments that are not close in the linear sequence but may be spatially associated through secondary structure. For epigenetic therapies that are expected to change DNA methylation, a methylation array assay to test for on-target/off-target changes in methylation at CpG-enriched sites combined with whole genome bisulfite sequencing can be conducted on relevant cell types. For epigenetic interventions that are expected to modulate chromatin state at target loci, studies using chromatin profiling techniques can be conducted to characterize chromatin modifications and evaluate off-target effects. Of these, the Cleavage Under Targets and Tagmentation (CUT&Tag) method was shown to be valuable for profiling histone modifications using small sample materials as well as on single cells, compared with standard chromatin immunoprecipitation with sequencing (ChIP-seq) [30]. It cannot be ruled out that epigenetic changes, especially sustained ones, could promote genetic changes through mechanisms such as

destabilization of chromatin or interference with DNA repair. Therefore, until proven otherwise, some testing for potential effects on genomic integrity (such as sequencing, PCR-based methods, or karyotyping) may be required. As with genome editing, off-target epigenetic editing of cells are likely to be inefficient and therefore impact only a small population of non-clonal cells, and as such the depth of analysis of the chosen assay is critical, in order to account for the few cells or even a single cell that harbor those unwanted modifications, as these can still be problematic even when present at low levels.

In addition, transcriptomic analysis using RNA sequencing to assess genome-wide transcriptional changes and proteomics can also provide a valuable unbiased insight into unexpected off-target changes in gene expression and potential associated toxic signals.

As with any biological/advanced biological therapy product, assessment of immunogenicity to the epi-editor/epi-effector components as well as the delivery systems employed, should be considered during non-clinical development, with additional monitoring implemented during clinical development using appropriate 'product-specific' bioanalytical assays in order to monitor immune responses and capture any relevant immunogenicity-related adverse events.

Product quality-related considerations

The quality considerations depend on the nature of the therapeutic material(s). These may include RNAs, LNPs, viral vectors and cellular components, for which the quality standards can be related to those described in guidelines for other gene therapies. Aspects related to the manufacturing process notably the quality of starting materials used in the production process, can have an impact on product quality, consistency, and safety. This, for instance, holds true when it comes to evaluating the purity of the guide sequences or the DNA-binding moieties, as any impurities in these with sequence variations can potentially increase the risk of epigenetic editing at off-target loci sites, which can subsequently result in unwanted modifications in the 'chemical' signature of DNA and its structure, hence causing unexpected dysregulation in gene expression. As with genome editing technologies, sequence design and specificity of the DNA-binding protein domains or guide RNAs, are critical in order to ensure quality and safety of the final epigenome editing-based product, whether this is designed for *in vivo* or *ex vivo* applications. This is of paramount importance, considering the inherent challenges and underlying complexities of the human genome, and that natural transcription factors may regulate the expression of multiple genes [31,32]. As such, efforts to enhance the specificity of epigenome editing construct elements, should continue, leveraging experience already gained from human genome editing-based modalities [33–35].

For CRISPR-Cas9-based epigenome editing systems employing enzymatically dead mutant Cas9 protein, any residual endonuclease activity must be assured to be eliminated, as this can potentially increase the risk of unwanted DNA cleavage events.

For *ex vivo* epigenetic therapies, while the effects are limited to the cell population being modified *ex vivo,* it is important to note that the degree of epigenetic changes/editing efficiency can be affected by the type of cells/cellular starting material used. In fact, the level of epigenetic editing effects observed, were previously shown to be impacted by the type of cells used and their chromatin plasticity. For instance, the use of a single epigenomic-modifying enzyme in differentiated cells (with reduced chromatin plasticity) was not sufficient to drive the desired changes, necessitating the simultaneous inclusion of a combination of factors [36].

Consideration should be given to establishment of an appropriate test for potency, which during development will become critical not only for product release but also for

stability and comparability studies. In the early stages of development, it may be sufficient to measure based on changes in target gene expression, but later more functional outputs will be expected if possible. Other quality-related considerations which can have an impact on product safety include residual levels of epigenetic components used to edit/modulate the cells *ex vivo*. These process-related impurities can be potentially immunogenic and/or impact host cells on administration, and efforts should be taken to minimize their levels and to demonstrate their clearance through testing of the final cell product.

TRANSLATION INSIGHT AND FUTURE PERSPECTIVE

The advanced therapy field is witnessing an unprecedented faster development with new emerging therapeutic technologies, building up from the ongoing technical and clinical progress acquired from first- and second-generation gene and cell therapies notably those based on genome editing.

Targeted epigenetic therapies based on 'epi-editors' and/or 'epi-effectors' mechanisms have the potential to precisely edit and/or modulate/tune the expression of target genes of interest, without changing host DNA sequences. These epigenome modalities are amenable to both a non-viral or a viral-based delivery system, which can be used *in vivo* or *ex vivo*, leading to a transient or a potentially sustained effect, depending on the type of epigenetic intervention employed and the transfer system used. In addition, the novel mechanisms of action brought by these epigenome-based technologies, add specific considerations to be addressed for clinical application. As discussed in this article these include characterization of epigenetic activity, product safety and other quality-related considerations.

Whether such epi-editor/epi-effector-based gene-targeted therapies are set to open new therapeutic possibilities, replace standard gene therapy approaches, where these are already effective, or even be used as an add-on treatment in combination with current pharmacological interventions in complex disorders, is yet to be demonstrated in clinical studies. Questions also remain with regards to the feasibility of delivery and optimal dosing strategies, and whether such therapeutic epigenetic manipulations can be reversed by endogenous mechanisms. Nevertheless, this article should assist developers by highlighting issues that need to be addressed in order to realize the potential of the novel technology to create new gene-targeted medicines.

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AUTHORSHIP & CONFLICT OF INTEREST

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

Acknowledgements: None.

Disclosure and potential conflicts of interest: The authors consult widely for the biotech/biopharma industry, especially in the field of gene and cell therapy. Houria Bachtarzi has received a small stipend to cover travel costs to attend a CMO summit (in Milan, Italy) organized by AGC Biologics.

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

Article source: Invited; externally peer reviewed.

Submitted for peer review: Sep 23, 2024; **Revised manuscript received:** Oct 10, 2024; **Publication date:** Oct 15, 2024.

CELL & GENE THERAPY INSIGHTS

GENE EDITING

SPOTLIGHT

REVIEW

Coming of age: an overview of the growing toolbox for gene editing and its use in CGT applications

Christina Fuentes, Jacob Staudhammer, Madison Pope, and Scott Cross

Genome editing has the potential to expand the number and types of indications that can be targeted by cell and gene therapies. In recent years, there have been significant advancements in the genome editing tools, technologies, delivery methods, and analytical techniques available to developers. This review covers available genome editing tools, their utility, safety profile, as well as high-level manufacturing and regulatory strategy considerations when using these advanced technologies.

Cell & Gene Therapy Insights 2024; 10(9), 1221–1236

DOI: 10.18609/cgti.2024.139

Cell and gene therapy (CGT) products represent a diverse class of advanced therapies with the potential to treat and cure the underlying cause of a disease. Over the last several years the number of FDA approved CGTs has steadily increased from two products in 2015 to a total of 28 products in 2024 (excluding cord blood derived therapies, off-market, and withdrawn licenses) [1]. These 28 approvals are spread across three major product classes: cell therapies, gene therapies, and gene-modified cell therapies. The majority of approved products in the field utilize viral vectors to deliver genetic material, with adeno-associated virus

(AAV) being the predominant platform for *in vivo* gene therapies and lentivirus (LVV) being the predominant modality used *ex vivo* for gene-modified cell therapies. AAV delivers genetic material that forms episomal DNA in a cell which largely does not integrate into the host genome. This leads to stable expression in non-dividing cells and transient expression in dividing cells, as each cell division will ultimately dilute the episomes [2]. In contrast, LVV delivers its genetic payload into both dividing and non-dividing cells leading to stable expression, and integration is typically random [3]. The desired target product profile drives selection of the appropriate modality for each indication. Both *in vivo* and *ex vivo* modalities can transform lives and provide durable and lasting efficacy to alleviate symptoms and cure diseases.

In parallel to the steady rise in cell and gene therapy product approvals, there have been major advances in genome editing (GE) technologies that have expanded the spectrum of indications that can be addressed by CGT. While introduction of genes is made possible by viral vector delivery via random integration with LVV or via episomal DNA with AAV, GE expands gene modification capabilities by allowing specific targeting of modifications to a precise location in the genome. GE enables a wide range of modifications, including permanent knockout (i.e., inactivation) of faulty genes and targeted gene insertion. Accordingly, the space is seeing a dramatic increase (>125 active clinical trials globally based on GlobalData information pulled in August 2024) in GE products entering the clinic as the next generation of gene modification *in vivo* and *ex vivo*. The 2023 approval of CASGEVY, the first CRISPR-Cas9 editing CGT product, has paved the way for other GE programs by demonstrating technical feasibility, safety, and efficacy [4]. This rise in GE programs has also led to efforts to advance the GE regulatory space across multiple jurisdictions, including a newly published GE guidance document by the FDA [5] as well as GE consortiums [6] to lower the risks associated with GE technologies. While GE poses unique safety risks, advances in sequencing platforms and bioinformatic pipelines are making it possible to analyze large datasets and characterize GE products and outcomes. Finally, continued advancements in *ex vivo* and *in vivo* delivery methods that maximize on-target editing and minimize off-target editing will further extend the possibilities for GE.

This review covers the current state of gene editing technologies, manufacturing and regulatory strategies with a particular focus on CRISPR-Cas based systems.

BACKGROUND

The concept of GE has been around since the 1970s and progressed into the 1980s with engineered nucleases (e.g., meganucleases). Meganucleases are homing endonucleases that have been re-engineered for gene editing. These enzymes exhibit high specificity, recognizing and cleaving DNA sequences between ~15–40 base pairs, creating double-strand breaks with a 3′ overhang, and facilitating gene modifications such as homology-directed repair [7]. Today, meganucleases are still in use for clinical applications (Table 1).

Similar to meganucleases, zinc finger nucleases (ZFNs) are protein-based nucleases that introduce double-strand breaks in DNA to produce targeted edits in the genome. While meganucleases have DNA binding and catalytic activity in one protein, ZFNs are created by fusing a zinc finger DNA-binding domain with the DNA-cleaving domain of the FokI enzyme. The zinc finger contains DNA binding motifs which bind to 3 base pairs each, allowing for multiple DNA binding motifs to be combined and target specific DNA sequences [8–11].

Transcription activator-like effector nucleases (TALENs) are another endonuclease technology introduced following the development of ZFNs. These engineered proteins consist of a TAL effector DNA-binding domain derived from plant pathogens fused

TABLE 1

Summary of genome editing technologies.

Technology	How genome targeting is achieved	Does it require a double-stranded break?	Example gene modification	First time in clinical trials (*ex vivo*)	First time in clinical trials (*in vivo*)
Meganuclease	Protein engineering	Yes	Gene knockout	Posted in 2019 NCT03666000	Posted in 2024 NCT06255782
ZFN	Protein engineering	Yes	Gene knockout	Posted in 2009 NCT00842634	Posted in 2005 NCT00110500
TALEN	Protein engineering	Yes	Gene knockout	Posted in 2016 NCT02808442	Posted in 2017 NCT03057912
CRISPR-Cas9 (e.g., Sp. Cas9)	Guide RNA	Yes	Gene knockout	Posted in 2016 NCT02793856	Posted in 2019 NCT03872479
Base editor	Guide RNA	No	Base edit	Posted in 2022 NCT05397184; NCT05456880	Posted in 2022 NCT05398029
Prime editor	guide RNA	No	Base edit	Posted in 2024 NCT06559176	Not yet in clinic
Mobile genetic elements (e.g., I-PGI and PASSIGE)	Recognition patterns in genome (recognition patterns introduced via guide RNA for I-PGI and PASSIGE)	Yes, but not exposed	Gene insertion	Not yet in clinic	Not yet in clinic
Epigenome editors (e.g., CRISPRi, CRISPRa)	Guide RNA	No	Gene silencing or activation	Not yet in clinic	Not yet in clinic

to a DNA-cleaving domain (Fok1 enzyme). The DNA-binding domain of a TAL effector contains a repeat variable diresidue, which specifies a single base pair for binding (i.e., target sequence), offering a more modular design than ZFNs, which utilize 3-base pair binding motifs [12–15]. This allows TALENs to possess a high degree of specificity, which is central to their safety and editing efficiency.

The advent of the CRISPR-Cas system has significantly reshaped the landscape of gene editing due to its ease of use, versatility, and simplicity as these systems can be easily engineered to efficiently and specifically target genes both individually, and in a multiplexed manner. Derived from a bacterial adaptive immune system, engineered CRISPR-Cas uses a guide RNA (gRNA), typically an 18–20 nucleotide targeting sequence, to direct the Cas nuclease to a specific DNA sequence, creating a double-strand break without engineering novel proteins like the previously mentioned nuclease systems [16]. This system is engineered as a two-component system, where the gRNA can be easily designed to target different genes, making it highly versatile. The most studied and understood CRISPR-Cas system is *Sp. Cas9* [17]. Many variants have since been discovered to be safer, have better on-target editing, and/or are small enough to fit within the size constraints of current delivery technologies (e.g., Nme2Cas9) [18–22]. Other proteins have also been fused to Cas protein to enable base editing, I-PGI or PASSIGE, transcriptional interference or repression, and transcriptional activation [23–32]. A summary of major GE technologies is provided in Table 1 and described in the next section.

COMPARATIVE TECHNOLOGIES IN GENOME EDITING BY APPROACH

With the rapidly evolving toolbox for gene modification, there can be more than one editing technology and delivery modality to treat a given indication. Developing a suitable approach should be informed by the target product profile (TPP) developed with input from an interdisciplinary team including clinical subject matter experts and patient advocacy groups. Figure 1 illustrates the potential approach and modality (i.e., GE tool and vector type) that can be used to achieve a desired therapeutic outcome. Each modality will have a different profile, such as whether the mechanism of action includes a double-stranded break in the genome or not. These profiles should be considered during modality selection as it will inform nonclinical, CMC, and clinical aspects of the program. Note, for a given application, multiple approaches exist, and each has a unique set of strengths and weaknesses to consider.

Gene knockout

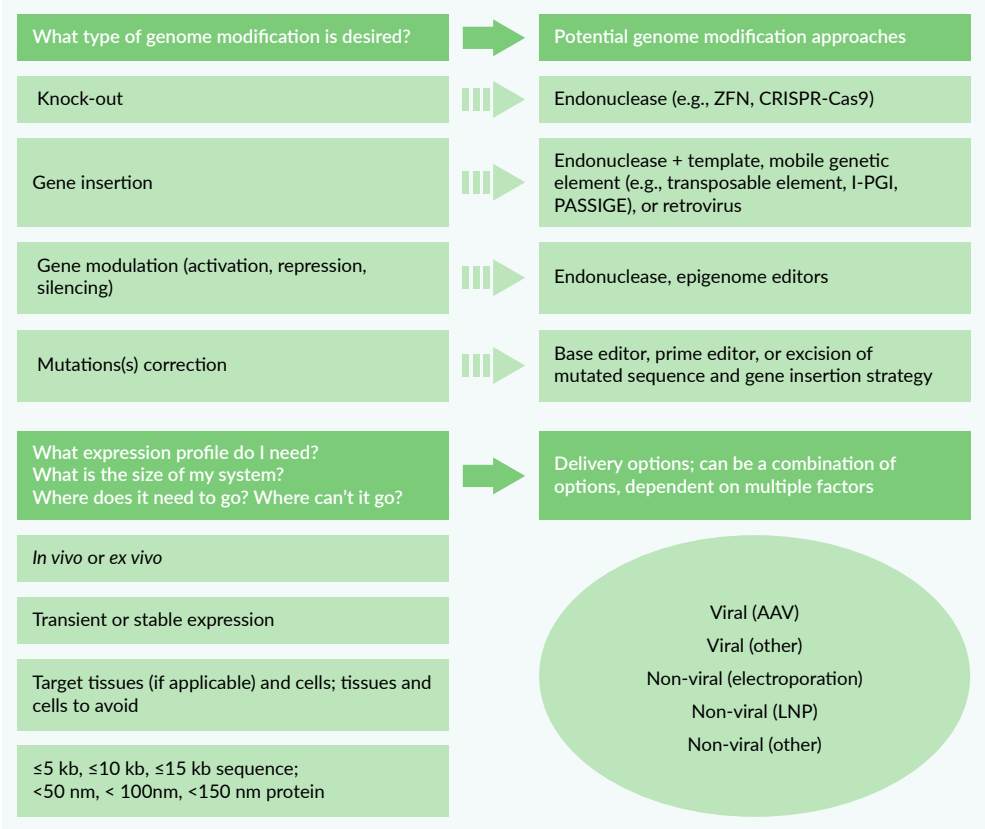
Gene knockout can be achieved using engineered nucleases such as meganucleases, ZFNs, TALENs, and CRISPR-Cas systems. CRISPR-Cas systems have gained traction because they can be readily targeted to specific genes through design of new gRNAs rather than protein engineering. Furthermore, CRISPR-Cas systems can be multiplexed (i.e., incorporating multiple gRNAs designed to target different sequences). Targeted gene knockout is used in CGT for various applications such as functional enhancement of cells, immune stealthing for allogeneic therapies, and reduction in protein levels which otherwise exacerbate disease progression. For example, CASGEVY utilizes CRISPR-Cas9 to knockout a repressor gene for fetal hemoglobin resulting in elevated fetal hemoglobin levels [4]. Fetal hemoglobin provides a replacement to the faulty adult hemoglobin. This strategy has been used in the treatment of sickle cell disease (2023 approval) and transfusion-dependent β-thalassemia (2024 approval) [4]. Additionally, engineered nucleases such as CRISPR-Cas9 are being used to knockout the endogenous T-cell receptor in allogeneic T cells to reduce the chance for graft-versus-host-disease [33]. Another noteworthy example is a CRISPR-Cas9 approach designed for *in vivo* delivery via a lipid nanoparticle (LNP) to knockout transthyretin protein to treat transthyretin amyloidosis where misfolded protein accumulates primarily in the nerves and heart and is ultimately fatal [34]. This program has begun Phase 3 trials and, if successful, could be the first approved *in vivo* CRISPR-Cas9-based therapy [35].

Gene insertion

Gene insertion involves the introduction of genes, often for a gain of function effect. For example, approved gene modified T-cell therapies for hematological malignancies rely on the insertion of a chimeric antigen receptor (CAR) to enable recognition and binding to cell surface markers on cancer cells. Most gene-modified cell therapies approved to date use retroviruses such as LVV to insert CARs into a patient's T cells to create a therapy. LVV allows for insertion of ~8 kb of payload which includes the gene of interest (GOI) and associated regulatory elements such as a promoter and polyA sequence. LVV has an insertion profile that is semi-random and with preference to intronic regions. The manufacturing process for LVV can be quite costly and a major drawback with LVV is the risk of insertional mutagenesis at oncogenic sites for which self-inactivating vectors have been designed to help reduce the risk [36–38]. Per FDA guidance [39], long-term patient follow-up of up to 15 years is needed after administration. However, to date the observed risk of oncogenesis due to insertional mutagenesis is still considered very low [40–42].

FIGURE 1

Options for genome modification and delivery.



With advances in gene editing techniques, engineered nucleases are being explored as alternative modalities for targeted insertion using a donor template. The endonuclease creates a double-stranded break at the target site, and a co-delivered donor sequence acts as a template for genome insertion. To facilitate insertion, the donor template often contains the sequence of interest for insertion flanked by homology arms which are homologous sequences to the site where the donor template is to be inserted [43]. The homology regions help facilitate recombination using the donor template as the reference sequence for repair mediated via the homology directed repair (HDR) pathway. The HDR pathway is active in the S and G2 phase of the cell proliferation cycle, and therefore, limiting application to actively dividing cells.

Genome insertion using an endonuclease system and donor template requires efficient delivery to the target cell(s). The delivery format is dependent upon the application, covered later in this review, and different modalities are currently being explored for delivery of donor template with an endonuclease.

Recombinases are being explored for large payload insertion (≥5.0 kb) as well. These large payload insertion technologies build on recombination reactions that occur in nature. This includes transposases and integrases that are part of transposable elements. Transposable elements, a class of mobile genetic elements, were first discovered in the late 1940s by Barbara McClintock [44]. There are two primary classes of transposons: Class I transposable elements (also known as retrotransposons) move using an RNA intermediate, whereas Class II transposable elements (also called DNA transposons) are segments of DNA that move using a 'cut-and-paste' mechanism [45]. Transposable elements are sometimes referred to as 'jumping

genes' because they are sequences that can move around (i.e., 'jump') the genome and insert at sites with a specific recognition pattern. The recognition patterns, however, can be quite common in the genome, so insertion is possible at multiple sites. In more recent years there has been significant progress identifying mobile genetic elements with recognition patterns less common in the genome while retaining activity in mammalian cells. In addition, there have been efforts to combine the targeting abilities of CRISPR-Cas systems with transposases or integrases for large payload insertion [46,47]. To reach the full potential of these technologies, delivery vectors or instruments are needed to introduce the GE tool and large donor template. If multiple delivery vector types are used (e.g., one for the GE tool and another for the template), then efficient co-delivery is needed for a successful edit.

Prime editors are an alternative CRISPR-Cas-based technology that allow for the insertion of small (<50 bp) sequences at a target site [25]. Prime editors rely on a CRISPR-Cas9 nickase, a reverse transcriptase, and a modified gRNA [26]. A Cas9 nickase is a modified Cas protein where one of the catalytic domains is inactivated via a mutation so that only a single DNA strand cut is made instead of cutting both strands. The modified gRNA, termed prime-editing guide RNA (pegRNA), serves two functions. First, it specifies the site for genome insertion, and second, it provides a template for insertion into the genome. The first IND cleared using prime editing technology is an *ex vivo* hematopoietic stem cell therapy where the prime editor is used to correct a mutation (Table 1).

I-PGI (formerly PASTE) and PASSIGE (formerly twinPE) are technologies that build on prime editing [23,24]. These technologies require multi-step gene edits to allow for targeted insertion of large payloads. They work by using a CRISPR-Cas9 nickase, a reverse transcriptase, and an integrase. The Cas9 nickase introduces a single cut, allowing for the reverse transcriptase to insert an integrase recognition site [15]. The integrase then recognizes the site and inserts a donor template that contains the same recognition pattern. Multi-step editing processes are complex and require analytical testing that evaluates the outcomes of each step in the process for both on-target editing and undesired off-target edits and translocations if exposed double-stranded breaks occur during editing.

The field is continuing to discover and engineer new modalities for gene insertion thereby diversifying the tools available for therapeutic development. As new editing tools become available, it is critical to understand their advantages and limitations as it relates to each potential therapeutic application [27].

Single point mutations

Base editors were developed from a modified CRISPR-Cas system where a Cas9 nickase is fused to a nucleoside deaminase that can remove an amino group from a nucleoside. In effect, Cas9 nickase and gRNA function to direct the fused protein to the target site where gRNA binds to its target strand, allowing the nucleoside deaminase to modify the non-target strand. The Cas9 nickase cuts the non-edited strand, and then DNA repair completes the process of introducing the base edit using the edited strand as the template. Cytosine and adenine base editors exist for both C to T and A to G substitutions, respectively [28,29]. Prime editors, described under gene insertion, can also be used for base substitutions, while base editors have also been used for gene knockout applications [48,49]. One approach to gene knockout with base editors is the introduction of a single point mutation which creates a premature stop codon, knocking out gene expression [50].

Gene modulation (activation, repression, silencing)

CRISPR-Cas systems can be modified to act at the transcription level through fusion of

nucleolytically inactive Cas (sometimes called dead Cas or dCas) protein with another enzymatic protein that can activate or repress transcription of a gene. By having an effect at the transcriptional level, no double-stranded breaks are required to modulate gene expression. The modified CRISPR-Cas system is directed to the promoter region upstream of a gene to modulate expression. CRISPR activation (CRISPRa) and interference (CRISPRi) have been useful research tools for genome-wide screens and cellular reprogramming among other applications [30–32]. Enzymes such as methyl transferases can be fused to a dead Cas protein for gene repression. This technology is often referred to as an epigenome editor since no edits are made to the genomic sequence. Therapeutic application of epigenome editors is in its infancy.

DELIVERY OF GENOME EDITING TECHNOLOGIES

Ex vivo GE CGTs are the most advanced in development (i.e., CASGEVY commercial approval [4]) and comprise the majority of GE CGTs in the clinic (GlobalData). In most cases, delivery is achieved via electroporation to the target cells *ex vivo* while *in vivo* GE CGTs utilize viral and nonviral delivery platforms. *Ex vivo* GE CGTs offer an advantage of greater control of editing, as the target cell population can be precisely selected, and analysis of any off-target edits can be performed prior to administration to the patient.

In vivo delivery of GE technologies can be categorized into viral and nonviral delivery platforms. AAV is the predominant *in vivo* gene therapy platform due to its ability to selectively target certain organs, low immunogenicity relative to other viral vectors, and its ability to transduce dividing and non-dividing cells. However, delivery of GE technologies by AAV is limited by the carrying capacity of the AAV vector, ~4.8 kb, and neutralizing antibodies which limit the treatable population and prevent redosing. Furthermore, treatment related adverse events have been observed at high systemic doses of AAV which remain a concern [51]. Nonviral vectors and delivery platforms seek to address the limitations of viral vectors, and typically deliver GE components through chemical or physical transfection of the target cells, which allows for greater payload capacity at the expense of reduced specificity and efficiency of transduction compared to viral vectors. In some cases, both viral and nonviral approaches can be used in combination for GE delivery. For example, AAV can be used to deliver the donor template while another approach, such as a lipid nanoparticle, can deliver the endonuclease system. One limitation with this approach is that the donor template, including the homology arms, must fit within the carrying capacity of AAV, limiting the ability to deliver genes coding for large proteins (e.g., full-length dystrophin for the treatment of Duchenne's muscular dystrophy) [52]. To overcome this limitation, several approaches are currently in use to meet packaging restraints of AAV such as the use of truncated forms of large genes (e.g., micro- or mini-dystrophin or the delivery of a CRISPR-Cas and two gRNA to allow for excision of a mutated dystrophin exon. In other cases, transgenes can be split across two AAVs for a dual delivery approach to mitigate the AAV capacity limitations.

One consideration with using AAV for delivery of endonucleases is the concern for persistent expression of the delivered GE component(s) in target cells if there is integration of the payload, even in very low frequencies or if non-dividing cells are targeted [52,53]. Persistent expression of an endonuclease such as CRISPR-Cas poses a safety risk as the opportunity for off-target editing, translocation events, and immunogenicity against the nuclease are all increased. Furthermore, the immunogenicity of the expressed gene editing product combined with the observed immune responses against AAV itself could exacerbate immunogenic responses. This is why transient expression of the gene editing product is often desirable for *in vivo* applications.

Due to these limitations with viral delivery systems, nonviral vectors and delivery platforms are becoming increasingly popular in the GE field. One class of nonviral vectors are lipid nanoparticles. LNPs have become increasingly prominent in recent years due to their success for vaccine applications during the COVID-19 pandemic [54,55]. LNPs have the advantage of delivering gene editing cargo for transient expression, can theoretically carry larger payloads relative to viral vectors, can be redosed, and generally require less complex manufacturing strategies [56]. However, non-viral platforms such as LNPs may have lower efficiency in delivery and targeting. When delivered systemically, most LNPs accumulate in the liver due to physiological factors such as size and surface charge. Novel LNPs that incorporate active targeting (e.g., ligand-based) and/or passive targeting (i.e., physical features such as particle size) are needed to improve *in vivo* delivery to specific organs but are not widely adopted yet in the clinic. Existing LNP formulations, such as those targeting intramuscular administration, are not optimal for delivery into other tissues. In most GE applications, endonucleases must be efficiently delivered to the correct tissue, cell type, and localized to the nucleus to effectively elicit the desired genome modification.

Table 2 summarizes major *ex vivo* and *in vivo* delivery methods and their relative strengths and weaknesses for GE delivery.

MANUFACTURING OF GENE EDITING COMPONENTS

Given the breadth of gene editing technologies, delivery methods, and cargo formats available, manufacturing processes can vary widely for CGT products using gene editing technologies. For example, the CRISPR-Cas based GE cargo can be a combination of gRNA co-delivered with Cas mRNA, DNA, or protein. In the case of *ex vivo* applications, the gene edit is often part of the process whereas for *in vivo* applications, the gene edit is part of the product's mechanism of action.

Manufacturing is often the rate limiting step when developing commercial gene therapy products. Incorporation of gene editing technologies into existing gene therapy applications can become increasingly burdensome when the gene editing technology requires multiple components and/or multiple delivery methods to achieve a successful edit, and when each pose a risk that must be mitigated. For example, many CRISPR-Cas based technologies may need multiple AAVs and/or LNPs to deliver all the necessary GE components. Since multiple manufacturing strategies exist for AAV and LNP manufacturing [57,58], this adds another potential layer of divergence in manufacturing strategy for similar products and thus makes it challenging to standardize manufacturing across the field. As with manufacturing viral and non-viral delivery platforms, multiple strategies may be considered for manufacturing the necessary raw and ancillary materials such as plasmids, RNAs, and nucleases. Whichever the manufacturing strategy is, the selected component should be manufactured in a consistent manner. Additionally, greater regulatory expectations may be placed on components used for *in vivo* technologies, such as increased purity expectations, which can increase cost of goods.

COMMON COMPONENTS (i.e., CARGO AND DELIVERY SYSTEM) OF A GENOME EDITED CGT PRODUCT

Plasmid DNA (common critical starting material)

Plasmid DNA is the starting material for some GE components and can be produced at small and large scales, often in gram quantities [5]. The sequence of interest is often cloned, or synthetically generated, depending on length, and incorporated into an *E. coli* plasmid backbone. *E. coli* strain selection should not be overlooked as selecting an optimal strain can result in production of larger and more consistent quantities of stable, high-quality DNA.

TABLE 2

Major delivery methods for genome editing CGTs.

Delivery method	Major GE application	Description	Strengths	Weaknesses
Electroporation	*Ex vivo*	Used to deliver GE cargo	Can accommodate different types of GE cargo (e.g., mRNA, DNA, protein)	Limited to *ex vivo* applications
Viral: AAV	*In vivo*	Used deliver GE cargo. Can also be used *ex vivo* for donor template delivery	Multiple capsid variants exists for preferential tissue targeting	Persistent expression of GE components in slowly dividing and non-dividing cells, which raises safety concerns; 4.6k b carrying capacity; can't be redosed
Nonviral: LNP	*In vivo*, *ex vivo*	Used to deliver GE cargo	Transient expression of GE component; can be redosed	LNP formulation development required to address poor extra-hepatic targeting *in vivo*

CGT: Cell and gene therapy; GE: Genome editing.

Once an *E. coli* strain is selected and shown to produce adequate quantities and quality of plasmid DNA, a master cell bank (MCB) is typically generated, tested, and utilized for routine manufacturing of plasmid batches. Working cell banks (WCB), produced from the MCB, are highly recommended and can ensure a lasting plasmid supply without the need to generate a new MCB when the initial stock is depleted [59].

In some cases, cell-free, chemical synthesis of DNA fragments of various formats can be used as a starting material for GE components in place of plasmid DNA. Smaller DNA fragments, such as those <200 bp can be chemically synthesized using technologies such as phosphoramidite synthesis, enzymatic oligonucleotide synthesis, and terminal deoxynucleotidyl transferase (TdT) [60]. Size limitation is a major drawback when using chemical synthesis of DNA as the technique can quickly become cost prohibitive for larger DNA fragments (>200 bp) and DNA synthesis error rates increase with larger fragments. However, the field continues to improve, and costs will become more reasonable as the technology further develops and competition increases.

The quality or grade of DNA template (i.e., plasmid or linear template) should be considered, as 'GMP' grade DNA templates can be costly and queues for manufacturing slots can be several months out. There is no regulatory requirement for the use of 'GMP' grade DNA templates when used as a critical starting material for early clinical development. However, it is highly recommended and becoming industry standard, to utilize a 'high-quality' grade DNA template (i.e., well documented and well controlled) during early phase trials before switching to a GMP grade DNA template for late stage and commercial applications.

mRNA (common critical starting material or drug substance)

Manufacturing of mRNA generally begins with plasmid DNA as a starting material. The sequence of interest is cloned into a plasmid and the plasmid DNA is then transcribed into mRNA within a bioreactor [61]. The mRNA is 'capped' at the 5′ end of the sequence, and the mRNA product is purified using chromatographic and filtration-based techniques [62]. Additionally, short RNA modalities (e.g., gRNA, siRNA) can all be manufactured using chemical synthesis methods.

RNPs and proteins (common critical starting material or drug substance)

The CRISPR complex, or RNP, is the amalgamation of a guide RNA (gRNA) fused to

a protein such as a nuclease, base editor or prime editor. The gRNA allows for site specific targeting within the genome and the protein (i.e., a nuclease acts as the molecular scissors to generate cuts in the genome). Alternatively, Cas and gRNA can be delivered separately. There is also flexibility with the format for Cas delivery as either DNA, RNA, or protein. Both Cas and gRNA components can be incorporated on the same platform (e.g., plasmid DNA, viral vector, LNP) or can be delivered individually using any of the platforms described, including electroporation.

Viral vectors (delivery system)

Viral vectors are often used to deliver gene editing components and have been used in gene replacement technology for over 30 years. AAV is the most popular viral vector platform chosen for delivering GE components and is capable of delivering up to 4.8 kb of single stranded DNA. The sequence for gRNA and nuclease is often encoded on the transgene plasmid and used to package the intended sequence into the AAV using transient transfection, baculovirus production, or an alternative strategy [63]. AAV has the advantage of having numerous capsid variants that are often used to target specific tissues and can be scaled to produce thousands of liters per batch. The disadvantages of using an AAV vector are that production methods are often time consuming and costly. Several AAV capsid variants can be challenging to manufacture (i.e., at high concentrations, high yield). There can also be IP issues surrounding the use of specific capsids that remain unresolved.

LNPs (delivery system)

Although viral vectors have long been the delivery vehicle of choice for gene therapies, non-viral methods are gaining traction and have recently been approved for commercial use for vaccine applications. Non-viral technologies, such as LNPs, can be more easily scaled for production, are less immunogenic than viral vectors, and can package large payloads, i.e., great for delivering a large transgene. LNPs are easier to produce than viral vectors and the raw materials (ionizable cationic lipids, PEG lipids, Zwitterionic lipids and cholesterol) are relatively inexpensive when compared to the production costs associated with viral vectors. However, the raw materials used to manufacture LNPs may require licenses for the IP [64,65] which could drive up costs.

REGULATORY STRATEGIES

The regulatory landscape for GE products is rapidly advancing and differs across jurisdictions and GE techniques. In the USA, the FDA published a Guidance for Industry in January 2024 titled 'Human Gene Therapy Products Incorporating Human Genome Editing' [5]. This guidance covers product development, CMC, nonclinical, and clinical considerations for GE products, and describes the agency's current thinking on the regulation of these products and their components. While this guidance is specific to GE components, other existing regulatory pathways should also inform a Sponsor's regulatory strategy. Specifically, if components such as viral vectors, plasmid DNA, small molecules, or other proven product classes are used, Sponsors should consider existing regulatory guidance.

Major considerations that Sponsors should consider during product development include the type of delivery system (i.e., viral vectors or non-viral delivery), the delivery method (i.e., *ex vivo* or *in vivo*), and the type of genome modification that is intended.

Regulatory strategies depending on product types

GE technology and delivery methods should inform the manufacturing strategy and testing of the GE components during clinical development. Gene editing components may be critical materials, drug substances,

or drug products depending on the product type and delivery method(s). For example, *ex vivo* products using non-viral electroporation-based delivery may use multiple GE components (e.g., a nuclease and a nucleic acid) to generate a final drug product (e.g., GE cells) for delivery to the patient, while *in vivo* products may deliver a single GE component (e.g., a single AAV) as the drug product itself. Sponsors should consider the delivery methods and justify their CMC strategy accordingly.

The type of edit the product makes will also inform the testing strategy of the product and clinical monitoring requirements. A key part of safety testing for genome modification is to monitor genome integrity and stability (e.g., insertional mutagenesis) as the modifications can result in genome instability. For genome editing applications, this often requires analysis of off-target edits and chromosomal rearrangements during nonclinical development, which should be used to inform the clinical monitoring and follow-up plan. The risk of chromosomal rearrangements is driven by GE technologies that induce double-stranded breaks. Alternative editing technologies that do not rely on double stranded breaks (e.g., epigenome editors) are in development to overcome this risk.

Analytics

Although many of the testing and release analytics for a GE product will be similar to other gene therapy techniques, there is an increased analytical burden for GE products to characterize any unintended editing that may carry safety risks. If a gene editing tool requires multiple steps to induce a desired edit, then off-target effects and safety risks associated with each step must be evaluated [66]. Off-target editing analysis is typically performed during nonclinical safety assessments using an NGS-based approach to identify areas within the genome that have undergone unintended edits due to the GE product. Current GE technologies all pose some degree of risk of off-target edits, and an assessment should be performed to understand where these off-target edits occur and the overall risk to patient safety because of them. If off-target edits are observed, the overall risk of these edits should inform additional analytics on a case-by-case basis.

TRANSLATION INSIGHT

The number of commercial cell and gene Ttherapy products relying on gene editing technologies is rapidly expanding and is expected to continue to increase. Since the first CRISPR-Cas GE clinical trial was initiated in 2016 [67], the pipeline of gene edited based therapies in the clinic has expanded significantly to >125 active clinical trials globally (GlobalData). Furthermore, the recent approval of the first ever CRISPR-Cas9 edited cell therapy has demonstrated a regulatory pathway for *ex vivo* gene edited cell therapy products while advancements in gene editing technologies and delivery methods are paving the way for *in vivo* gene editing applications in clinical studies. Dark Horse Consulting Group (DHC) is at the forefront of CGT and supports clients in both the GE space as well as the overall CGT field. The authors regularly support clients across a diverse modality spectrum, from academic to commercial, to develop and commercialize these products. This experience has highlighted a need for the field to better-understand the current tool kit that is available to clinicians, researchers, and sponsors for GE based CGT products. This review is intended to help the field more efficiently advance safe and effective products from proof-of-concept through commercialization. We are excited to see the field progress and look forward to continuing to support a wide range of sponsors, manufacturers, and investors in developing and commercializing their gene editing and CGT products.

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Acknowledgements: The authors would like to thank our internal reviewers Katy Spink PhD, Nathan Manley PhD, Brent Morse MS, and Tal Salz PhD, who graciously contributed their time and expertise to review this document. The authors would also like to thank Madeline St Onge MBA for contributing clinical information sourced from GlobalData.

Disclosure and potential conflicts of interest: The article is based on publicly available information. Some prior clients may be cited in the article, but the authors cannot disclose their names due to confidentiality obligations that they are bound by.

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

ARTICLE & COPYRIGHT INFORMATION

Article source: Invited; externally peer reviewed.

Submitted for peer review: Aug 27, 2024; **Revised manuscript received:** Sep 26, 2024; **Publication date:** Oct 7, 2024.

CELL & GENE THERAPY INSIGHTS

GENE EDITING

SPOTLIGHT

Gene delivery: persistent challenges call for a reference measure

Max Ryadnov
National Physical Laboratory

"Irrespective of what system is used, as any has its pros and cons...it is clear that the efficacy of gene therapies is defined by common events that apply to all gene delivery systems."

VIEWPOINT

Cell & Gene Therapy Insights 2024; 10(9), 1313–1316

DOI: 10.18609/cgti.2024.150

Genetic therapies constitute an evolving technology that holds promise for ultimate control over many diseases ranging from genetic disorders to metastatic cancers [1]. Nucleic acids (genes) and their derivatives are being developed as drugs to modulate genetic reactions by causing degradation of targeted genes, inhibiting their expression,

or promoting it. Such macromolecular therapeutics appear to have overcome the problems of stability, excretion, and uptake by phagocytes. However, a major barrier remains in their inefficient transfer into target cells, release from endosomes and entry into the nucleus when required.

Consequently, the technology finds itself at cross-roads [2]. On the one hand, therapeutics are available, the number of clinical trials grow, and the advent of gene editing technologies is expected to provide effective solutions to genome modifications of therapeutic relevance. Significant progress in all these areas over the past two decades bears witness to the demonstrable feasibility of the technology. Despite that, on the other hand, there remains a lack of clinical approvals, with only a handful of gene therapies approved for medical use.

THE ROOT OF THE PROBLEM

To exert a desired effect, genetic therapies require an effective means of delivery into the cell. Historically, viral vectors continue to represent the field of gene delivery systems. The value of AAV for gene delivery is demonstrated in recently approved gene therapies such as Luxturna® and Zolgensma® [3]. Such cases of success, albeit encouraging, remain in their infancy, which when compared against the sheer number of clinical trials for viral vectors prompts questions of their long-term and broader applicability in clinic [3,4].

Indeed, there persist several limitations that impede the systemic use of viral, virus-based, or virus-derived vectors. These include low gene loading capacities of viral capsids, safety concerns associated with insertional mutagenesis, off target effects and oncogene activation as well as adverse immune responses. As an example, many patients have pre-existing immunity to the AAV, which may be the case for the majority of the planet's population and hence cannot qualify for treatments involving the viruses. Technically, injecting a naked, i.e., free, DNA directly into a muscle tissue, especially when assisted by electroporation, provides an alternative gene delivery approach. Non-surprisingly, a significant practical and commercial interest in this approach due to its simplicity and accessibility has been maintained in clinical trials. Yet, this approach does not solve the problem of effective intracellular uptake, which is intrinsically low, gives low levels of gene expression as a result and introduces an additional issue of discomfort experienced by patients upon intramuscular injections.

THE VALUE OF EMULATING NATURE

All in all, these considerations stimulate the search for non-viral vectors [5]. These entities are based on different chemistries, can be low and high-molecular weight molecules, and at simplest form nanoparticles when complexed with nucleic acids. Typically, the resulting complexes are polyelectrolyte as non-viral vectors tend to be cationic, to package anionic nucleic acids, and are often polymorphic in size and morphology. Although structural polymorphism is common for viral vectors as well, non-viral gene carriers have a greater propensity to agglomeration, which can in turn lead to undesired side effects. Thus, non-viral systems relying on condensation of nucleic acids are not devoid of limitations either.

Most recent efforts have focused on developing non-viral gene encapsulators, which emulate viruses in their properties and functions, including their ability to assemble into discrete nanoscale shells, encapsulate genes, traverse cellular membranes, and induce endosomal egress to reach intracellular targets. These systems assemble from *de novo* polypeptide sequences, with no *a priori* biology, which allows them to effectively avoid the drawbacks and safety risks of viral vectors. They can be made polymorphic to package genes of virtually any size and complexity, without compromising on structural integrity

leading to aggregation, can be readily functionalized to target specific biology and are straightforward to manufacture. However, their self-attenuating and self-replicating capabilities alongside with potential safety concerns remain to be addressed.

CONCLUSIONS: CALL FOR A REFERENCE MEASURE

Irrespective of what system is used, as any has its pros and cons, and none provides an exhaustive answer to effective and safe gene transfer and targeting, it is clear that the efficacy of gene therapies is defined by common events that apply to all gene delivery systems. Such events cover critical stages of gene delivery including gene encapsulation, by the carrier, cell targeting and uptake, endosomal escape into the cytoplasm, transport to the nucleus, and gene expression. All these events translate into performance attributes of gene delivery systems which require quantitative descriptions reflecting their efficiency, i.e., to package genetic material, the metric of which is as a ratio of empty to loaded gene carriers, deliver the material into the cell, which is referred to as transfection efficacy and whose metric is often the count of transfected cells versus the total cell count, and like the efficacy of transcription, expression or knockdown can be quantified in terms of nucleic acid copy numbers. The attributes are also considered in combination with the impact delivery systems have on cell viability, proliferation and even cell morphology and phenotypes.

It is also clear that none of these metrics and events or any other related metric or event has a sufficient value individually to serve as a universal efficacy metric for gene delivery. Similarly, a mere combination of such metrics and events is of little use unless inter-relationships between them are established and quantified. Correlating the efficacy of gene encapsulation with those of cell uptake and gene expression, which are compounded by effects on cell viability, is a challenging, but yet necessary, task. The ultimate outcome of this task is a correlated measure of gene delivery. The measure itself once developed will constitute a significant advancement. However, it will remain as insufficient and arbitrary as individual events and their perceived metrics whose measured values are subject to variabilities from lab to lab, system to system and batch to batch, questioning their reproducibility and reliability. For these reasons, the correlated measure must integrate comparability, measurement uncertainty and traceability to translate into a reference measure of gene delivery—the highest point of reference supported by relevant reference materials and methods [6], which will provide an enduring and impartial solution for quantitative gene delivery allowing to benchmark the performance attributes of existing and emerging gene delivery systems regardless of their origin, chemistry, methods of manufacture and application.

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BIOGRAPHY

MAX RYADNOV is NPL Fellow in Biometrology and Professor of Biophysics from the UK's National Physical Laboratory, Teddington, UK and is currently heading up an engineering biology initiative aiming to develop reference standards for biological design and manufacturing. His research continues to focus on developing translational metrology for antimicrobial, cell, gene, and regenerative therapies, whilst his work continues to promote science beyond the bench receiving regular highlights in public and industry-oriented media.

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AUTHORSHIP & CONFLICT OF INTEREST

Contributions: The named author takes responsibility for the integrity of the work as a whole, and has given their approval for this version to be published.

Acknowledgements: None.

Disclosure and potential conflicts of interest: The author has no conflicts of interest.

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

ARTICLE & COPYRIGHT INFORMATION

Article source: Invited.

Revised manuscript received: Oct 18, 2024; **Publication date:** Oct 22, 2024.

CELL & GENE THERAPY INSIGHTS

GENE EDITING

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INTERVIEW

Advancing gene therapies for β-hemoglobinopathies with novel genome and epigenome editing tools

Charlotte Barker, Editor, BioInsights, speaks to **Annarita Miccio**, Group Leader, Imagine Institute of Genetic Diseases, discussing ongoing research aimed at developing gene therapies for β-hemoglobinopathies, focusing on transcriptional and epigenetic regulation. They also explore innovative tools such as CRISPR-Cas9, base editing, and epigenome editing to improve treatment safety and efficiency, with the goal of making gene therapies more accessible globally.

Cell & Gene Therapy Insights 2024; 10(9), 1163–1171

DOI: 10.18609/cgti.2024.133

Q What are you working on right now?

AM: My laboratory is working on transcriptional and epigenetic regulation in hematopoietic stem cells (HSCs) and their progeny to develop gene therapies for β-hemoglobinopathies.

"...it is essential to carry out genome-wide transcriptomic and epigenetic analyses to ensure that transplanted HSCs function properly, especially compared to unmanipulated cells."

Currently, we focus on developing treatments for two severe genetic anemias, known as β-hemoglobinopathies, which affect the production of the β-globin chain of the adult hemoglobin tetramer. This tetramer is composed of two α and two β-like globin chains. When the levels of β-globin or an altered β-globin are reduced, as seen in β-hemoglobinopathies, the function of red blood cells is affected, leading to anemia and multi-organ damage.

Our current research is focused on modulating gene expression to treat these conditions, utilizing our expertise in transcription and epigenetic regulation. The fundamental issue in these diseases is the altered production of the β-globin, which is the adult β-like chain. The idea is that we could potentially cure patients by reactivating the expression of the β-like fetal γ-globin subunit.

During fetal life, the body produces fetal hemoglobin, including the γ-globin chain, but it is usually silenced shortly after birth. Research has shown that patients who continue to express fetal hemoglobin into adulthood tend to have ameliorated clinical phenotypes. In some cases, people with persistent fetal hemoglobin expression, even those with mutations causing sickle cell disease (SCD), may not have symptoms and are hardly considered patients. Therefore, we currently focus on modulating gene expression as a potential therapy for β-hemoglobinopathies.

Q Can you tell me more about your work in researching the dynamics of transcriptional and epigenetic networks during stem cell development?

AM: We focus on the transcription and epigenetic regulation of HSCs and their erythroid progeny that give rise to red blood cells. HSCs are the target cell population in gene therapy approaches due to their ability to repopulate the patient's body and remain in the system indefinitely. Our approach involves extracting the cells from the patient, culturing them *ex vivo*, genetically modifying them, and re-infusing them into the patient. In the meantime, the patient receives chemotherapy or a conditioning regimen to create space in the bone marrow for the genetically modified cells.

One reason we study transcription and epigenetic regulation in erythroid cells is to understand how globin gene expression works, which will allow us to identify therapeutic targets. Another reason is safety. Since we manipulate these cells in the laboratory in an unnatural process, it is essential to carry out genome-wide transcriptomic and epigenetic analyses to ensure that transplanted HSCs function properly, especially compared to unmanipulated cells.

Q What is possible currently in the realm of epigenetic modulation, and what might the future hold in the context of both combinatorial and synergistic therapeutic approaches?

AM: Transcription and epigenetic modulation offer a promising approach to treating not only β-hemoglobinopathies but also a wide range of other genetic diseases. The ideal strategy for genetic diseases would be to directly correct the disease-causing mutation, though this is not always an easy approach. For example, β-thalassemia is caused by over 400 different mutations, meaning we would need to develop a separate therapy for each one, which is theoretically possible but not practical.

Fortunately, in the case of β-hemoglobinopathies, we have a promising alternative of using 'disease modifiers' which are usually genes that can be activated or inactivated, depending on the indication. This approach does not require a specific modification of genes and solely focuses on turning them on or off to potentially cure the disease. This technology offers a broader therapy, as it is independent of the specific disease-causing mutation, as exemplified by the treatment strategies for β-hemoglobinopathies.

For instance, β-thalassemia is characterized by a deficiency in β-globin expression, therefore we aim to activate the γ-globin genes, which encode for the fetal γ-globin genes that are the β-like fetal globin chain. This activation of fetal γ-globin genes compensates for β-globin deficiency. Similarly, in SCD, caused by the production of altered sickle hemoglobin, we also aim to increase the production of fetal γ-globin to counteract the effect of the toxic sickle hemoglobin.

Apart from hemoglobinopathies, we are also exploring other genetic diseases, such as α-thalassemia, a disease that affects the β-globin chain of the hemoglobin tetramer instead of the β-globin chain. Similar strategies could be applied to conditions beyond red blood cell diseases. For example, we are also developing therapies for spinal muscular atrophy (SMA), which is caused by mutations in the *SNM1* gene. Instead of targeting each mutation directly, we can focus on activating *SMN2*, a paralogous gene that is expressed at low levels, hopefully addressing various mutations in *SNM1* in a more universal approach.

We are already seeing some of these strategies being implemented in clinics. For example, Casgevy® was recently approved by the UK, USA, and EU regulatory agencies for 12–35-year-old patients with β-hemoglobinopathies, including both β-thalassemia and SCD, while pediatric patients are still not being treated with Casgevy. The primary goal is to reactivate fetal γ-globin, a universal strategy for all patients with SCD and β-thalassemia, considering γ-globin addresses both conditions and various mutations.

Our approach involves studying transcriptional regulation, which is essential for fetal γ-globin reactivation. This concept, initially developed by researchers at Harvard Medical School [1], is to inactivate a gene called *BCL11A*, which encodes for a potent transcriptional repression of fetal γ-globin expression. Normally in adults, BCL11A factor binds to the fetal γ-globin promoters, and represses fetal hemoglobin production. The strategy is to knock out *BCL11A* so that the γ-globin gene can be reactivated, stopping the repression of fetal hemoglobin. This

approach relies heavily on the CRISPR-Cas9 system, a nuclease-based system that generates double-strand breaks (DSBs) in the DNA at specific locations within the genome, acting as 'molecular scissors'. After the DSB, the cellular repair pathways try to repair the cleaved region, but the process is often imprecise, resulting in deletions. Based on research conducted by Vertex Pharmaceuticals and CRISPR Therapeutics [2], the approach involves cutting in the enhancer region of the *BCL11A* gene, specifically a binding site for transcriptional activator *GATA1*. Normally, GATA1 binds to the enhancer and activates *BCL11A* expression. Cutting out the GATA1 binding site in the enhancer with CRISPR-Cas9 nuclease leads to reduced expression of BCL11A which no is no longer there to repress γ-globin genes, leading to fetal γ-globin reactivation.

The CRISPR-Cas9-based strategy to target *BCL11A* is a pioneering example of how studying transcriptional gene regulation can lead to effective therapies. Additionally, this strategy of inactivating *BCL11A* could be combined with targeting other regulatory regions, potentially leading to even higher levels of fetal γ-globin expression and enhancing therapeutic benefits for patients with β-hemoglobinopathies.

However, typically, combining strategies that involve cutting into the genome is not recommended, as it might lead to unintended consequences. For example, cutting the *BCL11A* enhancer and another region on another chromosome simultaneously can cause genetic translocations, which could be harmful. However, novel tools such as base editing and prime editing can offer a safer alternative for combinatorial gene therapies, as they do not cut into the DNA, but rather change the nucleotide bases.

Q Can you expand on your work on the treatment of β-hemoglobinopathies and give a summary of your goals and how you approach them?

AM: Even though our current therapy for β-hemoglobinopathies is already in the clinic, it can still be improved. Our main goal is to develop safer and more efficient strategies to cure a larger number of patients.

The challenge with the CRISPR-Cas9 nuclease system is that it cleaves into the DNA, which HSCs do not always respond well to. The cleavage may be dangerous, as is comparable to breaks caused by UV radiation. In our lab, we have developed alternative approaches for β-hemoglobinopathies either to correct the disease-causing mutations [3] or to reactivate fetal hemoglobin expression [4]. In particular, we are using CRISPR-Cas9-based technologies that are DSB-free, such as base prime and epigenome editing.

CRISPR-Cas9-based base editing utilizes enzymes that can convert nucleotides–adenine into guanine, and cytosine into thymine. In our laboratory, we have used this approach to correct disease-causing mutations or reactivate fetal hemoglobin expression. The ultimate goal is to avoid DSBs that could trigger a DNA damage response, leading to apoptosis. Cutting into the genome can also cause large genomic rearrangements, such as translocations.

"We aim to introduce histone modifications on the γ-globin promoter that are typically associated with active transcription of fetal γ-globin genes."

Our idea is to use these safer base editing tools to create mutations that can reactivate fetal hemoglobin expression. Our approach, which we hope to bring to the clinics, targets the fetal γ-globin genes, in particular the fetal γ-globin gene promoter. By using a base editor, we can change just one base, creating a new binding site for a transcriptional activator. For example, by changing adenine into guanine, we generate a binding site for the potent transcriptional activator called KLF1 that binds to the γ-globin promoter and activates gene expression [4].

Even though base editors only generate single-strand breaks, they are not completely free of DSBs. Hence, we are also exploring epigenome editors, one of the latest technologies based on CRISPR-Cas9. This technology is promising because it allows us to target specific regions without cutting the DNA. Instead, we modulate the epigenetic modifications, such as histone modifications or DNA methylation. We aim to introduce histone modifications on the γ-globin promoter that are typically associated with active transcription of fetal γ-globin genes. To activate γ-globin genes, we would also need to remove DNA methylation, as this typically switches off genes.

Q What is the potential for gene therapy treatments for SCD and β-thalassemias, and where can lentiviral vector (LV) and genome editing approaches be applied here?

AM: Many studies, including our own, have shown that gene therapy using LV and genome editing approaches could be effective for patients with SCD and β-thalassemias. The LV-based approach involves permanently integrating a functional and active β-globin gene into the genome to rescue the phenotype in patients with β-thalassemias. However, this process is complex, because it also involves inserting a large enhancer to regulate and activate β-globin gene expression. The LV cannot accommodate the entire enhancer, therefore the natural chromatin conformation that occurs at the endogenous locus cannot be replicated. Consequently, each vector copy does not produce as much β-globin as the endogenous locus, which is why we are moving toward genome editing.

With genome editing, we can modify the endogenous locus directly and utilize natural enhancers to achieve high levels of β-globin expression. For example, humans produce approximately 15 g/dL of hemoglobin in the blood, meaning we would need a high level of expression. The challenge with LVs is that multiple copies of the vector are required to achieve sufficient expression for therapeutic doses. In patients with SCD, simply adding an extra β-globin gene is not enough as it does not sufficiently compete with the endogenous sickle β-globin, which is

quite toxic. In the most recent trial using LVs, we will include not only a functional β-globin gene but also a microRNA designed to downregulate the expression of the sickle β-globin.

There are currently over 20 registered trials for β-hemoglobinopathies, exploring various targets and tools. While base editors can only perform adenine to guanine or cytosine to thymine conversions, prime editing, a novel technology in the early stages of development, theoretically allows for all types of base conversions. This is a significant advantage as it can also address small deletions or insertions that are responsible for certain diseases. With prime editing, we can insert the missing nucleotides.

Q How are novel genomic and bioinformatic tools, such as RNA-seq, ChIP-Seq, and genome-wide analyses used in your work?

AM: Firstly, genome-wide bioinformatics tools are essential for discovering therapeutic targets. For example, to determine how BCL11A factor represses γ-globin expression, we can use ChIP-Seq technology to find its binding site.

Secondly, we are using bioinformatics tools to assess the safety of our approaches. For example, RNA-Seq experiments can demonstrate that after treating HSCs with our methods, the cell still correctly expresses the key genes that are necessary for stem cell engraftment and differentiation.

Thirdly, in the case of epigenome editing, genome-wide histone modification (by ChIP-seq) or DNA methylation analyses (by bisulphite sequencing or nanopore long read sequencing) can be used to confirm the specificity of our treatments and help ensure that intended epigenetic modifications are achieved without disrupting cellular functions. Other specific genome-wide techniques using next-generation sequencing such as GUIDEseq (and several other techniques) can be used to evaluate the potential off-target activity of the different gene editing tools. In fact, while CRISPR-Cas9 is believed to be specific, it relies on guide RNA that anneals to a specific region in the genome. As many regions in the genome can be similar, it is crucial to confirm the exact region that is being targeted.

Q What are the most pressing requirements for future innovations in the gene editing toolkit?

AM: The main requirements are to reduce the off-target activity of the different gene editing tools, reduce the DSBs in DNA, and optimize the efficiency of the most innovative editing tools such as prime editing and epigenome editing. Currently, we are exploring gene editing tools that generate less DSBs, such as base and prime editors. However, prime editing, while being a powerful technology in theory, still needs improvements.

Furthermore, epigenome editing would be an ideal strategy since it does not cut the DNA. We have achieved promising results so far, but sometimes the changes in histone modifications are not permanent, meaning multiple rounds of treatment would be required.

Additionally, targeting disease modifiers is advantageous because it offers a single therapy for a disease, regardless of the specific mutation involved. The challenge is that we do not have disease modifiers for all diseases as each patient often has a different mutation. For example, we are now working on developing a treatment for patients with chronic granulomatous disease (CGD) and we have 200 patients with different mutations. Since there is no universal disease modifier for CGD, we must choose whether to develop a specific base editor or a prime editor for each mutation. While it is technically possible, creating such highly personalized treatment is very complex.

Currently, many research groups are exploring novel tools, such as transposases, integrases, and other enzymes derived from bacteria. These tools could facilitate the integration of large genetic cassettes into the genome, and potentially entire genes as long as 20 kb. In fact, integrating a wild-type gene into the endogenous locus could potentially cure the disease for all patients, regardless of their specific mutation, offering a more universal solution. These approaches of targeted integration show low efficiency in primary cells, but optimizing these tools is the key goal for the future.

Q What's next for your team's work over the coming 12–24 months?

AM: We aim to start treating patients with the currently available therapies because there are so many people with β-hemoglobinopathies that need urgent treatments. There are over 20,000 patients with SCD in the region of Île-de-France alone. Over the next 24 months, we plan to finish the pre-clinical study to prepare for clinical trials. In parallel with these efforts, we will continue to optimize our prime and epigenome editing tools.

Additionally, we are starting to develop *in vivo* approaches, considering most treatments for β-hemoglobinopathies and other hematopoietic disorders are currently *ex vivo*, which are costly and less efficient. Currently, patients need to visit specialized treatment centers multiple times for us to collect enough HSCs to modify them with CRISPR-Cas9 nucleases. Therefore, we are working on developing an *in vivo* strategy by using nanoparticles or viral-inactivated vectors to inject therapeutics into the bloodstream, and directly target the HSCs in the bone marrow. The approach is similar to using AAV for other gene therapies, which are generally less costly and complex. If we could develop a single-injection method, it would expand the availability of gene therapy worldwide. SCD affects over 8 million people worldwide, with around 6 million patients in Africa, where there are not many specialized facilities for gene therapy.

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BIOGRAPHY

ANNARITA MICCIO is currently the Director of the Laboratory of Chromatin and Gene Regulation during Development at the Imagine Institute, Paris Cité University, located in the Necker Campus, Paris, France. Dr Miccio's main interests are the transcriptional control of hematopoiesis, and the development of therapeutic approaches to β-hemoglobinopathies. As a PhD student, she generated a lentiviral vector successfully used in an early clinical trial for β-thalassemia. As a post-doc and later as an Assistant Professor, she gained experience in the gene regulation during erythroid development and in evaluating the efficacy of gene therapy approaches for hematopoietic disorders. In 2014, she was appointed as a Lab Director at the Imagine Institute, where she pursued her studies on transcriptional regulation in normal and diseased stem cells, and their progeny. These basic research studies were instrumental in developing novel strategies for β-hemoglobinopathies. In particular, she optimized the design of lentiviral vectors currently employed in a clinical trial for sickle cell disease and developed CRISPR/Cas9 strategies for β-hemoglobinopathies.

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Contributions: The named author takes responsibility for the integrity of the work as a whole, and has given their approval for this version to be published.

Acknowledgements: None.

Disclosure and potential conflicts of interest: The author has no conflicts of interest.

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

ARTICLE & COPYRIGHT INFORMATION

Article source: Invited.

Interview conducted: Jul 18, 2024; **Revised manuscript received:** Aug 19, 2024;

Publication date: Sep 18, 2024.

CELL & GENE THERAPY INSIGHTS

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INTERVIEW

Beyond CRISPR: exploring the next frontier in gene editing technologies

Abi Pinchbeck, Editor, *Cell & Gene Therapy Insights*, speaks to **Devyn Smith**, CEO at Arbor Biotechnologies, about the company's innovative work in gene editing technologies developed to surpass the limitations of traditional CRISPR/Cas9 methods in addition to its pioneering AI/ML approaches. They also discuss the direction of regulatory guidance for gene editing and the overall future of the field as the era of commercial genome editing-based therapeutics arrives.

Cell & Gene Therapy Insights 2024; 10(9), 1149–1153

DOI: 10.18609/cgti.2024.130

Q What are you working on right now?

DS: Arbor Bio is a next-generation gene editing company, co-founded by Feng Zhang, a CRISPR pioneer, and David Walt, co-founder of Illumina. They founded Arbor with the idea that CRISPR/Cas9, while revolutionary, is not the only approach to gene editing. CRISPR/Cas9 has limitations, so Arbor aims to discover and develop novel gene editing tools that allow us to hit more targets and tissues.

We have a discovery platform that enables us to identify novel nucleases, bolstering our broad toolbox of editing technologies and opening up new genomic targets. With this expanded toolbox, we can tailor our selected editing approach to each unique disease. Our primary focus is on *in vivo* targeted programs in the central nervous system (CNS) and rare disease

"We have a discovery platform that enables us to identify novel nucleases, bolstering our broad toolbox of editing technologies and opening up new genomic targets. With this expanded toolbox, we can tailor our selected editing approach to each unique disease."

opportunities in the liver. Our lead program, targeting primary hyperoxaluria, is on track for IND and clinical trial application filings later this year. We also have multiple programs targeting amyotrophic lateral sclerosis in the CNS.

Q Can you elaborate on Arbor Bio's next-generation gene editing approach, including details of your gene editing toolbox?

DS: At Arbor, we use nucleases and editing technologies that can bind to any DNA sequence to vastly broaden our targeting capabilities beyond what is possible with Cas9. The main challenge with Cas9 is its large size, which restricts its use in many delivery systems beyond lipid nanoparticles. In addition, Cas9 has a single protospacer adjacent motif, a binding motif that tells it where to bind on the DNA, analogous to a zip code, which restricts its target range.

We work with smaller editing enzymes, making our tools more versatile and compatible with various delivery methods, including AAV which allows us to move outside the liver for new disease targets. The other piece of the equation is the need for approaches beyond simple cut-and-repair methods, or indels, which Cas9 primarily relies on. Our toolbox includes advanced editing techniques like reverse transcriptase-based editing and insertion approaches, enabling us to modify larger DNA segments. This differentiated toolbox allows us to tailor our approach to specific diseases.

Q How can AI/machine learning (ML) optimization methods, including high-throughput screening, be used to identify and optimize editing technologies?

DS: Our Chief Technology Officer, David Cheng, who has extensive experience in AI/ML, leads our platform development. He brings expertise from his time at Google and on Wall Street, where he worked on high-frequency trading algorithms and was one of the early inventors of autocomplete. At Arbor, we use AI/ML approaches to enhance the efficiency of our discovery platform in a few ways.

First, it allows us to quickly identify interesting potential editing approaches from the myriad of data in databases and characterize protospacer adjacent motifs via a high-throughput screening platform. This technology also helps us engineer these approaches to function effectively in human cells, as many have not evolved in mammalian systems, meaning we need to engineer proteins and enzymes to increase editing efficiencies. We approach this via an AI/ML-driven method to model crystal structures of new enzymes, allowing us to probe structure-activity relationships by

predicting which amino acid residues are critical for specific editing functions. We can alter those relationships much more efficiently because we know where they are and what changes to make.

Q How can we tackle remaining safety concerns as the era of commercial genome editing-based therapeutics arrives?

DS: Safety in editing is paramount. In addition to my role at Arbor, I serve as Chairman of the Alliance for Regenerative Medicine (ARM), a collective of over 400 companies in the cell and gene therapy (CGT) space. ARM aims to utilize this collective strength to help advance the field in areas such as regulatory, patient access, and reimbursement. One of the critical pieces is ensuring that we have clear regulatory guidance and clear approaches to reimbursement and market access, thus enabling the whole field so we can be successful collaboratively.

The US FDA has made significant strides in providing guidance for gene editing, giving the field some nice draft guidance to provide clarity to us as biotech companies. A group of gene editing companies enabled by ARM joined forces to provide feedback to the agency on this guidance to ensure the industry considerations and perspectives are represented.

Q What can we expect in the way of further guidance for the field of gene editing and genetic modification? What are the key areas of regulatory convergence or divergence to look out for?

DS: Peter Marks, director of the Center for Biologics Evaluation and Research (CBER) at FDA has done a fantastic job of making sure that the agency is providing safe medicines whilst eliminating the barriers to providing medications to patients.

From a CGT perspective, the Office of Tissue and Advanced Therapies has been reorganized and renamed to the Office of Therapeutic Products, overseen by Nicole Verdun. This group has been proactive in balancing safety with the need to expedite access to life-saving therapies to patients who often can only be treated with a CGT.

One of the FDA's goals is to move towards establishing a platform approach to CGTs. As an example, a gene-editing approach in two differing liver diseases often will use the exact same delivery vehicle and mRNA, and the only alteration is the guide RNA in the lipid nanoparticle, which tells the nucleus what to do with the site it binds to. A platform approach will remove the need to repeat everything performed for one indication when the only thing varying factor is the guide RNA. To further this work, we must partner with the regulatory agencies and ensure we can do this safely. Platform approaches will hopefully enable us to be much more efficient, so we can drive down the cost of drug development and make therapies accessible.

Q What is the current state of affairs surrounding gene editing-specific guidance for potency assays?

DS: Potency assays are particularly challenging for gene therapies and gene editing, and identifying the right approach is important. The FDA has done a number of workshops

working with ARM and other regulatory and legislative bodies to help identify how to more efficiently figure out the correct potency assay for a particular process.

The guidance here is improving, and although it is not perfect, it has been helpful for developers moving through the clinical phases towards market approval. While there has been significant progress, especially with the approval of gene-editing therapies like the one from Vertex Pharmaceuticals and CRISPR Therapeutics, more clarity is needed as *in vivo* editing approaches advance from clinical trials to commercial stages.

Q Finally, what's next for your work over the next 12–24 months? What are your key goals and priorities over that time period?

DS: Our immediate goals at Arbor Bio include transitioning into a clinical-stage company by launching our first clinical program, ABO-101, an investigational therapy for the treatment of primary hyperoxaluria type 1 (PH1). Additionally, we aim to advance our CNS programs toward clinical filings. We will continue to watch the broader space and monitor the great progress being made in both the regulatory and market access spaces for gene therapies, ultimately so patients can get access to these life-saving therapies.

Gene editing is still in its infancy, with fewer than 200 patients treated with a gene-edited therapy *in vivo* so far. As we are learning, there are a variety of technologies that can be deployed, including base editing, prime editing, and simple indel or deletion approaches. Progress in gene editing allows us to envision a future where all genetic diseases are treatable and, eventually, curable. The excitement of this modality lies in its potential to be completely transformative for patients. I look forward to seeing many exciting discoveries over the coming weeks, months, and years.

BIOGRAPHY

DEVYN SMITH is CEO at Arbor Bio, Cambridge, MA, USA. Smith has significant experience leading biopharma companies from product concept to clinical stage and bring novel therapies to market. Previously, Smith served as Chief Operations Officer and Head of Strategy at Sigilon Therapeutics, was head of Business Operations and Strategy at the Medicinal Sciences Division at Pfizer R&D, and previously served at Pfizer's Neusentis Research Unit in the UK as Chief Operating Officer. Prior to joining Pfizer, Smith was a principal for The Frankel Group, a boutique management consulting firm in New York City and Cambridge. Devyn currently serves as chairman of the Alliance of Regenerative Medicine, the industry group for nearly 400 gene and cell therapy companies and as an advisor/board member to several companies in the advanced therapies space. He received his PhD from Harvard Medical School, Boston, MA, USA, an MSc from Idaho State University, Pocatello, ID, USA, and a BSc from Brigham Young University, Provo, UT, USA.

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Contributions: The named author takes responsibility for the integrity of the work as a whole, and has given their approval for this version to be published.

Acknowledgements: None.

Disclosure and potential conflicts of interest: Smith D has exchanged consulting fees with AIRNA Biotech. Smith D has stocks/stock options in Arbor Biotechnologies, Aspect Biosystems, and AIRNA Bio.

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

ARTICLE & COPYRIGHT INFORMATION

Article source: Invited; this article is based on an interview.

Interview held: Jul 29, 2024; **Revised manuscript received:** Aug 14, 2024;

Publication date: Sep 18, 2024.

CELL & GENE THERAPY INSIGHTS

GENE EDITING

SPOTLIGHT

Pioneering the future of non-viral genome engineering

Hao Wu
Full Circles Therapeutics

"With the achievement of large DNA integration in a target-specific manner, many new therapeutic applications will be unlocked."

VIEWPOINT

Cell & Gene Therapy Insights 2024; 10(9), 1157–1162

DOI: 10.18609/cgti.2024.132

INTRODUCTION

On July 30, 2024 Abi Pinchbeck, Editor, *Cell & Gene Therapy Insights*, spoke to Hao (Howard) Wu, Co-Founder and CSO, Full Circles Therapeutics, in a discussion around the emerging innovations and challenges in genome and epigenome editing. This article is based on that conversation.

Full Circles Therapeutics is currently engaged in a monumental endeavor: realizing the final chapter of genome engineering. Their primary focus is on integrating large genetic payloads in a target-specific manner

within the genome, particularly in clinically relevant cell types, both *in vivo* and *ex vivo*. This approach aims to address the genetic root causes of rare genetic disorders, oncology, and autoimmune diseases such as systemic lupus erythematosus and acute myeloid leukemia.

INTRODUCING A NON-VIRAL GENOME ENGINEERING PLATFORM

At the heart of Full Circles Therapeutics' innovation is their viral-free genome engineering platform, a proprietary technology that utilizes a scalable, programmable mini-circular single-stranded DNA, trademarked as C4DNA™. This unique DNA molecule enables the integration of extra-large genetic payloads into the genome in locus specific manner, a feat previously not easy to achieve. C4DNA presents several advantages, including that it is non-viral, can be scalably manufactured to the milligram to gram level, and has a shorter half-life than conventional double-stranded (ds) DNA, hence lower cytotoxicity to the cells and less concerns for random integration.

C4DNA also enables highly efficient targeted genome integration, with a molecular weight of only half of dsDNA counterparts, leading to easy delivery of high copy numbers. C4DNA's versatility means that it can be used both with CRISPR and with various other genome editing systems such as transposases, integrases, transcription activator-like effector nucleases (TALENs), and zinc finger nucleases (ZFNs). This adaptability makes C4DNA a powerful tool across a wide range of genome engineering applications. Moreover, C4DNA's programmability allows for the incorporation of polycistronic cassettes, enabling genetic circuit engineering.

C4DNA can be used with payloads of up to 25 kb, and at Full Circles so far, developers are achieving 10–15 kb docking with satisfactory knock-in efficiency. The goal is to correct multiple mutations through the insertion of extra-large payloads. This platform will be useful in working to address diseases or genetic disorders with extra-large genetic root cause genes in a mutation agnostic manner, such as cystic fibrosis, Stargardt syndrome, and otoferlin-related hearing loss, as well as in immuno-oncology applications.

NON-VIRAL VERSUS VIRAL PLATFORMS

Cell and gene therapy is currently dominated by viral modalities, for instance, AAV-based therapies. Many CGT developers are working to engineer the capsid to produce more efficient and specific AAV. Lentivirus and other viral particles have already been used in early clinical trials, with some drug approvals seen. Viral particles are widely used in this way as they allow the efficient delivery of the genetic payload into the cell types used in clinical development.

However, viral modalities have many shortcomings, including their unavoidable immunogenicity, payload limitations, and manufacturing challenges. For example, over 50% of humans already show humoral immunity to AAV, meaning drugs developed using this modality offer limited dosing. AAV has a payload limitation of around 4.5–4.7 kb, which limits the number of genetic disorders that can be targeted. During manufacture, empty or truncated AAV capsids pose challenges for dosing and contribute to adverse effects, compromising the efficacy and safety of the final drug product. Lentivirus has been widely reported to be randomly integrated into genomes posing huge potential safety concerns.

To address these issues, Full Circles Therapeutics has opted to pursue a non-viral approach with C4DNA, eliminating those potential safety concerns, immunogenicity, and other manufacturing challenges, whilst allowing for the integration of much larger genetic payloads. Additionally, non-viral platforms like C4DNA promise to be more cost-effective, reducing manufacturing costs and ultimately making gene therapies more

affordable by reducing the cost of goods to 20% of current costs.

Non-viral platforms are not without their challenges, particularly in the realm of delivery. Effective delivery of nucleic acid modalities to specific tissues remains a significant hurdle. Innovative delivery methods and complementary technologies, such as lipid nanoparticles (LNPs) and ultrasound-guided delivery, are being explored to enhance the targeted delivery of genome editing therapeutics.

OVERCOMING DELIVERY CHALLENGES FOR GENE EDITING THERAPEUTICS

For genome engineering, solving efficient nuclear delivery is key to achieving highly efficient targeted genome integration. There are two levels of target specificity: tissue and genome targeting. Genome targeting specificity may be addressed more easily. For example, viral vectors possess the intrinsic nature of being untargeted, in that they can be integrated anywhere in the genome. Powerful genome engineering tools such as CRISPR, TALENs, ZFNs, and targeted integrases or transposons offer a more precise approach to genome targeting.

Achieving targeted tissue specificity remains a significant challenge, including for non-viral delivery systems. The field is witnessing substantial efforts from both academic and industry researchers to develop innovative approaches that can specifically target defined tissues or cell types. For instance, recent pioneering work from institutions like the Massachusetts Institute of Technology and various biotech companies has led to the development of LNPs designed to target non-hepatic tissues, such as the spleen and lungs.

Addressing these delivery challenges requires a multifaceted approach that takes into account both the chemical and formulation aspects of delivery systems. The strategy to overcome these challenges must be disease-dependent, considering the specific needs of different therapeutic indications. For example, certain genetic disorders in ophthalmology, such as Stargardt syndrome and retinitis pigmentosa, may not require highly innovative delivery methods, as they can be treated with targeted microinjections. The same principle applies to auditory conditions like otoferlin-related auditory neuropathy spectrum disorder or stereocilin-related hearing loss, where local delivery via microinjections of LNPs could be sufficient.

However, for most other indications, innovative targeted delivery methods are essential. Fortunately, the field has seen significant progress, particularly with the success of mRNA vaccines for COVID-19 and subsequent research into delivery technologies like LNPs. Emerging technologies such as ultrasound-guided delivery hold promise for achieving tissue-specific delivery of nucleic acids, offering a non-viral approach to advancing genome engineering therapies.

NOVEL TOOLS IN GENOME EDITING

One trend in the field of genome engineering is to identify smaller versions of genome editors such as mini versions of Cas9, integrase, or recombinase, for example. Companies like Metagenomi, Arbor Biotech, and Mammoth Biosciences have been at the forefront of this effort, uncovering novel, compact editing systems.

In this era of machine learning and AI, not only can we use generative AI and identify those smaller versions by mutagenesis, but we can also use the large databases being built to identify naturally occurring enzymes with potential applications in genome editing. Moreover, these tools could even help us discover unique editing systems within the human genome itself, which may have the added benefit of minimizing immune responses—a critical factor in the development of safer and more effective gene therapies.

Currently, the only approved drug in the field of genome engineering is CRISPR Therapeutics' and Vertex Pharmaceuticals' Casgevy, targeting sickle cell disease and beta thalassemia. There is confidence that this field will advance rapidly. Base editing and prime editing represent groundbreaking emerging technologies within genome editing. Base editing is capable of correcting single point mutations, while prime editing extends this capability by allowing the insertion of short DNA sequences, typically fewer than a couple of hundred nucleotides. Innovations such as genome writing and synthetic DNA, which allow for the insertion of large or extra-large payloads beyond the limitations of AAV payloads, are also gaining attention. Despite these advances, there remains a need for more innovative platforms, as the field is evolving swiftly.

Another area of interest is targeted transposase systems. Traditional transposon systems allow for semi-targeted or semi-random integration of large payloads, which could enable the integration of substantial genetic material. However, the non-targeted nature of this integration limits its transformative potential, although companies like Poseida Therapeutics are actively working to refine this technology. While promising, this technology is still in its infancy and has so far shown efficacy primarily in bacterial systems, with further data and testing needed in mammalian systems.

The field of genome engineering is in constant need of new technologies, and the current developments, though still in their infancy, are promising. The wide array of toolboxes emerging from numerous labs and biotech companies indicates a bright future for the field.

EXPLORING EPIGENOME EDITING

Unlike other genome engineering technologies, epigenome editing does not involve dsDNA breaks or alterations to the DNA sequence. Instead, it leverages a conservative mechanism of gene regulation to harmonize gene expression at the transcriptional level. This allows for the potential to unlock gene expression where it has been silenced in disease settings or to suppress disease-causing genes, such as oncogenes or those involved in neurological disorders. There is a broad spectrum of applications provided we can achieve precise, targeted epigenome editing, whether through DNA methylation or histone modifications.

However, there are significant challenges that need to be addressed. The first challenge is off-target effects at the genomic level. Similar to genome engineering, epigenome editing requires precise targeting within the genome. If the editing machinery affects regions outside of the intended target, it could lead to undesirable phenotypes, such as the inappropriate suppression or activation of other genes. This necessitates careful assessment and regulation, as in genome engineering.

The second challenge is tissue targeting—how do we ensure that the epigenome editor machinery is delivered specifically to the tissue of interest? For example, when targeting the liver to treat a disease like Pompe disease, addressing the *GAA* gene, it is crucial that the delivery system accurately targets the liver. If you deliver systemically, alterations to *GAA* in other tissues may lead to unwanted side effects.

The third challenge, which is somewhat unique to epigenome editing, concerns the formulation and the packaging of the editing machinery. Epigenome editing typically involves fusion proteins that are often large and bulky, such as those based on dCas9, TALENs, or ZFNs fused with effector domains like DNA methylation editors or histone modifiers. Delivering these large molecules into cells is a significant hurdle.

Fortunately, progress is being made, with companies like Chroma Medicine, OMEGA Therapeutics, and Tune Therapeutics demonstrating promising preclinical results. One trend in the field is the development of miniatures, such as a compact dCas9 versions

of these affected domains, which retain full functionality or even enhance genome targeting efficiency. AI and machine learning are also poised to play a crucial role in epigenome editing.

Another promising avenue that has been somewhat overlooked involves the use of small molecules in epigenome editing. Pyrrole-imidazole polyamides are small molecules with sequence-specific DNA-binding capabilities that have been studied for over a decade. These small molecules can be utilized in epigenome editing or even genome engineering without leveraging the external nuclease enzyme editor system. They can be covalently linked to small molecule binders that recruit endogenous enzymes like DNA methyltransferases or histone modifiers, creating a new class of programmable, small-molecule-based epigenome editors. This approach could offer an alternative to enzyme-based systems to potentially overcome some of the existing challenges. Whether using small molecules or traditional enzyme editors, off-target effects remain a critical concern that must be thoroughly evaluated to ensure safety.

THE FUTURE OF GENOME EDITING

One of the most important challenges in the life sciences field is genome engineering for large payload integration. The genome engineering field has already accomplished gene ablation by creating simple indel-like CRISPR technology, single-point mutation corrections by base editor, as well as tens of nucleotide insertions by prime editing.

The next chapter of genome engineering could revolve around large payload integration in a target-specific, safe, and efficient manner. Over the next 5–10 years, emerging technologies will be coming to play here. With the achievement of large DNA integration in a target-specific manner, many new therapeutic applications will be unlocked. This includes cystic fibrosis, a disease caused by the CFTR gene, which is >5 kb. Over the patient population, the disease has over 900 different mutation haplotypes. If a therapeutic strategy to insert a large payload the full length of the CFTR gene was developed, a universal approach to address all patients with cystic fibrosis could be established. This could also drive down costs and increase speed in bringing those medicines to patients.

Another trend in clinical applications, particularly on the regulatory side, is the need to tolerate n=1 clinical trials. Genome engineering is especially powerful for those genetic disorders for which there are no available treatments, usually in ultra-rare diseases. Within 5–10 years, there may be potentially 5–6 new drugs approved with this technology.

BIOGRAPHY

HAO (HOWARD) WU has almost 20 years experience in gene editing technology and new drug discovery. He specializes in overseeing R&D programs—new labs and research team set up within biotech start-ups.

Wu is the co-founder and CSO of Full Circles Therapeutics, Cambridge, MA, USA where he is dedicated to developing curative gene editing based gene and cell therapy. He is responsible for generating revenue through collaboration with MNC and biotech partners. Before founding Full Circles Therapeutics, Wu was leading multiple discovery biology programs and disease prioritization in the genetic disease space at Fulcrum Therapeutics, Inc. (NASDAQ:FULC), a Cambridge small molecule drug discovery biotech company. He had been with the company through the full development phases starting from the start-up, expansion, until post-IPO development, during which he led a cross-functional team for portfolio disease selection and prioritization of multiple disease programs including neuromuscular disease, cardiac disease, hematological, and metabolic diseases.

Before joining Fulcrum, Wu was a senior research fellow at Whitehead institute, MIT. His research focused on neurological disorders utilizing a combination of CRISPR/Cas9 mediated genomic and epigenomic editing technology and stem cell technology. He did his PhD in Biochemistry and Structural Biology at Hongkong University of Science and Technology (HKUST), New Territories, Hong Kong and Bachelor's degree in Chemistry from Fudan University, Shanghai, China before he did his postdoctoral research at Johns Hopkins University School of Medicine, Baltimore, MD, USA and Howard Hughes Medical Institute, Chevy Chase, MD, USA. Wu has more than 30 journal publications, patents, and research and industry grants. For his work, he has received fellowship award from human frontier science program (HSFP) and NARSAD young investigator award. He was also awarded the Alfred Blalock Young Investigator Award from JHMI and President's award from Whitehead Institute, MIT.

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Contributions: The named author takes responsibility for the integrity of the work as a whole, and has given their approval for this version to be published.

Acknowledgements: None.

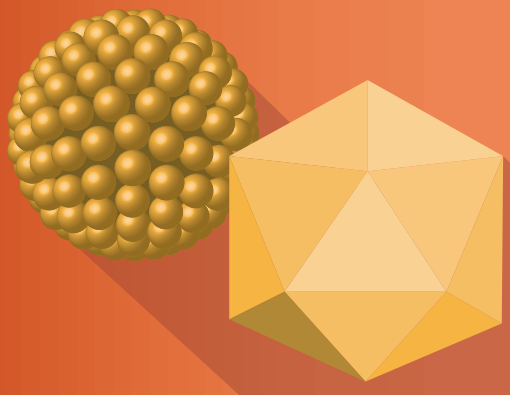
Disclosure and potential conflicts of interest: Wu H is a paid employee and equity shareholder of Full Circles Therapeutics.

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

ARTICLE & COPYRIGHT INFORMATION

Article source: Invited.

Revised manuscript received: Aug 16, 2024; **Publication date:** Sep 18, 2024.



CELL & GENE THERAPY INSIGHTS

VECTOR CHANNEL EDITION
Downstream processing

OCTOBER 2024
Volume 10, Issue 9

CELL & GENE THERAPY INSIGHTS

DOWNSTREAM PROCESSING

INNOVATOR INSIGHT

AAV process intensification using high-salt lysis and salt-tolerant endonuclease

Agnieszka Lass-Napiorkowska, Christina Toenjes, Michelle P Zoeller, Alexa Prager, Angeles Mecate-Zambrano, Sarah Lechat, and Dmitry Zabezhinsky

The increasing demand for recombinant adeno-associated viruses (rAAVs) in high-dose clinical trials and therapeutic applications requires increased efficiency and scalability. High salt concentrations (>150 mM) in production enhance rAAV yields and potency but can negatively impact DNA digestion [1,2]. This study evaluated the effect of four detergents (Polysorbate 20, Triton™ X-100, Deviron® C16, Deviron® 13-S9) at 150 mM and 500 mM NaCl concentrations on rAAV5 and rAAV2 vector yields and protein expression potency. Higher rAAV5 titers were observed with all detergents at 500 mM NaCl compared to 150 mM NaCl. Similarly, rAAV2 titer was 10-fold higher at 500 mM NaCl, than 150 mM. However, under these high salt conditions, standard endonuclease DNA digestion was ineffective. A protein-engineered, salt-tolerant endonuclease was developed to enable efficient DNA digestion in salt concentrations up to 1,000 mM NaCl. When combined with high-salt lysis, this endonuclease improved rAAV yields and titers while meeting requirements for DNA clearance. The use of high salt concentrations with Benzonase® Salt Tolerant endonuclease enhances the productivity of rAAV processes.

Cell & Gene Therapy Insights 2024; 10(9), 1137–1145

DOI: 10.18609/cgti.2024.128

INTRODUCTION

Recombinant adeno-associated virus (rAAV) is the preferred vector for many gene therapies due to its low pathogenicity and ability to establish long-term gene expression in various tissues [3]. The increased demand for rAAV therapies requires development of more efficient manufacturing methods at large production volumes that yield consistently high

product quality. In addition, safety concerns, including immune responses and potential genotoxic or neurotoxic effects related to high-dose viral therapies, need to be addressed [4–7].

One of the challenges in rAAV production is the removal of the residual DNA released from the host cell [8], especially given that clear FDA guidelines define allowed residual DNA content as under 10 ng per dose and the size of the DNA as below 200 bps. Therefore, the addition of the Benzonase® endonuclease is essential to ensure patient safety.

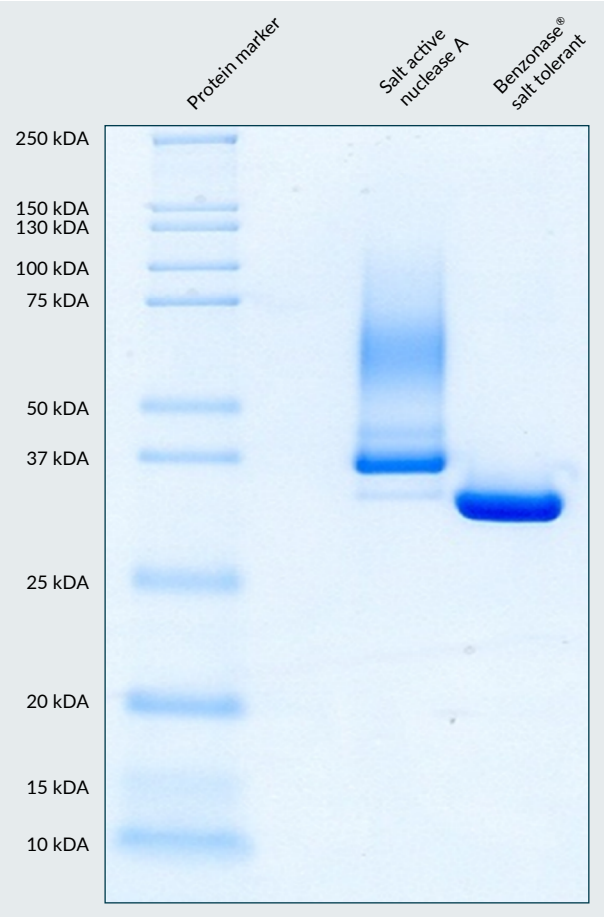
Historically, lysis buffers used for rAAV capsid release have contained a physiological salt concentration (150 mM NaCl). Several recent publications have reported that increasing the salt concentration to 500 mM increases the number of vector particles and infectious titers, and reduces AAV aggregation [5,6] as well as removing the sticky chromatic particles to the viral surface, as was shown with Measles virus [9]. However, high salt concentrations above 300 mM NaCl adversely affect endonuclease enzyme activity, posing a challenge to achieving the FDA-required DNA clearance.

We conducted studies with four commonly used detergents for cell lysis, including Triton™ X-100, which is banned in the European Union due to its endocrine and mutagenic effects, Polysorbate 20, Deviron® C16 detergent, and Deviron® 13-S9 detergent. Effects of the different detergents on rAAV yield and infectivity were monitored as well as the effect of salt concentration on cell lysis and vector yield.

In addition, these studies assessed the effectiveness of a newly developed salt-tolerant endonuclease at high salt concentrations, and benchmarked performance against standard and alternative salt-tolerant endonucleases.

FIGURE 1

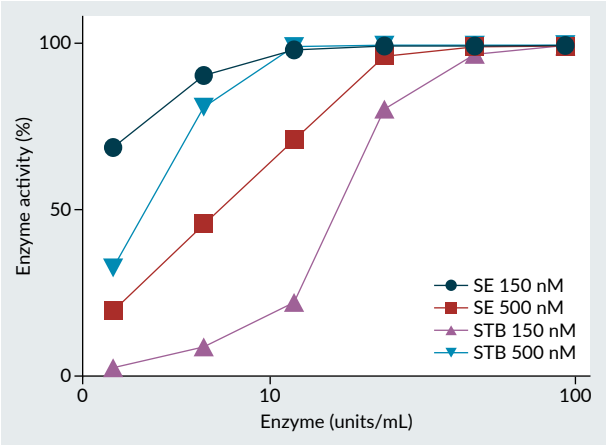
100 ng of a salt active nuclease A and Benzonase® Salt Tolerant endonuclease were loaded on SDS-PAGE and run under reducing conditions.



The gel was stained with Coomassie Brilliant Blue for protein detection.

FIGURE 2

Activity of different concentrations of standard endonuclease (SE) and Benzonase Salt Tolerant endonuclease (STB) enzymes in the presence of herring sperm DNA and 150 mM or 500 mM NaCl.



Relative enzyme activity was measured after 30 min of incubation at 37 °C using fluorescent readout.

MATERIALS AND METHODS

Enzyme activity assay

DNA digestion was performed with the enzymes, herring sperm DNA (hs DNA), 150 mM or 500 mM NaCl in the presence or absence of detergents at 37 °C for 30 mins. Following incubation, DNA concentration was measured using a fluorescent method (Thermo, Cat# Q33231:Qubit™ 1X dsDNA High Sensitivity [HS]).

HEK293 culture and rAAV production

The adherent and suspension HEK293 cell lines were grown according to the standard protocols. The cells were transfected with three plasmids system, cultured, then lysed to release rAAV2 or rAAV5.

ELISA for the rAAV titer determination

rAAVs particles were quantified using a sandwich ELISA kit with antibodies recognizing the viral capsids.

rAAV potency assay

Viral vector potency was evaluated by expression of the protein of interest in the 2D cultured cells. Different number of viral particles were incubated with the *in vitro* cells and potency (transfection units per mL) was assessed via fluorescent microscopy.

Protein size separation

Recombinant enzymes were separated using reducing SDS-PAGE, followed by staining with Coomassie Brilliant Blue.

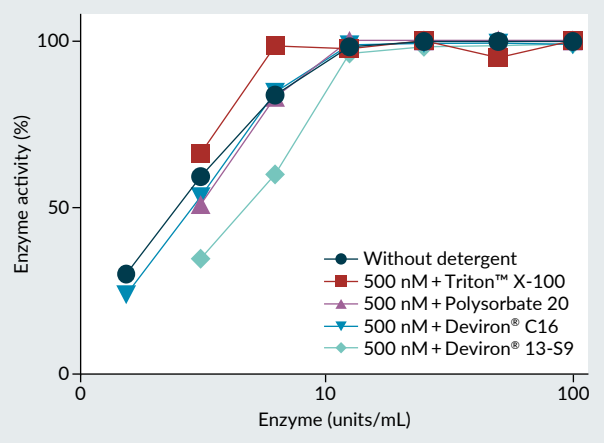
RESULTS

Development of a salt-tolerant endonuclease for rAAV vector production

Benzonase® Salt Tolerant endonuclease was developed to address the need for an endonuclease that is efficient at high salt concentrations.

FIGURE 3

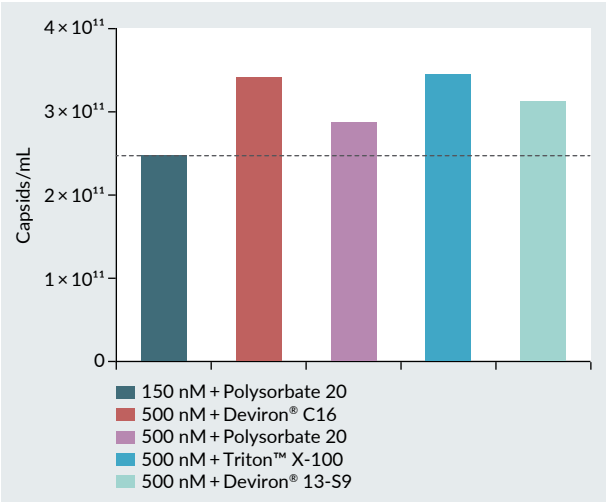
Activity of different concentrations of Benzonase Salt Tolerant endonuclease enzyme in the presence of detergents at 0.5% v/v, hsDNA and 500 mM NaCl.



Relative enzyme activity was measured after 30 min of incubation at 37 °C using fluorescent readout.

FIGURE 4

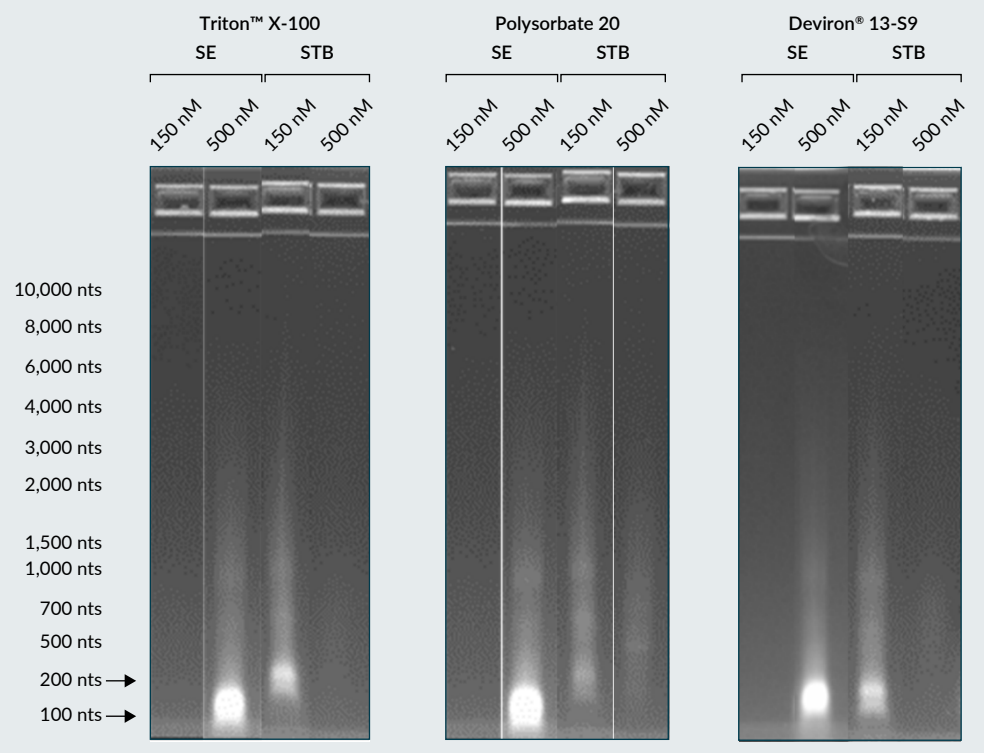
rAAV5 titers following lysis of HEK293 suspension cells with various detergents (0.5% v/v), 150 mM or 500 mM NaCl and 25 U/mL of the standard nuclease (SE) or Benzonase Salt Tolerant endonuclease (STB).



rAAV5 titers were measured using ELISA.

FIGURE 5

Agarose gel DNA profile of suspension HEK293 cells lysed with different detergents (0.5% v/v), 150 mM or 500 mM NaCl and 25 U/mL of the standard nuclease (SE) or benzonase salt tolerant endonuclease (STB).



Arrows indicate the undigested DNA contaminants.

This recombinant protein was developed in an *E. coli* expression system and is devoid of the post-translational modifications (e.g., glycosylation) typically observed when the protein is expressed in alternative hosts. By contrast, salt active nuclease A, an alternative commercially available product that was used for comparison, contains post translational modifications (Figure 1) [10], thus increasing the molecular weight (>37 kDa) and leading to formation of numerous protein with different glycosylation patterns and non-uniform weight species. This can result in variability in quantitation by ELISA, as recognition by the antibody is highly dependent on consistency of post translational modifications [11].

Benchmarking endonuclease activity with surrogate DNA

To assess the efficiency of nucleic acid digestion, different concentrations of Benzonase® Salt Tolerant endonuclease and standard endonuclease were incubated with Herrin Sperm DNA (hsDNA) for 30 mins at 37 °C in the presence of either 150 mM or 500 mM NaCl. Enzymatic activity was measured using fluorescent dye.

At low salt concentrations, standard endonuclease activity was higher than that of Benzonase Salt Tolerant endonuclease but at 500 mM salt concentrations, standard endonuclease activity was inhibited, while Benzonase Salt Tolerant endonuclease activity

remained high, even at relatively low concentrations (Figure 2).

Numerous detergents can be used to lyse cells and release rAAV viral particles. Consequently, it is important to ensure that any detergent used does not inhibit enzymatic activity of Benzonase Salt Tolerant endonuclease. Enzymatic activity of the Benzonase Salt Tolerant endonuclease was evaluated in the presence of 0.5% v/v of four detergents (the typical concentration used for cell lysis) and 500 mM NaCl. At enzyme concentration above 10 U/mL, enzyme activity was 100 % in the presence of all tested detergents. None of the selected detergents inhibited activity of the Benzonase Salt Tolerant endonuclease (Figure 3).

Effect of high salt concentration on rAAV yield

The effect of high salt concentration on rAAV5 yield was measured during cell lysis and midstream processing steps. HEK293 cells were grown in suspension and transfected with the vectors required for rAAV5 production. After virus propagation, cells were lysed using Polysorbate 20, Triton X-100, Deviron C16 or Deviron 13-S9 detergents at 0.5% (v/v) and salt concentrations of either 150 mM or 500 mM, and rAAV5 concentration was measured using ELISA (Figure 4).

Irrespective of the detergent used for lysis, under the 500 mM NaCl condition, higher rAAV5 titers were observed as compared to 150 mM NaCl condition, suggesting that the increased yield was most likely due to the higher salt concentration. By contrast, under these same high salt concentrations, host cell DNA was not effectively digested with standard endonuclease (Figure 5).

To maximize yield and impurity removal, the combination of high salt and Benzonase Salt Tolerant endonuclease is recommended to achieve both high AAV5 titers and effective removal of host cell nucleic acid impurities (Figures 4 & 5).

Effect of high salt concentration during midstream steps on rAAV2 infectivity

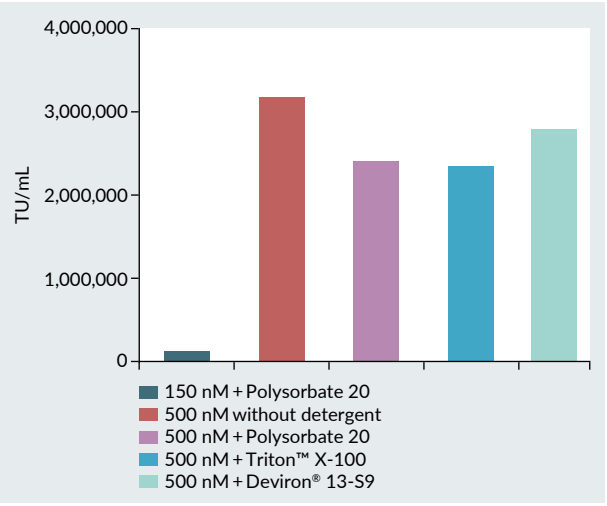
To assess the effect of 500 mM NaCl conditions on rAAV titers, a similar study was performed with adherent HEK293 cells transfected with the vectors required for rAAV2 production. The objective of the study was to evaluate protein expression in the target cells after rAAV2 infection using fluorescence microscopy.

After transfection and virus propagation, cells were lysed using the panel of three detergents at 0.5% (v/v) and salt concentrations of 150 mM or 500 mM; rAAV2 titer was measured using a proprietary potency assay.

rAAV2 potency was 10-fold higher in all high-salt conditions (Figure 6). Consequently, the combination of high salt and Benzonase Salt Tolerant endonuclease is recommended to achieve high rAAV2 yields and digest contaminating DNA as expected according to Figure 5.

FIGURE 6

rrAAV2 potency following lysis of HEK293 adherent cells with or without various detergents (0.5% v/v), at 500 mM NaCl as compared to lysis at 150 mM with Polysorbate 20 and 25 U/mL of the standard nuclease or benzonase salt tolerant endonuclease.



rAAV2 potency was measured using a proprietary potency assay.

CONCLUSIONS

The use of high salt concentrations during cell lysis in rAAV production has been shown to result in higher titers and improved potency. Although beneficial, adopting higher salt concentrations has long been deemed impractical due to the inhibitory effect of these conditions on midstream purification steps that include endonuclease-mediated nucleic acid digestion. Despite the potential for more efficient processes, high salt conditions were not generally adopted due to the risks associated with insufficient impurity removal that could result in noncompliance with regulatory requirements, or potential safety concerns for patients.

The newly developed Benzonase Salt Tolerant endonuclease has higher activity than standard endonucleases in the 500 mM salt conditions that favor increased rAAV titers during cell lysis. Results from these studies confirm compatibility of this salt-tolerant endonuclease with multiple detergents, including Deviron detergents, demonstrating the broad suitability of Benzonase Salt Tolerant endonuclease with bioprocessing cell lysis steps. The availability of this new endonuclease enables more efficient bioprocesses, facilitated by improved midstream lysis and DNA digestion steps, to meet the increasing yield demands for rAAV-based therapeutics.

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

Acknowledgements: None.

Disclosure and potential conflicts of interest: The authors have no conflicts of interest.

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

ARTICLE & COPYRIGHT INFORMATION

Article source: Externally peer reviewed.

Submitted for peer review: Jul 17, 2024; **Revised manuscript received:** Aug 27, 2024;
Publication date: Sep 16, 2024.

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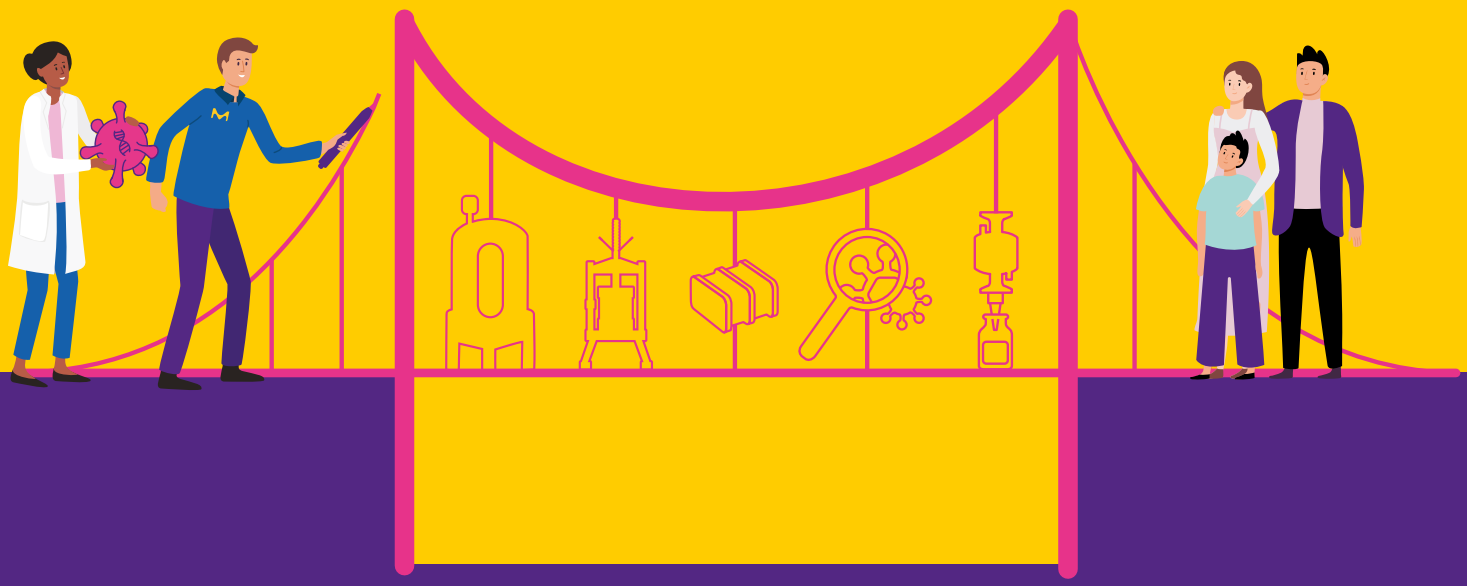

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AAV downstream challenges: expert insights

Nathalie Clément, William Kish, Nicolas Laroudie, and Matthew Roach




Cell & Gene Therapy Insights 2024; 10(9), 1353–1366

DOI: 10.18609/cgti.2024.154

Q What are some of the recent advancements or innovations in AAV manufacturing that you have found particularly promising?

MR: Firstly, on the upstream side, there has been a push to understand what is happening inside the cell during vector production. Multiple publications have focused on

investigating both the proteome and transcriptome while AAV is being produced. Additionally, researchers are comparing high- and low-producing conditions to identify which genes and proteins are abundantly expressed in high-producing scenarios. The findings from these studies are exciting, as they could help us identify producer genes to be modified chemically or through gene editing, potentially leading to much higher yields or potency.

I have also noticed an increase in offerings from vendors in the AAV space. Compared to 5 years ago, there has been a huge influx of new reagents and products focused on increasing yield, particularly upstream products like transfection enhancers. A great aspect of this is that companies are now providing data upfront, allowing users to compare it with their own results. This is relatively new. These are two developments that I am really excited about.

WK: One of the most significant challenges in downstream processing (DSP) is the separation (removal) of empty capsids, and there has been a lot of great progress in this area. Removing empty capsids is crucial for increasing potency. We are now reaching the point where we can separate not only empty from full capsids, but also begin separating partially packaged capsids as well.

I want to highlight some work presented at the American Society of Gene and Cell Therapy (ASGCT) annual meeting this year by a leading gene therapy biotech company. They developed an anion exchange chromatography method using a combination of resin chemistry optimization, column overloading, and fine-tuning of elution salts and cosolvents, which allowed them to remove over 95% of empty capsids and 40% of partially packaged capsids, all while achieving a respectable yield of 80%. This is a significant step forward, particularly if this approach can be applied to other serotypes.

Looking beyond manufacturing, I am excited about combining modalities, such as conjugating antibodies to AAV. Another industry leader presented on this at ASGCT, demonstrating how they were able to fine-tune the tropism of an AAV capsid to cross the blood–brain barrier while de-targeting the liver. This has great potential for improving safety and efficacy but also brings new challenges as we take an already complex modality like AAV, and make it even more complex by coupling an antibody to it. I am eager to work on these kinds of challenges in the future.

NL: In recent years, we have generally seen an increase in production efficiency. Optimized plasmid design has enhanced production systems, leading to higher yields and more consistent AAV output, which is crucial. From an upstream processing (USP) standpoint, we have also seen the release of optimized media for cell culture. While this isn't entirely new, there has been a systematic shift towards suspension culture and away from adherent culture, and a greater focus on suspension cells over systems like insect cells.

From a purification perspective, the introduction of immunoaffinity for capture has been a major advancement in large-scale vector production from an industrial standpoint. In my opinion, this was a key development and a barrier-breaker. New technologies for separating full and empty capsids have already been mentioned, which remains one of the major challenges we face. Additionally, we have seen the introduction of new analytical methods and equipment

"With over 40 years of accumulated data...there is now an opportunity to use machine learning platforms to analyze this vast body of information and determine the best approaches."

in recent years, such as mass photometry, which provides a quick and accurate understanding of what is happening during production. This has definitely supported further development.

NC: I am most excited by the emphasis on optimizing upstream conditions. There are many new reagents, cell lines, helper plasmid designs, and Rep/Cap plasmid designs that are being developed and evaluated. The key here is not just increasing the vector genome yield, which for the past 20 years has been the primary goal, as we were focusing on 'the more the better', but other critical quality attributes. We are now prioritizing better quality as well.

As my colleagues have mentioned, there are significant efforts to increase the yield of full capsids while reducing the number of empty capsids and decreasing the amount of plasmid co-packaged in the empties, which ties back to the plasmid design. This also involves reducing other impurities or residuals in the harvest process. The focus on optimizing not just quantity but also quality right from the start is something that truly excites me.

In addition, in an effort to optimize constructs and manufacturing conditions, AI is now here to help us design the next generation of AAV vectors from multiple angles, whether it is the vector genome, the helper plasmid, or the cell line. With over 40 years of accumulated data, including many production successes and failures, there is now an opportunity to use machine learning platforms to analyze this vast body of information and determine the best approaches. Although I have not yet witnessed the direct impact of these new 'AI-engineered' constructs on AAV manufacturing, I am eager to see what will come next.

Q What for you are the key current challenges in the downstream purification of AAV vectors, and what strategies or techniques have you found most effective in overcoming them?

NL: I will divide my answer into three parts: the capture step, polishing, and analytics. Each plays a key role in DSP.

Firstly, on capture: currently, immunoaffinity is the standard for 99% of AAV producers. One major challenge with using this resin is processing time - this step can take hours or even days. The issue lies in the discrepancy between the extremely high capacity of immunoaffinity resins and the relatively low titers of the feedstock. Despite recent increases in titers, this means loading times remain very long, which poses risks for manufacturing. The extended timeframe increases the risk of equipment failure, leakage, or other problems, requiring personnel to constantly monitor the process. There is also a risk to the product itself—stability can decrease

"For polishing, the main challenge remains the separation of full and empty capsids."

over time, and prolonged processing increases the chance of contamination. Addressing this challenge requires changes such as optimizing the geometry of the column, implementing single-pass tangential flow filtration (TFF) ahead of the column, using multi-column chromatography or rapid cycling, and exploring other technologies. We are also hoping for systems like membranes that could enable rapid capture, although such options are not available on the market yet.

For polishing, the main challenge remains the separation of full and empty capsids. As my co-panelist mentioned, there are numerous initiatives, trials, and application notes addressing this, but we still lack a universal method to separate full from empty capsids. Each new serotype, construction, and even lot or batch, requires redevelopment of separation steps, which is a significant bottleneck. This is an area where progress is urgently needed in the coming years.

Lastly, on analytics: one of the long-standing challenges in this field is the lack of accuracy and high variability in analytical methods. These methods are time-consuming, costly, and resource-intensive, with variability often reaching 20–25%. This makes development challenging, as multiple assays and analytics are needed to determine the correct direction. Addressing this bottleneck requires the development of new tools to improve speed and accuracy from an analytical standpoint, which I believe is crucial for advancing the field.

MR: Nicolas did a great job covering the empty-full—a longstanding issue in the field. We are definitely not at the point where separation can be fully standardized, but hopefully, we will get there. Right now, people have found suitable strategies for individual capsids, but having a more broadly applicable solution, which would also shorten the development timeline, would be ideal.

Beyond that, our focus has been on studying post-translational modifications (PTMs) to better understand the product at each stage of the process. We have been conducting process hold and degradation studies to identify the conditions that drive PTMs, such as deamidation. Additionally, we are correlating these modifications with *in vitro* and *in vivo* potency to determine what could potentially lead to a drop in efficacy. Together, these efforts have allowed us to identify critical parameters for maintaining potency throughout the process, even for steps that might have been overlooked in the past.

WK: As we move towards a more mature modality, cost will increasingly become a key challenge. One major focus is identifying the biggest cost drivers in DSPs and finding ways to reduce them. Benzonase™ or other nucleases used to clear host cell DNA, while highly effective, are also quite expensive. Alternatives like flocculation, using either low pH or quaternary ammoniums, have shown promise in reducing the cost of DNA clearance before harvest.

Another significant cost driver, as Nicolas mentioned, is affinity chromatography, which is a major advancement but comes with high costs. Affinity resins are among the most expensive components in DSP. To address this, improving the number of times an affinity resin can be cycled, through effective clean-in-place procedures, is crucial.

NC: One of the key bottlenecks we all agree on is the separation of full and empty capsids, but I would like to frame this in a broader context. Each AAV product is unique and finding universal approaches for upstream or downstream will remain challenging unless we standardize the vector design itself. The complexity arises not only from the capsid but also from what is inside the capsid, which makes downstream particularly difficult. Even if you do not necessarily need to reinvent the wheel for each construct, it does often require making adjustments to the platformed processes, which can be time-consuming and costly for every drug product.

In terms of separating full and empty capsids, it has become clear that it is not simply about separating fulls from empties, or even from partially packaged capsids. These are not discrete species; rather, there is a continuum between full and empty capsids, with all sorts of intermediates. Current techniques are not designed to specifically target and bind to fully packaged capsids, and whoever solves that, will make a major breakthrough in the field.

Cost is also an important factor, but a significant challenge is the overall recovery, especially in downstream steps. While tremendous progress has been made upstream, with yields multiplied by a factor of 10 or even 100, downstream remains a bottleneck. At best, a 30% final recovery in the vialed drug product is considered good, which makes me think, 'What can we do better?' One approach could be to simplify DSP processes. We know that every additional step taken to make the product cleaner and purer, results in the loss of full capsids. Therefore, I believe we should focus on reducing the number of steps or developing more powerful but fewer steps to improve efficiency and recovery.

Q What are the most important upstream process (USP) techniques, tools, or strategies to focus on with the goal of helping DSP?

NC: Optimizing upstream with downstream in mind is so important. It is often overlooked that we need to develop a platform where both USP and DSP work together to optimize not only the yields but also the product quality. I am a strong believer that quality starts upstream. With that in mind, it is crucial to develop upstream conditions that optimize several critical quality attributes, such as improving vector genome titers, reducing empties, and minimizing contaminating residual plasmids, host cell DNA and host cell proteins.

One impurity that has been overlooked for quite some time, but is now getting more attention, is residual host cell DNA and plasmid DNA; I have heard many times how excessive DNA in the harvest can significantly impact DSP. DNA is viscous, which can affect the filtration and/or binding steps. DNA can even bind to capsids and cause aggregation in unpredictable

ways. Therefore, improving upstream by minimizing DNA from the harvest is crucial, and there are several platforms and solutions to explore in this area.

Regarding cell lysis, one key aspect for downstream is receiving a homogenous and consistent product from the beginning. If upstream can consistently provide the same type and quality of material, downstream will not have to reinvent the wheel for every batch. They will be able to predict outcomes more accurately based on titers and capsid counts, for example.

Consistency in production—whether by transfection or infection, depending on the platform—and homogeneity of the clarified material is critical. Whether it is through Benzonase digestion or other methods to ensure complete cell lysis and AAV release, these considerations will also support downstream steps like TFF or filtration. In summary, providing downstream with material that is both homogenous and of consistent quality will greatly simplify the process.

NL: We have seen cases where, even with the same serotype, affinity can vary depending on the specific construct being used, and this affects steps like chromatography. The polishing step in particular is highly influenced by these variations.

Regarding nuclease use, this is a crucial point. I see many customers adding large amounts of endonucleases or nucleases to their feedstock without checking if the enzyme is actually effective, or if they still have nucleic acids at the end. Often, they spend a lot of money on nuclease but still end up with high residual DNA levels, because they do not consider that most of these enzymes are inhibited by salt or other conditions common in cell culture. This renders the enzyme inefficient. Fortunately, we now have enzymes on the market that are salt-tolerant, and my recommendation would be to switch to one of these, but always pay attention to your nuclease treatment.

I also want to emphasize clarification. It is key in a DSP process, and it is an area where we need to put the most effort. It may seem obvious, but the cleaner and clearer the feedstock you load onto capture chromatography, the better the resin's performance in terms of yield, consistency, and step reproducibility. Clarification also impacts the reusability of the resin. If the feedstock is cleaner, the resin is easier to clean and can be reused more times. Investing time in optimizing USP will ultimately make DSP easier and save considerable time, money, and resources.

MR: I completely agree with both sets of comments. In past years, the primary focus has been on yield, but there is now a definite shift towards prioritizing percent full, capsid even early on in USP. We have found that while our purification processes are capable of substantial enrichment, the lower the starting percent full, the more challenging it is to achieve a higher percent full at the end of purification. For example, if you are aiming for a two- to three-fold enrichment, starting at 5% full is going to be much more difficult for the DSP—ideally, we want at least 20% full from the outset.

When conducting Design of Experiments (DoE) studies or screening experiments in upstream, we still prioritize yield, but we also set a threshold for percent full. On the bright side, as USP continues to improve, we are seeing more conditions with higher percent full

"On the bright side, as upstream processing continues to improve, we are seeing more conditions with higher percent full becoming more common."

becoming more common. Another strategic point is ensuring that impurities are properly considered. It may sound obvious, but sometimes in the excitement of increasing yield by two- or three-fold, people overlook impurities, leading to downstream issues—particularly with residual host cell DNA.

During process intensification, if you are increasing viable cell density significantly, you could be in a situation where yield has gone up two- or three-fold, but cell density has increased five-fold, making downstream purification (especially at the anion exchange step) much harder due to the extra residual host cell DNA. Therefore, our focus has been on cell-specific productivity rather than just viral genome titer or capsid titer alone.

WK: As a downstream scientist, I completely understand the excitement when yield goes up two- or three-fold! But my first question is always: what has happened to the total capsid level and percent full capsids? These factors can significantly impact the DSP. One key element affecting the percent full ratio in upstream is the transfection process—specifically, the cell health at transfection and the scalability of the mixing and delivery of the transfection cocktail.

Upstream teams might do a great job developing a process at a 2 L or 10 L scale, but when scaling up to 500 L or 2,000 L, delivering a consistent transfection cocktail with the same type of mixing becomes very challenging. This is something I always watch for during scale-up, as changes to the feed stream and percent full capsids can impact what we see downstream. It is not an easy task and often requires significant effort because you cannot simply do process development at scale. Utilizing modeling or thoughtful experiments can be a way to address these challenges without heavy investments of time and plasmid. I realize I have pointed out a problem for upstream to solve here, without providing a solution—a typical downstream perspective!

Q What approaches or techniques do you use to determine the optimal purification tools for a given AAV process?

WK: Focusing on chromatography, I prefer conducting early-stage development in a high-throughput or semi-high-throughput manner. This approach does not necessarily require an expensive robotic liquid handler—it can be done with well-plates and resin, using a multichannel pipette for sample handling and collection. This setup allows for much more data generation compared to using individual columns connected to a traditional chromatography system.

Once you generate all this data, you may face a bottleneck at the analytics stage. To address this, high-throughput, directional analytical tools can be used. While they may not provide precise values like droplet digital PCR (ddPCR), they do give useful directional insights. Several analytical instruments are very efficient for this purpose, providing data on dynamic light scattering, capsid titer, empty/full ratio, and aggregation. You can also use absorbance-based readings from well plates, which are common in analytical labs. This approach gives quick readouts for A_{260} and A_{280}, providing a preliminary empty/full ratio. After screening conditions with these tools, you can proceed to more detailed analytics like ddPCR and, eventually, analytical ultracentrifugation for deeper characterization.

This high-throughput approach can be adapted to study affinity chromatography by evaluating binding, washes, and elution in a loose resin format. For anion exchange (AEX), high-throughput studies can be conducted on dilution/load preparation, and binding steps. However, when you get to developing gradient elution over AEX, it is best to transition from loose resin to small columns that can provide smooth gradient formation. Thermo Scientific™ MiniChrom™ columns of 200–500 µL, pre-packed and connected to a downstream chromatography system with low flow capabilities, work well here. This setup allows for efficient screening of elution salts or cosolvents, which is particularly impactful for empty/full separation over AEX. This is my go-to approach to chromatography in a semi-high-throughput fashion.

NC: I want to emphasize how crucial it is to have strong analytics at these steps. I completely agree with what Nicolas mentioned earlier—you will be making decisions by comparing many different conditions, whether it is binding buffers, wash, or elution buffers, and submitting these samples to various assays, including ddPCR. What often gets overlooked is the impact of the matrix on these analytics.

Fortunately, with ddPCR, the impact of the matrix is reduced, but it is still not negligible. You will be comparing samples that vary significantly in pH, salt concentration, and titer, from very diluted to highly concentrated, depending on the steps being evaluated. It is essential to trust your analytics and ensure they are capable of making accurate comparisons.

To achieve this, it is important to have a set of assays specifically designed for evaluating the various conditions during screening. Additionally, multiple critical quality attributes should be assessed when selecting optimal conditions. Vector genome titer and total capsid titers are key attributes, but do not overlook potency or infectivity—these can be evaluated in a relatively simple, high-throughput manner. As mentioned previously, residual DNA is another critical factor. It is crucial to screen for multiple quality attributes rather than focusing solely on one, such as vector genome, as was often the case in the past.

NL: Working for a supplier of chromatography resins, my choice is quite straightforward. I, of course, recommend using Thermo Scientific™ POROS™ and Thermo Scientific™ CaptureSelect™ resins for both capture and polishing. As William mentioned earlier, high-throughput is important, and it is worth noting that these resins come in various formats, ranging from Thermo Scientific™ RoboColumns™ to 96-well screening plates, pre-packed

columns, and even magnetic beads for quick evaluation of AAV purification or determining if a method will work effectively. It can be beneficial to utilize these different formats.

Additionally, I encourage people to contact their local suppliers and application specialists for support in developing their purification steps. This can save a significant amount of time and effort. I may be advertising myself here, but I strongly recommend reaching out to your local application support for assistance.

Q How do you navigate the complexities of AAV purification to ensure both high purity and yield in your downstream process?

MR: This can be a challenge, particularly for the empty/full separation. I think of it as a balancing act, trying to achieve a high degree of purity while maintaining yield. Fortunately, affinity chromatography has been extremely helpful in reaching high purity levels early in the DSP. Our main strategy is to identify options and levers within the process, particularly in the post-affinity chromatography steps, that we can adjust to change purity or yield if needed. This approach allows us to maintain good process understanding, which is hopefully applicable across multiple products.

One example of this is increasing the salt concentration of a wash during anion exchange to remove more empty capsids if we are starting with a lower percent full capsids for that particular construct. It might also involve changing the salt concentration in the load or the buffer you are diluting with, or using TFF to exchange into a different solution. My advice here is to ensure that you are not only focused on empty/full capsids but also giving appropriate attention to residuals. Especially when developing a pooling strategy for AEX, it is important to observe how residual levels change across pools. Ideally, conduct a study where you are fractionating individually to analyze residual host cell DNA or plasmid DNA at different points of your peak (start, middle, and end), which provides valuable insights into these levels.

NC: This is a challenging question, and it is something we have all encountered at some point when developing drug products for clinical use. We have discussed recovery: if achieving the highest purity means a 99% loss, making it impossible to reach the clinic because there is not enough product, that becomes a significant challenge. While purity is paramount and we should strive for it, safety must also guide the development of a platform that meets your needs for clinical trials. Always remember that you are also evaluating product safety during your IND-enabling toxicology studies, which play a crucial role in assessing how well your processes are performing in terms of ensuring production of a safe product.

WK: When preparing to file, the focus should be on SISPQ: strength, identity, safety, purity, and quality. Yield is not part of an IND filing requirement, meaning purity, potency, and safety are prioritized over yield. Ensuring that safety and potency come first is crucial. One factor that can impact potency is the rate of deamination—an impactful post-translational

"To effectively approach scale-up, it is essential to begin addressing scalability early in development."

modification on the capsid surface that can reduce potency. Finding ways to improve stability during in-process holds to maintain potency is an important aspect of development.

To echo what Matt said, most yield loss occurs during AEX. Understanding the balance between yield and purity by analyzing different fractions across AEX—not just focusing on empty and full capsids but also considering other post-translational modifications, deamination, and residual host cell proteins or DNA—provides a clearer understanding of what needs to be compromised to achieve a particular yield. Having these kinds of insights is critical for making good decisions.

Q How do you ensure the scalability and reproducibility of your AAV purification process in large-scale manufacturing?

WK: This is particularly important as gene therapy moves toward larger scales and doses, especially for applications beyond rare disease indications. For instance, Nathalie works at Siren, which focuses on treating cancer, indicating the need for substantial vector quantities. To effectively approach scale-up, it is essential to begin addressing scalability early in development.

If you receive a process from an academic partner that involves non-scalable unit operations, such as freeze-thaw cycles or ultracentrifugation for separating empty from full capsids, it is crucial to *immediately* start developing scalable alternatives, like detergent lysis or AEX for that separation. It is important to manage expectations regarding initial performance, as investing in scalable unit operations early will pay off later during scale-up.

In terms of chromatographic scale-up for AAV, a key strategy is to size your chromatography columns by fixing the load challenge across scales. This involves knowing the dynamic binding capacity of your affinity column and ideally, matching the column bed height when moving from small- to large-scale, by increasing the column diameter. This bed height matching is particularly vital for AEX, as it requires high-resolution separation, while affinity chromatography can tolerate some variation without significant impact.

It is also essential to maintain consistent residence times and match gradient slopes and lengths between small and large scales. A crucial *watch out* during AEX scale-up is ensuring proper gradient formation in larger-scale chromatography systems. Working at the lower range of pump capabilities can lead to instability and result in nonlinear gradients. This can adversely affect separation quality. Additionally, variations in the upstream empty-full ratio can occur during scale-up; therefore, early-stage process development should include testing the robustness of the AEX process against different percent full capsid levels.

Lastly, while somewhat tangential, having scientists present in the GMP suite during tech transfers is crucial. Observing chromatography and filtration steps firsthand provides insights that are far more valuable than hearing about them later. These tips are key for ensuring reproducibility and setting the stage for successful large-scale operations.

NC: My approach begins with selecting scalable methods right from the start of the process development phase and Phase 1 clinical studies. It is essential not to adopt a mindset of 'we'll figure it out later', as this can lead to significant complications in Phase 2 and at the BLA submission stage. Understanding the concept of scaling out is critical: to produce sufficient product, you will need to multiply the number of bioreactors or centrifuges.

If you have the opportunity, choose methods that are inherently scalable, enabling a smooth transition to subsequent clinical trials and BLA submissions. For example, although cesium chloride gradient purification method may have historical significance, they are not scalable, so it is best to avoid them when possible. Focusing on USPs in suspension culture instead is also a more effective strategy for achieving scale-up or scale-out.

MR: The key to our success has been thoroughly understanding each part of the process, including the smallest details, as we transition to large-scale manufacturing. In the past, process development teams often assumed factors like process hold times would not significantly impact outcomes, especially in early-stage processes, and underestimated their importance. While AAV can appear to be robust, we now recognize the necessity of delving deeper into these aspects as our product candidates mature.

Moreover, when working with a CDMO, it is essential to cultivate a collaborative and positive environment. This fosters effective process transfers and ensures scalability and reproducibility. Establishing clear lines of communication and making collaboration as seamless as possible is critical to achieving our goals.

NL: As Nathalie emphasized, it is crucial to think about large-scale processes right from the beginning of development. Connecting with experts who understand the constraints of running chromatography and DSP techniques at scale is vital. This collaboration allows you to develop a process that is both scalable and robust enough for successful transfer.

When considering chromatography, selecting the right format and media is essential. Not all materials have the same properties, and managing back pressure becomes a key concern when scaling up. Non-compressible materials, for example, make scaling easier since their back pressure is influenced solely by flow and bed height, not diameter.

Additionally, as William pointed out, accuracy in gradients should be a priority. Whenever possible, I recommend minimizing the use of gradients, as they can complicate the robustness of your process. If a gradient is necessary, ensure that initial separations do not occur at the beginning of the gradient, as pump inaccuracies can significantly alter results. By focusing on these elements and collaborating with large-scale manufacturing experts, you can develop processes that are well-suited to successful scale-up.

BIOGRAPHIES

NATHALIE CLEMENT has over 25 years of experience in the field of gene therapy with industry-leading expertise in AAV vector manufacturing. Since January 2022, Nathalie has held the role of Vice President of Vector Development for Translational Gene Therapies at Siren Biotechnology, San Francisco, CA, USA a startup focused on delivering a cure to cancer using AAV-mediated gene transfer.

WILLIAM KISH is a Purification Scientist with over a decade of experience across industry and academia. As a Principal Scientist at Taysha Gene Therapies, Durham, NC, USA he leads their downstream CMC activities, including process development, characterization, tech transfer, CDMO oversight, and the authoring of regulatory documents. He holds PhD and Master's degrees in Chemical Engineering from North Carolina State University, Raleigh, NC, USA.

NICOLAS LAROUDIE is a Senior Field Application Scientist at Thermo Fisher Scientific, Strasbourg, France supporting the technical implementation of POROS™ and CaptureSelect™ chromatography products in southwestern Europe. A biochemist by education, he has over 20 years of experience in downstream processing purification, from lab-scale R&D to commercial manufacturing.

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AUTHORSHIP & CONFLICT OF INTEREST

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

Acknowledgements: None.

Disclosure and potential conflicts of interest: William Kish holds stock options in Taysha Gene Therapies, Inc., a gene therapy company focused on developing and commercializing adeno-associated virus-based gene therapies for the treatment of monogenic diseases of the central nervous system.

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

ARTICLE & COPYRIGHT INFORMATION

Article source: This article was based on a webinar, which can be found here.

Webinar conducted: Sep 17, 2024; **Revised manuscript received:** Oct 22, 2024;

Publication date: Nov 4, 2024.

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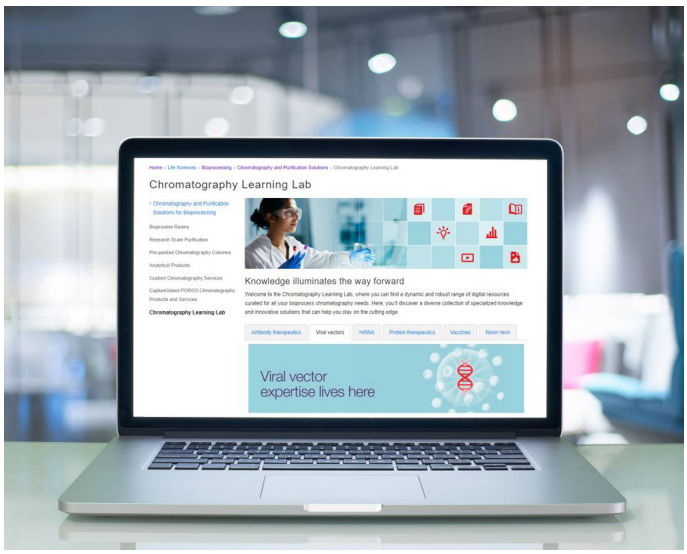

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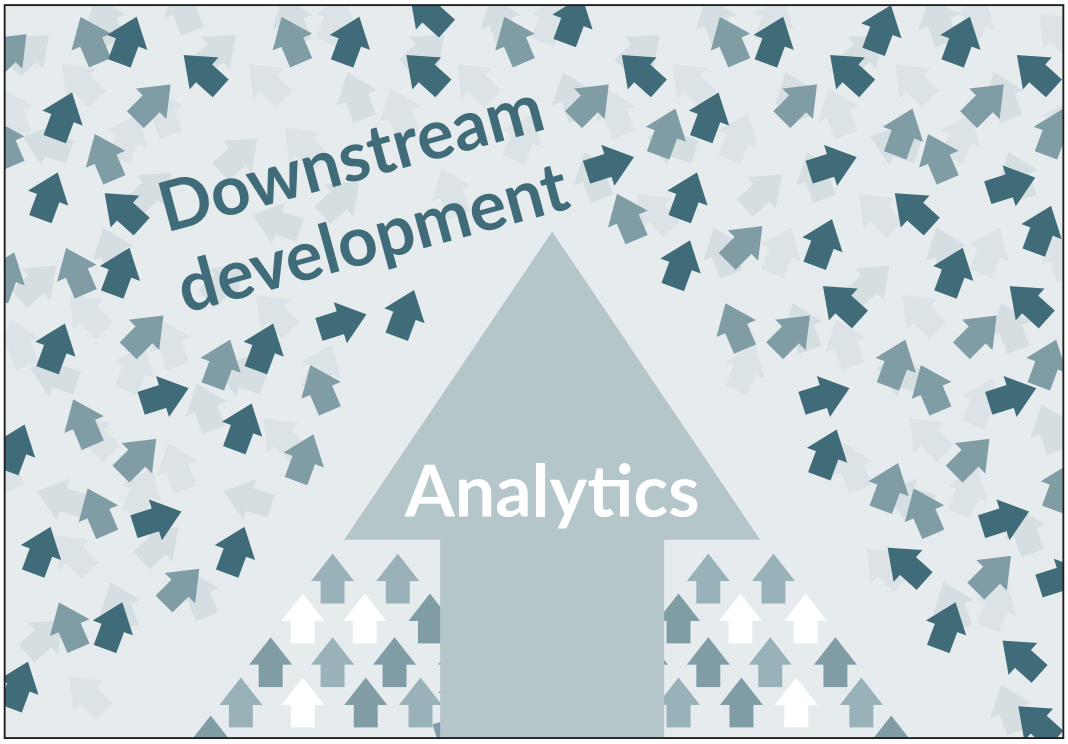
Enhancing AAV process quality and efficiency: three case studies highlighting the benefits of upgraded analytics on downstream process development

Elissa Hudspeth, Isabel Green, Teresa Dewosky, and Suleiman Sweilem

The success of downstream process development for biopharmaceuticals, especially in gene therapy viral vectors, relies heavily on effective analytical tools. These vectors have unique impurities and physicochemical properties along with condensed timelines that require robust analytical support. Increased demand for clinical safety and efficacy, which ties directly to product quality, further drives the need for a deep understanding of the manufacturing process. This article explores three case studies on downstream purification development for AAV vectors, highlighting the importance of analytical resources. Key findings include (1) optimized sample preparation enabled agarose gel analysis of cell lysate nuclease digestion; (2) size exclusion high performance liquid chromatography (HPLC-SE) identified hidden impurities in affinity eluate; and (3) dynamic light scattering (DLS) monitoring mitigated AAV loss during anion exchange chromatography. Overall, enhanced analytical techniques improved process understanding, quality, and efficiency, while also shortening development timelines and cutting costs.

Cell & Gene Therapy Insights 2024; 10(9), 1377–1392

DOI: 10.18609/cgti.2024.158



Gene therapy products have experienced rapid growth over a short period of time. The approvals of Glybera®, Luxturna® and Zolgensma® in the 2010s ignited hopes of cures for many genetic-based diseases. Thus, a significant influx of products under development followed [1]. As of June 2024, the number of *in vivo* viral vector-based products has reached 1,897 and 510 in pre-clinical and clinical development, respectively [2]. This surge in development has intensified competition for limited patient pools and, combined with clinical and business priorities, has fueled efforts to significantly shorten process and product development timelines. This progress has sparked new hope for patients to receive much needed, often previously non-existent, treatments sooner. However, short process development timelines have also placed significant challenges on manufacturing process development groups, along with the added hurdle of using technologies that are still in their nascent stages, evolving concurrently with the therapies themselves.

Initially, researchers transferred processes out of academic labs directly to current cGMP production runs, bypassing the process development phase [3]. This approach resulted in less-than-optimal 'large' scale operations but ones that still met necessary objectives: accelerated manufacturing of limited product quantities to support extremely small clinical trials. As the field rapidly evolved and the needs grew, teams first transferred production processes from academic research labs to process development units, which began developing more manufacturing-friendly large-scale unit operations prior to final cGMP manufacturing runs. This evolution has led to the emergence of a standardized downstream process for AAV products, as depicted in **Figure 1**. The unit operations focus on biomanufacturing-friendly techniques, utilizing depth filtration to clarify the cell lysate followed by affinity and anion exchange (AEX) chromatography, and ultrafiltration/diafiltration prior to bulk fill. The most notable change from the original academic labs was the replacement of ultracentrifugation with chromatography. The case studies presented in this article generally follow this same schematic.

The challenge of developing processes for novel products is further compounded by

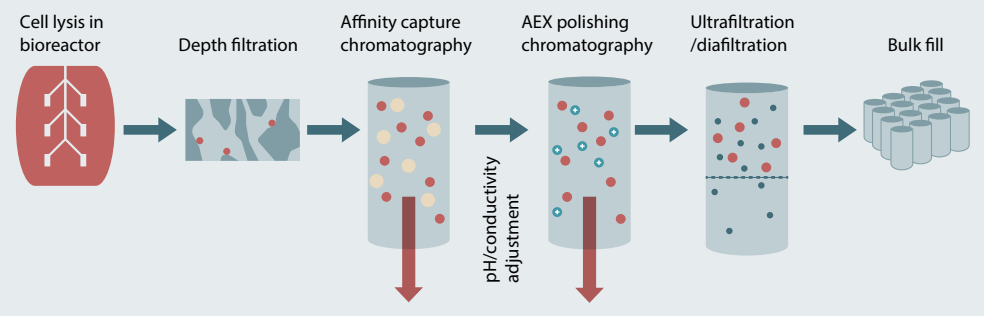
the physiochemical nature of gene therapy products, which consist of both protein and nucleic acid forming molecules significantly larger than most current therapeutic proteins. Most traditional assays in biopharmaceutical manufacturing were developed to evaluate products that are either protein or nucleic acid and smaller in size, such as monoclonal antibodies, hormones, enzymes, and blood clotting factors [4]. For example, monoclonal antibodies are 150 kDa or less and measure ~12 nm in diameter or less [5]. In contrast, gene therapy viral vectors range from ~25–100 nm in diameter [6]. Specifically, AAV measures ~25 nm while adenovirus and lentivirus are ~100 nm. Consequently, analytical technologies are evolving to meet the unique chemical and physical properties of viral vectors.

Lastly, the regulatory expectations have increased in tandem with the growth of the gene therapy industry [7]. The requirements for quality attribute testing have increased as knowledge of safety improves and new analytical capabilities emerge [7]. For instance, aggregation was initially uncharacterized and not reported as an impurity [8,9]. As it became included in the QC testing repertoire, the technology advanced. Size exclusion columns for analytics were characterized and optimized for viral vectors, especially AAV [10–12]. In addition, advanced detection methods, such as multi-angled light scattering (MALS) on HPLC-SE, now have customized capabilities to provide data on multiple quality attributes, including quantity, capsid content, and size [13]. Additionally, advancements in DLS equipment, such as the Malvern Zetasizer Ultra with multi-angle DLS, have also reduced sample volume requirements and increased the sensitivity [14].

Most importantly, all of the factors discussed above, combined with considerations of clinical and market access factors, which are beyond the scope of this article, highlight the pressing need to deliver better gene therapy products that are more commercially viable, effective, and safe [15,16]. On the drug process side, this calls for improvements in manufacturing costs and quality. To achieve higher quality, those in process development must gain a more comprehensive understanding of the product's critical quality attributes (CQAs) and the corresponding impact of critical process parameters (CPPs) earlier on in development. The only way to ensure this understanding is by enhancing the analytical capabilities during early development to ensure success. While this may increase the analytical cost investment, it can result in a more focused and streamlined downstream development process that delivers a higher

FIGURE 1

Generic AAV downstream process.



AAV produced in either mammalian or insect cell culture is lysed in the bioreactor with detergent. The cell lysate is clarified with a depth filter followed by affinity chromatography to capture AAV. Conductivity and pH adjustments are made followed by AEX, which primarily serves to remove empty capsids. The eluate is exchanged into the formulation buffer using ultrafiltration/diafiltration and processed through bulk fill.

quality and lower cost process, ultimately leading to a favorable investment.

In this article, we examine three case studies of downstream purification development for gene therapies using AAV as a vector for genetic payload delivery. These examples illustrate the interdependence of downstream development with analytical resources. Using representative information from past experiences in the industry along with actual data generated at the Biomanufacturing Training and Education Center (BTEC), this work demonstrates that incorporating carefully selected analytical capabilities can enhance process development activities. This led to three key scientific discoveries, which enhanced process understanding and quality as well as reduced development timelines, net resources, and manufacturing costs. In the first case study, we discovered a critical sample preparation step that enabled the replacement of a more resource-intensive qPCR assay with a less-resource intensive agarose gel assay. The second case study demonstrates the potential pitfalls of limited in process sample analysis by showing how one additional analytical technique, HPLC-SE, revealed a 'hidden' impurity co-eluting with AAV on affinity chromatography resulting in misinterpretation of chromatography data. For the final case study, the root cause of AAV loss was identified by adding one key analytical technique, DLS. We also leveraged the added analytical technique to perform downstream development and discover a new optimal buffer condition that mitigated product loss and improved product purity.

CASE STUDY 1: MISSING IN ACTION—DNA DETECTION

Manufacturers typically produce AAV in mammalian or insect cell-based systems, where it is present both intracellularly and extracellularly. To recover AAV, cells commonly undergo lysis with detergent, releasing both intracellular virus and host cell genomic DNA (hcDNA). Manufacturers must remove the residual DNA, which includes baculovirus, helper virus, plasmid, and host cell DNA. These process impurities pose a risk to safety and downstream operations. Safety concerns include immunogenicity, oncogenicity, and infectivity. Downstream operations concerns include the high viscosity created by large strands of hcDNA in solution. Furthermore, the WHO and US FDA guidelines recommend limiting residual host cell DNA to 10 ng per dose at a size of <200 base pairs for biological drug products [17,18]. However, the FDA has since acknowledged that achieving these limits may not always be feasible for gene therapies and each drug product's limits should be justified with a risk-based assessment [19,20].

To reduce the safety and viscosity risk of residual DNA, a nuclease is typically added to digest the DNA into smaller fragments and facilitate clearance. In this case study, the team initially chose Benzonase® since it was the only GMP-grade nuclease available. They selected the quantity on a theoretical basis (i.e., literature and manufacturer guidance) and set at 100 units per milliliter of lysate (U/mL), translating to a cost of US$85,000 for enough nuclease to process a 200 L suspension bioreactor.

Due to competing priorities, limited knowledge on lower nuclease concentrations, and the significant investment required to develop and execute the 'gold' standard assay, qPCR, further optimization was not performed. However, if a widely accepted and established method for analyzing DNA digestion in cell lysate had been available that required minimal development and resources, it could have tipped the scales toward a favorable development option.

Newer publications indicate that this development may have led to a reduction in nuclease concentrations to 12.5 U/mL [21]. Based on the current cost of Benzonase, this would have resulted in a raw materials cost savings of US$74,000 per 200 L run (US$85,000 for 100 U/mL vs US$10,625 for 12.5 U/mL).

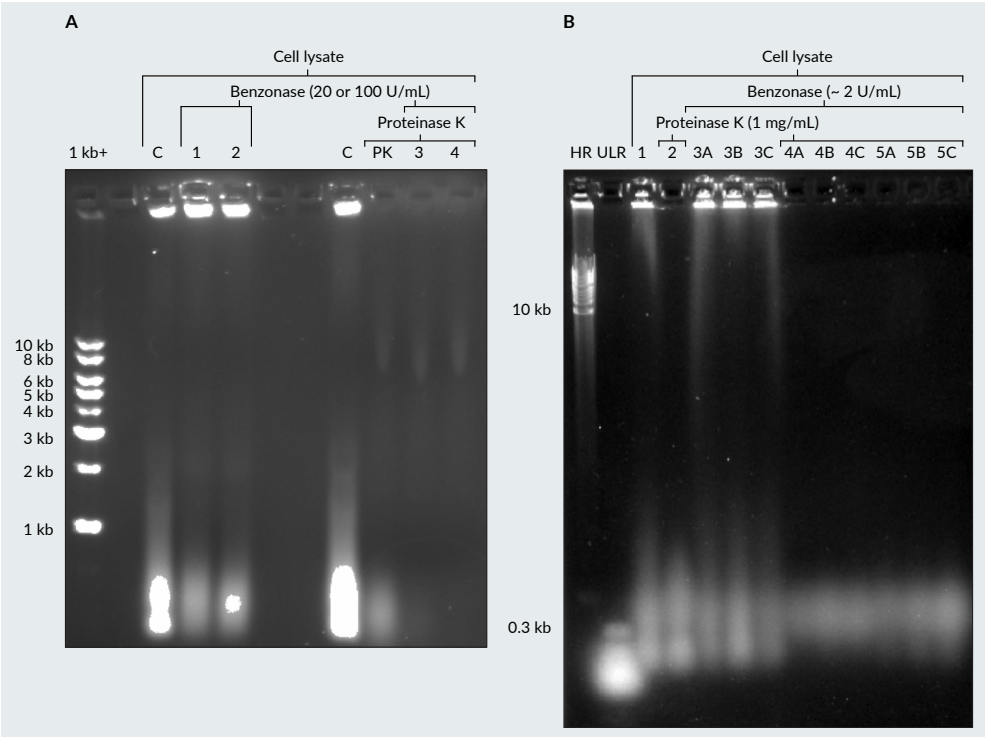
Among the available analytical techniques, qPCR is the current 'gold' standard for release and process monitoring assays. However, newer digital droplet PCR (ddPCR) is becoming more common due to its improved consistency. Both assays provide quantitative data on residual DNA present in the sample that is equal to or greater than the PCR primers' target sequence (often <200 base pairs). Despite their advantages, developing or outsourcing these assays can be resource-intensive. New commercially available kits, such as resDNASEQ® from Thermo Fisher Scientific, offer improved consistency and decreased development time, but the tradeoff is a high cost of US$5000 for one 96-well kit. Additionally, these assays only detect DNA targeted by the PCR primers and do not provide information about the size of the DNA beyond confirming that detectable fragments exceed the primer target size, while the non-detectable fragments are smaller.

Next-generation sequencing can overcome these limitations but is significantly more resource-intensive and may provide less accurate quantitation.

An alternative strategy could involve using less technical, more economical assays for screening and development, while reserving PCR-based assays for confirmatory testing. Examples of such assays include PicoGreen™ (a double-stranded DNA fluorescent dye) and DNA agarose gels. At BTEC, we have explored the use of DNA agarose gels to

FIGURE 2

DNA agarose gels.



Agarose gels were performed to assay the extent of cell lysate DNA digestion by Benzonase®. The cell lysate was Sf9 cell culture that was lysed with detergent and stored at ≤-60 °C. All samples are labeled accordingly if they contain cell lysate (C), proteinase K (PK) or Benzonase (B). Gel A: used ethidium bromide and a 1 kb pair DNA ladder (first lane). DNA digestion and gel were performed by Operator A. Lanes: C: cell lysate, PK: lysate + PK, 1: lysate + Benzonase (20 U/mL), 2: lysate + Benzonase (100 U/mL), 3: lysate + PK + Benzonase (20 U/mL), 4: lysate + PK + Benzonase (100 U/mL). Gel B used SYBR Gold and both, a high range (HR) and ultra low range (ULR) DNA ladder, in the first and second lanes, respectively. DNA digestion and gel were performed by Operator B. DNA digestion was performed in triplicate with replicates indicated by A, B, and C for labeling the lanes. Lanes: 1: cell lysate, 2: lysate + PK, 3: lysate + B, 4: lysate + B + PK, 5: lysate + PK + B.

quantify genomic DNA in cell lysates and are working to develop a simplified protocol that can mitigate the challenges of running crude cell lysate on an agarose gel to achieve accurate and robust results.

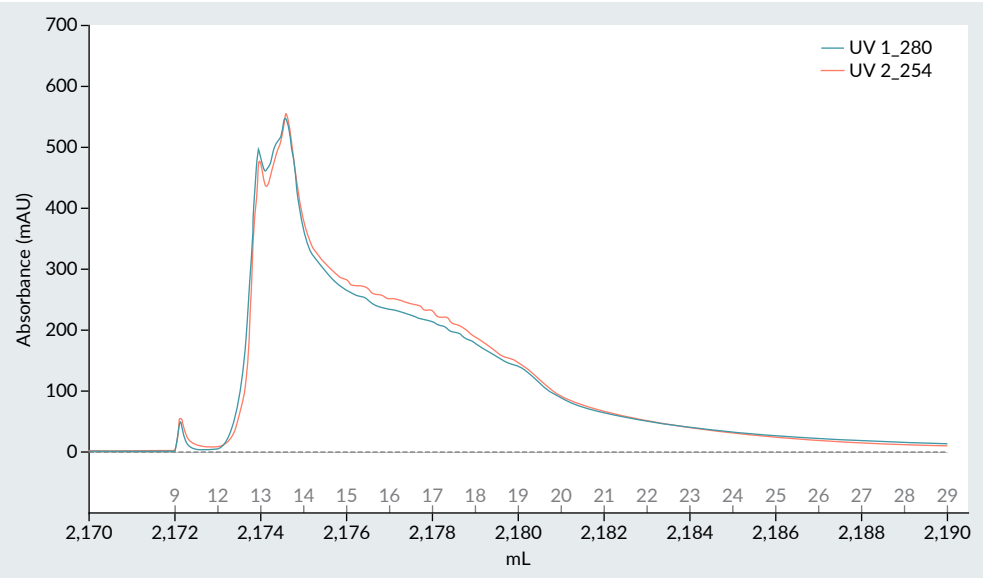
Initial data suggest that the procedure is more complex than simply running cell lysate on a gel and observing changes from a high molecular weight band to a smear of lower molecular weight bands, and finally to a broad, very low molecular weight band at the bottom of the gel. However, universal applicability requires a protocol that provides detailed guidance on running the assay and analyzing the results.

Figure 2 demonstrates a few of the initial findings. For the undigested samples (see well C in Gel A and well 1, Gel B), the results showed some evidence of small nucleic acids, possibly due to DNA shearing from freeze-thawing or the presence of RNA. However, the majority of cell lysate nucleic acid did not migrate into the gel; instead, it retained its position at the top of the gel. We hypothesized that proteins, such as chromatin, might have caused the nucleic acid to aggregate into protein/DNA complexes. To test this hypothesis, we assessed the effect of Proteinase K addition to digest the proteins and, thereby, liberate the nucleic acids from these complexes. In both gels, run by different operators under differing conditions, the high molecular weight band at the top of the gel disappeared for samples treated with Proteinase K (see well PK in Gel A and well 2 in Gel B). These results indicate that adding Proteinase K to cell lysate is essential for evaluating the DNA digestion.

Furthermore, when operators applied Proteinase K treatment and allowed the DNA retained in the well to migrate into the gel, the digested DNA in the nuclease-treated samples seemed to disappear (or was no longer detectable) when using ethidium bromide stain (Figure 2: Gel A wells 3 and 4). However, using SYBR gold staining, along with an increased DNA load, allowed for the visualization of some smaller molecular weight nucleic acids (Figure 2: Gel B wells 4A, B, and C, and 5A, B, and C). This underscores the importance of selecting the appropriate stain and DNA load amount to provide conclusive evidence of digestion.

FIGURE 3

Affinity elution peak.



Elution peak from affinity capture chromatography of clarified AAV cell lysate (Sf9 cell-generated). The UV absorption at 254 nm and 280 nm is shown in red and blue, respectively. The fractions collected are denoted by the gray bars and numbers.

Further work intends to investigate the minimum size of DNA detectable using agarose gel electrophoresis. Literature suggests that detection down to at least 25 base pairs is possible [22]. During associated experiments (data not shown), detection of DNA sizes of 3 and 5 base pairs was unsuccessful, even when loading up to 2 ug and staining with SYBR Gold. An alternative method for tracking lower molecular weight DNA digestion could involve using SDS-PAGE gels.

CASE STUDY 2: WHAT YOU SEE ISN'T ALWAYS WHAT YOU HAVE

Affinity chromatography is now a standard method for the initial purification step of AAV. UV-Vis absorbance is a standard online method built into most preparative chromatography systems to monitor nucleic acids and proteins, which have an absorbance maximum at wavelengths of 260 nm and 280 nm, respectively. The 260/280 ratio provides an estimation of the relative proportions of protein and nucleic acids in a sample, where a ratio of ~0.57 indicates 100% protein and a ratio of ~1.8–2.0 indicates pure DNA and/or RNA [23].

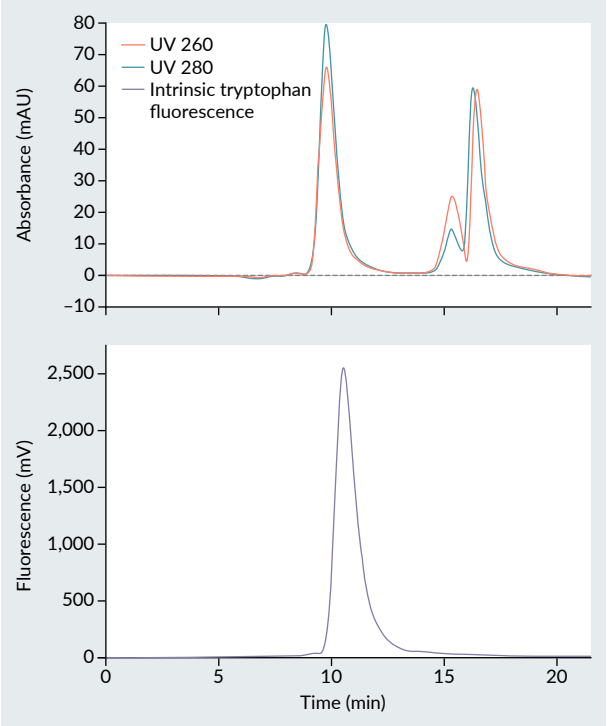
By accounting for the DNA payload sequence and capsid serotype, molecular extinction coefficients can be estimated and used to generate a theoretical relationship between the 260/280 ratio and the percentage of full and empty capsids [24]. It is important to note that this relationship of A260/280 ratio and protein:nucleic acid content is exponential rather than linear, and that the data is most accurate when generating an empirical curve using pre-prepared mixtures of the specific AAV full and empty capsids being quantified.

In this case study, clarified Sf9 cell lysate containing AAV from an unoptimized upstream process was purified through an affinity column. The affinity elution peak, as shown in Figure 3, yielded a 260/280 ratio of ~1.00, which corresponds to roughly 50–70% full capsids [25].

A new high-performance liquid chromatography (HPLC) method using a size exclusion column was developed. The main peak fraction from the affinity column (#13) was analyzed with a combination of two online detectors on the HPLC unit, photo-diode array (PDA) and fluorescence. As shown in Figure 4, the intrinsic tryptophan fluorescence from AAV revealed a main peak comprising ~99% of the protein, alongside a smaller front shoulder peak, likely representing AAV dimers due to the earlier elution time. Interestingly, the UV 260 nm and 280 nm signals, measured by the PDA, revealed that the 260/280 ratio of the main peak was 0.80 in contrast to the value of 1.00 observed for the affinity column (Figure 4). This ratio would correlate with ~15–30%

FIGURE 4

HPLC-SE.



HPLC was performed on the affinity eluate (fraction 13) using a large pore size exclusion column (700–1000 Å). The top chromatogram shows the UV absorbance data collected by the PDA. The UV absorbance at 260 nm and 280 nm is shown in red and blue, respectively. The bottom chromatogram shows the intrinsic tryptophan fluorescence in purple. HPLC-SE: size exclusion high performance liquid chromatography.

full capsids as opposed to the previously calculated 50–70%. Additionally, the UV trace exhibited two additional, significantly sized peaks eluting much later with ratios of 1.70 and 1.00. These peaks suggest the presence of non-protein impurities in the affinity eluate. This provided key information about some unknown impurity binding to the affinity column and co-eluting with AAV, thus obscuring the true AAV 260/280 ratio and the corresponding percentage of full capsids.

This information helps facilitate further development efforts to enhance AAV purification from clarified lysate. Further analytics could be performed to identify the impurity, and their clearance can be optimized on a subsequent polishing chromatography and/or affinity wash step development. Separation of empty and full capsids can be achieved through anion exchange chromatography; however, the performance is significantly influenced by the percentage of full capsids in the starting material as well as the serotype and production method. Thus, one might reasonably expect to achieve 70 to >90% full capsids if starting with 50–70% full capsids, but this expectation diminishes significantly to approximately 40–80% if starting with 15–20% full capsids [26,27].

Notably, re-purifying the affinity eluate was explored to evaluate if loading a purer feed stream would facilitate removing the impurity, as shown in Figure 5. An impurity was observed in the load flow-through with an A260/280 ratio of 1.31. The elution peak was sharper with an A260/280 ratio of 0.78, comparable to HPLC-SE data. The total area of the flow-through and elution had an A260/280 ratio of 1.02, similar to that of the original affinity eluate peak. This suggests there is potential to optimize the original affinity method to separate the originally co-eluting impurity.

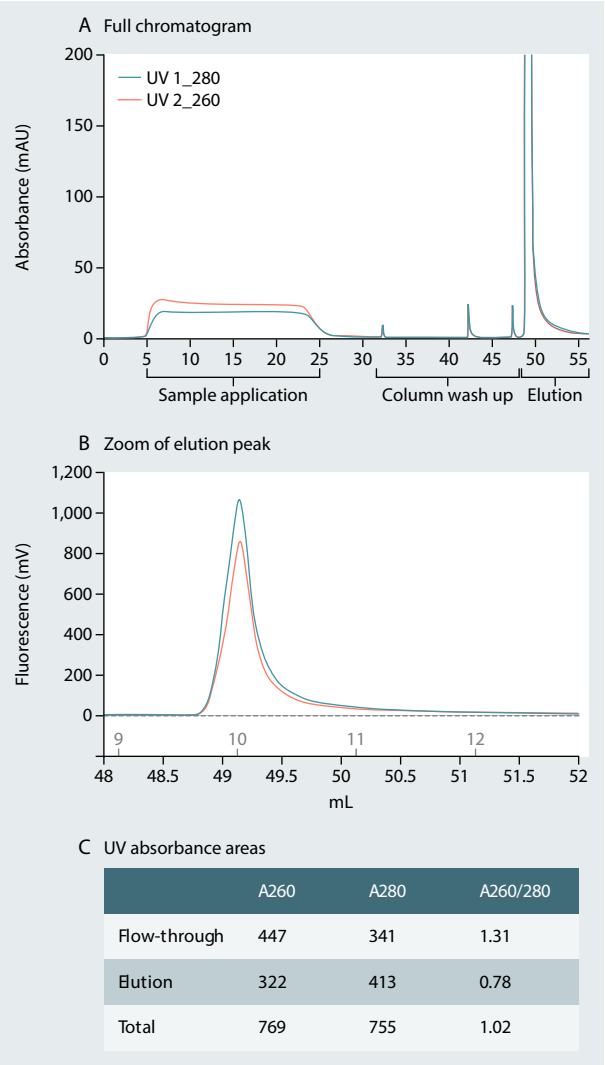
CASE STUDY 3: SHINING THE LIGHT ON MISSING AAV

Following affinity capture chromatography, viral vector capsids that were assembled without encapsulating the genetic payload (empty capsids) must be separated and removed. Anion exchange chromatography has emerged as one of the predominant methods for this separation.

In this case study, AAV affinity eluate was diluted into a low salt, high pH buffer condition and purified using anion exchange

FIGURE 5

Affinity re-run elution peak.



C UV absorbance areas

	A260	A280	A260/280
Flow-through	447	341	1.31
Elution	322	413	0.78
Total	769	755	1.02

Entire chromatogram (A) and zoom of elution peak (B) from affinity chromatography of the previously run affinity eluate peak, shown in Figure 3. The UV absorption at 260 nm and 280 nm is shown in red and blue, respectively. The fractions collected are denoted by the grey bars and numbers. UV absorbance areas (mAU*mL) of the flow-through or sample application and elution peak are provided along with the A260/280 ratio of the corresponding areas. The total area and ratio of the flow-through and elution peak is also shown.

chromatography. We analyzed the performance of this process with a ddPCR assay, which indicated a significant loss of AAV full capsids.

Initially, ddPCR was the only analytical technique made available for downstream sample testing. These results indicated minimal loss of AAV full capsids during the dilution with few AAV full capsids present in the column flow-through. However, the majority of the AAV full capsids remained unaccounted for. It is possible that they were bound strongly to the column in a manner that destabilized them during elution under harsher conditions. With this information in mind, the available options were to take a non-specific approach by evaluating different buffers, salts, pH levels, and resins in the hope that one would provide improved performance.

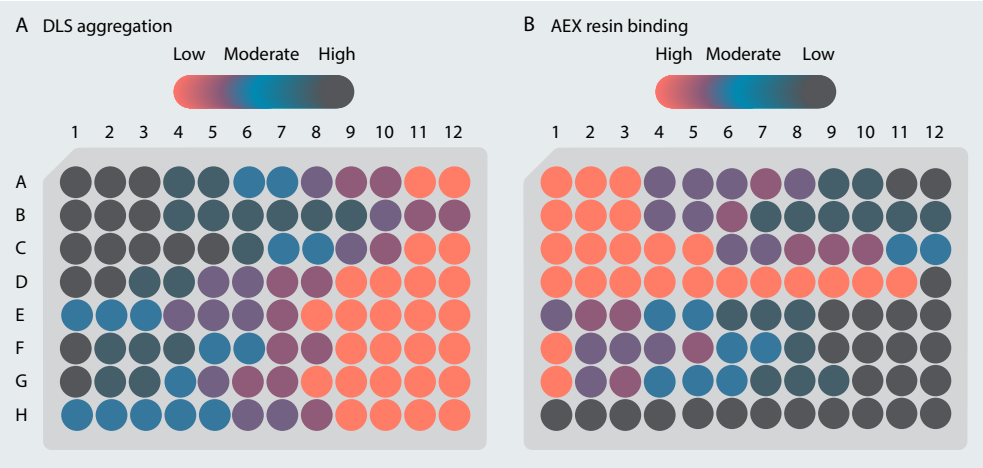
Fortunately, a DLS instrument was eventually acquired to allow additional analysis. The DLS revealed the presence of AAV aggregates, which ddPCR cannot detect, in the column load sample. Protein aggregates are known to bind more strongly to ion exchange chromatography than their corresponding monomers and, in some cases, cause denaturation and 'irreversible' binding to the column [28]. In addition, certain AAV serotypes tend to aggregate under low conductivity or osmolality [29].

We further leveraged the DLS to screen several buffer conditions for their effect on AAV aggregation. The 96-well plate format facilitated high throughput testing and required significantly less AAV feed material. To confirm conditions that would allow AAV binding in each buffer system, we used a filter-plate based static binding system with a plate-based UV-Vis spectrophotometer.

With the aid of these enhanced analytical assays, we quickly identified optimal conditions that mitigated the AAV aggregation and significantly increased AAV product recovery from the anion exchange column (Figure 6). The experiments to determine the cause of the low recovery and to find the solutions could be executed in less than 2 weeks with minimal feed material. By adding one additional analytical capability in a high-throughput format, the development team determined the root cause of the low recovery of full capsids, pursued a mechanistic solution, and screened conditions in a high-throughput format with minimal sample consumption leading to enhanced recovery of full capsids.

FIGURE 6

DLS (representative data).



Representative data is shown from an experiment testing various buffer conditions for aggregation by DLS and AAV binding to AEX resin. The relative quantity of AAV binding was assessed by measuring 280 nm absorbance with a spectrophotometer. AEX: anion exchange, DLS: dynamic light scattering.

MATERIALS AND METHODS

AAV was produced in either HEK293 (mammalian) or Spodoptera frugiperda (Sf9; insect cell) expression systems using triple transfection or baculovirus, respectively. For examples from BTEC, AAV was expressed in an Sf9 system obtained from the University of Florida [30–32]. Cells were lysed with a non-ionic detergent and clarified by depth filtration or centrifugation. Affinity chromatography was performed using AVB resin, either Capto or Sepharose High Performance (Cytiva, #17372201 or #28411210, respectively). Polishing chromatography was performed with a quaternary ammonium-based anion exchange resin or column, such as POROS 50 HQ (Thermo, #1255911) or CIMmultus® QA (Sartorius, #311.5113-2). An AKTA Pure (Cytiva) was used to execute chromatography.

Dynamic light scattering was performed using equipment such as the Wyatt DynaPro plate reader or similar. The HPLC system

TABLE 1

Case studies summary.

	Case 1	Case 2	Case 3
Problem	DNA digestion step not optimized	Incorrect interpretation of high % full capsids in affinity eluate (~50–70%) based on A260/280	Loss of AAV during AEX purification
Business challenge	'Gold' standard assay cost†:benefit ratio not favorable	Extensive development (time and resources) required and likely will be unproductive to obtain expected purity	Extensive development (time and resources) required to solve; level of AAV yield=unacceptable productivity
Scientific challenge	Alternative lower cost† assays not well-developed for this application	Potentially unable to achieve expected AEX chromatography results	Unknown cause based on available chromatography and ddPCR data
Scientific discovery/ breakthrough	Universal development of DNA gel agarose assay—critical proteinase sample prep step	Analysis on HPLC-SE revealed 'novel hidden' impurity that can co-elute with AAV on affinity chromatography=corrected to ~15–30% full AAV capsids=potential to improve affinity purity and to adjust AEX expectations	Analysis on DLS revealed root cause of AAV loss=AAV aggregation; DLS used for high-throughput screening to find a novel buffer condition to mitigate aggregation
Business benefit	Availability of alternative assay with a favorable cost†:benefit ratio=optimization of DNA digestion earlier in development=manufacturing process raw material COGS reduction	Avoidance of unproductive development path=decreased development timelines and resources	Decreased development timelines and resources
Overall impact	Greater process and CQA understanding, overall project cost savings	Greater process and CQA understanding, potential for improved process quality and productivity	Greater process and CQA understanding, improved process quality and productivity
Analytical change	Development of 'new' alternative assay	Addition of accepted assay	Addition of accepted assay and use as a high-throughput screening tool
Downstream impact	Could not proceed without assay	Development path altered and more focused	Development path altered, more focused, and streamlined

†Cost here includes financial (capital equipment and consumables), personnel, and timelines.

was a Sartorius PATFix, and the size-exclusion column was a 700–1000 Å pore size resin, such as the Sepax SRT SEC-750 A (#2157504625) or similar.

Agarose gels were prepared in-house and varied from 0.5–1.0% agarose. Gels were prepared with either ethidium bromide (Thermo Fisher, #15585011)or SYBR Gold (Thermo Fisher, #S11494) stain. Gels were run at 70 volts and imaged with a Bio-Rad imager. Cell lysate was prepared from an Sf9 culture that was lysed with detergent and stored at <-60 °C. The DNA ladders were 1 kb Plus DNA Ladder (Thermo Fisher, #10787018), GeneRuler High Range DNA ladder (Thermo Fisher #SM1351), and GeneRuler Ultra Low Range DNA Ladder (Thermo Fisher #SM1211).

DISCUSSION

With the current speed to market demand driving gene therapy drug product and process development, strategies that deliver optimal drug product material with available resources are crucial.

Here, three case studies demonstrate the advantages of strategically bolstering analytic capabilities to support both a deeper understanding of downstream processes and expedited development timelines. A summary appears in Table 1.

The first example illustrates potential raw materials cost savings of up to US$74,000 per 200 L run that could have been realized by adding an analytical assay and optimizing the DNA digestion step of the manufacturing process. This results in 7–15% savings on the overall manufacturing costs, estimated to be between US$0.5 and 1.0 million [33]. However, these cost savings did not outweigh the perceived cost to perform the development, including financial, personnel, and timeline investments. The available 'gold' standard quantitative PCR-based assays would have required a significant investment, which opened the door to alternatives, such as the DNA agarose gel method BTEC is developing. While the gel-based method may lack specificity and is only semi-quantitative, it has the advantages of simplicity and cost–effectiveness while still providing enough information to enable DNA analysis and decision-making for in process samples during the early stages of process development. Establishing a publicly available protocol and data set that gains acceptance could offer a lower cost option streamlining the development of a DNA digestion step and allowing for its integration during earlier phases.

The second example highlights the risk of misinterpreting data without supporting orthogonal assays. UV-vis absorbance can provide a rough estimate of the percentage of full capsids by evaluating the ratio of absorbance at 260 and 280 nm wavelengths; however, it has significant limitations. In this case, HPLC-SE revealed an impurity present in the affinity elution peak that significantly influenced the A260/280 ratio. Had development proceeded directly to the polishing step, without the HPLC-SE data, significant effort could have been misspent on development due to an inaccurate assessment of the product intermediate's intermediate purity from the affinity chromatography step.

In the final example, a newly acquired analytical tool, DLS, not only identified the root cause of AAV loss during the polishing step development, but also streamlined process development activities. This approach resulted in a significantly shorter timeline requiring fewer resources and led to a more solid and robust solution.

These examples demonstrate how early incorporation of additional analytical methods can bolster process understanding, optimize development activities, and improve process robustness, product quality, and/or manufacturing cost-savings. Furthermore, they highlight how streamlining downstream development by reducing timelines, costs, and potential pitfalls, can offset the drawbacks of investing in more analytical capabilities. Finally, continued advancement in analytical assays and protocols can further broaden the

options for earlier implementation, resulting in the aforementioned benefits.

While acquiring the equipment and technical capabilities to support a wide array of specialized analytical techniques can be challenging, several approaches exist that can lighten the burden. Large biopharmaceutical companies often have the resources in-house. However, the complexity of their large networks can often make utilizing them untenable across sites and departments. Techniques can be challenging, several approaches establishing more of a core support-based system may facilitate better leverage of these resources. Conversely, small and emerging biopharmaceutical companies face the challenge of having to establish new labs. One approach could involve leveraging the resources and expertise available at established CDMOs and CROs. Another approach involves developing a co-operative based lab with other small companies, although this poses business challenges related to intellectual property, legal considerations, and ownership assignments.

BTEC recognizes the unique challenges in gene therapy analytics and has developed a unique course for industry professionals that provides a 3-day in-depth overview. The course, "AAV Quality Attributes: Theory and Practice" benefits a wide array of backgrounds and job roles, spanning analytics and downstream to sales and quality. While providing comprehensive material across the breadth of techniques required for viral vector gene therapy products has been challenging, BTEC has pooled the expertise of a team of experts to ensure course material remains at the cutting edge of the field.

CONCLUSION

In an ideal world, all currently available tools would be readily accessible to develop the most efficient and robust processes. However, gene therapy is currently far from that reality due to its novelty, complex nature, and development speed.

This paper has shown how transformative early investment in analytic capabilities can be for gene therapy process development. The case studies presented highlight remarkable gains in both time and cost efficiency: potential cost savings of up to US$74,000 per 200 L run through DNA digestion optimization, avoidance of costly downstream rework by using orthogonal assays to correctly assess intermediate purity, and the use of DLS to streamline development activities and reduce timelines while utilizing fewer resources.

Strong analytics do not just prevent costly mistakes; they drive faster development, reduce costs, and improve product quality. Even with limited resources, integrating these tools early can significantly enhance efficiency. Despite ongoing challenges, the power of analytics lies in turning limitations into opportunities—helping to achieve better outcomes with smarter, more efficient processes.

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AUTHORSHIP & CONFLICT OF INTEREST

Contributions: Elissa Hudspeth was involved in the conception of the ideas for the work presented; directing the acquisition, analysis, and interpretation of all data for the work; and drafting the work and reviewing it. Isabel Green was involved in leading the second stage of development for the DNA agarose work (designing, executing, analysis, and interpretation); acquisition, analysis, and interpretation of re-run affinity chromatography; and drafting the work and reviewing it. Teresa Dewosky was involved in leading the first stage of development for the DNA agarose work (designing, executing, analysis and interpretation); acquisition, analysis and interpretation of initial affinity chromatography and HPLC-SE data; and drafting the work and reviewing it. Suleiman Sweilem was involved in contributing ideas for the DNA agarose work presented; managing the acquisition, analysis, and interpretation of the DNA agarose work and generating the cell culture specifically for use in that work; producing AAV cell culture used for the affinity work; and drafting the work and reviewing it. The named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.

Acknowledgements: The authors thank Laurie Overton and Chris Cummings, who produced the AAV in cell culture for BTEC's Hands-On cGMP Biomanufacturing of Vectors for Gene Therapy short course, the leftovers of which were used for a portion of this work. They also thank Gary Gilleskie, Sara Siegel, Danny Schmitt, and Dennis Coss for critical reading of the manuscript and valuable suggestions. Jason Whitley's effort to generate the design concept for the graphical abstract is also appreciated.

Disclosure and potential conflicts of interest: The authors have no conflicts of interest.

Funding declaration: All authors of the manuscript are BTEC employees or students and the work was supported by funding from BTEC.

ARTICLE & COPYRIGHT INFORMATION

Article source: Invited; externally peer reviewed.

Submitted for peer review: Sep 13, 2024; **Revised manuscript received:** Oct 23, 2024; **Publication date:** Nov 11, 2024.

CELL & GENE THERAPY INSIGHTS

DOWNSTREAM PROCESSING

RESEARCH ARTICLE

Displacement chromatography for enrichment of rAAV genome-containing capsids using weak organic acid

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Separation of empty and genome-containing particles is a challenging requirement for recombinant adeno-associated virus gene therapy vectors. Strong anion exchange chromatography operated with a linear salt concentration gradient to achieve a high degree of resolution has emerged as the replacement for traditional density-gradient ultracentrifugation to improve efficiency, consistency, and scalability. Herein, an innovative mechanism of anion exchange chromatography using weak organic acid present in the load is shown to selectively displace empty capsids into the flowthrough, enabling an isocratic elution and eliminating operational challenges associated with linear gradients. This separation technique was applied to AAV2 and AAV8 serotypes using three chromatographic media (monoliths, resin, and membranes) and showed comparable genome-containing capsid enrichment levels to that of density gradient ultracentrifugation. Processes for both serotypes were successfully transferred from lab to production scale.

Cell & Gene Therapy Insights 2024; 10(9), 1317–1335

DOI: 10.18609/cgti.2024.150

Recombinant adeno-associated viruses (rAAV) have emerged as promising vectors for transferring therapeutic recombinant genes to humans with several new therapies transitioning to commercialization [1,2]. As advances in upstream processing continue to improve rAAV productivity, traditional downstream purification using density gradient ultracentrifugation processing to enrich genome-containing capsids presents a bottleneck in large scale rAAV purifications [3]. Empty capsids that do not contain the therapeutic gene are an inevitable by-product of the rAAV vector production process and can result in clinical safety and dosage impediments [4,5]. Historically, cesium chloride, sucrose, or iodixanol-based density gradient ultracentrifugation methods have been utilized to separate genome-containing and empty capsids in a serotype-independent manner based on differences in density, as shown in Figure 1. These manual processes are operationally complex at large scale and could result in altering quality attributes important for product and process validations [6–8].

Highly similar size, charge, and accessible surface morphology between the empty and genome-containing capsids makes chromatographic separation of empty capsids challenging for downstream processing of rAAV gene therapy products [9–12]. Ion exchange chromatography has commonly been used in the manufacture of biotherapeutics and the purification of rAAV vectors. Separation of empty from genome-containing capsids has been demonstrated using different modalities of anion exchange (AEX) chromatography such as conventional quaternary amine ligands on resins, monolithic media, and membranes [5,6,13,14]. However, these chromatographic methods primarily use bind and elute strategies with linear salt concentration gradients to resolve impurities like empty capsids [14–16]. A gradient elution strategy can be challenging to implement at commercial scale [17]. Recent developments have shown progress toward a step elution process, but would require minimizing the variation in buffer conductivity, pH, and component additions to achieve a manufacturing ready procedure [13,18–20]. A step elution method capable of separating empty and full capsids in a robust and scalable manner is thus desirable, ensuring consistent product quality between manufacturing batches is attained.

Herein, the AEX load was modified with specific concentrations of a weak organic acid to promote separation based on slight differences in capsids charge and hydrophobicity [21]. This combination of interactions can create different binding strengths for empty capsids and charged stationary phase. Addition of weak organic acid with intermediate binding strength enabled preferential displacement of empty capsids and scalable step elution. Ongoing experiments, utilizing modeling approaches, will help gain further insight into the mechanism of separation.

This method was successfully applied to AAV8 and AAV2 serotypes. Multiple AEX chromatographic media were investigated for each serotype. Substrates with larger pore size or open pore structures were chosen to allow accessibility and faster transport of AAV to binding sites inside the pores of the substrate, which may result in a faster flow rate, higher binding capacity and resolution. Enrichment of genome-containing capsids achieved with this method was comparable to that of density gradient ultracentrifugation for vector manufactured at lab (3 L), pilot (50 L), and production scales (250 L or 500 L).

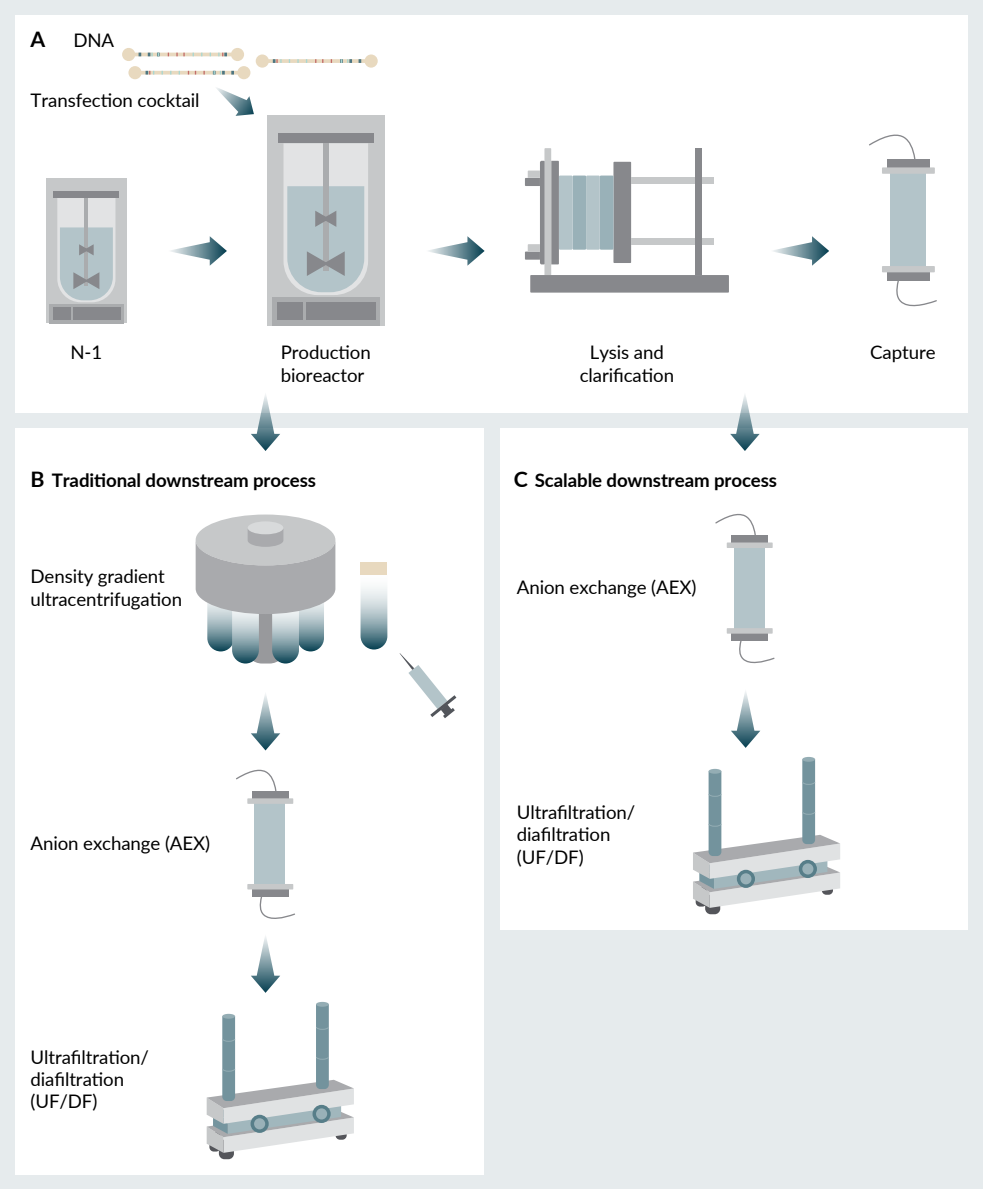
MATERIALS AND METHODS

Cell culture and rAAV production

Both AAV2 and AAV8 manufacturing processes were initiated with the thaw of a single vial of the suspension HEK293 Pro10 master cell bank (MCB), in chemically defined medium and were expanded through a series of shake flasks to produce sufficient cells to seed a bioreactor at the 3 L scale, 50 L scale,

FIGURE 1

Process flow diagram for both traditional downstream processes and a scalable downstream process without density gradient ultracentrifugation.



(A) Upstream process, transfection, lysis and clarification, and capture chromatography remains the same for both traditional and scalable processes. **(B)** The traditional process to separate genome-containing capsids from empty capsids includes density gradient ultracentrifugation, AEX to remove the density gradient material such as iodixanol, then UF/DF for buffer exchange. **(C)** A scalable process eliminates the density gradient ultracentrifugation step and uses AEX for separation of genome-containing capsids from empty capsids, then UF/DF for buffer exchange.

or a sequential 50 L and 250 L or 500 L stirring production bioreactor. After expansion of the cells to the target production volume and viable cell density, cells were transfected with a cocktail consisting of adenovirus helper, rep/cap, and transgene-containing neDNA cassette (TAAV Biomanufacturing Solutions, S.L.). Transgene cassettes for AAV8 and AAV2 were single stranded DNA at 4779 kb and 2705 kb lengths, respectively. Cells were harvested approximately 72 hours post-transfection.

Clarification, capture, and anion exchange load preparation

The downstream purification process for both AAV2 and AAV8 was initiated by chemical lysis to release the viral vector from the transfected cells in the bioreactor. Primary clarification was accomplished using 20 µm pore size depth filtration and 0.2 µm sterile filtration. Intact rAAV particles were further purified via capture chromatography using CaptureSelect™ AAVX resin (Thermo Fisher). The affinity-purified eluate was prepared for AEX separation by diluting the product with buffers containing specific amounts of weak organic acids [22]. Buffer compositions were developed to facilitate the interaction between chromatographic media, product, and empty capsids. The load material was filtered through a 0.2 µm filter prior to applying onto the AEX chromatography media.

Density gradient ultracentrifugation

Enrichment of genome-containing capsids was separated by iodixanol gradient ultracentrifugation using a Beckman Ti70 fixed angle rotor. The affinity-purified material was under-layered with 25%, 40% and 60% iodixanol and centrifuged at 505,000 rcf for 1 hour. Genome-containing capsids were extracted from the tube using a syringe at the interface between 40% and 60% iodixanol layers. The iodixanol fractions were pooled and diluted with buffer to reach the desired pH and conductivity required for binding to the AEX media. The genome-containing product was processed over AEX, in a bind-and-elute mode, using a sodium chloride step elution.

Lab scale AEX chromatography

Small-scale AEX chromatographic purifications were performed using 1 mL POROS™ 50HQ prepacked columns (5 cm bed height, Thermo Fisher), 1 mL CIMmultus® QA (2 µm) monolith devices (BIA Separations), or 1 mL Mustang™ Q (Cytiva) membrane devices. Each AEX chromatographic media was evaluated using the same methods, buffers, and load material. Residence times were 1.5 minutes for POROS 50HQ resin, 0.2 minutes for CIMmultus QA monolith, and 0.29 minutes for Mustang Q membrane. Chromatography was performed and 0.25 CV fractions automatically collected by an AKTA Avant™ 25 skid (Cytiva). AKTA skid cleaning in place (CIP) method was performed using 0.5 M sodium hydroxide before each cycle and stored in 20% ethanol.

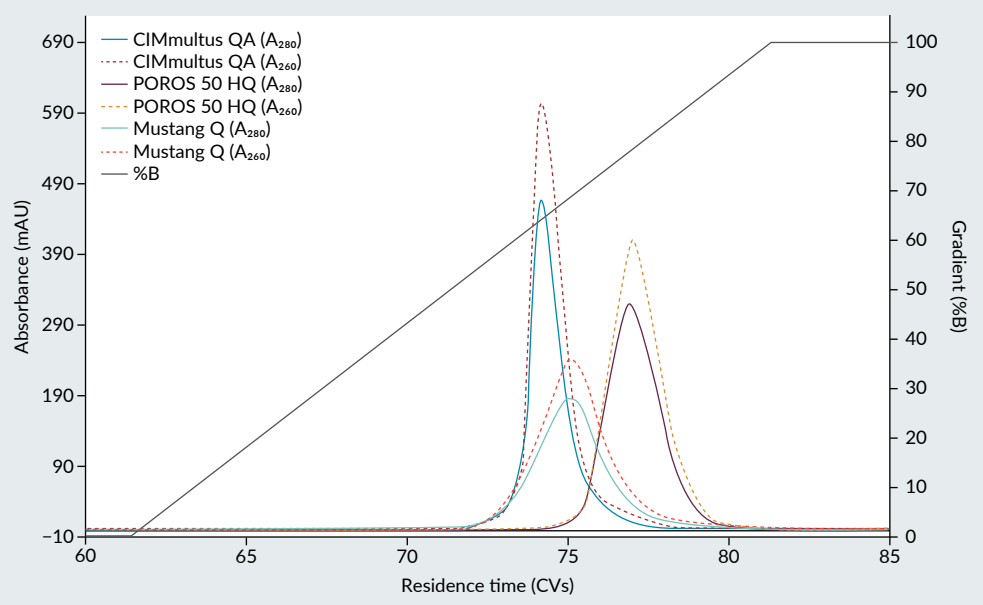
Initial development of the AEX method included screening different buffer components in the AEX load and gradient elution phase. AEX load was prepared by diluting the affinity eluate with a pH 9.0 buffer containing varying amounts of weak organic acid. Conductivities during the weak organic acid concentration screen ranged from 2.022–2.450 mS/cm for the AAV8 serotype (2.4 mM–6.4 mM weak organic acid) and from 2.276–2.725 mS/cm for the AAV2 serotype (6.4–11.2 mM). The AEX column was equilibrated with the pH 9.0 buffer excluding weak organic acid. Following buffer selections, a 20 CV gradient elution was converted to a step elution, keeping the same buffer components as developed in the gradient elution. During initial development runs, AEX elutions at pH 9.0 were titrated to a neutral pH prior to sample storage and analysis.

AEX purification scale up

At the 3 L scale, the final AEX process for enriching genome-containing capsids utilized POROS 50HQ resin (5 mL, 10 cm bed height) or CIMmultus QA monolith devices (8 mL). POROS 50HQ resin was operated at a 1.5 minute residence time while CIMmultus QA monolith flow rate was at a 1.4 minute residence time to accommodate scaleup requirements.

FIGURE 2

Chromatogram of 20 CV elution gradient using the same prepared AAV8 load over CIMmultus QA monolith, POROS 50HQ resin, and Mustang Q membrane.



AAV8 was produced at a 50 L pilot scale and a 500 L production scale. An 80 mL CIMmultus QA (2 µm) monolith device was used for 50 L pilot production, and an 800 mL device was used at the 500 L scale on an AKTA Process chromatography skid (Cytiva). AAV2 produced at a 50 L pilot scale used 196 mL (10 cm bed height) POROS 50HQ column, while 250 L production scale used 1.5 L (10 cm bed height) column on an AKTA Process™ chromatography skid (Cytiva).

Elutions during scale up runs were already at a neutral pH and did not require any additional adjustments.

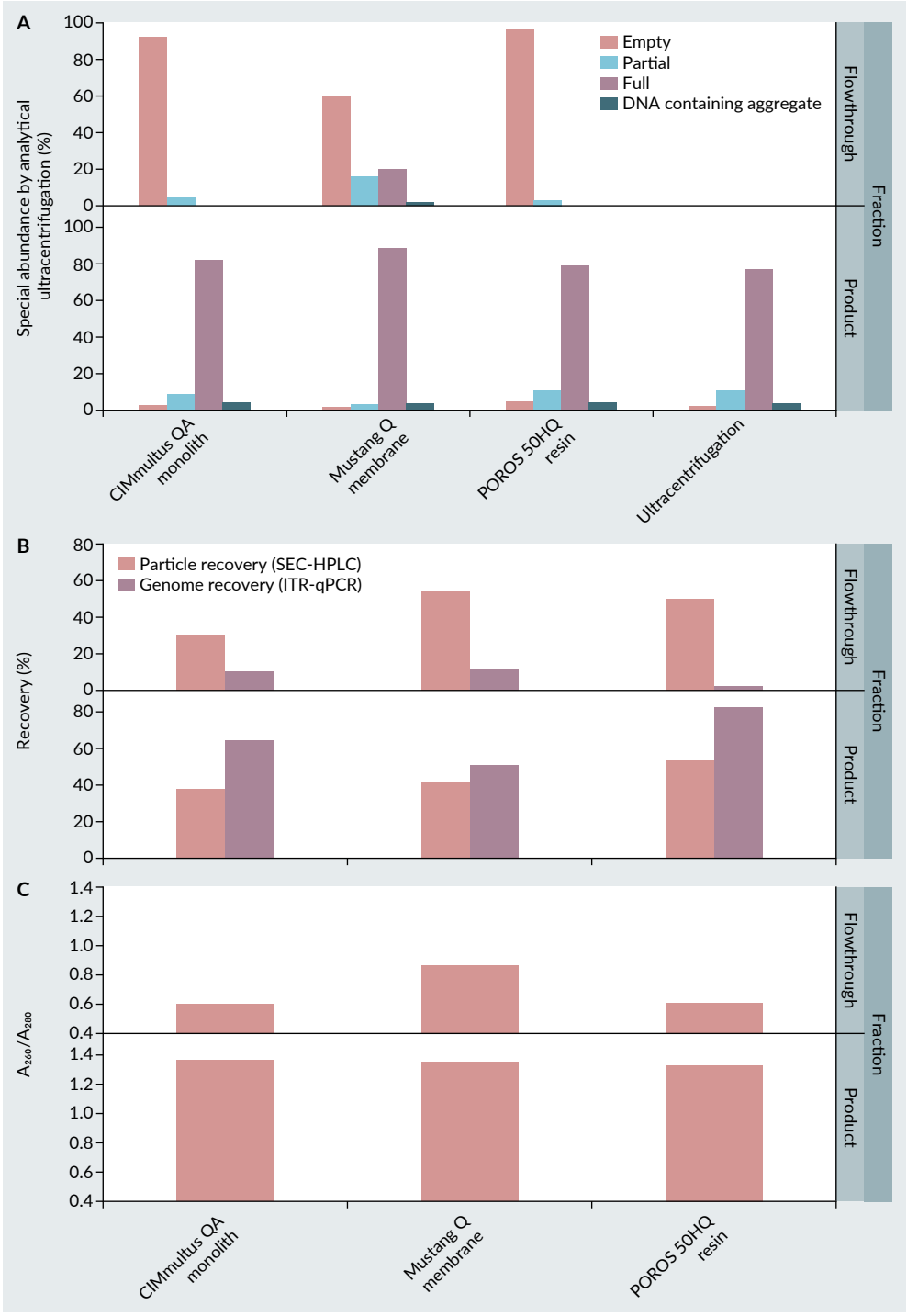
Analytical methods

Viral particle (vp) titers were assessed using an Agilent 1260 Prime II HPLC system (Agilent, Sata Clara, CA) with Agilent Bio SEC-5 4.6 × 150 mm, 500 Å column (Agilent, Santa Clara, CA) to perform size exclusion high performance liquid chromatography (SEC-HPLC). A DAD detector was used to collect traces at 214, 260, and 280 nm. Analysis was performed using Waters® Empower™ 3 software. Viral particle (vp) titers were determined via qualified SEC-HPLC method by integrating the 214 nm peaks and comparing peaks against a standard curve. Standard curves were generated using purified AAV8 or AAV2 for their respective products. The standards had been previously titered by ELISA. Titers generated by SEC-HPLC were used to determine particle recoveries. SEC A_{260}/A_{280} ratios were generated using integrated peak data to provide an estimate of purity for samples not analyzed by analytical ultracentrifugation. All scale up run analyses were performed with qualified SEC-HPLC methods.

Vector genome (vg) titers were assessed with quantitative real time PCR (qPCR) using QuantStudio™ Flex 6 (Thermo Fisher) to determine step recoveries. Custom primers and probes (IDT, Coralville, IA) targeted the ITR region. The forward primer sequence was 5′-GGAACCCCTAGTGATGGAGTT-3′, and the reverse primer sequence was 5′-CGGCCTCAGTGAGCGA-3′. TaqMan™ Fast Advanced Master Mix (Thermo Fisher) facilitated the PCR reaction performed in the thermocycler. The

FIGURE 3

(A) Empty and genome-containing AAV8 capsid distribution in the product (elution pool) by fraction and media type. (B) Recovery of vp and vg in the product (elution pool) by fraction and media type. (C) A260/A280 ratios by both fraction and media type.

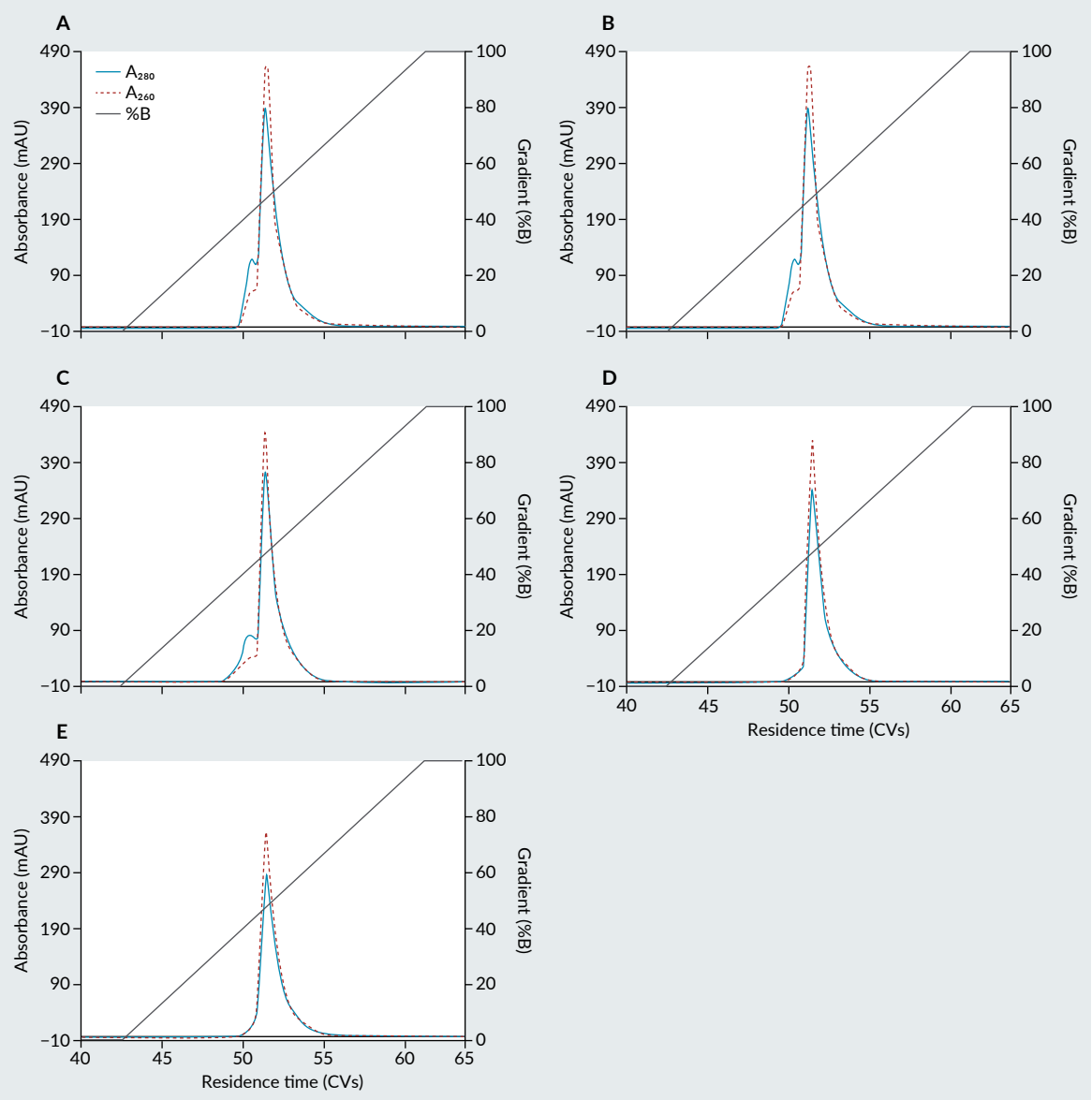


TaqMan probe sequence was 5′-/56-FAM/CACTCCCTCTCTGCGCGCTCG/3BHQ_1/-3.

Qualified ITR-ddPCR was performed for large scale runs using QX200 Droplet Digital™ PCR (ddPCR) system (BioRad, Hercules,

FIGURE 4

Separation of empty and genome-containing AAV8 capsids was modulated by addition of a weak organic acid into the load.



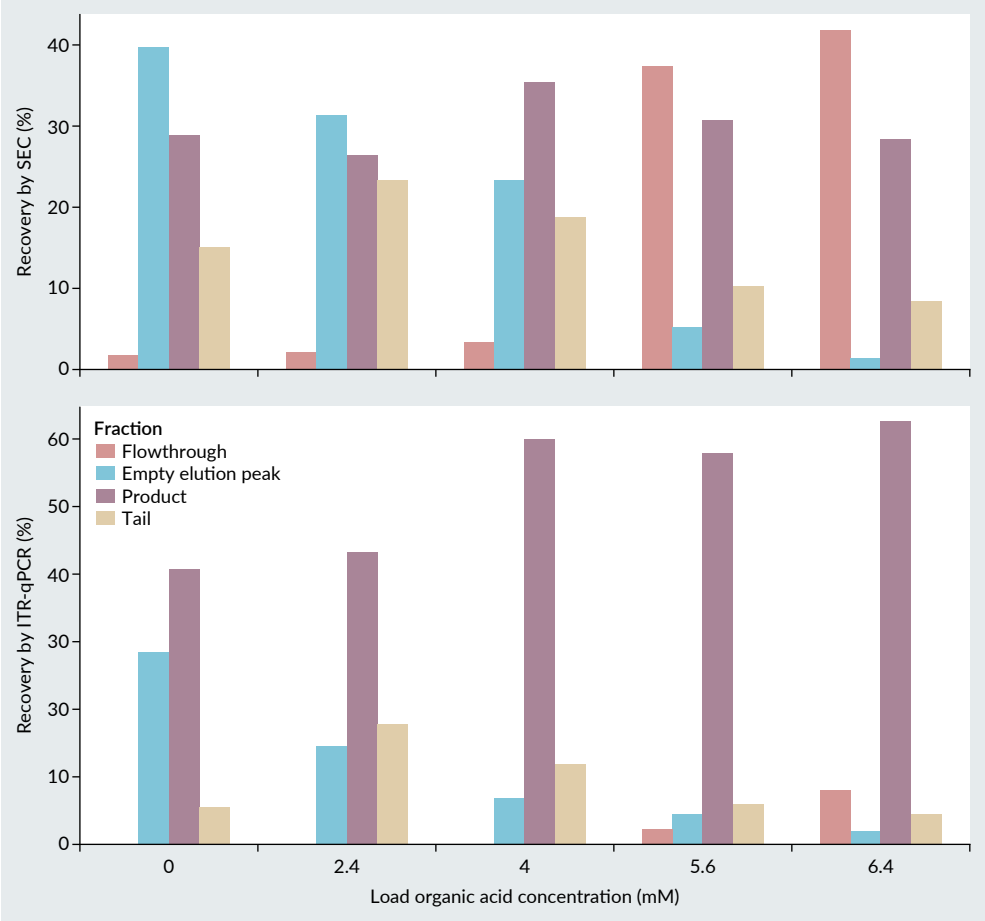
Elution peak chromatograms are shown for loads containing **(A)** 0 mM weak organic acid, **(B)** 2.4 mM weak organic acid, **(C)** 4.0 mM weak organic acid, **(D)** 5.6 mM weak organic acid, and **(E)** 6.4 mM weak organic acid.

CA). Custom, proprietary primers and probes (IDT, Coralville, IA) and TaqMan Fast Advanced Master Mix (Thermo Fisher) were used to perform PCR on droplet samples using a C100 thermocycler (BioRad, Hercules, CA). The QX200 Droplet Reader was then used to detect PCR products and analyzed using QuantaSoft™ RE software (BioRad, Hercules, CA). Trends in recovery were comparable between qPCR and ddPCR assays.

Sedimentation velocity analytical ultracentrifugation (SV-AUC) was analyzed by KBI Biopharma. SV-AUC is a first-principles hydrodynamic technique that determines macromolecular size and conformation directly from a sample in solution. This method is used to assess the viral size distribution and analyze capsid content through the difference in buoyant density between empty and genome-containing capsids. Due

FIGURE 5

Recovery of AAV8 vg by ITR-qPCR and vp by SEC-HPLC titer of pooled fractions with genome-containing capsid enrichment above a SEC A_{260}/A_{280} ratio by SEC-HPLC of 1.20.



to a greater buoyant density, genome-containing capsids sediment more quickly through solution, compared to empty capsids. Capsids sedimentation is accomplished through centrifugation at high angular velocity. The concentration of each capsid distribution is measured as a function of time and radial position using absorbance optics, and then the concentration profiles can be analyzed to provide information about the different capsid size distribution, plotted as a c(s) distribution. The method works by measuring how much of a particular capsid content is present in a sample, by modeling the expected rates of sedimentation and the rates of diffusion. Each peak in the c(s) distribution is integrated and its area (as a percentage of the total area) represents the relative amount of empty, full, and partially packaged rAAV in a sample [23–25].

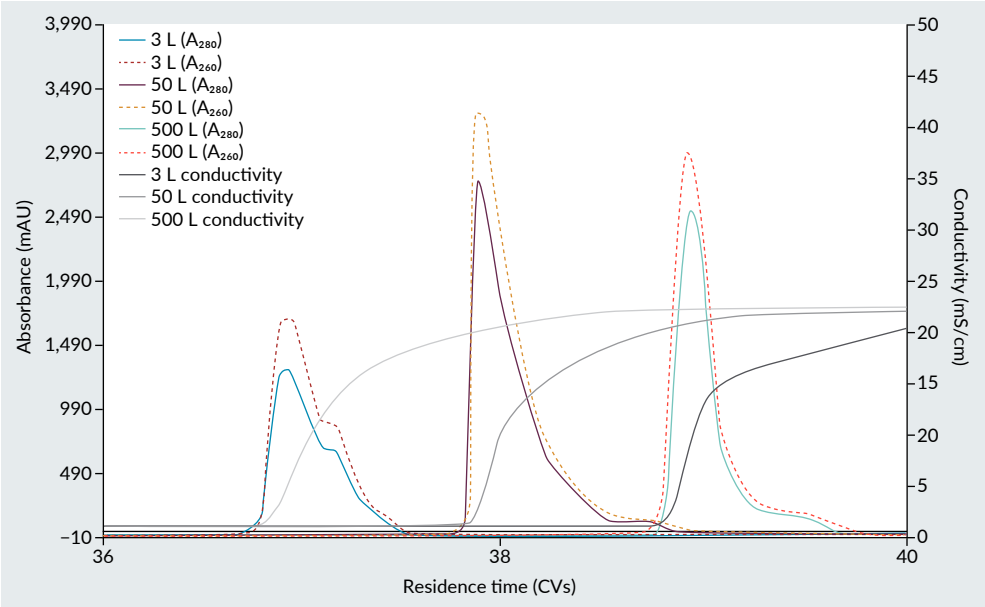
RESULTS

AEX media screening selection for removal of empty AAV8 capsids via impurity flowthrough chromatographic method

POROS 50HQ resin, CIMmultus QA monolith, and Mustang Q membrane modalities were evaluated for separation of empty from genome-containing AAV8 capsids. AEX starting materials were generated by diluting affinity eluate (composed of 32%–44% genome containing capsids by AUC) with a

FIGURE 6

Chromatogram showing overlay of 3 L, 50 L, and 500 L purification runs for an AAV8 capsid with a CIMmultus QA substrate.



pre-defined concentration of weak organic acid (<10 mM), titrating to pH 9.0, and loading at 2×10^{14} vp per mL of media. As shown in Figure 2, each modality exhibited a similar chromatographic elution profile, indicating genome-containing capsids eluting as single peaks. CIMmultus QA monolith showed the lowest band broadening, followed by POROS 50HQ and Mustang Q membrane. AEX elution fractions from the three modalities were analyzed by SEC-HPLC. Fractions greater than 1.20 by SEC A_{260}/A_{280} were collected to generate the AEX elution pool. The A_{260}/A_{280} ratio from each chromatographic modality elution pool was comparable with the A_{260}/A_{280} ratio of the iodixanol enriched genome-containing capsids.

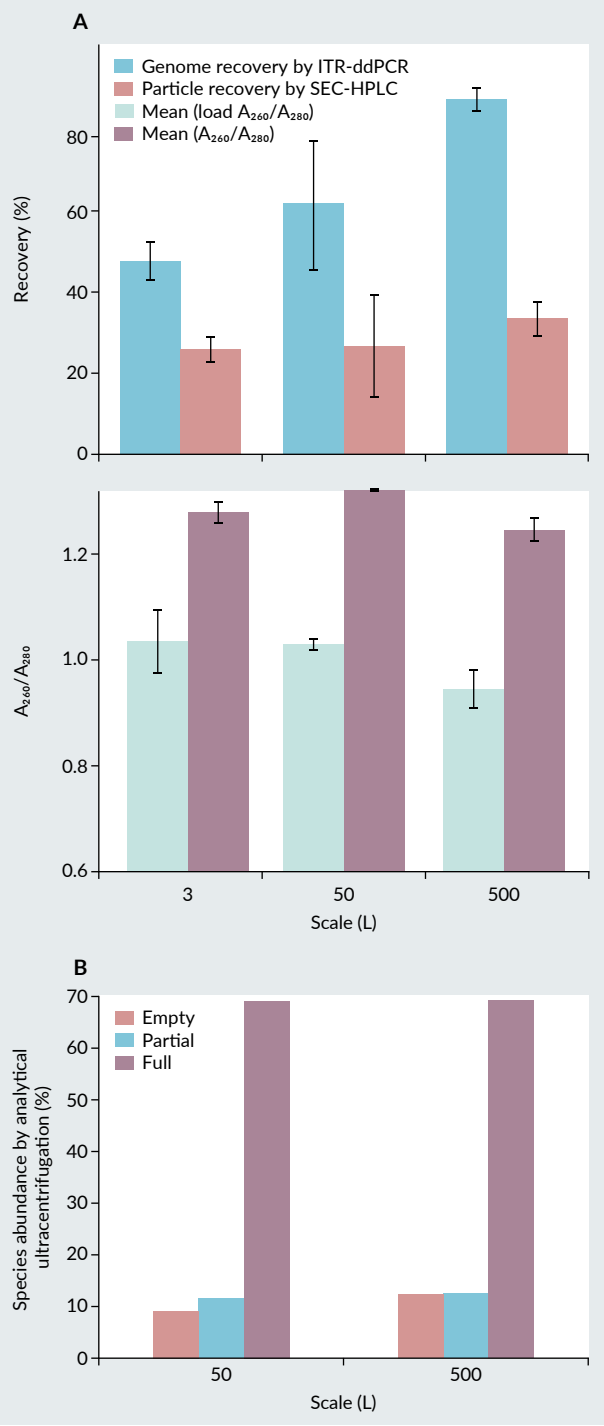
SV-AUC was used to analyze capsid content through the difference in buoyant density between empty, partial and full capsids. Figure 3A shows equivalent enrichment of full capsids in the AEX elution for the three chromatographic media. Capsid distribution (Figure 3A and C) as well as step recoveries (Figure 3B) were comparable between the chromatography-based and the iodixanol-based gradient enriched full capsids.

Optimization of AAV8 empty and genome-containing capsids separation using CIMmultus QA monolith at lab-scale purification (3 L)

Bioreactors were transfected and processed as shown in Figure 1A. CIMmultus QA monolith was chosen as a chromatographic modality due to the lowest band broadening observed during screening studies, comparable product recovery, and comparable enrichment of genome-containing capsids to iodixanol gradient ultracentrifugation. AEX starting materials were generated by diluting affinity eluate with different concentrations of weak organic acid (0 mM–6.4 mM), titrating to pH 9.0, and loading a 1 mL CIMmultus QA monolith at 2×10^{14} vp per mL of media. A 20 CV linear salt concentration gradient was used to elute the product as shown in Figure 4. Elution profile chromatograms indicated a gradual reduction of empty capsids as the concentration of weak organic acids increased in the AEX load, observed by A_{260}/A_{280} changes in the front half of the elution peak (referred to as empty elution

FIGURE 7

(A) AEX step recoveries (elution pool) of AAV8 by ITR-ddPCR and SEC-HPLC, respectively, for 3 L, 50 L, and 500 L bioreactor using CIMmultus QA monolith. Runs were performed with n=6 for 3 L scale, n=3 for 50 L scale, and n=2 for 500 L scale. Error bars show standard deviation as calculated in JMP. (B) Purity of final product (elution pool) by analytical ultracentrifugation. No 3 L bioreactors were analyzed by SV-AUC due to sampling requirements.



peak). AEX fractions greater than 1.20 by SEC A_{260}/A_{280} were collected to generate the product (AEX elution pool). Post product elution fractions with SEC AA_{260}/A_{280} <1.20 were collected and defined as the tail.

The AEX step recovery was assessed by SEC-HPLC (vector particle recovery), and ITR-qPCR (vector genome recovery) as depicted in Figure 5. The highest reduction of empty capsids and maximized product recovery was achieved for the condition that contained the highest concentration of weak organic acid in the AEX load.

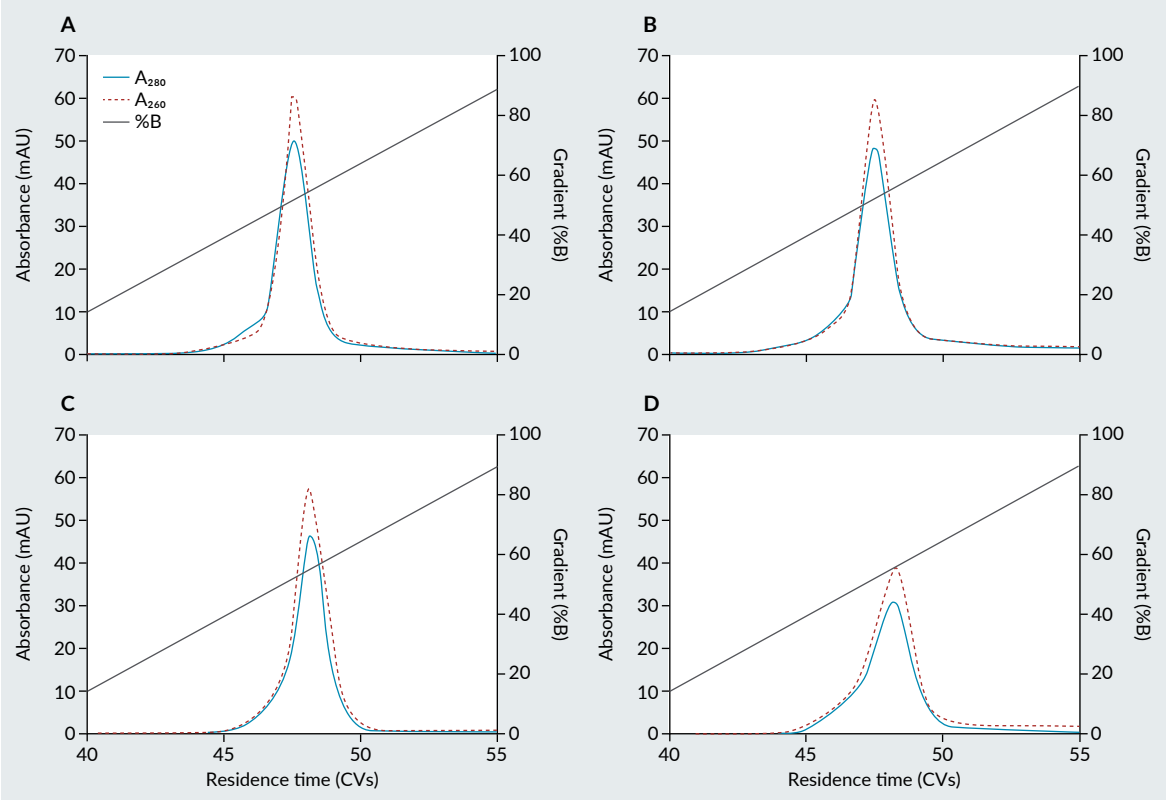
Scaleup of AAV8 empty and genome-containing capsids separation using CIMmultus QA monolith

An isocratic method for AAV8 elution was developed using a CIMmultus QA monolith device after selecting the final concentration of weak organic acid to ensure optimized removal of the empty capsids was achieved during the AEX sample loading phase. Processes that include a step elution are inherently more scalable compared to a linear salt concentration gradient elution due to equipment limitations, buffer mixing from the stationary and mobile phase, and robust product collection criteria. Optimized AEX conditions were evaluated at 3 L, 50 L, and 500 L bioreactor scales, using 8 mL, 80 mL, and 800 mL monolith devices, respectively. Figure 6 shows chromatographic overlay of three comparable elution profiles and consistent enrichment of genome-containing capsids from the 3 L to 500 L purification scale.

AEX step recoveries, assessed by SEC-HPLC for vector particle and ITR-ddPCR for vector genome, were compared across three scales as shown in Figure 7A. CIMmultus QA monolith step recoveries (by ITR-ddPCR) for AAV8 serotype varied from 48% for 3 L scale (n=6) to 89% for 500 L scale (n=2). Genome-containing capsid enrichment, assessed by SV-AUC and SEC A_{260}/A_{280} ratio,

FIGURE 8

Effects of modulating weak organic acid content in the AAV2 load for a CIMmultus QA monolith using a gradient elution method at a concentration of (A) 6.4 mM, (B) 7.2 mM, (C) 8.0 mM, and (D) 8.8 mM.



was equivalent between 50 L and 500 L scales as indicated in Figure 7B.

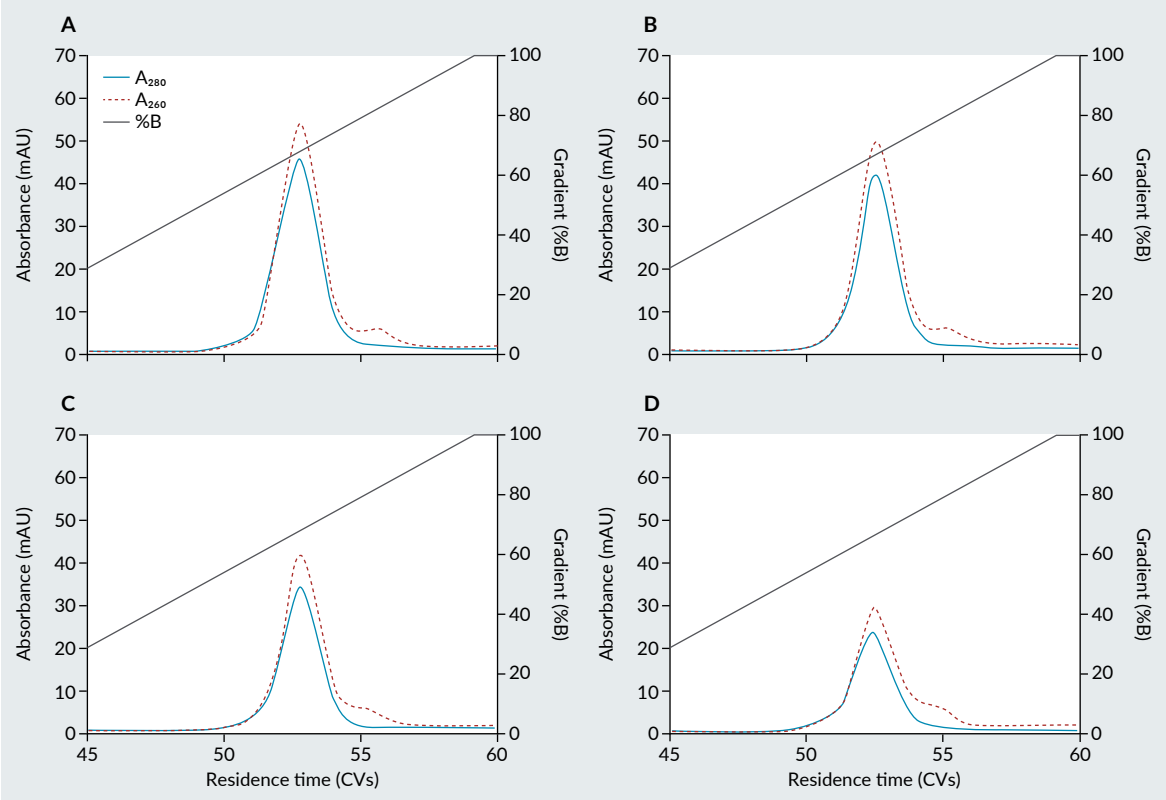
Enrichment of genome-containing AAV2 capsids using POROS 50HQ and CIMmultus QA Monolith

The AEX method developed for enrichment of the AAV8 genome-containing capsids was adapted to an AAV2 vector. Due to differences in charge and hydrophobicity between AAV2 and AAV8 serotypes [21], the AAV2 serotype required modification of the weak organic acid concentration in the AEX load to achieve optimal separation of empty and genome-containing capsids. Three-liter bioreactors were transfected and processed as shown in Figure 1A. AEX starting materials were generated by diluting affinity eluate (composed of 30%–43% genome containing capsids by AUC) with different concentrations of weak organic acid (6.4 mM–11.2 mM), titrating to pH 9.0, and loading on a 1 mL POROS 50HQ or a 1 mL CIMmultus QA monolith at 6×10^{13} vp per mL of media. A 20 CV linear salt concentration gradient was used to elute the product from the CIMmultus QA monolith (Figure 8) and POROS 50HQ (Figure 9). Elution profile chromatograms indicated a gradual reduction of empty capsids as the concentration of weak organic acids increased in the AEX load, observed by A_{260}/A_{280} changes in the front half of the elution peaks.

AEX step recoveries, assessed by SEC-HPLC for vector particle and ITR-qPCR for vector genome, are shown in Figure 10 for CIMmultus QA Monolith and in Figure 11 for POROS 50HQ. AEX fractions greater than 1.20 by SEC A_{260}/A_{280} were collected to generate the AEX elution pool. The highest product recovery was achieved for the condition containing 6.5 mM concentration of weak organic acid in the AEX load

FIGURE 9

Gradient elution profile for increasing concentration of the weak organic acid in the AAV2 load of POROS 50HQ from (A) 8.8 mM, (B) 9.6 mM, (C) 10.4 mM, to (D) 11.2 mM.



for CIMmultus QA monolith or 9.5 mM concentration for POROS 50HQ. Due to slightly higher product recovery and comparable enrichment by SEC A_{260}/A_{280} achieved with POROS 50HQ, this resin was selected for scale up to 50 L and 250 L bioreactors.

Scaleup of AAV2 empty and genome-containing capsids separation using POROS 50HQ

Prior to AAV2 POROS 50HQ scaling, a 20 CV linear salt elution gradient was converted to isocratic step elution by selecting an optimal elution conductivity to minimize operational challenges at the large manufacturing process. Isocratic step elution was performed at the 3 L, 50 L, and 250 L scales, using 5 mL, 200 mL, and 1500 mL POROS 50HQ columns, respectively. The 250 L purification was performed using an oversized column to match operational parameters and be within the loading specifications at 500 L manufacturing scale. Comparable elution profile chromatograms for all three scales are shown in Figure 12.

Comparable recoveries by SEC-HPLC and ITR-ddPCR across three scales are presented in Figure 13A. Greater than 70% AEX step recovery by ITR-ddPCR was conserved between the 3 L and 250 L scale. Large scale runs, 50 L and 250 L, were analyzed for capsids distribution using SV-AUC as shown in Figure 13B. Greater than 80% genome-containing AAV2 capsid enrichments were achieved across all scales with less than 6% high and low molecular mass species content.

DISCUSSION

Experimental results collectively led to successful separation of empty and genome-containing capsids for both AAV8 and AAV2 serotypes. Weak organic acid present in the

FIGURE 10

AEX step recoveries (elution pool) of particle capsids and genome-containing capsids by SEC-HPLC and ITR-qPCR and A260/A280 by SEC-HPLC for the CIMmultus QA monolith modality.

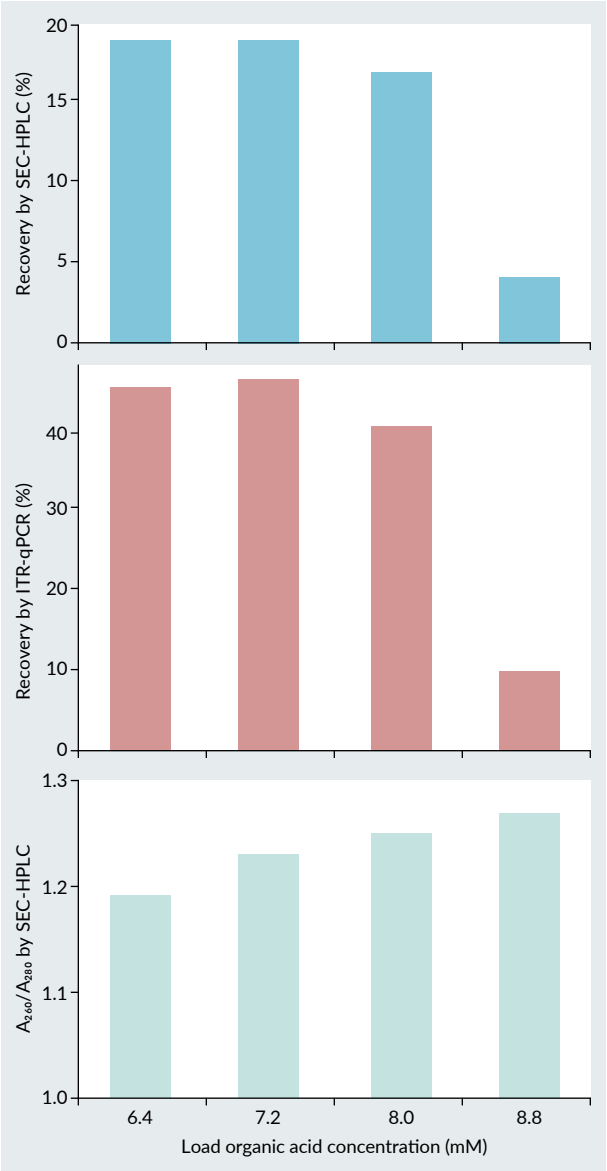
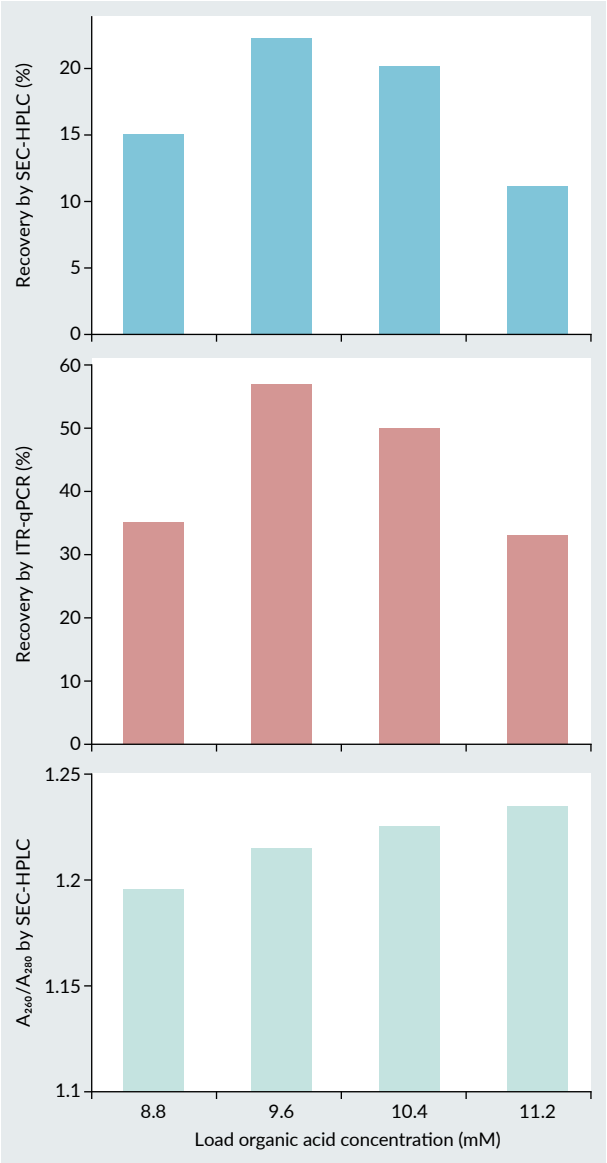


FIGURE 11

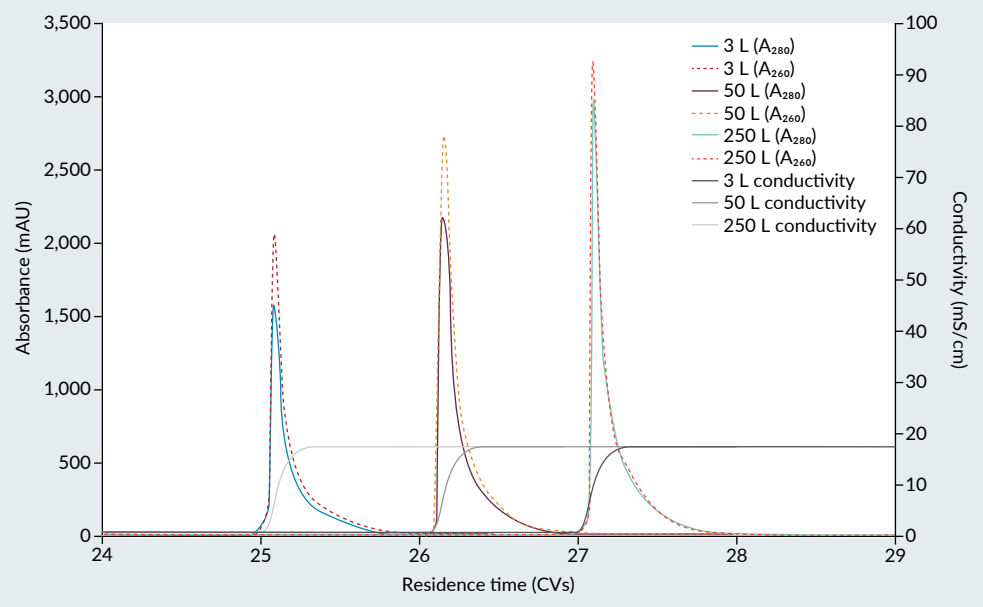
Recoveries of total capsids and genome-containing capsids by SEC-HPLC and ITR-qPCR and A260/A280 by SEC-HPLC for the POROS 50HQ resin modality.



AEX load excluded empty capsids from binding to the media and provided sufficient resolution for chromatographic capsids separation. The hypothesis of the mechanism is that, as the load material is applied to the media, only empty capsids are selectively displaced by weak organic acid due to its higher affinity to the positively charged anion exchange groups. Data from Figure 5 supported the separation was driven by changes in the concentration of weak organic acids and not by changes in conductivity. Increasing concentration of weak organic acid from 2.4 mM–6.4 mM for AAV8 serotype raised the conductivity of the load from 2.022–2.450 mS/cm, and increasing the weak organic acid concentration 6.4 mM–11.2 mM for AAV2 serotype raised the conductivity of the load from 2.276–2.725 mS/cm. These small conductivity differences between increased

FIGURE 12

Chromatogram showing overlay of 3 L, 50 L, and 250 L purification runs for an AAV2 capsid using POROS 50HQ resin.



concentration of weak organic acid are unlikely to be the determinative factor in binding selectivity. Further studies are ongoing to understand the mechanism of separation by utilizing modeling approaches.

AAV8 development and scaleup

As shown in the elution chromatograms in Figure 4, increased weak organic acid concentration diminished the early-eluting empty capsid peak during the 20 CV gradient elution. The reduction of front shoulder peak intensity indicated the concentration of weak organic acid in the load was inversely proportional to the amount of empty capsids in the eluate fractions, as shown by SEC-HPLC analysis. The recovery of genome-containing product by ITR-qPCR also increased as the weak organic acid in the load improved resolution between empty and genome-containing capsids. An optimal concentration of weak organic acid was identified by monitoring the loss of genome-containing capsids in the flowthrough fraction and maximizing capsid enrichment. These results confirmed tuning the concentration of weak organic acid in the load was an effective method to achieve empty and genome-containing capsid separation by displacing the empty capsids into the flowthrough during loading of the AEX column.

Mustang Q membrane showed the lowest recovery during the initial modality screening and was not selected for scale up of AAV2 or AAV8 serotype. Lower step recovery could be due to unoptimized processing parameters or peak broadening, which is observed with membrane geometries [26,27]. However, by modulating the weak organic acid in the AEX load (Figures 5, 10, and 11), the ratio of empty and genome-containing capsids could be tunable for all modalities tested, including Mustang Q membrane. As shown in Figure 2, CIMmultus QA monolith provided a higher resolution and comparable step recovery for AAV8 compared to POROS 50HQ. It was selected for further development of AAV8 empty and genome-containing capsids.

The AAV8 serotype was scaled from 3 L to 50 L pilot and 500 L production process. As shown in Figure 6, the chromatographic elution peaks showed similar shape at all three scales. The 3 L runs were performed at a challenge of

3.2×10^{13} vp per mL of media, while 50 L and 500 L runs were performed at 1.0×10^{14} and 7.2×10^{13} vp per mL of media, respectively. However, lower challenge at the 3 L scale did not affect the chromatographic separation and enrichment of genome-containing capsids.

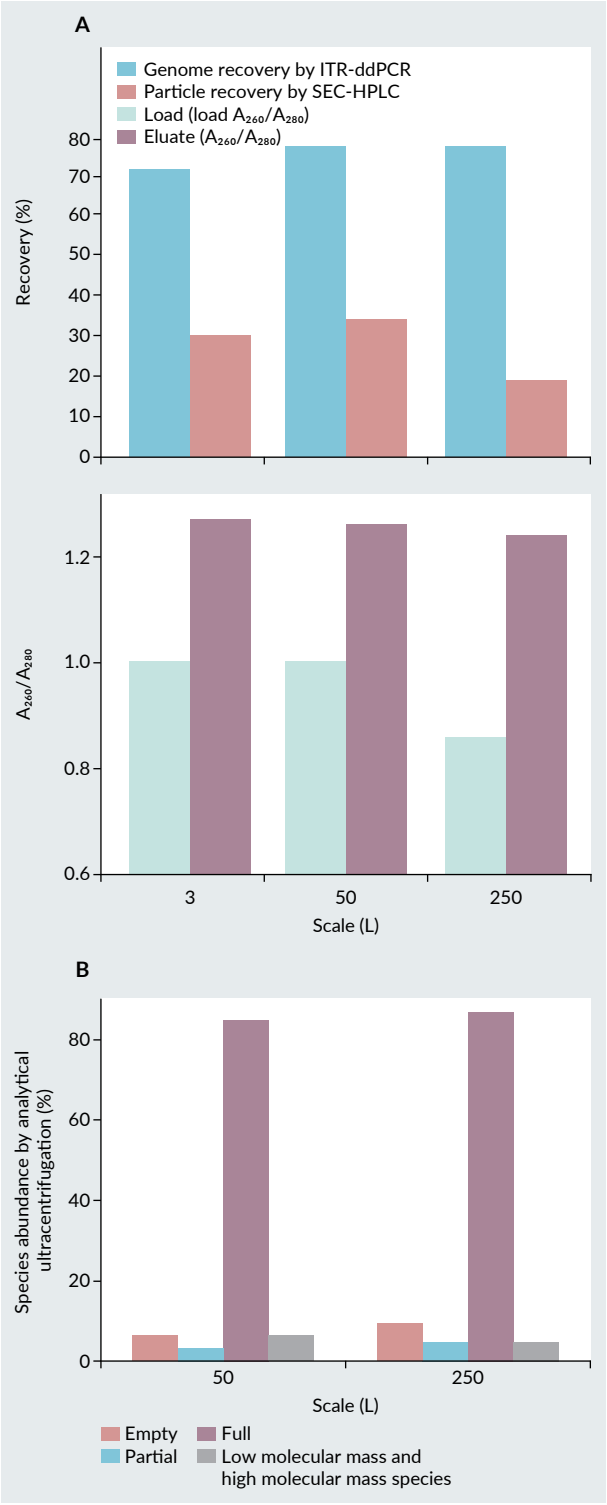
CIMmultus QA monolith step recoveries (by ITR-ddPCR) for AAV8 serotype varied from 48% for 3 L scale (n=6) to 89% for 500 L scale (n=2). This variability may be caused by column loading challenge differences or higher product loss during sampling. Additionally, work with monoliths (data not shown) appeared to demonstrate lot-to-lot variability which may explain some of the difference across various scales of CIMmultus QA monolith, and recent monolith developments may address this variability [28]. As shown in Figure 7, chromatographic separation could effectively remove empty capsids and achieve comparable distribution of empty and genome-containing capsids compared to traditional separation using density gradient ultracentrifugation. This demonstrated the scalability of the AEX method which included the use of weak organic acid to displace the empty capsids into the flowthrough while selectively binding genome-containing capsids.

Adapting AAV8 process to an AAV2 serotype

With an AAV2 product, both CIMmultus QA monolith and POROS 50HQ resin AEX modalities demonstrated separation of empty and genome-containing capsids under the conditions explored. The two modalities showed adjusting the concentration of organic acid in the load could be a suitable approach to improve separation of empty and genome-containing capsids. Due to the higher product recovery provided by POROS 50HQ resin (CIMmultus QA monolith results in Figure 10 and POROS 50HQ resin results in Figure 11) during the initial media screen, this modality was selected for further refinement of weak acids in the AEX load and scaled to a

FIGURE 13

(A) Recoveries of different particle species for 3 L, 50 L, and 250 L bioreactor scale AEX purifications of an AAV2 capsid with POROS 50HQ resin. Runs were performed with an n=1. (B) Analytical centrifugation results for AEX eluates of 50 and 250 L scale runs.



250 L bioreactor. Both the pilot and the production POROS 50HQ run showed comparable elution chromatograms (Figure 12), AEX step recoveries (Figure 13A) and enrichment of genome-containing capsids (Figure 13B). The vector genome (ITR-ddPCR) AEX step recovery varied from 71% for 250 L to 78% for 50 L bioreactor scale. Enrichment of genome-containing capsids was measured by SV-AUC and was consistently above 80%, ranging from 85% for the 50 L scale to 87% for the 250 L scale. This was comparable to the 89% genome-containing capsids achieved by traditional iodixanol density gradient ultracentrifugation preparation. This successful development and scalability mimicked the approach and purification results shown by the AAV8 AEX process.

CONCLUSION

The addition of weak organic acid during AEX load preparation led to a scalable, robust separation of empty and genome-containing capsids. Sufficient removal of the empty capsids into the AEX flowthrough fraction was induced without causing early product release of the genome-containing AAV2 or AAV8 serotype. Selective binding of the genome-containing capsids allowed elution in a stepwise manner while achieving a high AEX step recovery and capsid enrichment. Product enrichment was accomplished across different chromatographic media and scaled to 250 L (AAV2) or 500 L (AAV8) by modulating weak organic acid concentration in the AEX load. AAV8 serotype was enriched from 25%–65% genome containing capsids, while AAV2 serotype was enriched from 40%–80% genome containing capsid. This chromatographic method was successfully converted from linear to step elution and scaled for two serotypes to achieve comparable level of separation to that obtained with iodixanol density gradient ultracentrifugation. This innovative AEX method can serve as a scalable platform approach for separation of empty and genome-containing capsids for other rAAV serotypes.

Chromatographic removal of empty capsids during the AEX load and conversion from linear gradient to step elution can significantly simplify the control strategy for large scale manufacturing. This approach offers a robust, scalable, and cost-effective process with possibilities to utilize versatile elution buffers optimized for different rAAV gene therapy products. This advancement in chromatographic displacement for the enrichment of genome-containing rAAV removes the need to use density gradient ultracentrifugation at large manufacturing scales.

TRANSLATIONAL INSIGHT

An isocratic step-elution based chromatography method that elutes genome-containing rAAV from AEX after displacing empty capsids into the AEX flowthrough can be achieved using any chromatographic skid while achieving product quality requirements. The chromatographic displacement technology can be adapted to all current well-established rAAV scalable manufacturing platform processes. This technology will have a significant impact on future high cell density stable rAAV producer cell line manufacturing processes that transition to continuous processing. Improvements in manufacturing process and technologies will ultimately lead to improved yields, quality attributes, efficiencies and lower COGs for rAAV gene therapy vectors for patients with genetic diseases.

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AUTHORSHIP & CONFLICT OF INTEREST

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

Acknowledgements: The team would like to acknowledge Process Development and Analytical Development groups at Asklepios Biopharmaceutical, Inc.

Disclosure and potential conflicts of interest: Tamara Zekovic, Paul Greback-Clarke, Eric Vorst, Eva Graham, Jordan Hobbs, Robert Tikkanen, Hunter Reese, Rashmi Bhangale, Carlos Cruz-Teran, Christian Denis, Amith Naik, Jacob Smith, David Knop, and Joshua Grieger are employees of Asklepios Biopharmaceutical, Inc. Tamara Zekovic and Joshua Grieger hold the patent: Method for purifying recombinant viral particles, publication number: 20240084268. Paul Greback-Clarke and Eric Vorst hold patent PCT/US2022/013279. Eva Graham holds patent publication 2022/159679. Jordan Hobbs holds patent WO2022159679. Robert Tikkanen has an AskBio associated patent with this technology (publication number: 20240084268). Thomas Guarinoni is an employee of Viralgen, which was partly involved in this work. Jacob Smith holds patent publication number 20240084268, and received stock options from AskBio, Inc. Joshua Grieger is an equity holder of Asklepios Biopharmaceutical, Inc.

Funding declaration: The work included in this article was funded by AskBio by way of employing the authors.

ARTICLE & COPYRIGHT INFORMATION

Article source: Invited; externally peer reviewed.

Submitted for peer review: Jul 23, 2024; **Revised manuscript received:** Oct 9, 2024;
Publication date: Oct 25, 2024.

FASTFACTS

AAV9 capsid enrichment by optimization of anion exchange chromatography

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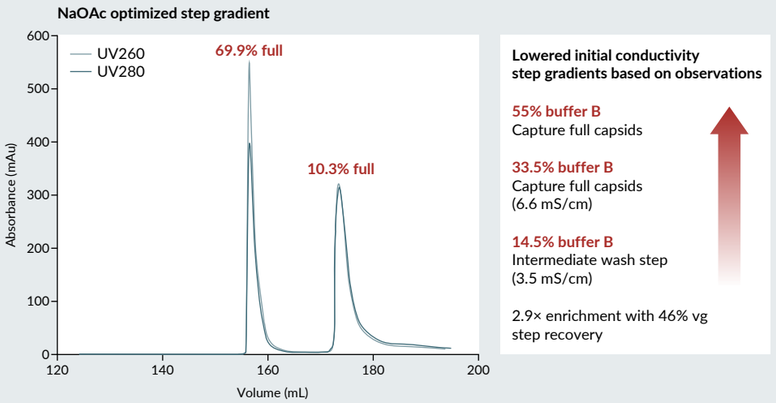
Anion exchange chromatography is widely recognized as an effective technique for achieving the required degree of purity in large-scale AAV manufacturing. This poster describes the development and optimization of an AAV9 full capsid enrichment process utilizing an anion exchange chromatography (AEX) resin.

In this case study, AEX was optimized for AAV9 full capsid enrichment using the Thermo Scientific™ POROS™ HQ AEX Resin. The affinity eluate material contained approximately 25% full capsid and was used as the starting material for the AEX optimization study.

AAV9 CASE STUDY

A screening of elution buffer salt systems to enrich AAV9 full capsids was performed. The salts chosen for the study were 50 mM magnesium sulfate, 200 mM sodium chloride, and 200 mM sodium acetate. The criteria for success for this experiment was to achieve >50% full capsid enrichment with a step recovery of >30%. A 5 mL column was used for testing with POROS HQ AEX resin. The loading buffer contained a mixture of Bis-Tris Propane and Pluronic F-68, and 10 mM $MgCl_2$, and target salt condition was added to the elution buffer. The column residence time was consistent across conditions.

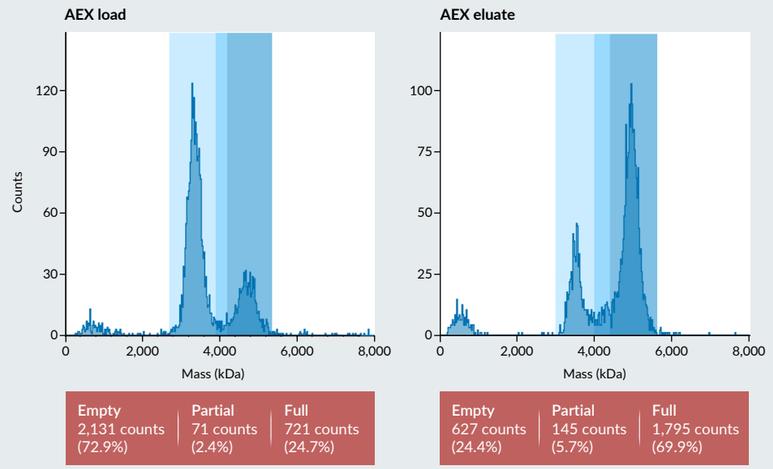
Figure 1. Sodium acetate step elution.



Run parameters	
Column	POROS GoPure HQ, 0.8 × 10 cm, 5 mL
Load conductivity	<2 mS/cm
Column load density	9.7×10^{12} vg/mL-resin or 9.9×10^{13} cp/mL-resin
Buffer A	20 nM Bis-tris propane (BTP), 0.01% (v/v) Pluronic F-68, pH 9.0
Buffer B	Buffer A + 10 mM $MgCl_2$ + 200 mM NaOAc, pH 9.0
Column residence time	2-minute (load), 1-minute (all other phases)
Gradient	Isocratic (14.5% B for wash, 33.5% B for full capsid, 55% B for empty capsids)

Adjusting earlier step gradients increased enrichment and recovery in primary eluate fraction

Figure 2. Mass photometry analysis of samples.



Magnesium sulfate was found to provide insufficient full capsid enrichment (37.3% full) with the operating parameters. Sodium chloride was found to provide good full capsid enrichment (67.7% full) but low vector genome recovery; step yield recovery was only ~10%, so this condition was also insufficient. Sodium acetate was found to provide equivalent full capsid enrichment to sodium chloride (65.9% full) with greater vector genome recovery, meeting the success criteria for both full capsid enrichment and step recovery. Following this optimization, the elution mode method was switched from a linear gradient to an isocratic elution mode to ensure the process was manufacturing-friendly.

A 3-step elution gradient was built out and tested. The original step gradient chosen resulted in lower enrichment than the target results. Therefore, the initial conductivity step gradients were lowered based on observations, shown in **Figure 1**. This adjustment increased enrichment and recovery in primary eluate fraction to above the target.

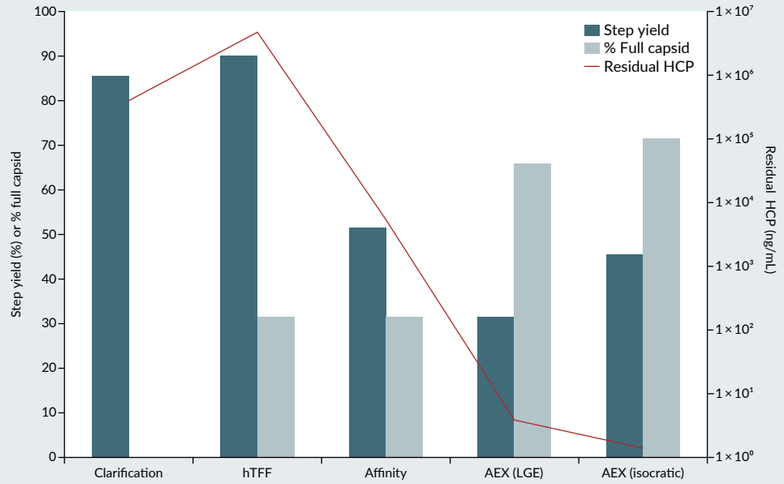
The initial conductivity step gradients were lowered based on observations. This adjustment to earlier step gradients increased enrichment and recovery in primary eluate fraction. Results were analyzed via mass photometry and previous findings were confirmed (**Figure 2**).

SUMMARY

In summary, >2.5× full capsid enrichment via AEX with 46% recovery utilizing a step gradient with sodium acetate as the primary buffer salt was achieved. Residual host cell protein level was effectively reduced by ~6 logs over the entire process (**Figure 3**), demonstrating robust impurity clearance.

POROS resins: pharmaceutical grade reagent. For manufacturing and laboratory use only.

Figure 3. Overall downstream summary.



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Cell & Gene Therapy Insights 2024; 10(9), 1337; DOI: 10.18609/cgti.2024.152

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INTERVIEW

Process optimization for AAV-based gene therapy: insights on downstream purification

In this article, **Srivatsan Ramesh**, Scientist, Downstream Process Development, BridgeBio discusses the critical challenges in downstream purification for AAV-based gene therapies, emerging technologies for viral vector characterization, in addition to methods for the robust translation of pre-clinical programs to clinical/commercial manufacturing. The importance of a holistic approach to process development—where both upstream and downstream processes are co-optimized—is highlighted.

Cell & Gene Therapy Insights 2024; 10(9), 1173–1178

DOI: 10.18609/cgti.2024.134

Q What are you working on right now?

SR: As part of the Technical Development and Operations (TDO)/CMC team at BridgeBio Gene Therapy, we are developing clinical and preclinical AAV-based gene therapies in the BridgeBio pipeline. The workflow presently involves (i) process and analytical development activities and (ii) supporting clinical manufacturing to prepare clinical resupplies to move

"An industry-wide purification challenge is characterizing and clearing mispackaged DNA impurities without affecting the yield of full capsid particles."

the programs further along in the clinical trials. Additionally, through focused analytics and improved characterization techniques, we are deepening our understanding of these vectors and the effects of processing conditions on critical quality attributes (CQAs) and key performance attributes (KPAs). This improved understanding has helped us progress our efforts to develop a platform process for scaled-up AAV cGMP production.

Q Can you tell us more about BridgeBio's pipeline of AAV-based gene therapies?

SR: BridgeBio is advancing AAV-based gene therapies, including BBP-812 for Canavan disease, which is currently in a Phase 1/2 clinical trial. BBP-812 aims to deliver functional copies of the *ASPA* gene to treat Canavan disease, a severe condition beginning in infancy that leads to rapid neuromuscular decline and early mortality. Other AAV-based programs under development include gene therapies for tuberous sclerosis complex (TSC) types 1 and 2, cystinuria, and LMNA-associated cardiomyopathy.

Q What in your view are the key current challenges in the downstream purification of AAV- based gene therapies?

SR: Separating full AAV capsids from empty and partial ones remains a critical challenge in downstream purification for AAV-based gene therapies. Understanding capsid and gene stability/heterogeneity during processing is also essential to prevent post-translational modifications or loss of vector functionality. Versatile platform purification processes that efficiently purify various AAV serotypes are needed, accelerating process development timelines and reducing time to the clinic. Additionally, new technologies must be developed to selectively purify full capsids of novel hybrid viral vectors designed to enhance therapeutic effects. An industry-wide purification challenge is characterizing and clearing mispackaged DNA impurities without affecting the yield of full capsid particles. Compared to monoclonal antibodies, bioreactors' relatively low productivity requires high concentration factors through the downstream purification process.

Q What does the current purification toolkit look like? What advancements would you like to/do you expect to see in the coming few years?

SR: The existing purification toolkit for AAV employs a range of unit operations designed to ensure high purity, yield, and quality of viral vectors used in gene therapy applications. The current toolkit typically includes a combination of ultrafiltration/diafiltration for concentration and buffer exchange and chromatography/ultracentrifugation steps to facilitate impurity reduction and enriching full particles. There are ongoing efforts across the industry to load bioreactor harvest, depending on stability and resin characteristics, directly on the capture chromatography, thereby reducing operation timelines and total effort. The chromatography step either employs monolith columns or resin-based columns. As the industry develops higher-producing AAV bioreactors, the onus would be on the downstream process to transition away from relatively expensive and often caustic-intolerant affinity resins for the capture step. Various mixed-mode chromatographic resins can separate biomolecules or enrich empty and full capsids based on multiple physicochemical properties. They combine interactions like ionic, hydrophobic, hydrogen bonding, and van der Waals forces within a single chromatography resin. Membrane-based chromatography for AAV purification is another promising technology on the commercialization horizon and will allow for reduced processing times and costs. High-throughput screening with automation and AI algorithms will accelerate these efforts. As AAV bioprocessing matures, it is foreseen to take the same developmental path as functional proteins and monoclonal antibodies (mAbs) to go to a continuous processing setup. Although there is proof-of-concept emerging out of academic research labs for using different technologies in AAV downstream purification—namely peptide-based affinity ligands, crystallization, etc.—I am excited for these technologies to mature and demonstrate commercial readiness.

Q What is the role of the upstream and the downstream, including the way they interact, in solving the empty/full/partial problem for AAV? How do you expect the empty/full separation challenge to evolve over the next 5 years?

SR: The interactions between upstream and downstream process development and analytical characterization are essential for understanding and addressing AAV production's empty/full/partial capsid problem. The upstream process influences the proportion of empty, partial, and full capsids generated in the bioreactor, depending on factors such as the choice of transfection agents, optimized plasmid designs, plasmid ratios, cell lines, and production

"Adopting a holistic approach to process development, where both upstream and downstream processes are co-optimized, can substantially increase the efficiency of AAV production and reduce other product-related impurities."

systems (e.g., triple transfection). According to existing literature and current process knowledge, AAV serotypes, DNA vector constructs, and production systems significantly impact the distribution of empty, partial, and full capsid distribution.

When bioreactor harvests provide limited characterization data, data obtained from downstream purification can serve as feedback to refine the upstream process. This feedback can help optimize parameters like DNA input ratios and transfection conditions to better control the encapsidation profile. Improving the selectivity and specificity of purification ligands in the downstream process can enhance the proportion of full capsids in the final drug substance or product.

Adopting a holistic approach to process development, where both upstream and downstream processes are co-optimized, can substantially increase the efficiency of AAV production and reduce other product-related impurities. Developing and integrating more sensitive analytical techniques will enable more precise monitoring and characterization of empty, partial, and full capsids. Moreover, devising processes and potency assays to separate and evaluate the therapeutic effects of empty, partial, and full capsids could transform how the industry approaches this challenge. As the field evolves, control and validation strategies will become more robust, guided by increasing regulatory oversight and explicit guidelines regarding acceptable levels of empty and partial capsids.

Q How can novel technologies enable quicker and more robust translation of therapies to clinical/commercial manufacturing?

SR: Translating AAV-based therapies from research to process development to clinical and commercial manufacturing is a complex process that requires consideration of scalability, quality, safety, and cost–effectiveness. Novel technologies enable quicker and more robust translation across these stages. This can be attained by improving workflows, starting with advanced vector design, aided by next-generation sequencing, to improve transduction efficiency and, thereby, the therapeutic effect while minimizing immunogenicity. More potent vectors could result in smaller dosages, reducing overall manufacturing requirements and the cost of goods. Capsid evolution is another workflow with immense potential to improve the targeting of cells/tissues and transduction efficiencies. As the industry moves forward, we see the adoption of producer cell lines optimized with the machinery to produce genome encapsidated vectors with high productivity. All this will be accelerated and made more accessible with

high-throughput screening platforms for the rapid assessment of variants and AI/ML to identify patterns and predict optimal AAV designs from large datasets of genomic data. Developing a cell line development and purification platform and an arsenal of well-understood characterization techniques will allow for a seamless, fast, and robust translation of pre-clinical programs to clinical/commercial manufacturing. A well-defined and robust platform minimizes variability and rework, enabling efficient scaling by reducing timeline delays during later stages of development. Additionally, minimizing changes to the process from Phase 1 or early animal models will reduce rework for comparability and validation. Approaching the program development life cycle through all these aspects ensures safe and effective AAV therapies can reach patients quickly.

Q What are your key goals and priorities, both in your own work and for BridgeBio as a whole, over the next 12–24 months?

SR: Over the next 12–24 months, my key goals and priorities focus on ensuring the successful execution and expansion of our clinical manufacturing capabilities for our internal AAV programs. This includes supporting clinical resupply and advancing our programs through critical milestones, such as Process Performance Qualification (PPQ) runs and achieving Biological License Application (BLA)-readiness.

Additionally, I am committed to strategically developing a platform purification process to seamlessly integrate the next wave of programs emerging from our research and development pipeline. Ensuring a smooth transition from R&D to clinical and commercial stages is crucial.

For BridgeBio, a future priority is the commercialization of acoramidis, an investigational medication that if approved will be an orally administered small molecule for patients with transthyretin amyloidosis. This will involve developing and executing a commercialization strategy for sustained commercial success, ensuring that all aspects are fully aligned and optimized for long-term impact. These efforts contribute to our overarching mission of delivering transformative therapies addressing genetic diseases to patients.

BIOGRAPHY

SRIVATSAN RAMESH is a versatile scientist specializing in biologics process development and high-throughput analytical method development, with a demonstrated ability to drive innovation and accelerate therapeutic development pipelines. With expertise in viral vector purification, Srivatsan has played a pivotal role in designing, developing, and optimizing scalable downstream processes for therapies treating various genetic disorders and rare diseases. He holds a PhD in Chemical and Biomolecular Engineering from North Carolina State University, Raleigh, NC, USA where his research focused on two areas: the synthesis of stimuli-responsive polymers for applications in membrane purification and drug delivery and the development of biosensors for protein quantification.

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AUTHORSHIP & CONFLICT OF INTEREST

Contributions: The named author takes responsibility for the integrity of the work as a whole, and has given their approval for this version to be published.

Acknowledgements: None.

Disclosure and potential conflicts of interest: The author is an employee of BridgeBio Gene Therapy LLC, an affiliate of BridgeBio Pharma, Inc.

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

ARTICLE & COPYRIGHT INFORMATION

Article source: Invited.

Interview conducted: Sep 5, 2024; **Revised manuscript received:** Sep 11, 2024;

Publication date: Oct 17, 2024.

OCTOBER 2024
Volume 10, Issue 9

CELL & GENE THERAPY INSIGHTS

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INNOVATOR INSIGHT

Extracellular vesicles: setting your path to IND with advanced characterization packages

Davide Zocco

Extracellular vesicles (EVs) are an increasingly promising modality in the cell and gene therapy (CGT) field due to their ability to leverage the body's natural delivery mechanisms. EVs exhibit several unique advantages, such as being immune-silent, having a broad delivery potential for diverse drug molecules, and the ability to cross the blood-brain barrier. Additionally, engineered EVs can carry therapeutic payloads, as demonstrated by successful clinical trials such as exoIL-12™ for treating cutaneous T cell lymphoma (CTCL). However, several challenges remain, particularly in characterization, scalable GMP manufacturing, and establishing functional assays. Advanced characterization techniques such as nano-flow cytometry and omics approaches enable a quantitative assessment of an EV sample at a single particle level, which can help to ensure product quality, streamlining clinical and commercial development.

Cell & Gene Therapy Insights 2024; 10(9), 1273–1287

DOI: 10.18609/cgti.2024.145

THE ADVANTAGES OF EVs FOR DELIVERING THERAPEUTICS

Despite being an emerging field, extracellular vesicles (EVs) could be the next modality in cell and gene therapy (CGT) due to their ability to mimic cells' natural delivery system and carry therapeutic payloads. Firstly, based on scientific data and some promising clinical trials, EVs are immune-silent. For instance, Codiak BioSciences has extensively investigated both preclinical and Phase 1 clinical trials [1], demonstrating that EVs have good tolerability in patients, making them suitable for repeated dosing. Secondly, EVs have broad delivery potential for multiple drug-like molecules. Thirdly, certain types of EVs exhibit specific tropism for various tissues and can

cross the blood–brain barrier. These features can be leveraged to exploit the targeted cell delivery potential of EVs. In essence, the concept revolves around exploiting the body's natural delivery system to design engineered EVs.

The use of EVs as therapeutics or delivery systems has been explored in three approaches. The first approach involves using naïve EVs, purified from a specific cell type, such as mesenchymal stem cells (MSCs), and utilized for their therapeutic potential and regenerative properties.

In other cases, EVs can be engineered specifically to carry specific payloads, either on the surface or within their lumen, even accommodating particles as large as AAVs. This can be achieved through the engineering of the producer cell line.

INNOVATIVE PLATFORM FOR ENGINEERING EVs WITH SPECIFIC PAYLOADS

A novel Xcite® EV platform provides advanced capabilities for engineering exosomes with specific payloads by two key approaches—surface engineering with PTGFRN protein and luminal loading with BASP-1 protein. PTGFRN may be utilized to payload exosomes with antibodies, cytokines, enzymes, and other types of proteins [2]. BASP-1 allows for luminal loading of micromolecules, such as various enzymes, antigens, peptides, and nucleases. Additionally, for smaller molecules, such as oligonucleotides or small peptides, Xcite EV platform also supports efficient surface linkage to exosomes using non-covalent interactions.

Various drug candidates were developed with engEx® platform technology, and are in different development stages, as shown in Figure 1.

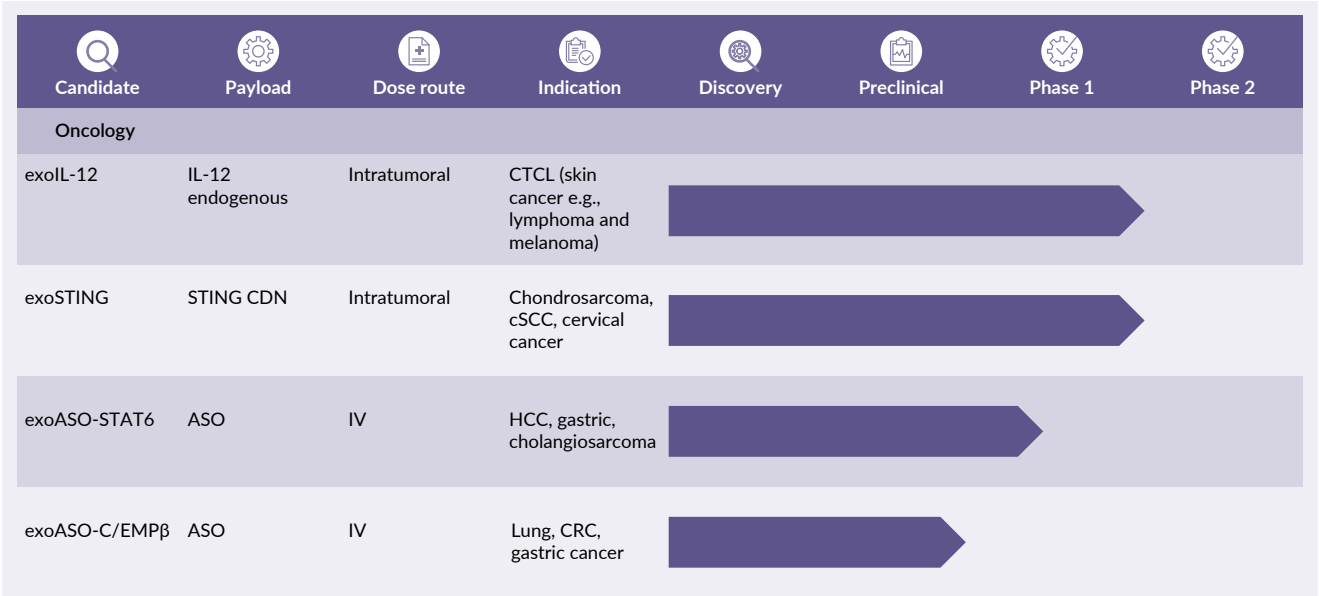
CASE STUDY: EV-BASED DRUG FOR TREATING CUTANEOUS T CELL LYMPHOMA

Although there are currently only a few examples of engineering EVs to deliver therapeutic molecules, one notable example is the first-in-human Phase 1 study of utilizing EV-based exoIL-12™ drug in patients with CTCL [3].

ExoIL-12 contains EVs engineered to express interleukin 12 (IL-12) on their

FIGURE 1

Extracellular vesicles (EV)-based oncological drug candidates engineered using engEx® platform technology.



surface—a crucial cytokine that activates the CD8 cytotoxic immune response. This activation can in turn target and attack cancer cells.

In this case study, Codiak BioSciences observed not only a promising and safe Phase 1 data set but also partial to complete responses in a few treated patients. For example, 3 weeks after the administration, a reduction in lesions was observed in response to the drug. Another important factor is that the IL-12 molecule specifically remained in the tumor, with no detected systemic exposure or inflammatory response. This is significant because IL-12 has previously shown high toxicity levels in clinical trials. In this clinical trial, no adverse events were observed, highlighting the potential of EVs as a promising therapeutic modality.

KEY CHALLENGES IN MANUFACTURING EV-BASED THERAPIES

The development of EV therapeutics poses many significant challenges that must be addressed. Firstly, there is a substantial lack of characterization methods. Although there have been improvements in analytical techniques over the past few years, it remains unclear which tools should be employed for effective EV characterization. Another hurdle is the limited or non-existent access to end-to-end GMP manufacturing expertise, which in turn makes it challenging to scale up the production of EVs. In addition, there is a lack of EV-specific or exosome-specific processes, including those for purification, engineering, formulation, and functional assays. The complexity of EVs makes it particularly difficult to establish functional assays or a matrix of functional assays indicative of a mechanism of action.

OVERCOMING EXOSOME CHARACTERIZATION CHALLENGES WITH NOVEL ANALYTICAL TOOLS

From a molecular standpoint, exosomes are highly complex and heterogeneous particles. Therefore, to fully characterize exosomes, a multi-assay approach is essential. Different assays can be ranked or categorized based on their assessment of primary, secondary, and tertiary attributes. Primary attributes pertain to the biophysical properties of an EV sample, such as size distribution, particle concentration, particle zeta potential, and integrity. Secondary attributes focus on the molecular characteristics of an EV sample. Assays of these secondary attributes help determine the purity of the sample by differentiating EV and non-EV particles, even within the same size range. They also help characterize different EV subpopulations, such as identifying CD9-positive EVs, and molecular cargo of an EV sample. Tertiary attributes address the potency of an EV drug product to a specific mechanism of action and help ensure lot-to-lot consistency, which is crucial for the reliable production of EV-based therapeutics.

A comprehensive set of methods, tools, and analytics dedicated to the characterization of exosomes can allow for a much clearer definition of upstream and downstream processes, ensuring the ability to determine the quality of exosome drug products or stem cells and fully characterize an EV sample. Thorough characterization is a critical success factor that would enable the development of a fully controlled and scalable EV upstream technology, and the selection of unit operations for purifying the exosomes, scaling up, and potentially linking and loading to achieve a specific EV drug product.

More specifically, one key application of analytical tools is in-process monitoring. For example, characterization tools can help determine how to control the process effectively, such as deciding between different unit operations based on whether a specific subpopulation of EVs is being lost, damaged, or not intact anymore. Characterization is also crucial for defining the critical quality attributes of the final EV product. This includes developing release assays that meet regulatory requirements for identity, safety, purity, and potency of the drug product. Lastly, analytics

are required for assessing the stability of drug products and informing product formulation.

NANO-FLOW CYTOMETRY FOR SINGLE-PARTICLE ANALYSIS

One of the advanced technologies for EV characterization is nano-flow cytometry designed specifically for nanoparticles. This technology enables quantitative assessment of an EV sample at a single particle level. Traditional flow cytometry instruments often suffer from the 'swarm effect' which may occur when the laser hits a sample with multiple nanoparticles and detects them as one unique particle. Nano-flow cytometry addresses this issue by utilizing microfluidics and applying appropriate sheath pressure to align nanoparticles within the size range of exosomes or EVs (40–200 nm). This technology allows for obtaining definitive answers when characterizing samples. For instance,

FIGURE 2

EV staining with a mix of anti-tetraspanin antibodies before (left) and after purification (right), visualized using nano-flow cytometry.

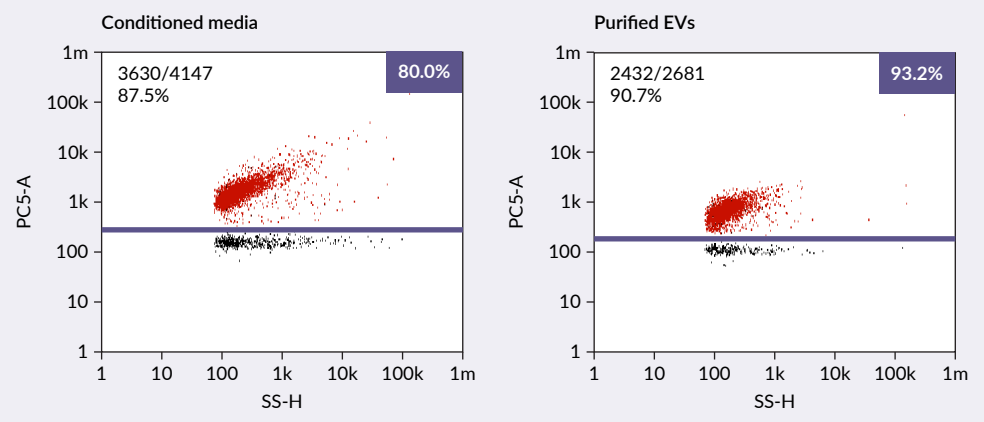


FIGURE 3

Qualitative assessment results of EV integrity using TEM/cryoEM and quantitative analysis results using nano-flow cytometry.

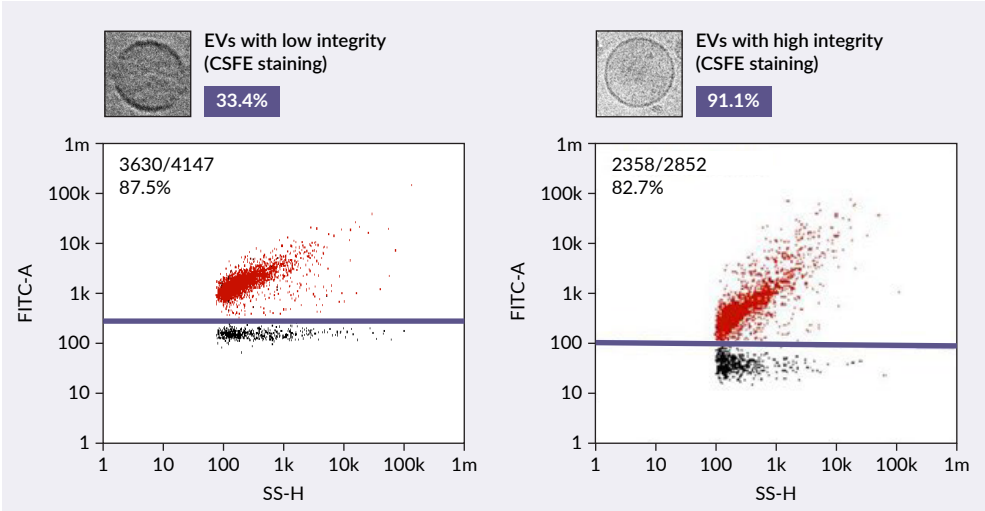


Figure 2 shows how a sample was characterized using anti-tetraspanin antibodies on conditioned media, resulting in 80% staining. After purification, the protein aggregates that typically co-purify or are present in the condition media were removed, as highlighted by the staining of the canonical EV markers, the tetraspanins, increasing from 80–93.2%. This demonstrates the enhanced accuracy and specificity that nano-flow cytometry provides in distinguishing EVs from other components in the sample.

Quantitative analysis of EV integrity with nano-flow cytometry

Another crucial attribute that must be addressed–and is likely to become a requirement from regulatory bodies in the future—is the integrity of EVs. Transmission electron microscopy (TEM) or cryogenic electron microscopy (cryo-EM) may be utilized for a qualitative assessment of EV integrity while nano-flow cytometry may be used for a quantitative analysis of EV integrity, as shown in Figure 3.

In this experiment, an EV with a damaged membrane was assessed using nano-flow cytometry with a CFSC dye, which is only activated in intact EVs by an esterase enzyme. The quantitative results show that in the low-integrity sample, only 33.4% of the EVs were stained with CFSC, indicating a low percentage of intact EVs. In contrast, the CFSC staining percentage in a high-integrity EV sample is 91.1%, which is also visually evident through imaging. Maintaining a highly intact EV sample is crucial for preserving its biological functions. Therefore, having an analytical platform that can quantify the integrity of an EV sample is essential for ensuring its efficacy and safety.

Characterizing EV subpopulations with nano-flow cytometry

Nano-flow cytometry also enables the characterization of different EV subpopulations. Figure 4 shows EVs stained with antibodies targeting an MSC-specific marker CD90 and a more canonical marker CD81. Utilizing HEK 293 EVs, which are not MSC-derived, resulted in minimal staining for CD90 (0.2%), and significant staining for CD81 (65.2%). In contrast, utilizing bone marrow-derived MSC-EVs resulted in 43.8% staining for CD90 AND 67.4% for CD81, with a combined total percentage of 80.8%. As a result, this study suggests that nano-flow cytometry allows for characterizing different subpopulations of EVs present in the sample.

Double-staining at a single-particle level with nano-flow cytometry

Another key feature of nano-flow cytometry is the ability to perform double staining at a single-particle level. This capability allows for detailed analysis of EV subpopulations based on multiple biomarkers.

In Figure 5, an EV sample was stained with an anti-CD9 antibody labeled with Alexa 488 dye and co-stained with an anti-CD81 antibody labeled with phycoerythrin (PE). The results of single staining showed 49% positivity for the anti-CD9 antibody and 70% positivity for the anti-CD81 antibody. When both markers were combined in a double staining procedure, 50.5% of the particles were positive for both CD9 and CD81. The study results show that nano-flow cytometry enables precise identification of particles positive for both biomarkers, providing a clearer understanding of the subpopulations present in the sample.

OMICS APPROACHES FOR CHARACTERIZING EV SUBPOPULATIONS

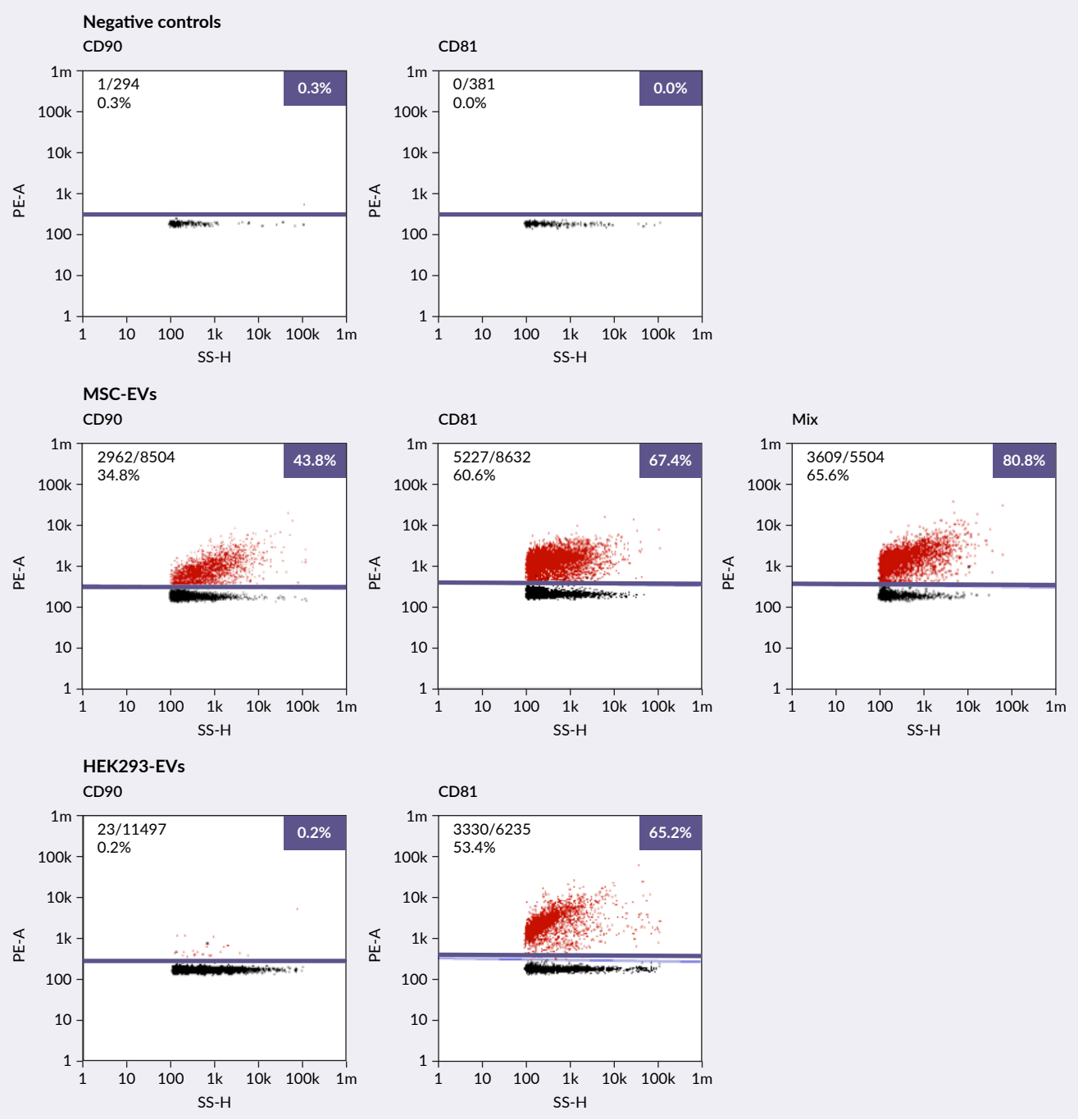
Aside from single-particle analysis for the characterization of an EV sample, omics approaches are becoming increasingly important for initial product characterization. For instance, a nanobeads-based platform can capture specific EV subpopulations, and omics techniques,

such as next-generation sequencing, can be applied to analyze small RNAs, including microRNAs, within the isolated subpopulations. An RNA-extraction-free protocol can be applied to avoid losing RNA in the process and characterize the specific subpopulation.

A comprehensive approach is recommended when characterizing EVs. In an internal study with bone marrow-derived MSC-EVs, two research methods were compared for EV purification: density gradient ultracentrifugation and size exclusion

FIGURE 4

Nano-flow cytometry results showing MSC-derived (middle) and HEK293-derived EVs (bottom), as well as negative controls (top) stained with antibodies targeting CD90 and CD81.



chromatography (SEC). The characterization study involved single-particle analysis using different antibodies targeting CD9, CD63, and CD81, and two different omics platforms developed in-house. One platform was nano-liquid chromatography-mass spectrometry, designed specifically for small amounts of proteins obtained from EV samples. The other platform focused on microRNA sequencing. The results indicated that the samples were similar, with a correlation of 0.76 for proteins, and 0.92 for microRNA. This demonstrates the efficiency of innovative omics technologies in EV characterization.

COMMERCIALIZATION CHALLENGES OF EV-BASED THERAPIES

Companies developing EV-based therapies face multiple challenges during the path to commercialization. Without sufficient proof of concept data needed to generate and raise further funding, companies cannot develop reproducible processes necessary for scaling up to GMP clinical production. As a result, non-GMP processes can lead to substantial rework and delays in obtaining Biologics License Application approval. The key to overcoming these issues is to ensure that the correct steps are taken early in the development process.

Case study: perfusion-based process for continuous manufacturing of EV-based therapies

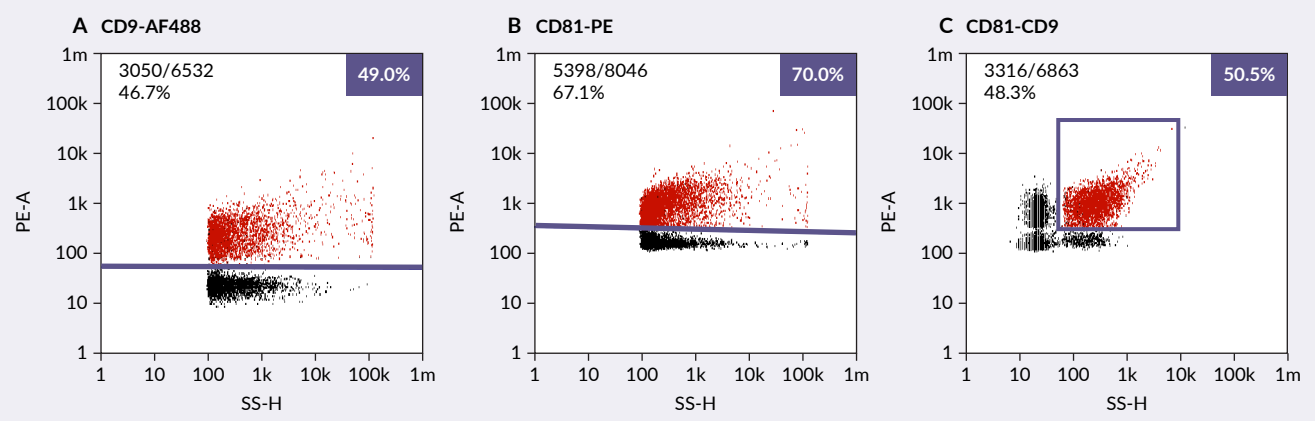
One strategy to overcome commercialization challenges is to utilize advanced characterization and purification technologies to support scalable purification processes. In a case study, exosomes were produced from HEK 293 cells utilizing a perfusion-based bioreactor. The platform supports cell densities of <60 million cells/mL in the continuous bioreactor while maintaining high cell viability and ensuring that apoptotic bodies are minimized in the purified sample. Additionally, the system is designed with a compact footprint and operates with a low residence time. Perfusion occurs daily, with each bioreactor volume being replaced once per day. This system mitigates the re-uptake of EVs by the cells, optimizing the yield of exosomes, and delivering 10-fold higher productivity than traditional fed-batch systems at the same volume.

Establishing qualified release assays for GMP-grade EVs

Qualified release assays are also crucial for qualifying GMP-grade EVs and meeting regulatory requirements. The assays

FIGURE 5

Nano-flow cytometry results showing EV sample stained with an anti-CD9 antibody labeled with (A) Alexa 488 dye, B) EV sample stained with an anti-CD81 antibody labeled with PE, and (C) double-staining with both CD9 and CD81.



should cover essential attributes, including physicochemical properties of the sample (visual inspection, pH, osmolality), EV concentration (measured with nanoparticle tracking analysis [NTA]), size, and aggregation (assessed with dynamic light scattering [DLS]), purity (evaluated with qPCR, residual benzonase assay, or separation ultra performance liquid chromatography [SEP-UPLC]), identity (confirmed through SEP-UPLC, host cell protein ELISA, or western blotting), microbiological testing (assessed with bioburden and bacterial endotoxin assays), and potency assay.

Stability studies for EV-based therapies to meet regulatory compliance

Another crucial aspect, while often less discussed, is the design of stability studies for EV-based therapies. For instance, the

FIGURE 6

Stability study results of PTGFRN-loaded exosomes over 24 months at −70 °C using three different techniques for concentration and size: (A) NTA, (B) WES, and (C) DLS.

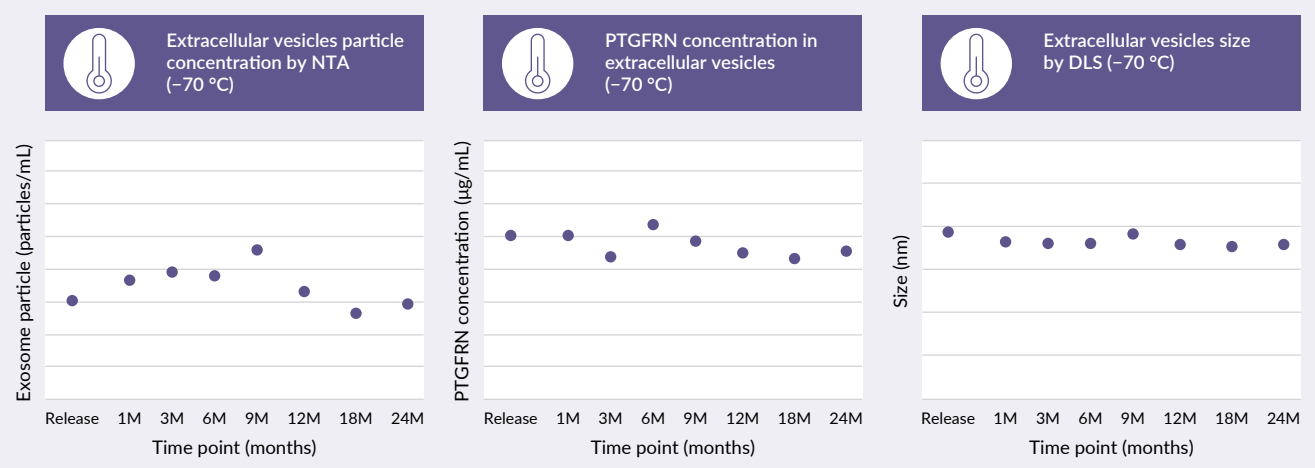
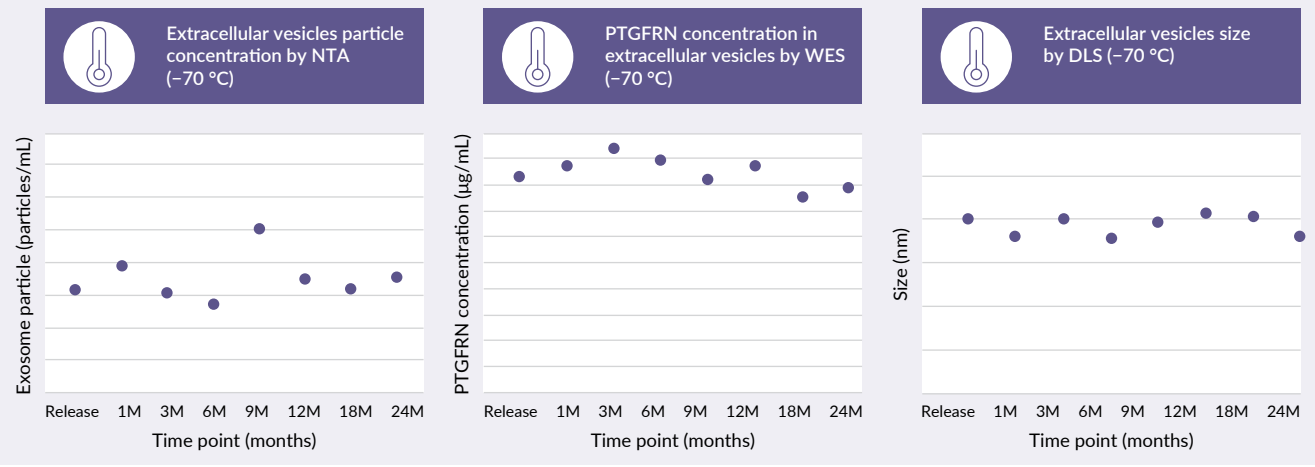


FIGURE 7

Stability study results of PTGFRN-loaded exosome drug substance intermediate over 24 months at −70 °C using three different techniques for concentration and size: (A) NTA, (B) WES, and (C) DLS.



stability study conducted on an EV batch with a loaded drug product developed by Codiak BioSciences utilized various analytics to assess the stability of the PTGFRN protein-loaded exosomes within a 24-month timeframe. It was discovered that PTGFRN-loaded exosomes remained stable over 24 months at −70°C based on data obtained through NTA, Western Epitope Specificity (WES), and DLS studies (Figure 6).

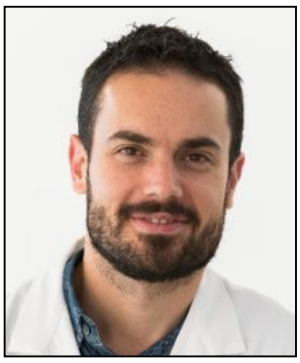
The same stability study was applied to the drug substance intermediate, specifically to the EVs immediately following the purification before loading with the antisense oligonucleotide. As seen in Figure 7, the drug substance intermediate was also stable over 24 months at −70 °C.

This expertise can be leveraged to design stability studies and release testing, which are critical for regulatory compliance.

SUMMARY

Comprehensive EV characterization tools enable the establishment of critical baselines necessary for the development and optimization of upstream and downstream GMP-grade processes, helping to overcome the challenges associated with the therapeutics' path to clinical and commercial success.

Q&A



Davide Zocco

Q Does Lonza offer development services outside GMP manufacturing contracts?

DZ: Our end-to-end platform can serve a wide range of clients, whether they require non-GMP development services or full GMP manufacturing. Lonza has invested significantly not only in analytical tools and small-scale processes, but also in manufacturing facilities supporting both GMP and non-GMP needs. Additionally, our comprehensive platform includes advanced technologies such as perfusion-based cell culture and chromatography-based purification processes. Furthermore, Lonza also has a quality control laboratory and capabilities for linking and loading EVs.

Q Why is it critical to assess the integrity of EVs?

DZ: There is growing evidence that the integrity of EVs is closely linked to their biological activity. Our observations indicate that a suboptimal process can result in poor integrity, even if some level of bioactivity is present. While regulatory bodies have not specifically mandated how to address EV integrity, we believe it will become an important topic in future discussions.

Q Is it possible to purify and characterize EVs from biological fluids or matrices?

DZ: We have experience purifying EVs from biological fluids and complex matrices, such as plasma, milk, and urine. Although the purification technologies for these complex matrices present additional challenges, our technologies and analytical tools can be applied to purify EVs from these sources as well.

Q Why is 3D manufacturing the preferred method for EV development?

DZ: It is well established in the field that achieving high productivity and sufficient amounts of EVs necessitates utilizing 3D bioreactor-based systems. This approach is crucial not only for HEK 293-based EV platforms but for other cell types as well, including MSCs and adrenal cells. The 3D method using micro-carriers ensures the process can be scaled up, which is crucial for clinical-stage manufacturing.

Q Can Lonza provide manufacturing and characterization services for cosmeceutical companies?

DZ: The previously mentioned analytical platforms and purification processes can be adapted for the cosmeceutical industry. Both upstream and downstream technologies can be effectively applied to the cosmeceutical sector for high-quality production and analysis.

Q Can Lonza offer regulatory support specific to EVs?

DZ: We have a dedicated regulatory team at Lonza and can also leverage regulatory experience from Codiak BioSciences. Given the early stage of the EV field, it is crucial to engage with regulatory agencies, such as the US FDA and EMA to ensure they are well-informed about EV therapeutics. Our approach involves initiating early discussions with the regulatory bodies to assess the safety and efficacy of EV therapeutics.

Q Which nano-flow cytometry instruments for EVs are available on the market and do any of them have sorting capacity?

DZ: Currently, there are at least three specialized nano-flow cytometers designed for EV analysis that can effectively meet sorting capacity needs. One good example is NanoFCM NanoAnalyzer, but we there are also other emerging technologies, such as the nano-flow cytometer from Beckman Coulter. In any case, none of these instruments have sorting capabilities.

Q Can a perfusion bioreactor system be utilized to scale up the production of EVs?

DZ: By utilizing the perfusion bioreactor system, it is possible to scale up to 500 L in our GMP facility. For smaller-scale needs, we can operate down to 3 L, with intermediate scales commonly including either 10 L or 50 L.

Q Is it possible to use the linking-loading platforms to evaluate if they work for a specific target?

DZ: The assessment is straightforward and can be adapted depending on the type of the loaded molecule. We will determine the most suitable linking-loading technology from those available, tailoring the approach to achieve the highest efficiency and bioactivity. Maximizing payload on an EV is crucial because bioactivity aligns with the desired effect on target cells.

Q Can EVs be manufactured from any cell line?

DZ: While our current platform is based on HEK 293 cells, it is also adaptable to other suspension cell lines. Additionally, the process could be modified to support the growth of adherent cells such as MSCs and iPSCs, by using microcarriers. Our Bioscience Tissue Acquisition team has expertise in different cell types, their growth, and expansion. From a platform and capacity perspective, we can provide a viable path for most cell types, though specific process optimization may be required for some.

Q What is the sufficient amount of high-quality EVs for standard characterization in early-stage research?

DZ: Firstly, it is essential to review NTA or nano-flow cytometry data and assess particle concentration. If available, providing MicroBCA™ data for protein concentration would

also be helpful for an early evaluation of the sample purity index as a proxy. Based on this initial assessment, we can estimate the EVs required for each assay selected by the customer

Q Is western blot an appropriate method for semi-quantitative analysis of EV markers?

DZ: We utilize WES for digital western blotting, which operates through capillaries and provides a quantitative assessment of protein bands. Traditional western blotting, often quantified using ImageJ, can be semi-quantitative. For accurate results, it is essential to avoid overexposure of the blot to ensure fair comparison. Conventional western blotting remains a valuable and cost-effective tool, and we recommend incorporating it in the initial phase of protein assessment. For proteins located in the lumen of EVs, which cannot be assessed using nano-flow cytometry, we can assist in selecting the appropriate antibodies and advise on the protocols for western blotting.

Q What is a typical in-process testing for EV manufacturing?

DZ: For in-process testing, we currently employ a combination of NTA, nano-flow cytometry, and SEC UPLC. NTA provides a robust method for assessing size and concentration. Though it is slightly less precise than nano-flow cytometry, NTA can still be utilized for getting results from in-process samples. SEC UPLC is utilized for evaluating protein aggregates and contaminants by analyzing the shoulders of protein peaks and their elution profiles at 280 nm. This technology can help to monitor the depletion of impurities and confirm the presence of a pure EV peak as the purification process progresses.

Q Can you perform characterization assays for different types of nanoparticles in addition to EVs?

DZ: Nano-flow cytometry can be applied to various types of nanoparticles, including lipid nanoparticles (LNPs), viral vectors, and synthetic particles. We have established a strong relationship with nano-flow cytometry, as they have developed specialized protocols for comprehensive analysis of LNPs and viral vectors.

Q Are there specific conditions where EV therapy is more suitable than other CGT therapeutics?

DZ: One of the major challenges that EV therapies face today is their lack of tropism—the ability to target specific tissues or organs. There is not enough evidence showing that EVs go beyond liver or spleen absorption, which is similar to other nanoparticle-based systems used in intravenous (IV) applications. Therefore, understanding and improving EV tropism is

crucial for expanding their therapeutic potential. Nonetheless, there are still promising applications for EVs that do not rely on IV administration, including vaccine development for viral infections, topical treatments for wound healing and regenerative therapies, and intratumoral injections, particularly for skin cancer. While systemic IV delivery remains a challenge, focusing on these non-IV routes could provide valuable insights and pave the way for broader use of EV therapies.

Q In general, what are the therapeutic advantages of EVs over other existing therapies?

DZ: The primary advantage of EVs is their safety and limited adverse events compared to other therapeutics, which could in turn allow for repeated dosing. Furthermore, EVs offer the potential to deliver vaccines that can activate mucosal immunity more effectively than LNPs, for example. Finally, a naturally complex composition of EVs and their ability to interact with various biological pathways can be leveraged to deliver multifunctional effects beyond the capabilities of simpler therapeutics like antibodies or small molecule drugs.

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BIOGRAPHY

DAVIDE ZOCCO, Head of Exosomes Development and Site Head of the Lonza site in Siena, Italy, leads R&D programs and process development services to support companies developing exosome-based therapeutics. He was board member at his former company, Exosomics, Lonza's strategic R&D partner. He is an author of peer-reviewed publications in high profile journals which include *Nature Chemical Biology*, *Proceedings of the National Academy of Sciences*, and *American Journal of Pathology* (>5000 citations), and of four patents in the extracellular vesicle field. He achieved a PhD from University College Dublin, Dublin, Ireland and previously a post-doctoral research fellow at Harvard Medical School, Boston, MA, USA.

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AUTHORSHIP & CONFLICT OF INTEREST

Contributions: The named author takes responsibility for the integrity of the work as a whole, and has given their approval for this version to be published.

Acknowledgements: None.

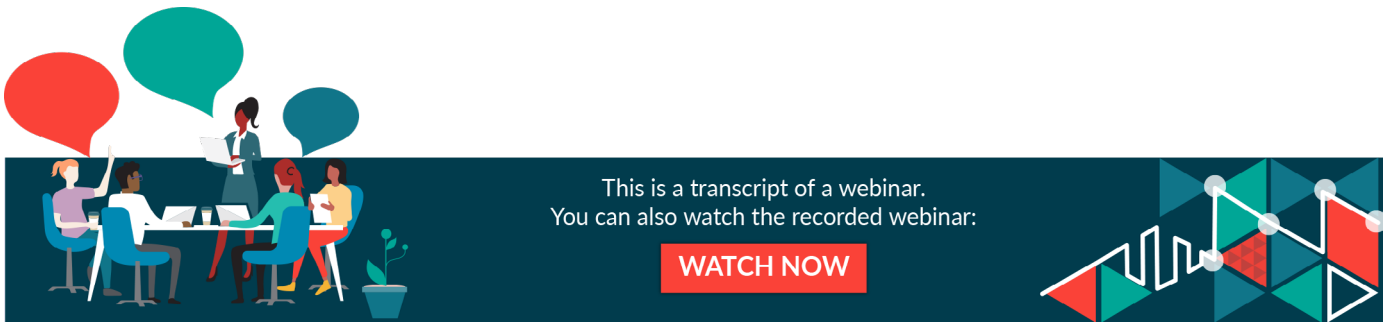
Disclosure and potential conflicts of interest: Zocco D is a Lonza employee and stock options holder, and a Exosomics SpA board member.

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

ARTICLE & COPYRIGHT INFORMATION

Article source: This article was based on a webinar, which can be found here.

Interview conducted: Aug 28, 2024; **Revised manuscript received:** Oct 7, 2024;
Publication date: Oct 28, 2024.



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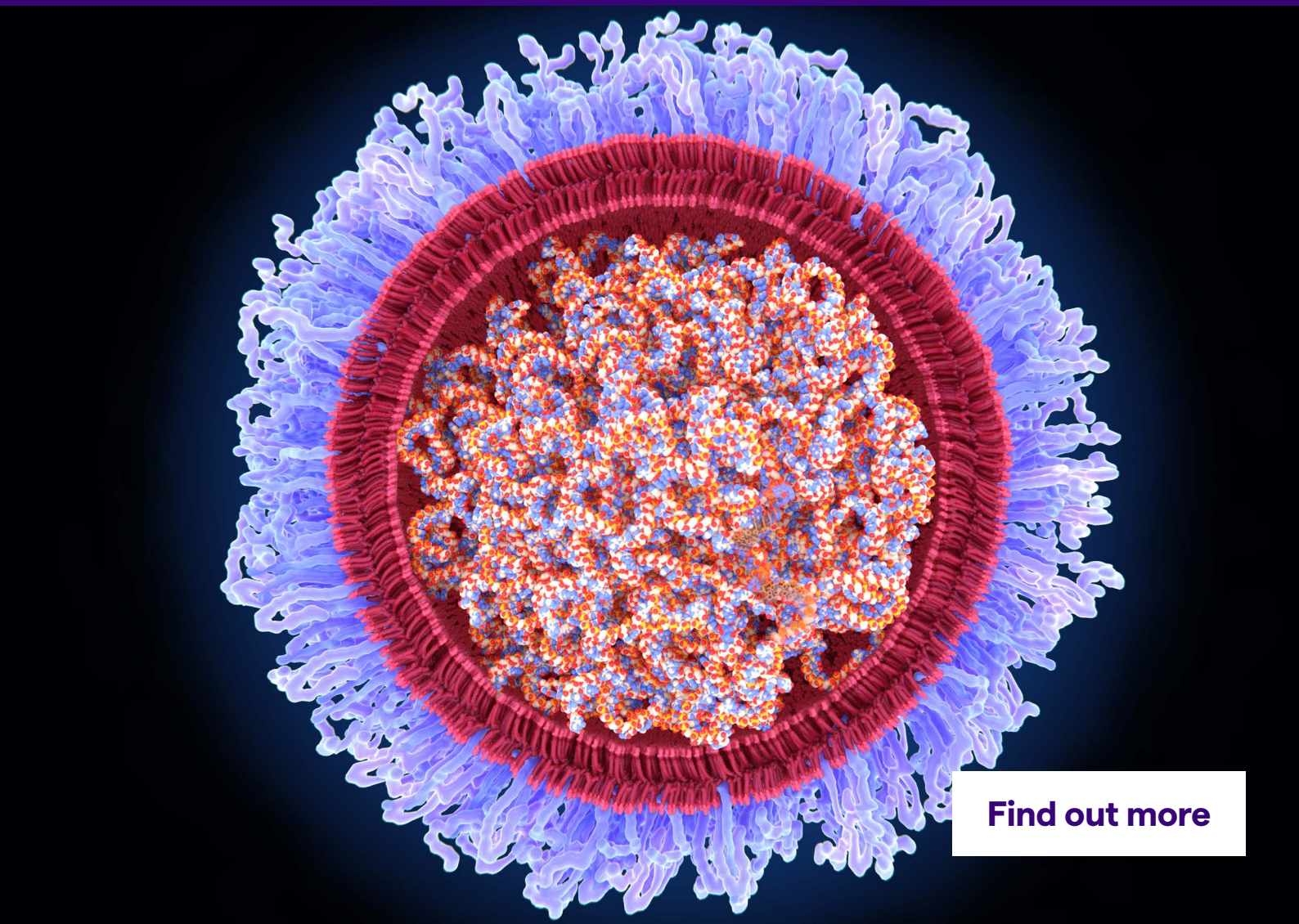

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Control your AAV titers with in-line UV-Vis analysis and PAT-driven UF/DF systems

Brandon Goldberg

Ultrafiltration/diafiltration (UF/DF) is a critical step in bioprocessing, especially in gene therapy. However, traditional methods often face significant challenges due to process variability and a heavy reliance on off-line titer analysis. This dependency can lead to delays and inconsistencies, hindering the ability to achieve precise and reproducible results. This article explores a novel tangential flow filtration system that leverages real-time, in-line titer measurement through in-line variable pathlength spectroscopy to control the UF/DF process.

Cell & Gene Therapy Insights 2024; 10(9), 1121–1133

DOI: 10.18609/cgti.2024.127

TANGENTIAL FLOW FILTRATION SOLUTION

Biomanufacturers face a number of challenges when utilizing current mass dependent tangential flow filtration (TFF) systems, including fragmented processes, variability in measurements, and a high risk of human error (Figure 1). Solving these challenges requires novel methods with features such as real-time in-line product samples and testing, automation instrumentation, analytical assays, continuous bioprocessing, and validation services. Two existing Repligen products—the KrosFlo® KR2i system and the CTech™ FlowVPX® System (Figure 2)—can successfully be used in combination to achieve these goals and address the challenges of TFF systems.

The KrosFlo KR2i TFF system is an automated lab-scale TFF system used in downstream applications, while the CTech FlowVPX system is an in-line ultraviolet-visible (UV-Vis) spectrometer with the unique ability to vary pathlength. Together,

they make up the 'real-time process management' (RPM™) System (Figure 3), which provides process management to the ultrafiltration/diafiltration (UF/DF) process through concentration measurement control, all from a single control platform.

This integrated system strengthens process controls, generating high-quality and highly reproducible results. In addition, the RPM system increases process efficiency and reduces cycle time through in-line analytics with the KrosFlo RPM software platform. This newly developed flexible plug-and-play software platform provides real-time data for every step of the filtration process, automatically generating graphs, charts, and trends.

The flexibility of the RMP system is further demonstrated with the integrated solutions either having the KR2i TFF system, which is a peristaltic pump, or the KrosFlo FS 15 TFF system, which is a diaphragm pump. Users can put the FlowVPX on either system with either a hollow fiber or a flat sheet cassette, along with single-use flow paths and

FIGURE 1

TFF challenges.

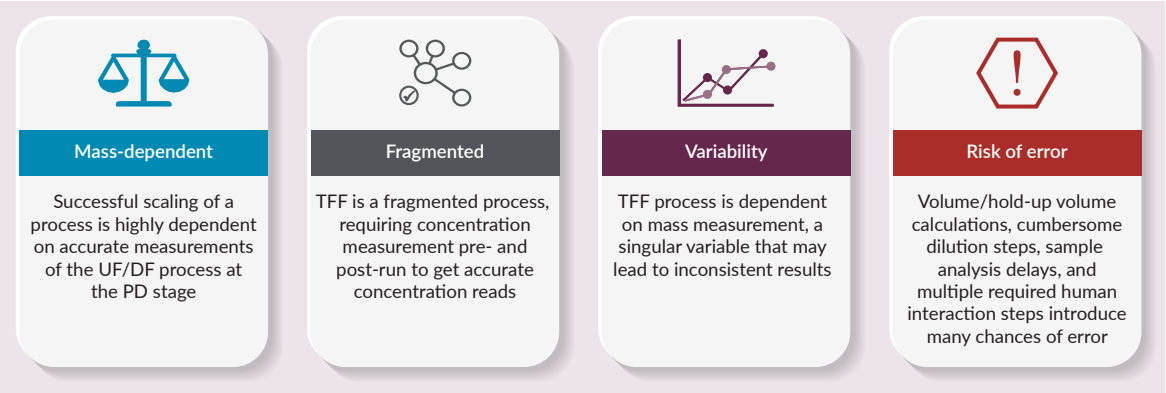


FIGURE 2

The KrosFlo® KR2i system and the CTech FlowVPX system.

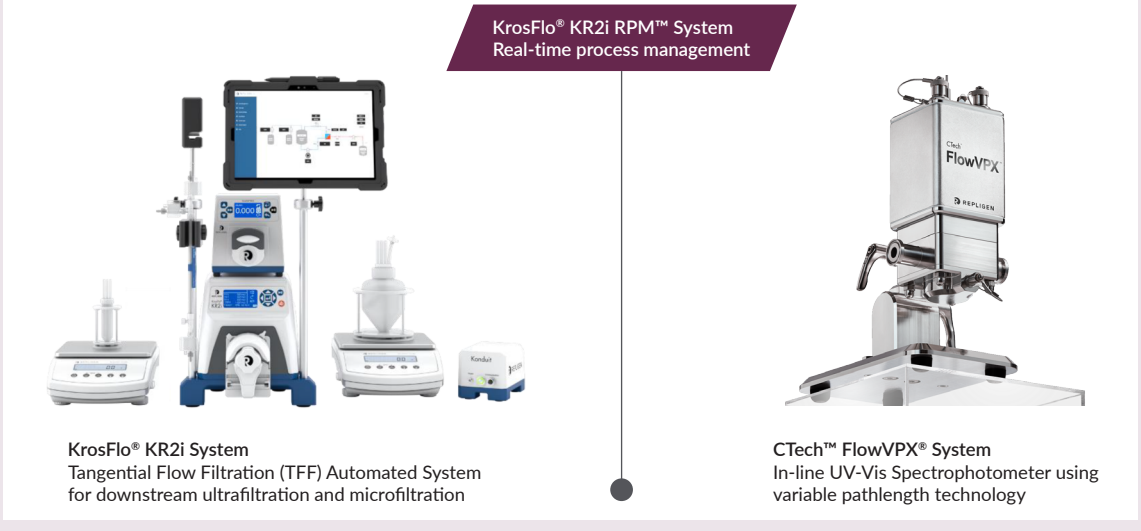


FIGURE 3

KrosFlo KR2i RPM system.

single-use components. These components are scalable whether you are in lab scale, pilot scale, or commercial scale.

KROSFLO KR2i SYSTEM OVERVIEW

The first part of the KR2i RPM system is the KrosFlo KR2i system, a turnkey benchtop TFF system with walk-away automation. Thirteen automated process control modes help with concentration, dye filtration, and cleaning or membrane studies. The system's robust processing allows for processing volumes from 10 mL–10 L, meeting both lab and clinical production requirements and enabling low volume and high concentration

FIGURE 4

Traditional UV-Vis spectroscopy, based on Beer-Lambert law.

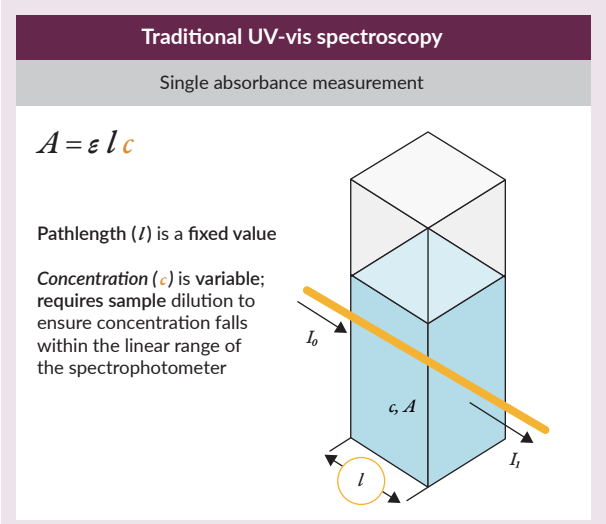


FIGURE 5

Variable pathlength spectroscopy.

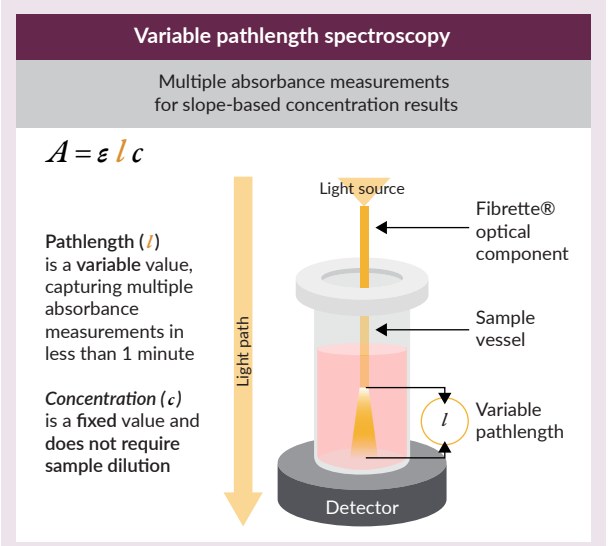


FIGURE 6

The Beer-Lambert equation, rearranged to solve for slope.

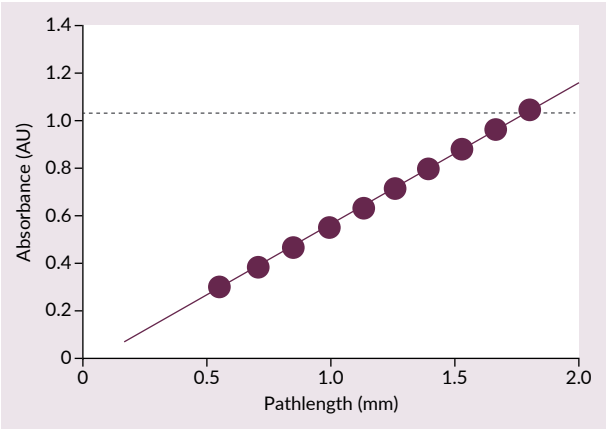
Beer's Law

$$A = \varepsilon l c \longrightarrow m = A / l \longrightarrow m = \varepsilon c$$

A = Absorbance ε = Extinction coefficient l = Pathlength
c = Concentration m = Slope

FIGURE 7

Determining slope using ten data points.



applications. Further, the system minimizes risk with fully enclosed ProConnex® custom flow paths as part of the automated TFF process.

The KrosFlo KR2i system also has a flexible configuration. It is compatible with flat sheet cassettes and hollow fiber filters, has plug-and-play accessories, utilizes a customizable flow path, and has a small footprint.

CTECH FLOWVPX SYSTEM OVERVIEW

Traditional UV-Vis spectroscopy is based on the Beer-Lambert Law (Figure 4), wherein absorbance is proportional to solute concentration. This causes measurement variability, introducing error into the process.

To overcome these challenges, the CTech FlowVPX System—the second part of the KR2i RPM system—uses Variable Pathlength Technology (VPT), a different approach to spectroscopy analysis (Figure 5). Variable pathlength spectroscopy, following a rearranged equation for Beer's Law (Figure 6), allows users the ability to change the pathlength from 5 mm–1 µ so that a wide range of concentrations can be measured. Additionally, with this method, concentration is a fixed value so it does not require sample dilution.

Another difference in variable pathlength spectroscopy is that it takes multiple absorbance measurements, finding

FIGURE 8

Concentration measurements obtained using the FlowVPX system.

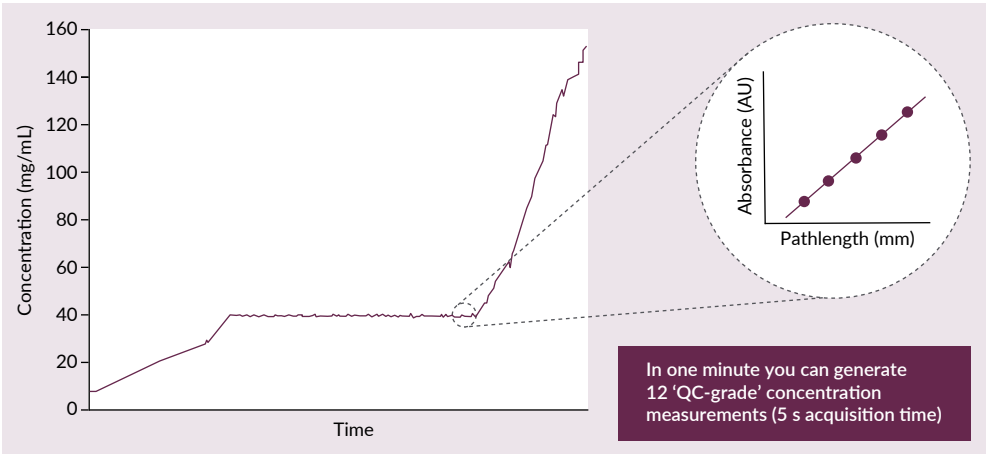


FIGURE 9

AAV viral titer determination using in-line VPT technology.

Objective

Evaluated variable pathlength spectroscopy method for AA viral titer

Assessment utilized the FlowVPX System to monitor the concentration of AAV during a UF/DF run; the initial and final concentration was compared to qPCR

Results

Initial titer
Flow VPX=6.06×10^{12}/qPCR=5.07×10^{12}

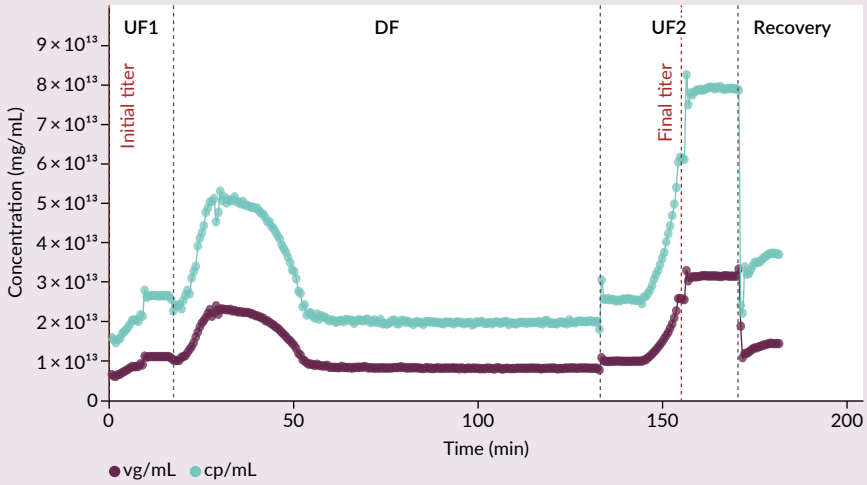
Final titer
Flow VPX=2.55×10^{13}/qPCR=1.54×10^{13}

~12 readings per minute

Precipitation shown during diafiltration

No sample dilution required

No background correction needed



The FlowVPX System is able to accurately determine the viral titer of AAV. The system's ability to monitor concentration during a UF/DF processs allows users to make real-time decisions.

FIGURE 10

AAV viral titer process monitoring using in-line VPT technology as compared to ELISA and ddPCR.

Objective

Evaluated variable pathlength spectroscopy method for AA viral titer monitoring

Assessment utilized the RPM System to monitor the concentration of AAV2 during a UF/DF run; each stage in the UF/DF was compared to ddPCR, ddPCR GOI, and ELISA

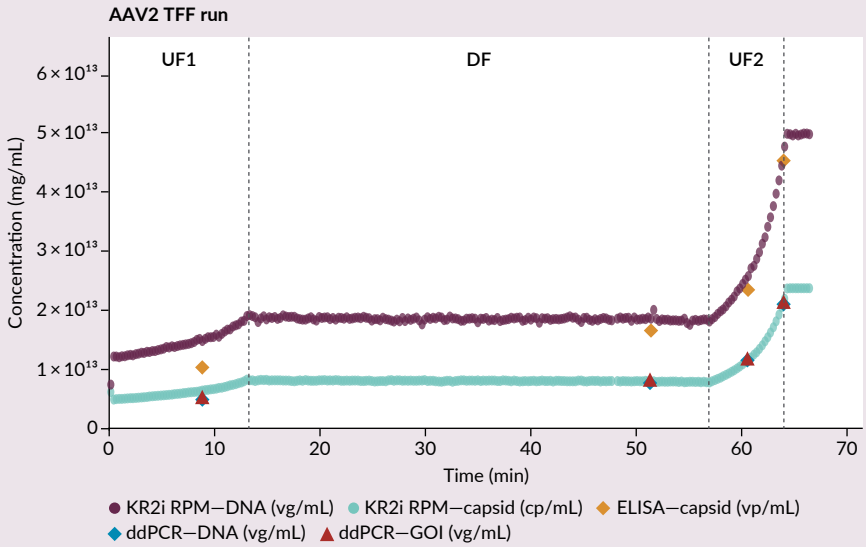
Results

Run met expected values and final UF2 target

RPM System results were successfully acquired in real time

Off-line methods were generated by two different users in the analytical group with a two-week turnaround time

Average variation found to be >15% for capside titer and >7 for genome titer



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

the absorbance change between different pathlengths and generating an accurate slope regression. The algorithm in the VPT software finds a suitable pathlength, with a range of 5 µ–15 mm, based on the concentration of the sample in the first couple of seconds of sample measurement. Once a suitable initial pathlength is chosen, ten data points are collected at different pathlengths to generate a slope (Figure 7). Using this data, the system quickly calculates concentration for a sample using the rearranged Beer's Law equation.

Furthermore, traditional UV has many areas where the process steps are time-consuming, which are further exacerbated by a highly error-prone dilution step that is variable from analyst to analyst. The CTech VPT system removes some steps of the process, as in the case of dilutions and estimations, and automates other steps down to a 'measure' and 'report' process. Plotting, calculating, and establishing the acceptance criteria are all automated within the VPT software, taking a 1–2 hour process and reducing it to 1–2 minutes. The simplicity of operation has made at-line testing on the manufacturing floor completely feasible and facilitates process efficiencies.

Although the CTech™ SoloVPE® System is not integrated into the KR2i RPM System, many users often compare it with the FlowVPX and KR2i RPM systems. While the SoloVPE utilizes variable pathlength technology at-line, the FlowVPX takes this a step further by implementing it in-line. Both systems employ VPT, but they differ in how they capture and process data. The FlowVPX stands out for its rapid and accurate

FIGURE 11

AAV5 viral titer determination using in-line VPT technology.

Objective 2

Assessing robustness of the FlowVPX System for in-line titer measurement independent of standard serotypes

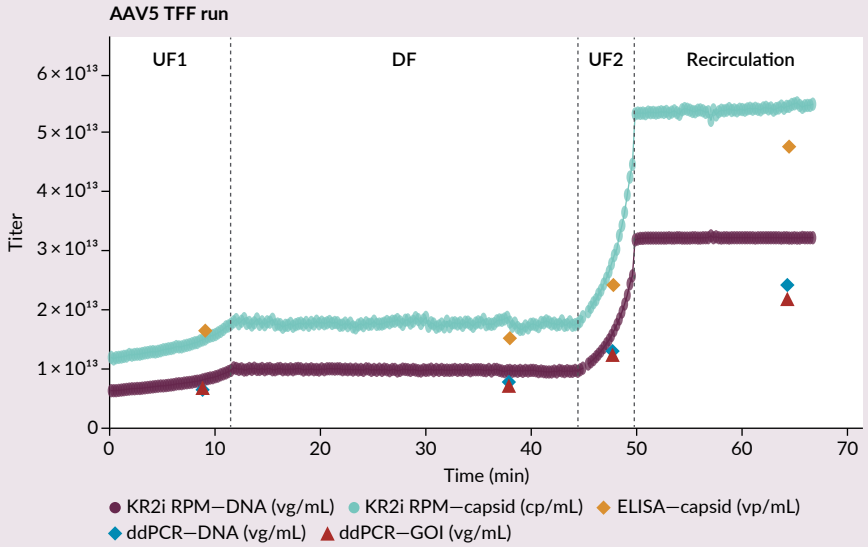
Assessment utilized the RPM System to monitor the concentration of AAV5 during a UF/DF run; each stage in the UF/DF was compared to ddPCR, ddPCR GOI, and ELISA

Results

RPM System results were successfully acquired in real time

Off-line methods were generated by two different users in the analytical group with a two-week turnaround time

Higher percent difference was seen between methods but remained consistent and linear



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

Monitoring concentration during a UF/DF process allows users to make real-time decisions. The high comparability of the FlowVPX readings to the analytical lab's methods gave the client confidence in the capabilities of the RPM System

data acquisition, delivering twelve QC-grade concentration measurements in just one minute. As shown in Figure 8, the system identifies an absorbance, then takes five to ten data points to generate a slope, which represents the QC-grade concentration measurement over time. Additionally, the FlowVPX offers a broad dynamic range, capable of reading concentrations from 0.1 mg/mL–300 mg/mL, depending on the extinction coefficient.

RPM SYSTEM CASE STUDIES

In order to demonstrate how the RPM System can provide key insights to ultimately make the UF/DF process more efficient, a variety of case studies were performed. In the first case study, the FlowVPX was used to monitor AAV titer results through a UF/DF process (Figure 9). The viral titer (vg/mL) was compared to qPCR during the initial and final concentration step. As shown in Figure 9, there is great comparability between the two systems. More importantly, this case study revealed that the FlowVPX is able to give more insight to the user throughout this process. During the diafiltration step, you can see that the AAV starts to aggregate, causing an increase in concentration. This would not be captured during any traditional UF/DF process. The end user now has more knowledge about how this buffer interacts with their molecule.

In the second case study, a standard a CDC TFF run was performed, and the data was compared to ELISA and ddPCR data. As shown in Figure 10, FlowVPX titer readings were comparable to standard ddPCR and ELISA data but generated instantly instead

FIGURE 12

AAV viral titer process monitoring using in-line VPT technology as compared to SoloVPE, qPCR, and Octet.

Objective

Succesfully monitor AAV output using variable pathlength spectroscopy method and capture insight for AAV viral titer monitoring

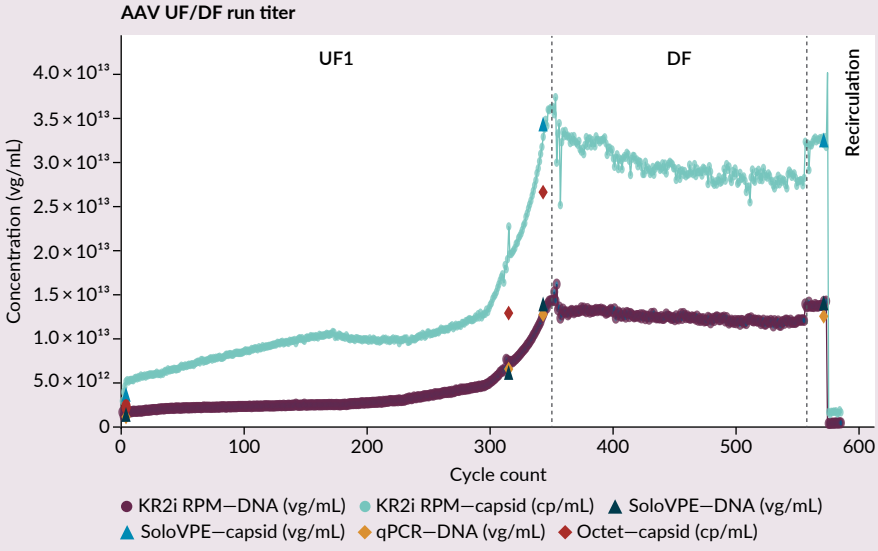
Assessment utilized the RPM System to monitor the concentration of AAV2 during a UF/DF run; each stage in the UF/DF was compared to qPCR, Octet, and the at-line SoloVPE System

Results

RPM System results were successfully acquired in real time and compared to SoloVPE System data collected on same day

Real-time process insights allowed for modification of run to address unforeseen filtration issues and adapt accordingly

Octet and qPCR data generated by AD group



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
6×	6.94×10^{12}	2.26×10^{13}	6.52×10^{12}	1.3×10^{13}	6.51	73.75	13.10	72.60
12×	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

of taking 2 weeks. The FlowVPX successfully captured real-time, in-line titer values and tracked and ended the run at the final UF2 target.

The third case study followed the same logistics as the second, but used a different AAV serotype: AAV5. As demonstrated in Figure 11, the FlowVPX recorded data independent of serotype, successfully tracking and ending the run at the final UF2 target. Though there was a percent difference in result between methods (RPM, ELISA, and ddPCR), that is likely due to the fact that two users conducted off-line testing over 2 weeks versus the VPX's real-time data. The multiple users and turnaround time could have caused variation in the results. However, FlowVPX titer readings were still comparable to ddPCR and ELISA and the percent difference operated within a predictable output.

The fourth case study focused on a concentration step with a DF step at the end. FlowVPX titers were compared to SoloVPE, Octet, and qPCR. As illustrated in Figure 12, the FlowVPX successfully tracked and ended the run, providing real-time process management that captured the formation of a gel layer (titer drop) in the DF stage. Although the data may look variable at times, it is still trackable with comparability graphs. Further optimization is required to avoid the formation of a gel layer and to optimize off-line collections.

SUMMARY

The KrosFlo KR2i RPM System is able to strengthen process control by allowing for real-time monitoring, producing high quality and highly reproducible results. It increases process efficiency by providing accurate results on your chosen endpoint, without extra runs or yield. Lastly, the system greatly reduces risk of error though the use of automated controls.

Q&A

Brandon Goldberg

Q If I already have a TFF system, can I purchase the FlowVPX by itself and integrate that with my system? Is there help available to optimize a custom integration with my existing TFF system?

BG: The FlowVPX is a standalone system, just like the SoloVPE. That means that you do not always have to connect it to the Repligen UF/DF kits. You can use the FlowVPX

with any UF/DF or chromatography instrument. You can either integrate the systems using OPC Unified Architecture, or just by manually putting the FlowVPX in-line on your feedline.

As far as help goes, Repligen has full application teams around the world that can help you integrate your system and be there whenever you need help with a run.

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

BG: You can do it manually, but we recommend the automated option through our software. We have two auxiliary pumps that come with the KR2i system that are built to do your diafiltration automatically. However, you could go into manual mode if you would like to control your process manually. A manual process does come with more risks.

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

BG: With in-line measurements, you get the correct concentration reading right away and as long as you have mixing in-line, the product is going to be homogeneous. When taking off-line measurements, you first have to sample the product, store it somewhere, and then physically bring it to the off-line measurement system. The product could settle, separate, evaporate, or any other number of problems.

Also, when you are reading measurements in-line, you know the concentration that you are comparing to at that minute. In contrast, if you sample off-line, depending on where you pull the sample, the concentrations might not match perfectly. If you pull the sample directly after the membrane, for example, it will have a higher concentration than a sample reading on the feed line, say, where the FlowVPX would be.

Q Can you integrate FlowVPX for upstream bioreactor processes?

BG: You can use the FlowVPX for upstream bioreactor processes. You can put the FlowVPX in-line anywhere you need it. However, when you are doing upstream integration of the FlowVPX, most of the time, your sample will not yet be purified. We usually try to integrate the FlowVPX downstream after chromatography, since your sample is usually pure after chromatography. Like any UV-Vis instrument, the FlowVPX reads the complete UV signal at whatever wavelength you are interested in. So, if you are reading at 280 and you have your proteins there along with your impurities, then you are going to read the absorbance as a whole. You have to be careful of that.

Q The FlowVPX looks like it is a stainless steel system. Is there a single-use version or a planned future version that will be single-use?

BG: The FlowVPX comes with the option of stainless steel flow cells or single-use flow cells. The single-use flow cells are X-ray radiated and made out of polyphenylsulfone (PPSU). The stainless steel flow cells are also autoclavable and cleanable with a clean-in-place (CIP) system.

Q Can the FlowVPX be used with just the KrosFlo KR2i system?

BG: We have a software called the RPM software where the FlowVPX is especially integrated into the KR2i system. However, you can use the FlowVPX with any system. If you want to connect the software to the FlowVPX, you will need more automation. However, if you just want to physically put the FlowVPX in-line, then you can use any system right away.

Q Does the VPX use disposable fabrics? Have you seen issues with protein absorption in the probe during processing, and can this be recovered by CIP?

BG: Unlike the SoloVPE, the FlowVPX does not use disposable fabrics. The FlowVPX uses a specific flow cell that is encased in stainless steel or the single-use option, PPSU. The flow cell does not absorb any of the material, so it can be reused for over 500,000 cycles. Once you are done with that flow cell, you can use CIP or you can take the flow cell off-line and use an autoclave. I usually recommend following the same procedure that you use for columns or membranes.

Q What is the highest flow rate FlowVPX can be used with? Are there different piping sizes that can be installed on FlowVPX for production scale?

BG: The FlowVPX has a one-system head that can fit all scales of production. We have different flow cells from 3 mm all the way up to a custom-made 2 in cell. The size of the flow cell determines the speed of flow. Our slowest flow, which is for lab scale, has a maximum of 1.5 L for the 3 mm flow cell. Our largest flow cell is 2 in, which uses a couple hundred liters per minute.

Usually, achieving a faster flow rate is not a problem. There may be challenges if a user wants to stop the system and still run. You are able to do this, but you have to ensure that your sample stays homogeneous when you are reading analytics in-line.

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

BG: With the FlowVPX, viscosity does not matter too much as long as you are able to get the sample flowing. Some of our users have had to heat up their tubing just to keep their sample moving, but it has not caused any problems.

For protein concentration, we normally aim for anywhere from 0.1–300 mg/mL at a 1.5 extinction coefficient, but that range is very extinction coefficient-based. For example, in terms of AAV, there would be no maximum protein concentration since a sample would never reach a maximum concentration. The minimum concentration would be around e^{12} viral particles.

Q Does it make more sense to use FlowVPX in the feed line or in the TFF retentate line?

BG: It really depends on if you want to know your concentration before or after you hit your membrane. There are benefits to both. You can either use the FlowVPX to control how long the material is going to go through the membrane and also control the concentration of the material, or you can read the concentration at the backend of the process. Or to rephrase, it does not matter if you put the FlowVPX on the feed line or on the retentate line, it just depends on how you use concentration to control your continuous process.

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

BG: Very robust. Due to the fact that the FlowVPX is a fully stainless steel instrument, precipitates do not have an effect. Also, the FlowVPX has little pressure and does not cause any precipitates in the solution because of the fact that it is a very thin fibrin moving up and down very gently.

You have to worry more for your own material. If your material is very fragile and you have to be careful with it, then I would lower the mixing and lower your feed flow rate.

Q During the DF step, is mixing necessary?

BG: When you have analytics in-line, like with the FlowVPX, I highly recommend mixing throughout the whole run. You really have to make sure that your solution is homogeneous or else it will start to short circuit. So, for example, if your line short-circuited and you are reading just buffer, then you are going to see a very low concentration. Then the concentration will jump up once you get, say, your protein or AAV in there.

Q What is the minimum process volume using both the VPX and cross-flow RPM systems?

BG: We normally say you can go down to 10 mL and up to 10 L. However, depending on your process and how much you want to cut your tubing, you are able to get it shorter. You really have to see with your own system. If you use shortened size 14 tubing, a 3 mm flow cell, and hollow fiber, you can get the volume very low, which is a huge benefit of the KR2i.

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

BG: Currently in the software, you can only put in a specific concentration for things like when to switch over to diafiltration or when to end the run. You cannot put in a range. I would usually tell my customers that, if you have a range, to target the middle of that range. Of course, the FlowVPX is usually very accurate, so you will hit at or very close to your targeted concentration.

BIOGRAPHY

BRANDON GOLDBERG is a Senior Bioanalytical Application Specialist at Repligen, Bridgewater, NJ, USA where he has dedicated 7 years to advancing bioanalytical technologies and ensuring that customers can effectively implement and utilize advanced bioprocessing technologies, specializing in process analytical technology (PAT) solutions. In his role, Brandon combines technical support, training, implementation and validation assistance, and ongoing collaboration to help customers achieve their bioprocessing goals efficiently and effectively. He focuses on helping customers implement and validate both at-line and in-line analytical solutions, driving process optimization with the FlowVPX and SoloVPE Systems. A graduate of Central Connecticut State University, New Britain, CT, USA in May 2016, Brandon has been active in the pharmaceutical industry ever since. He remains committed to his professional network in Connecticut, regularly connecting with colleagues and old friends to share insights and foster collaborations.

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AUTHORSHIP & CONFLICT OF INTEREST

Contributions: The named author takes responsibility for the integrity of the work as a whole, and has given their approval for this version to be published.

Acknowledgements: None.

Disclosure and potential conflicts of interest: The author has no conflicts of interest.

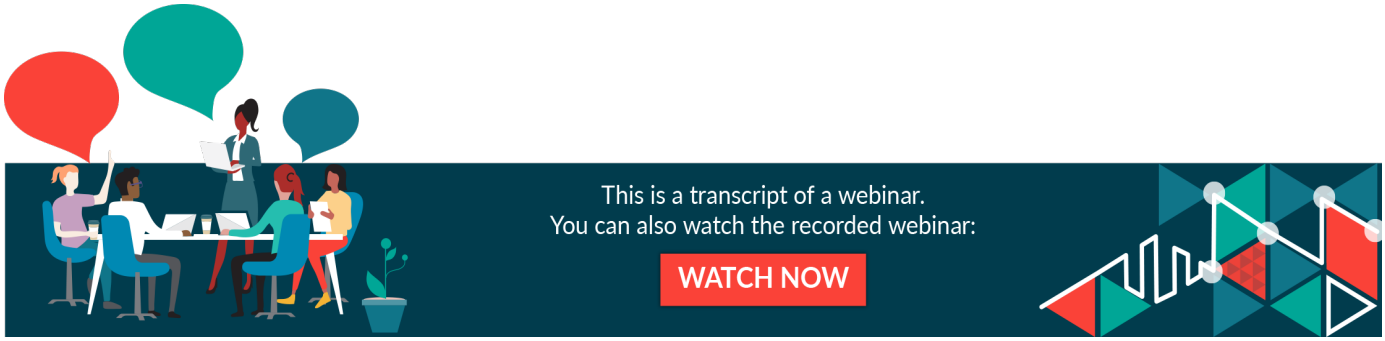
Funding declaration: The author received no financial support for the research, authorship and/or publication of this article.

ARTICLE & COPYRIGHT INFORMATION

Article source: This article was based on a webinar, which can be found here.

Webinar conducted: Jul 11, 2024; **Revised manuscript received:** Aug 21, 2024;

Publication date: Oct 14, 2024.



CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Supporting efficacy and scaling with a next-generation T cell AOF formulation medium

Alan Gutowski and Joanna Kern

Cell culture media are critical to successful cell therapy production, serving as a source of essential nutrients and maintaining the physiological conditions necessary for optimal cell growth and function. The use of well-defined, animal origin-free (AOF) media is increasingly recognized as being pivotal in ensuring experimental reproducibility, minimizing contamination risks, and addressing stringent regulatory requirements. This article explores the CTS™ OpTmizer™ One serum-free medium, an innovative AOF formulation tailored for T cell expansion. This medium is designed to enhance the early memory T cell phenotype, improve cell viability, and facilitate scalability, positioning it as a crucial tool for both preclinical research and clinical manufacturing.

Cell & Gene Therapy Insights 2024; 10(9), 1203–1219

DOI: 10.18609/cgti.2024.138

Cell culture media play a crucial role in cell therapy applications, providing essential nutrients that allow cells to grow, divide, and function properly. They also maintain physiological conditions such as pH and osmolarity, and enhance reproducibility and thus, standardization. Well-defined cell media compositions, which are animal origin-free (AOF), are crucial for ensuring reproducible results. An AOF medium reduces the risk of contaminants and variability from animal source components, thereby decreasing regulatory concerns.

Understanding the need for AOF components, Thermo Fisher Scientific pioneered the first AOF vaccine production medium in 1997. In the early 2000s, under the Invitrogen name, the Gibco™ brand became the world leader in feed, supplements, and serum media. Today, Gibco products have been integrated into hundreds of clinical and commercial products, including multiple approved cell therapies. The Gibco catalog media consist of several cell therapy systems (CTS™), which between them provide end users with extensive safety testing of raw materials, the support of expert quality and regulatory teams, and GMP-manufactured products with robust traceability that meet

the latest cell and gene therapy guidance for ancillary materials.

CTS™ OPTMIZER™ SERUM-FREE MEDIA FAMILY

The CTS OpTmizer family comprises fit-for-purpose T cell media that are used in the production of clinically proven and commercialized cell therapies. The legacy OpTmizer media comes in kits with basal and supplement components. The complete medium is prepared by mixing the entirety of the supplement with the corresponding volume of basal media. There are two versions of this legacy T cell medium: the CTS OpTmizer serum-free media (SFM) and the CTS OpTmizer Pro SFM.

The CTS OpTmizer SFM perform across a variety of culture vessels, from static plates to dynamic bioreactors. As an example, Figure 1 depicts performance data utilizing a rocking bioreactor. The CTS OpTmizer SFM, supplemented with CTS immune cell serum replacement (ICSR), expands cells as efficiently as a serum-containing medium, as shown in the panel on the left. The CD8/CD4 ratio is maintained, as shown in the middle panel. Furthermore, the CTS OpTmizer SFM yields a desirable T cell phenotype with low levels of effector cells, as depicted in the panel on the right.

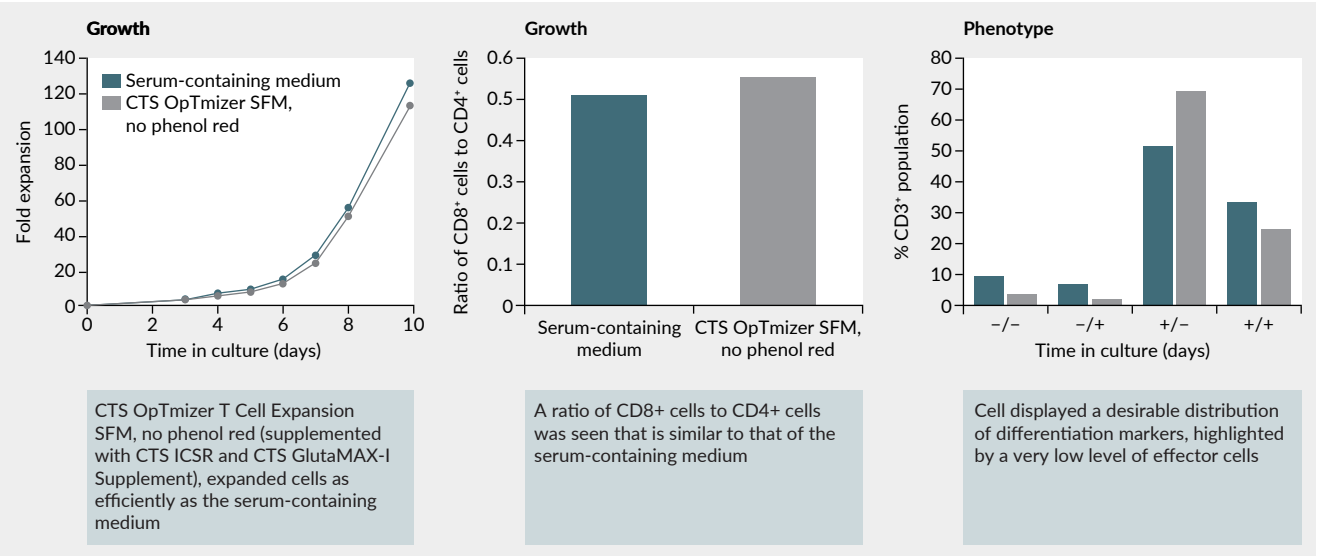
While the CTS OpTmizer SFM is recommended for autologous cell workflows, the CTS OpTmizer Pro SFM is the first T cell medium designed for allogeneic cell workflows. The purpose of the CTS OpTmizer Pro SFM is to drive towards early memory T cell phenotypes, thus delaying differentiation. This process is important when considering longer-duration allogeneic cell workflows. The benefits of the CTS OpTmizer Pro SFM are demonstrated in Figure 2, which shows a simple comparison of performance in terms of preservation of memory T cells, enhancement of T cell proliferation, and production of cytokines between the CTS OpTmizer Pro SFM and the parent CTS OpTmizer medium in healthy donor cultures. The improvements shown in Figure 2 are most consistently seen with healthy donors and become more pronounced over time.

INTRODUCTION TO CTS OPTMIZER ONE SFM

A new addition to the CTS OpTmizer SFM family, the CTS OpTmizer One SFM is a

FIGURE 1

Performance of the CTS OpTmizer SFM in a rocking bioreactor.



CTS OpTmizer T Cell Expansion SFM, no phenol red (supplemented with CTS ICSR and CTS GlutaMAX-I Supplement), expanded cells as efficiently as the serum-containing medium

A ratio of CD8+ cells to CD4+ cells was seen that is similar to that of the serum-containing medium

Cell displayed a desirable distribution of differentiation markers, highlighted by a very low level of effector cells

novel AOF formulation for T cell expansion. It enhances drug function by improving early memory T cell phenotype, enhancing cell growth, and increasing cell viability. The CTS OpTmizer One SFM is easy to use with the simple addition of L-glutamine, and is available in a 1 L bottle as well as 1 L, 5 L, and 10 L bioprocessing containers that are compatible with automated systems.

The larger volume containers not only address the need for scale but also have both C-Flex™ and polyvinyl chloride line sets that allow for closed system integration. The increased volume and the availability of weldable line sets help reduce the number of open steps in the process, thus reducing contamination risks. In addition, these characteristics allow for integration with consumables for Thermo Fisher Scientific's CTS instruments, including the DynaCellect™, the Rotea™, and the Xenon™ Systems. Furthermore, the larger formats all come with a 12-month shelf-life to enhance supply chain security for cell therapy manufacturing.

CTS OPTMIZER ONE SFM CASE STUDIES

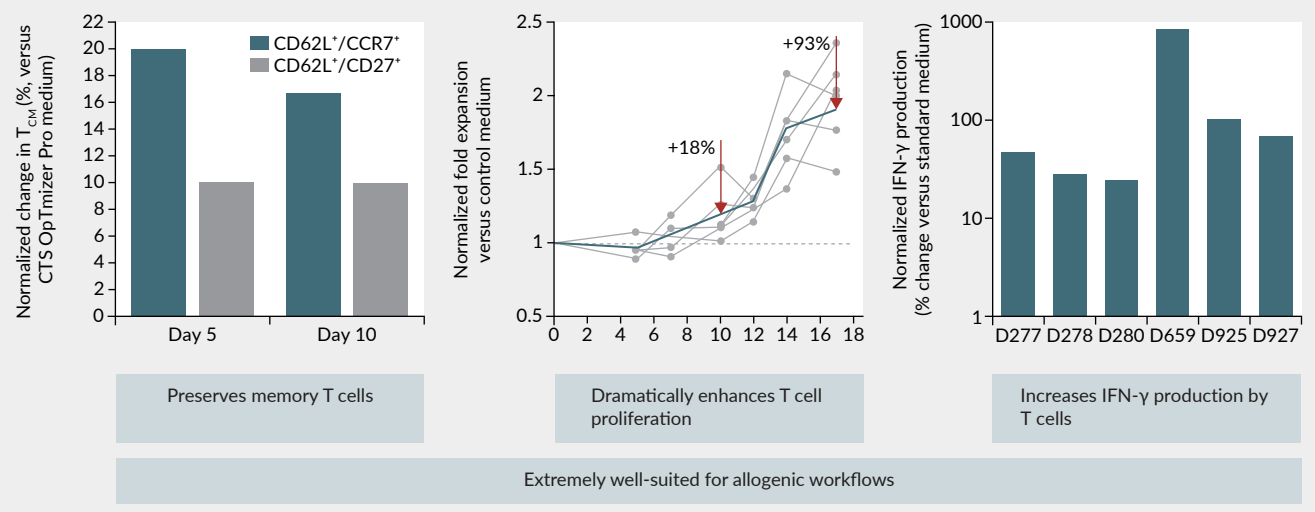
To demonstrate the capabilities of this medium, T cell expansion data from the CTS OpTmizer One SFM was compared with that of competing T cell expansion media (Figure 3). The CTS OpTmizer One SFM was supplemented with 4 mM L-glutamine, while the other media used were supplemented according to each individual product's recommendations. Peripheral blood mononuclear cells (PBMC) from 5–11 healthy donors (represented by dots on the left-hand graph) were seeded in 24 G-Rex plates. Cells were activated using Dynabeads™ and were then cultured until day 10 of the process. Each condition was performed in technical duplicates or triplicates.

It was found that, by day 10, average cell viabilities remained comparably high at 84% with CTS OpTmizer One SFM, and from 76–88% with the other media tested (data not shown). This demonstrates that OpTmizer One delivered consistently higher cell counts compared to CM1, CM2, and CM3 on day 5, day 7, and day 10 of the culture process. Furthermore, the right-hand graph shows a relative cell count increase when compared to the competing media.

Although clearly vital, cell viability is not the only important metric for T cell expansion success. Another important aspect is the

FIGURE 2

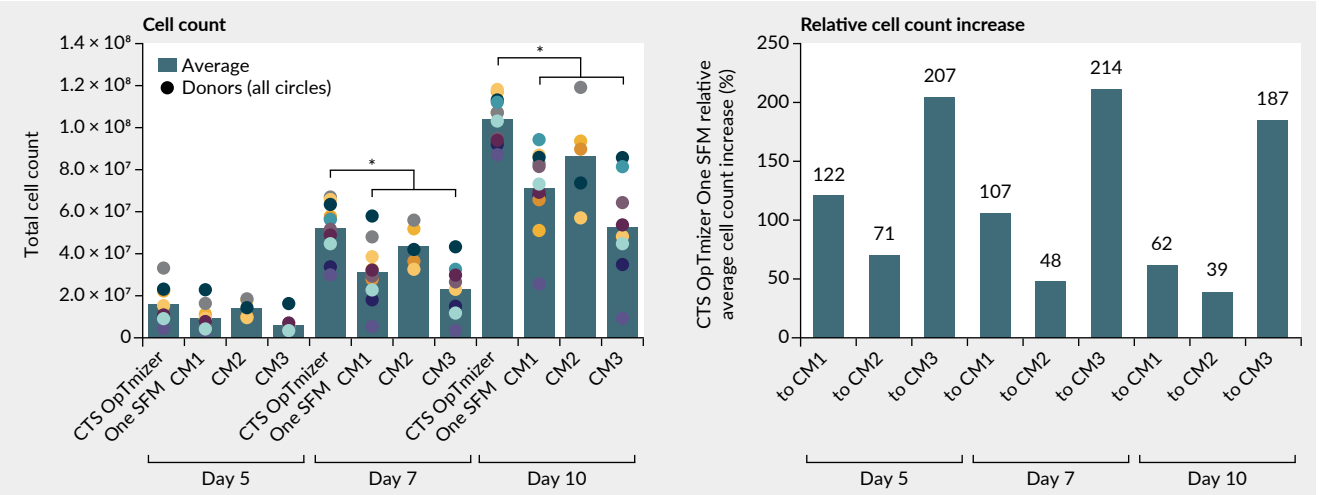
Benefits of the CTS OpTmizer Pro SFM.



phenotype of the cells at harvest, as early memory or stem cell memory markers are most desirable due to their effectiveness. Figure 4 displays the expression of early memory markers CD62L, CCR7, CD27, and CD45RO in the cells expanded using the CTS OpTmizer One SFM and the media competitors from the T cell expansion experiment in Figure 3. The levels of expression of early memory markers were similar or higher with the use of the CTS

FIGURE 3

Comparison of T cell expansion data from multiple media, including the CTS OpTmizer One SFM.

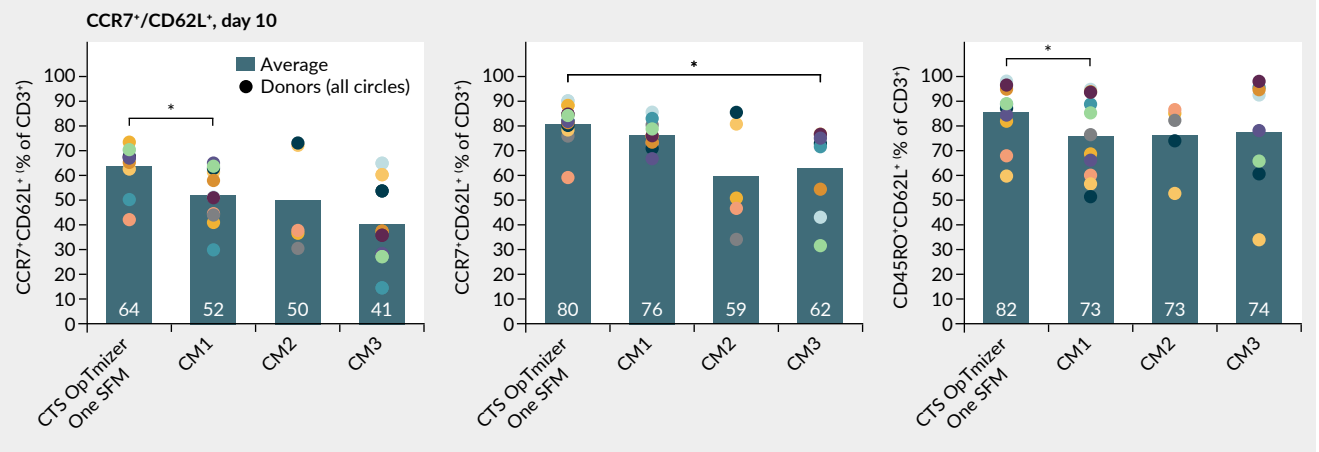


Cell growth was consistently comparable to or higher than with the commonly used formulations on the market

CTS OpTmizer One SFM: n=11 donors, CM1: n=11 donors, CM2: n=5 donors, CM3: n=8 donors. *p<0.05.

FIGURE 4

Comparison of early memory phenotype marker expression from multiple media, including the CTS OpTmizer One SFM.



CTS OpTmizer One SFM maintained higher or comparable expression of early memory phenotype markers

CTS OpTmizer One SFM: n=11 donors, CM1: n=11 donors, CM2: n=5 donors, CM3: n=8 donors. *p<0.05.

OpTmizer One SFM versus the competing media.

These media were further tested for the functionality of CD4 and CD8 T cells at harvest on day 10 (Figure 5). As illustrated here, the CTS OpTmizer One SFM provided on average a 1.2 ratio of CD4:CD8 cells with the donors tested. This ratio was similar to that achieved by other chemically defined AOF formulations. Interestingly, a higher percentage of CD8 cells was observed with the CTS OpTmizer One SFM than with the CM3 xeno-free formulation, which supported the expansion of more CD4 cells. From this data, it can be concluded that the CTS OpTmizer One SFM supports the expansion of both CD4 and CD8 cells.

The right-hand graph of Figure 5 shows T cell functionality. Here, IFN-γ production was measured by the Luminex™ platform. T cells were activated with Dynabeads and cultured in the CTS OpTmizer One SFM or the CM1 media competitor, which is also an AOF formulation. Figure 5 shows that the T cells expanded in the CTS OpTmizer One SFM are functional and produce IFN-γ at a similar level to the chemically defined media competitor.

T cell expansion media must also be scalable, flexible, and automation-friendly. Figure 6 demonstrates that the CTS OpTmizer One SFM is scalable and supports expansion in a rocking bioreactor with perfusion. In this experiment, a 1 L bioreactor was used to test three PBMC donors. 8 mL and 40 mL G-Rex plates were used as a control.

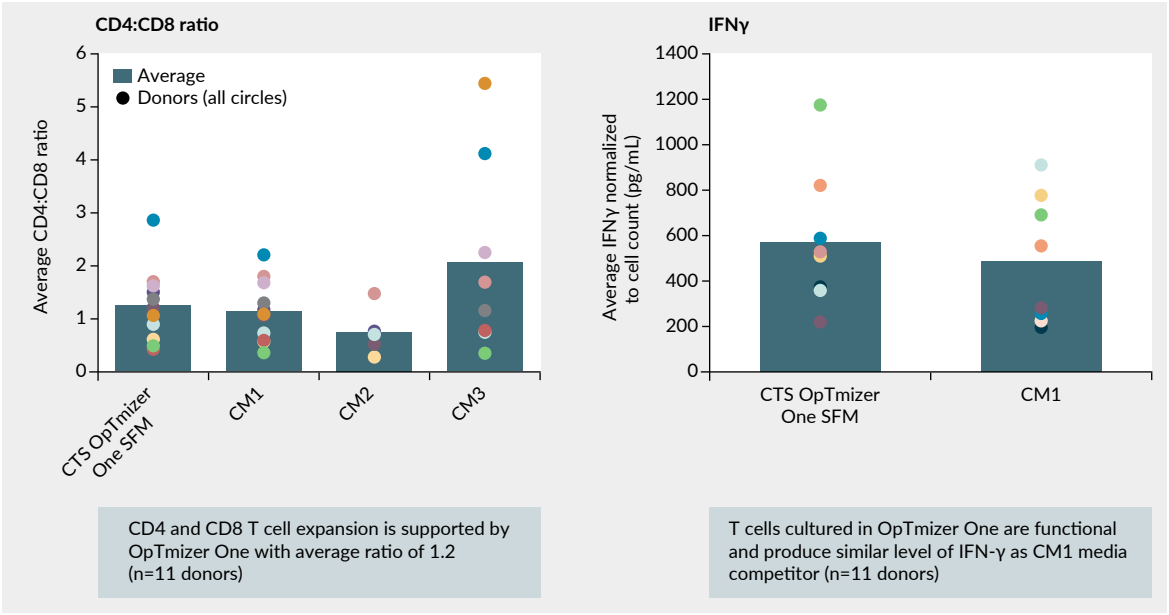
T cells were activated with Dynabeads on day 0 and transferred to the bioreactor or G-Rex plates on day 2. They were cultured in the bioreactor or G-Rex plates until day 14. Addition of fresh media was performed on day 3 or 4, and the perfusion process for the bioreactor started on day 6 or 7, depending on cell density. It was found that the CTS OpTmizer One SFM supported higher-fold expansion in the bioreactor. At over 85%, viability was high in both types of vessels.

Additionally, CTS OpTmizer One SFM was found to support high-cell density cultures of over 40 million cells per mL, but this result can only be achieved with the right medium perfusion process.

The capability of CTS OpTmizer One SFM to support the expansion of diseased donor cells was also tested (Figure 7). Cells

FIGURE 5

Comparison of functional CD4 and CD8 T cell expansion using multiple media, including the OpTmizer One.



CD4 and CD8 T cell expansion is supported by OpTmizer One with average ratio of 1.2 (n=11 donors)

T cells cultured in OpTmizer One are functional and produce similar level of IFN-γ as CM1 media competitor (n=11 donors)

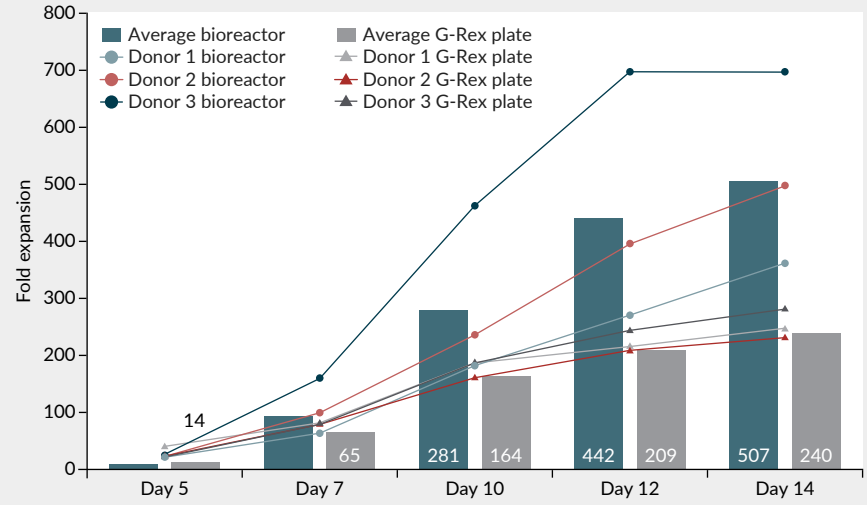
were obtained from two patients diagnosed with acute myeloid leukemia (AML), and two patients diagnosed with chronic lymphocytic leukemia (CLL). BPMCs were activated with Dynabeads and were cultured in G-Rex plates until day 10. Fold expansion was observed on day 7 and on day 10. The CTS OpTmizer One SFM was shown to outperform the chemically defined (CM1) and xeno-free (CM3) media competitors at the tested time points.

Seeding density for the donors was very low, with less than 200,000 cells per well, but the CTS OpTmizer One SFM still showed strong cell expansion performance.

In addition to expansion, the phenotype of the diseased donor cells was also tested.

FIGURE 6

Scale-up in a rocking bioreactor using the CTS OpTmizer One SFM.



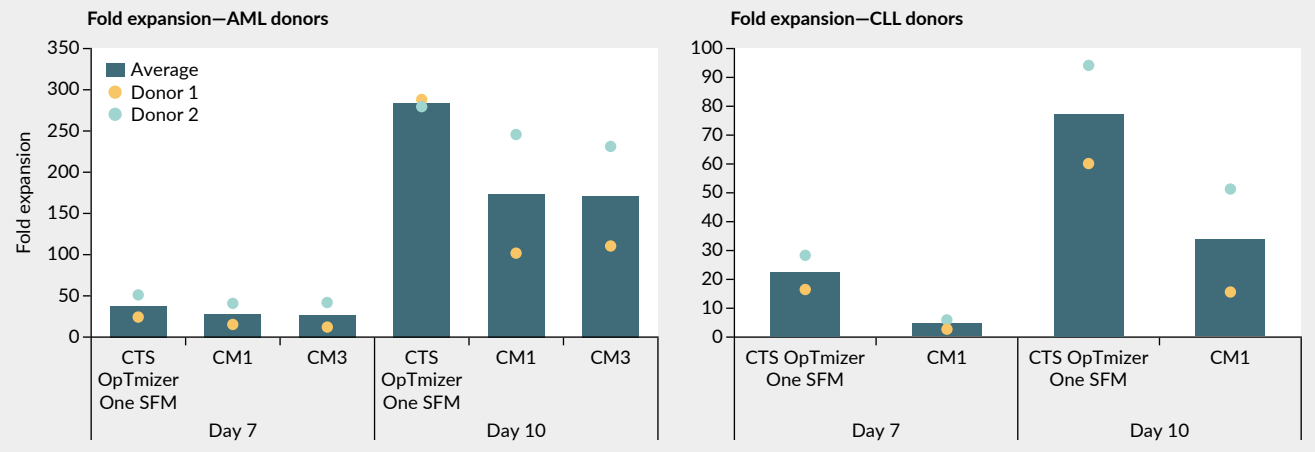
Scale-up in the rocking bioreactor with perfusion with CTS OpTmizer One SFM showed higher average expansion of 507-fold at day 14, with up to nearly 700-foldexpansion for donor 3

In comparison, the average expansion in the G-Rex™ plate was 240-fold at day 14

By day 14, the average cell viability remained high at 93% in the bioreactors and 86% in the plates (data not shown)

FIGURE 7

Comparison of diseased T cell expansion data from multiple media, including the CTS OpTmizer One SFM.



By day 10, AML donor cells achieved more consistent and robust (284-fold) expansion with CTS OpTmizer One SFM than with similar media from other suppliers

Cell viability remained comparably high at approximately 85%

By day 10, CLL donor cells showed nearly 80-fold expansion with CTS OpTmizer One SFM, compared to >40-fold expansion with a medium from another supplier

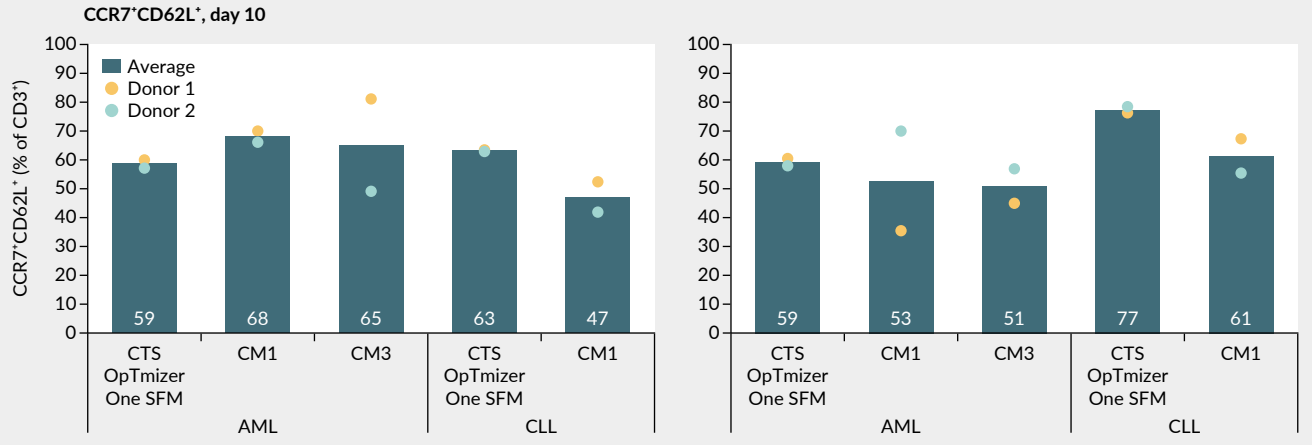
High cell viability of 84–88% was maintained

The phenotype of AML and CLL donor cells on day 10 is shown in Figure 8. The expression of early memory markers was similar, or even higher than that of the media competitors CM1 and CM3, and was above 50% for each donor tested. This data shows that the CTS OpTmizer One SFM supports the maintenance of early memory marker expression, despite a much higher-fold expansion for both of these donors, as was shown in Figure 7.

Furthermore, the expression of exhaustion markers of the expanded diseased T cells was also tested on day 10. As shown in Figure 9, the expression of LAG-3, PD-1, and TIM-1 was the lowest in the CTS OpTmizer One SFM. This shows that the CTS OpTmizer One SFM supports the expansion of T cells from AML and CLL PBMC donors with high viability, high expression of memory markers, and low expression of exhaustion markers.

FIGURE 8

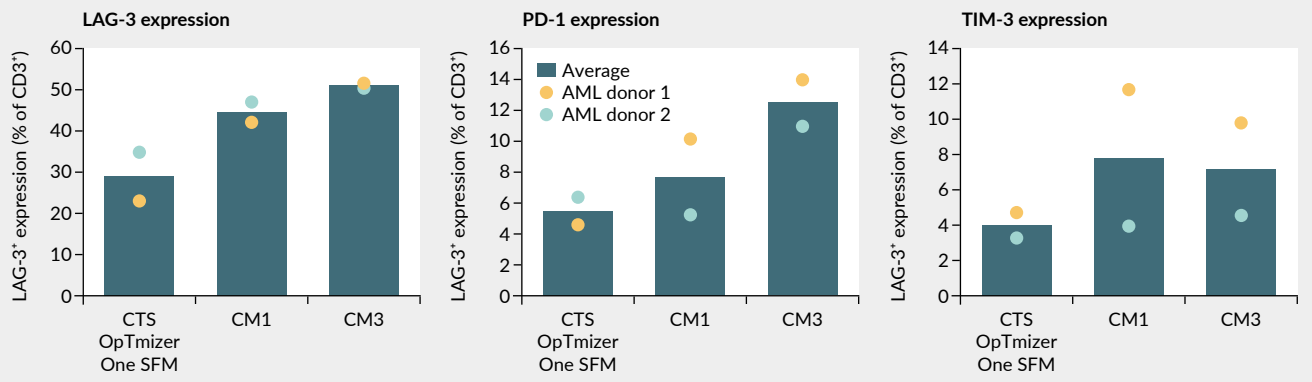
Comparison of early memory phenotype marker expression in diseased T cells from multiple media, including the CTS OpTmizer One SFM.



Early memory markers expression in AML and CLL T cell donors cultured in OpTmizer One remains similar or higher than CM1 and CM3 despite greater fold expansion

FIGURE 9

Expression of exhaustion markers in diseased T cells.



Exhaustion markers expression in AML and CLL T cell donors cultured in OpTmizer One is lower than CM1 and CM3 despite greater fold expansion

Another critical aspect of a medium's performance is the degree of support it provides both for gene editing and for expansion post-editing, the capability of the CTS OpTmizer One SFM to support lentivirus (LV) transduction and expansion of CD19 CAR-T cells was also tested (Figure 10). In this experiment, cells from two donors were isolated using the CTS DynaCellect with CD3/CD28 detachable Dynabeads. Transduction was performed with CD19 CAR-T cells on day 1 at a multiplicity of infection (MOI) of 5. Beads were removed on day 2 and the cells were transferred to a rocking bioreactor where they were cultured until day 10. Fresh medium was added on day 3 or 4, and the perfusion process began on day 6.

Figure 10 shows that the CTS OpTmizer One SFM supported the expansion of transduced cells, with an average of 275-fold expansion. The cells had a high viability of over 90% throughout the culture. Additionally, as shown on the right-hand graph, the transduction efficiency was high on day 6, at >50% for both donors. The CTS OpTmizer One SFM supported the enrichment of CAR-T cells during the culture and bioreactor steps, reaching over 80% CAR-T positive cells on day 10.

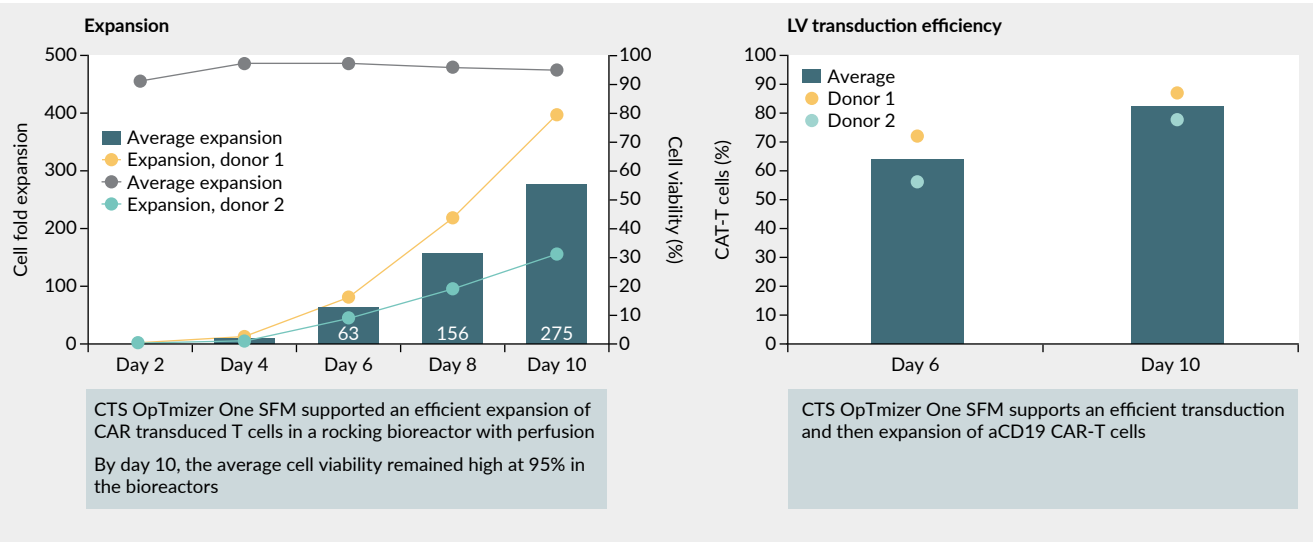
A phenotype of CAR-T cell was tested on days 6 and 10 of this experiment. As shown in Figure 11, the expression of CD62L and CD45RO was maintained at >80%. Expression of CCR7 and CD27 was ≥50% on day 10 of the culture. Additionally, as shown in the right-hand graph, CD4 and CD8 cells comprised 50% of the cell population on day 10 of the culture. This data shows that the CTS OpTmizer One SFM supports the efficient expansion of LV-transduced CD4 and CD8 CAR-T cells in a bioreactor with a perfusion process.

Lastly, the CTS OpTmizer One SFM was tested for gene editing and expansion in a stirred-tank bioreactor. The experiment shown in Figure 12 was performed with CAR construct delivery with a CRISPR-Cas9 system through electroporation. The CTS OpTmizer One SFM performance was tested in a stirred-tank bioreactor, and additionally, both CTS OpTmizer One SFM and CTS OpTmizer Pro SFM were used in G-Rex plates as a control.

T cells were isolated with CD3/CD28 Dynabeads, with the DynaCellect System. Electroporation was conducted with the Xenon System on day 2. The transfected cells were transferred to a stirred-tank bioreactor

FIGURE 10

LV transduction and expansion of CD19 CAR-T cell in a rocking bioreactor with perfusion.



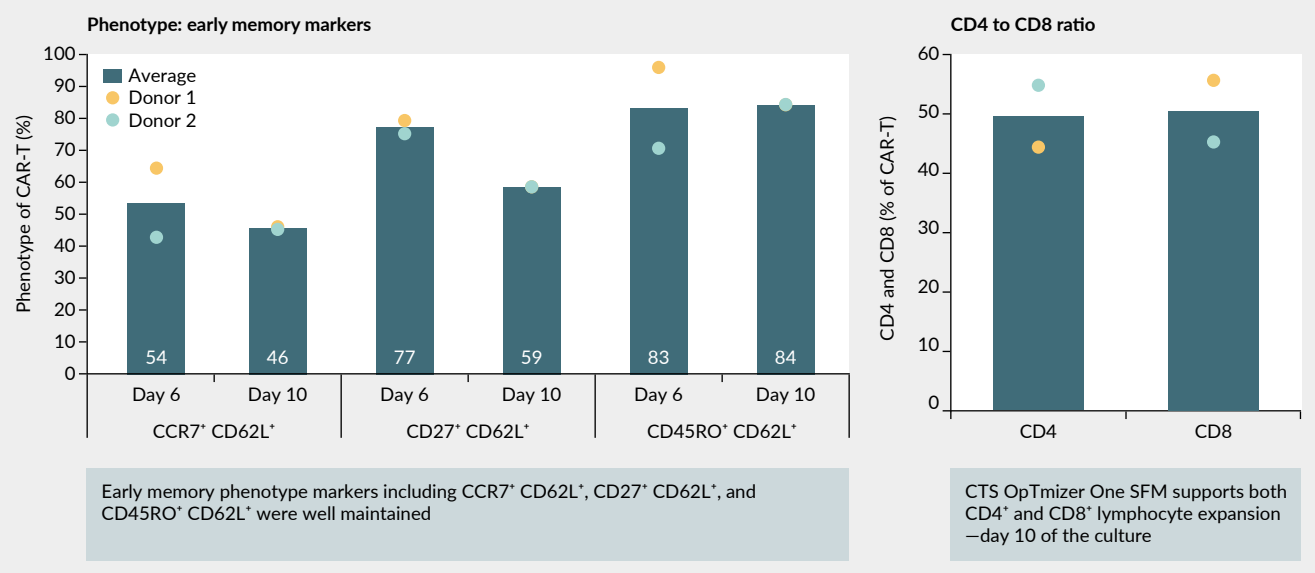
CTS OpTmizer One SFM supported an efficient expansion of CAR transduced T cells in a rocking bioreactor with perfusion

By day 10, the average cell viability remained high at 95% in the bioreactors

CTS OpTmizer One SFM supports an efficient transduction and then expansion of aCD19 CAR-T cells

FIGURE 11

Early memory T cell phenotype and CD4:CD8 ratio of different CAR-T cells using the CTS OpTmizer One SFM.



Early memory phenotype markers including CCR7⁺ CD62L⁺, CD27⁺ CD62L⁺, and CD45RO⁺ CD62L⁺ were well maintained

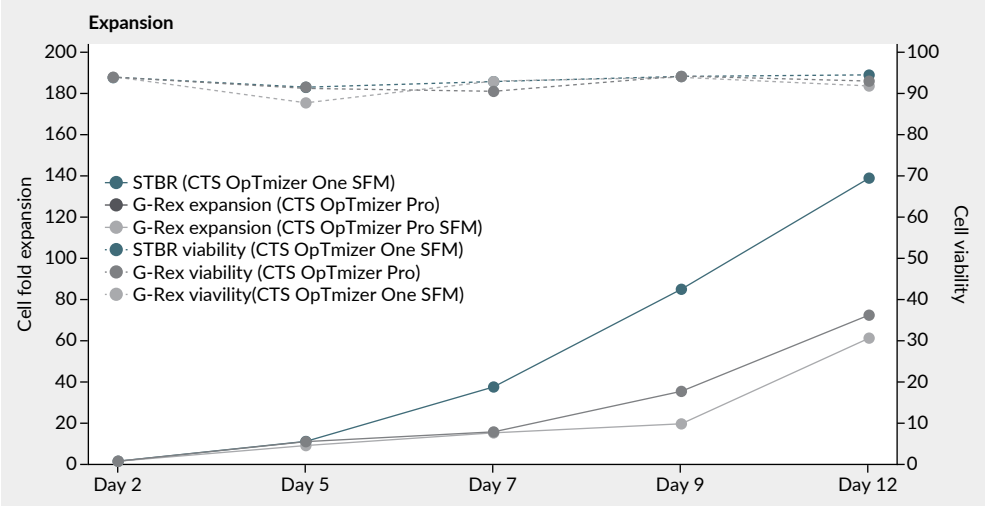
CTS OpTmizer One SFM supports both CD4⁺ and CD8⁺ lymphocyte expansion —day 10 of the culture

and the G-Rex plates on the same day and were cultured until day 12. A similar expansion was observed in the CTS OpTmizer One SFM and the CTS OpTmizer Pro SFM in G-Rex plates. However, there was a much higher expansion of transfected cells in the stirred-tank bioreactor using the CTS OpTmizer One SFM. Both media supported high viability of cells in both vessels, at >90% throughout the culture.

Furthermore, the data shown in Figure 13 shows transfection efficiency in both the CTS OpTmizer One SFM and the CTS OpTmizer Pro SFM, together with post-expansion enrichment. In this figure, knock-in represents CD19-CAR expression, and knockout

FIGURE 12

Expansion of CD19-CAR transfected T cells in a stirred-tank bioreactor with CTS OpTmizer One SFM.



represents the TCR-α/β- population. As illustrated on the far right-hand of Figure 12, >80% of cells have knockout of TCR-α/β- for days 5 and 12. For both media and for both vessels tested, knock-in expression was expectedly lower, at 20–30% on day 5. CD19 CAR-expressing T cells expanded well in both CTS OpTmizer media tested. Enrichment of approximately 50% was also observed in the G-Rex plates. However, a better enrichment of CAR+ cells of >80% was observed on day 12 in the stirred-tank bioreactor.

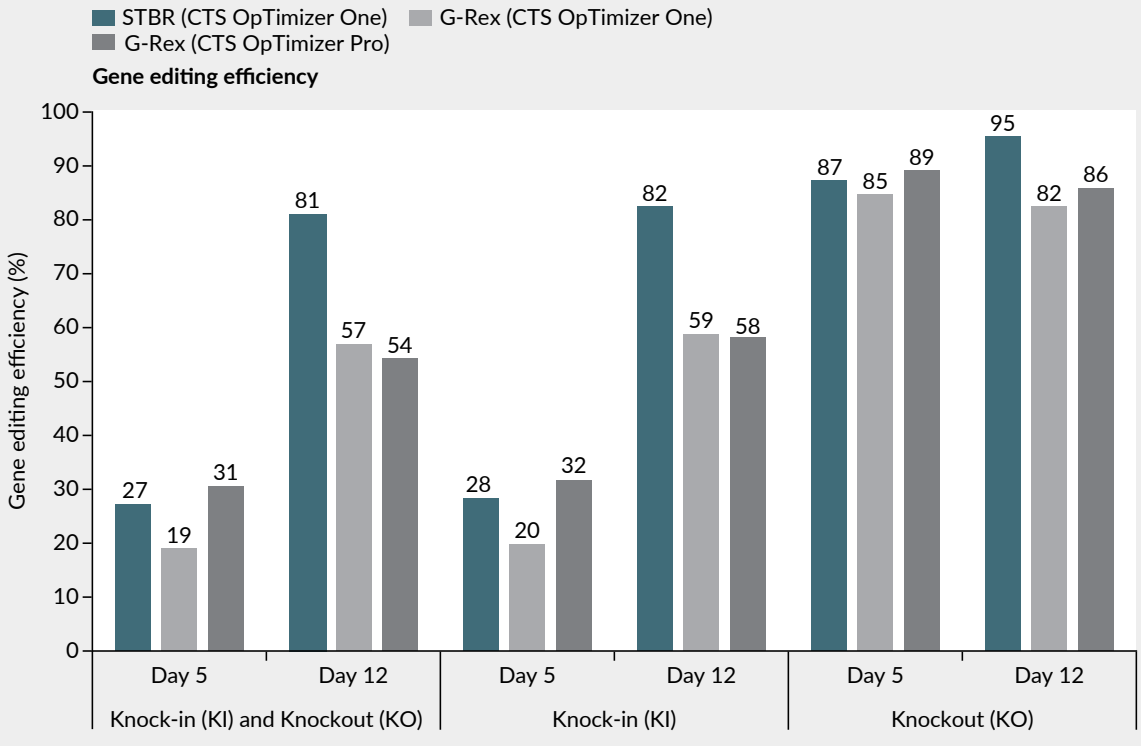
Next, the phenotype of CAR-T cells was checked on day 5 and day 12. As shown in Figure 14, the expression of stem cell memory markers was similar in the bioreactor and the G-Rex plates in the CTS OpTmizer One SFM. Additionally, central memory marker expression was similar across the vessels and the media tested on day 5 and day 12.

The CTS OpTmizer One SFM supported a higher percentage of CD8 cells than the CTS OpTmizer Pro SFM. This was observed in both vessels, but the percentage of CD8 cells was slightly higher in the stirred-tank bioreactor than in the G-Rex plates.

Finally, CD19-CAR-T cells were tested for their functionality using a cytotoxicity assay (Figure 15). CAR-T cells were cultured in either the CTS OpTmizer One SFM or the CTS OpTmizer Pro SFM, and target cells were plated together at different ratios. Both media produced functional T cells that were able to kill target cells. However, in both vessels tested, the CAR-T cells cultured in the CTS OpTmizer One

FIGURE 13

Transfection efficiency in the CTS OpTmizer One SFM and the CTS OpTmizer Pro SFM, together with post-expansion enrichment of CD19-CAR-T cells in a stirred-tank bioreactor.

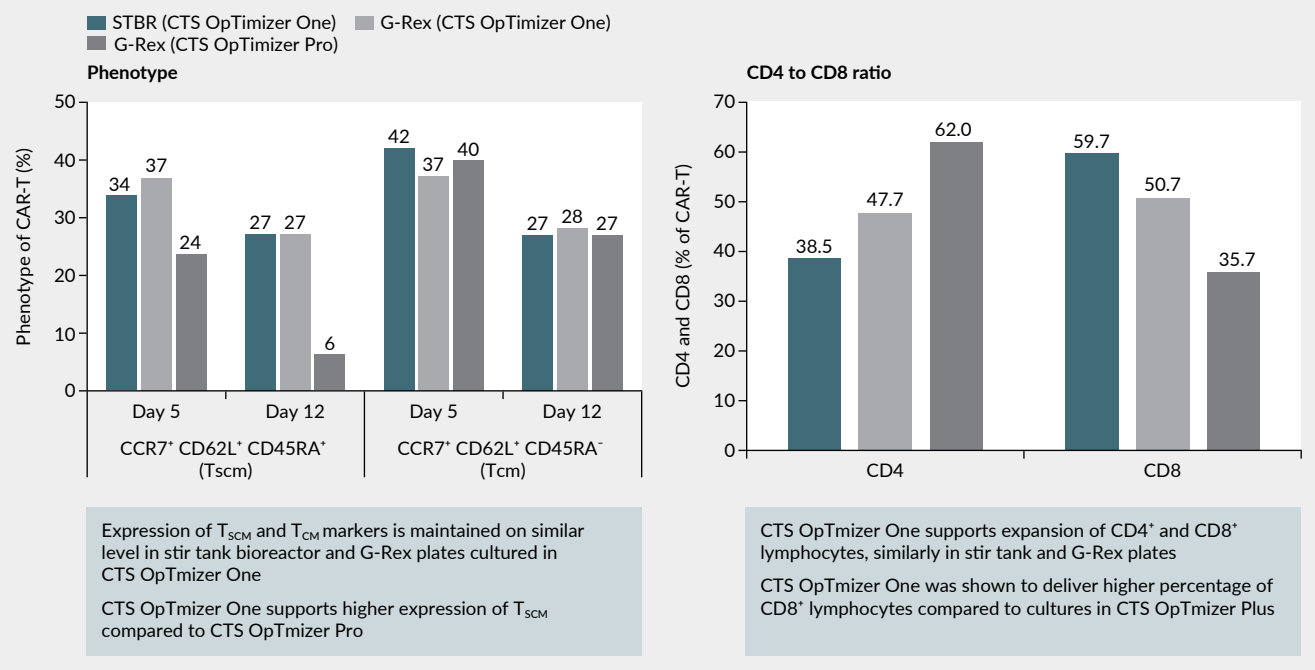


CTS OpTmizer One supports efficient anti-CD19-CAR CRISP/CAS9 transfection and enhanced enrichment of CAR+ lymphocytes in stir tank bioreactor and G-Rex plates

KI=CD19 CAR-T expression; KO=TCRαβ−

FIGURE 14

Expression of early memory phenotype CAR-T cells cultured in stirred-tank bioreactor.



Expression of T_{SCM} and T_{CM} markers is maintained on similar level in stir tank bioreactor and G-Rex plates cultured in CTS OpTmizer One

CTS OpTmizer One supports higher expression of T_{SCM} compared to CTS OpTmizer Pro

CTS OpTmizer One supports expansion of $CD4^+$ and $CD8^+$ lymphocytes, similarly in stir tank and G-Rex plates

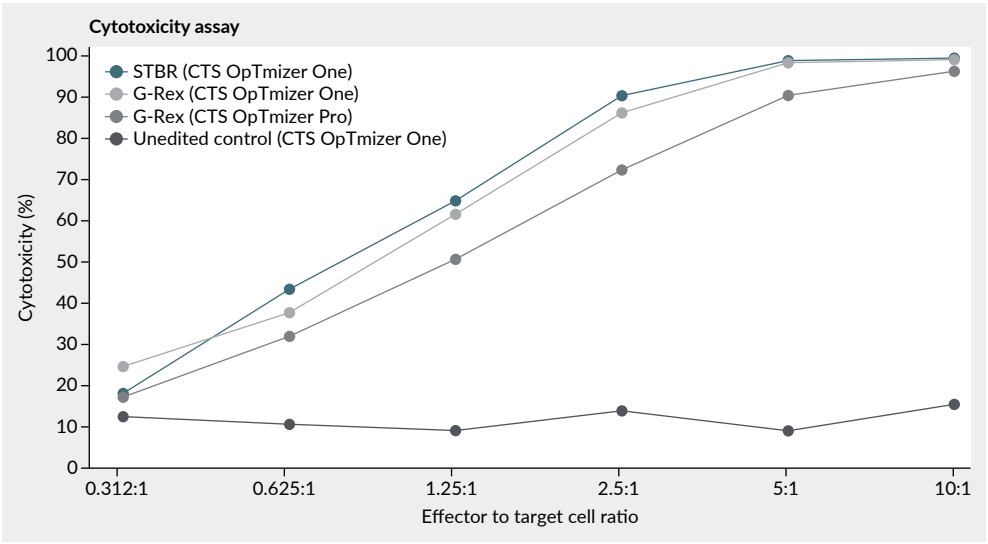
CTS OpTmizer One was shown to deliver higher percentage of $CD8^+$ lymphocytes compared to cultures in CTS OpTmizer Plus

SFM had a higher cytotoxic effect throughout the assay. This could be a result of the higher percentage of CD8 cells observed in the CTS OpTmizer One SFM, as shown in Figure 14.

In summary, the new AOF CTS OpTmizer One SFM has demonstrated a high level of performance in comparative studies, resulting in healthy, functional T cells at the end of production.

FIGURE 15

Expansion of functional and effective CD19-CAR-T cells cultured in different media, including the CTS OpTmizer One SFM.



Q&A


Alan Gutowski and Joanna Kern

Q Is the CTS OpTmizer One SFM just an AOF version of legacy CTS OpTmizer SFM?

AG: No, even though the CTS OpTmizer One SFM is within the OpTmizer family, which is composed of fit-for-purpose T cell media, the CTS OpTmizer One SFM is a brand new, next-generation formulation. It is a one-part formulation, while the legacy CTS OpTmizer has two components—a basal component and a supplement component, which you mix. The CTS OpTmizer One SFM does not require any mixing. It is available off the shelf and ready to go, with the sole addition of glutamine.

Q Does the CTS OpTmizer One SFM contain L-glutamine?

JK: The CTS OpTmizer family media do not contain L-glutamine. We add L-glutamine at the point of use, which provides for consistent performance of the media.

Q What is the difference between the CTS OpTmizer One SFM and the CTS OpTmizer Pro SFM in terms of ingredients?

AG: Our CTS OpTmizer formulations are highly protected as far as their constituents and our IP disclosures, so we cannot comment on the similarities or differences between the two. If there are any specific formulation questions, though, feel free to reach out to Thermo Fisher Scientific's tech support group. They can go through a series of IP triage steps to get an answer for you regarding formulation.

Q During prolonged expansion, why does the CD4 cell population sometimes expand with this media?

JK: The expansion of CD4 and CD8 depends largely on what activation system is used. We have noticed that with the CTS OpTmizer One SFM, the percentage of CD8 cells is slightly higher after approximately seven days of expansion. However, both CD4 and CD8 do expand well in the media. The ratio does lean slightly towards CD4 at the beginning, but then moves towards CD8 with longer expansion times.

Q Does the CTS OpTmizer One SFM contain protein?

JK: Yes, recombinant proteins are in the formulation.

Q Why is the CTS OpTmizer One SFM not a chemically defined medium?

AG: This question goes back to the harmonization of the nomenclature between chemically defined AOF. At Thermo Fisher Scientific, we take a rather conservative approach due to the breadth and scope of media that we offer. Since the CTS OpTmizer One SFM does contain recombinant protein, we are not calling it chemically defined.

However, if the CTS OpTmizer One SFM was manufactured by another organization, it may be called a chemically defined medium.

Q What was the reason behind designing the CTS OpTmizer Pro SFM?

JK: The CTS OpTmizer Pro SFM was designed specifically for the lengthy expansion of T cells with allogeneic therapies. When you have a longer time frame and want the phenotype to remain in the early memory stage, the CTS OpTmizer Pro SFM can be used.

Q Does the CTS OpTmizer One SFM contain phenol red?

AG: It does not. Only the legacy CTS OpTmizer, our first formulation, contains phenol red. Following that launch, we introduced a non-phenol red-containing serum-free version of the CTS OpTmizer. We have demonstrated analogous performance between the phenol red-containing version and the non-phenol red-containing version.

We made these two different versions for a few reasons. For example, at certain concentrations, phenol red can interfere with an analytical method in the manufacturing workflows. In addition, phenol red can also be carcinogenic at higher concentrations. So, to ease some regulatory oversight around phenol red, we removed it from our latest formulations.

Q What film is used in the 1 L, 5 L, and 10 L bioprocessing containers?

AG: In the larger BPCs or the bag versions of our media, we incorporate our own film. It is the Thermo Fisher Scientific Aegis™ 5–14 film. If we compare this film against, say, an older generation ethylene vinyl acetate (EVA) film, the Aegis5–14 has lower additives, which essentially means a cleaner fluid contact surface. It also has lower gas and vapor transmission, which leads to better maintenance of pH and solute concentrations. Additionally, because it is a Thermo Fisher-owned film structure, we have great control over formulation, manufacturing, and the supply chain.

Q How does the CTS OpTmizer One SFM compare to the legacy CTS OpTmizer?

AG: The CTS OpTmizer One SFM has enhanced performance, with a higher chance of achieving a desired phenotype with a potential increase in the CD8 population.

JK: From a technical point of view, it is a tricky question because both media are completely different in terms of the components and how they were designed. We have consistently seen that the CTS OpTmizer One SFM offers similar or better expansion than the legacy CTS OpTmizer. Additionally, while the expansion does depend on the workload, the CTS OpTmizer One SFM improves stem cell memory phenotype for a majority of the workloads tested. It also usually offers higher CD8 expansion in the tested workload. Lastly, we have consistently seen higher transduction efficiency in the CTS OpTmizer One SFM.

Q Can the CTS OpTmizer One SFM be frozen?

JK: Yes, it can be stored frozen, or it can be frozen when it is prepared. We have data to support the freezing of the media for approximately six months, but it can probably be frozen for a longer period of time.

Q Does the CTS OpTmizer One SFM work with IL-7 or IL-15, instead of IL-2?

JK: Yes, IL-2 works similarly to IL-7 and IL-15 in the CTS OpTmizer One SFM.

Q What was the scale of the bioreactors used for T cell expansion in the experiments shown?

JK: In those experiments, we were using two types of bioreactors. One was a rocking bioreactor with a 1 L bag. This bioreactor was used with perfusion so that the culture would contain a very high density of cells. The other bioreactor was a stirred-tank bioreactor. This had a smaller scale (300 mL) and did not utilize the perfusion process.

Q Is the new medium GMP-grade or is it available as research use only (RUO)?

AG: All of the media within the CTS OpTmizer family include GMP manufacturing. There is no RUO version of any of the CTS OpTmizer media right now, it is all CTS-designated.

Q Is there a stable supply for this medium?

AG: All formats of the CTS OpTmizer One SFM are available as off-the-shelf catalog products, meaning that they currently have on-demand availability. For supply assurance, we rely on accurate demand forecasting. We suggest that you work with your sales representative so that, through our reserves team, we can maintain that supply assurance.

Additionally, even though this is a catalog item, we do have a custom media option. This option would allow you to have full control over manufacturing dates, formats, and discrete lots.

BIOGRAPHIES

ALAN GUTOWSKI is a Product Manager at Thermo Fisher Scientific, Dearborn Heights, MI, USA. He is responsible for the global CTS OpTmizer SFM portfolio, including the recently launched, novel formulation of CTS OpTmizer One SFM. He is dedicated to helping manufacturers alleviate pain points in their workflow, across scale and modality, to bring potentially curative cell therapies to market. Alan holds degrees in biological sciences and business from the University of Buffalo, Buffalo, NY, USA.

JOANNA KERN is a Staff Scientist at Thermo Fisher Scientific, Waltham, MA, USA. She currently works on the new product introduction for cell therapy. Joanna has a PhD in Immunology and over 10 years of experience studying T cells biology and using flow cytometry, and is currently focused on solving challenges in cell therapy field.

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AUTHORSHIP & CONFLICT OF INTEREST

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

Acknowledgements: None.

Disclosure and potential conflicts of interest: The authors have no conflicts of interest.

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

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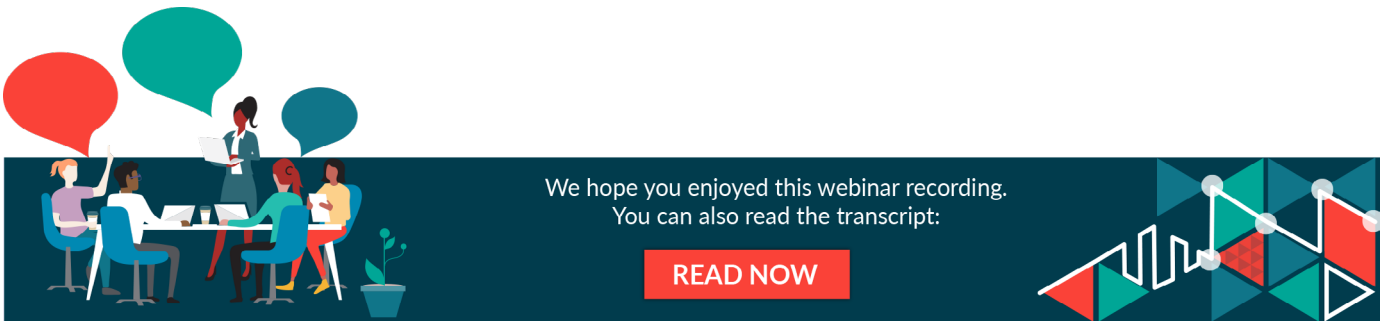
Article source: This article was based on a webinar, which can be found here.

Webinar conducted: Jul 23, 2024; **Revised manuscript received:** Sep 17, 2024;
Publication date: Oct 2, 2024.



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Optimizing bioprocess purification: effective nucleic acid removal in high salt environments

Eleonora Turco and Joanna Niska-Blakie

Efficient cell lysis and host cell DNA removal are crucial in biopharmaceutical purification, especially for viral vectors. This article will discuss how an advanced salt-active endonuclease can enhance bioprocess purification workflows by effectively removing nucleic acid impurities and meeting stringent regulatory standards.

Cell & Gene Therapy Insights 2024; 10(9), 1291–1302

DOI: 10.18609/cgti.2024.147

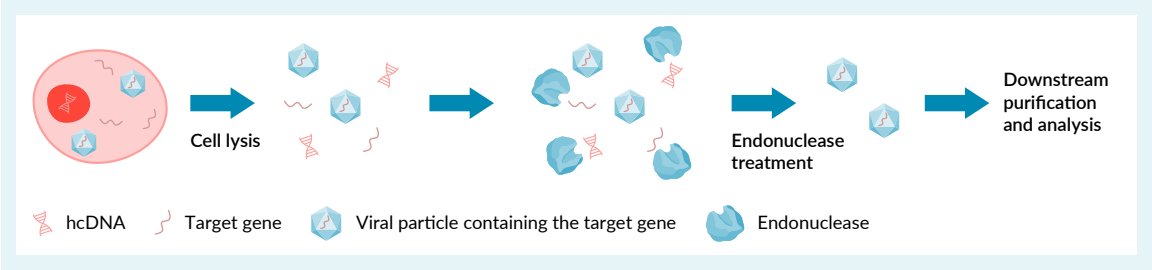
CELL LYSIS IN BIOPHARMACEUTICAL PURIFICATION PROCESSES: AAV PRODUCTION

The requirements of the biopharma industry for cell lysis in viral vectors, specifically the removal of host cell DNA, are extremely stringent, especially when related to various enzyme properties. For an enzyme to be suitable for bioprocesses, it must be highly pure, predominantly in its monomeric active form, free from animal origin, endotoxins, and antibiotics, and have a low bioburden. Additionally, the enzyme should exhibit robust and consistent activity across various batches to avoid costs associated with lot testing.

Ideally, enzymes should be versatile and maintain high activity across buffers and environmental conditions, which facilitates the development of a cost-effective bioprocess. For example, a versatile enzyme minimizes the need for additional buffer exchange steps, which can lead to longer processing times, increased material costs, and potential product loss. Moreover, constant technical and

FIGURE 1

Purification process of the cell lysis of viral vectors.



hcDNA: high fidelity DNA.

scientific support, along with the development of tailored solutions and reliable supply chains, are critical factors considered by customers and are integral to the enzyme development process.

The first step in the purification of viral vectors, as shown in Figure 1, involves the lysis of eukaryotic cells in a lysis buffer. A typical lysis buffer comprises buffering agents to control the pH, with slightly alkaline buffers commonly used. The pH may be adjusted according to the properties of the target molecule. Detergents are used to solubilize the cell membrane, and salts may be included at varying concentrations depending on the process.

While many applications utilize salts at physiological concentrations, viral vector purification often benefits from lysis in high salt concentrations. Following lysis, viral particles and cellular contents, including genomic DNA and target DNA not encapsulated in the viral capsid, are released. At this stage, treatment with endonuclease during the lysis step aids in eliminating contaminant DNA and achieving a purer product.

PURITY AND SAFETY: RISKS ASSOCIATED WITH RESIDUAL DNA AND REGULATORY REQUIREMENTS

The removal of DNA by endonucleases is crucial due to several associated risks posed by residual DNA in the final product. Firstly, there is the risk of oncogenicity. Residual DNA may introduce dominant oncogenes into normal cells, potentially transforming them into tumorigenic cells. Additionally, residual DNA fragments could integrate into the genome and cause mutation in oncogenes, further contributing to oncogenic potential.

Another significant concern is infectiousness. If the viral genome is inserted into the cell, it can generate infectious viral particles, which pose a potential hazard. Additionally, there are technical issues related to viscosity. Released genomic DNA can increase the

TABLE 1

Regulatory requirements of various regulatory bodies on the maximum size of residual DNA fragments permissible in a final product following cell lysis.

Regulatory body	Product	Maximum size requirement
WHO and US FDA	Finished products	Size: <200 bp; amount: <10 ng/dose
US FDA	Host cells of biologics	Amount: <100 pg/dose
	High-dose biologics	Amount: <100 ng/dose
EMA	Finished products	Usual amount: <100 pg/dose; hepatitis A vaccine amount: <100 pg/dose; hepatitis B vaccine amount: <10 pg/dose

viscosity of the solution, resulting in reduced process efficiency. For example, higher viscosity can lead to increased membrane fouling during downstream filtration steps.

Lastly, there are compliance issues. Regulations from the US FDA, WHO, and EMA specify acceptable levels of residual DNA per dose, and the presence of residual DNA in the final product can result in compliance problems. Adhering to these regulations is essential to ensure the safety and acceptability of the biopharmaceutical product.

Various regulatory bodies specify the maximum size of residual DNA fragments permissible in any preparation or product, with the intention that the residual fragments should be short enough not to contain viable genes. However, as illustrated in Table 1, regulations differ across countries and product types. Given this variability and the evolving nature of regulatory standards, the most prudent approach is to err on the side of caution by removing as much DNA contamination as possible. In this context, treatment with endonucleases becomes essential.

KEY CHALLENGES IN HOST CELL DNA REMOVAL DURING CELL LYSIS AND ENDONUCLEASE ACCESS TO DNA

Using endonucleases to remove host cell DNA following cell lysis presents several challenges, one of which is caused by the structure of chromatin. Chromatin is tightly packed within the nucleus, with DNA wrapped around histones. This creates a strong interaction that makes it difficult for endonucleases to access and degrade the DNA effectively.

Another challenge is the viscosity created by the DNA itself, which hinders its digestion. In viscose solutions, enzymes struggle to reach their target. However, research has shown that higher salt cell concentrations can reduce viscosity, leading to an increased yield of viral vectors. Furthermore, high salt concentrations have been found to minimize vector aggregation, which poses an additional obstacle to DNA removal during viral vector production and purification.

Finally, cost considerations must be taken into account. Endonucleases are relatively expensive enzymes, making the development of enzymes with high specific activity essential. By using enzymes that require smaller quantities to achieve efficient digestion, the overall process can be made more cost-effective.

Several studies have demonstrated that cell lysis buffers containing high salt concentrations not only increase the yield of viral vectors but also enhance the production of more infectious viral particles.

SALTONASE® ENDONUCLEASE OVERCOMES HOST CELL DNA REMOVAL CHALLENGES

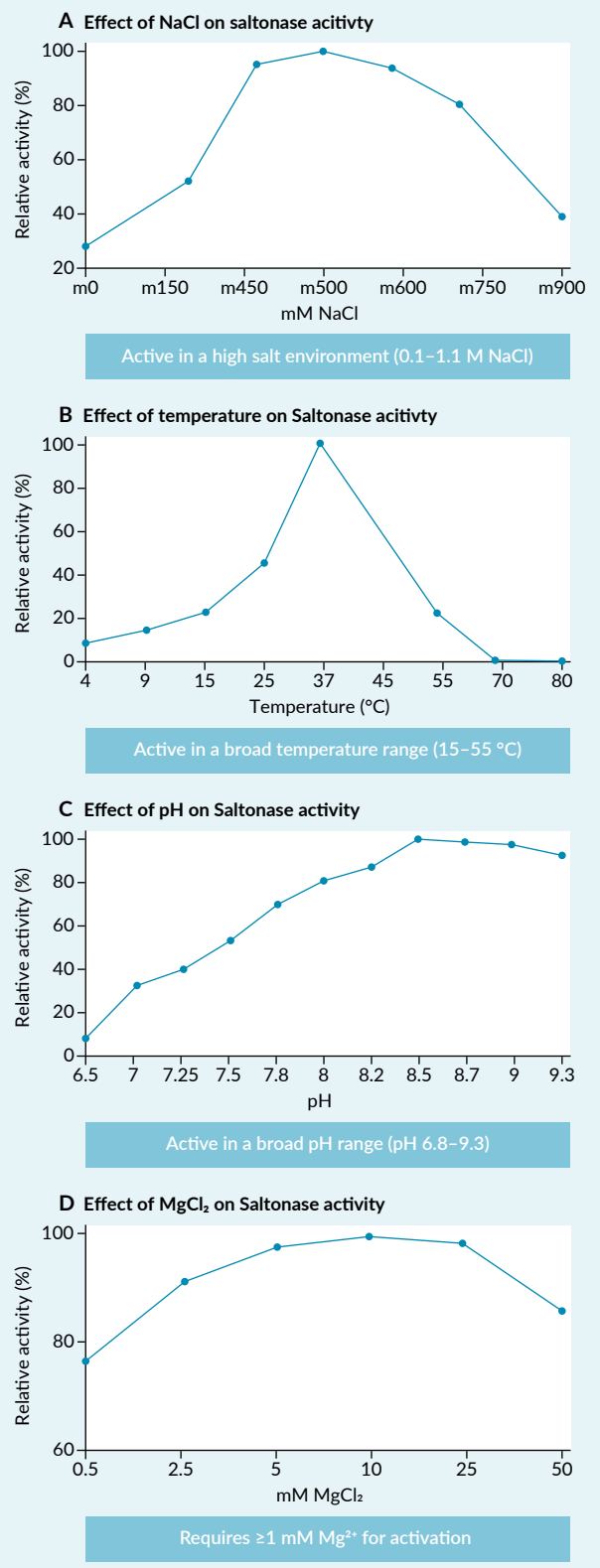
As discussed above, high salt concentration plays a critical role in the depletion of host cell DNA. To address the challenges associated with enzymatic digestion, QIAGEN developed a salt-active endonuclease named Saltonase. As the name implies, it is active in high salt conditions. Unlike most other endonucleases, it is derived from psychrophilic bacteria and expressed in *E. coli*. The enzyme has a molecular weight of 40 KDa and possesses several unique features, including activity across a broad range of pH, salt concentration, and temperatures.

What differentiates Saltonase from other endonucleases is its ability to remain relatively active under diverse conditions. Relative activity, in this scenario, refers to the ratio of activity measured under a specific parameter, such as NaCl concentration, compared to the enzyme's maximum activity. Saltonase achieves optimal performance under conditions of 500 mM NaCl, a pH of 8.5, a temperature of 37 °C, and 5 mM magnesium, as seen in Figure 2.

The operating range of Saltonase is defined as retaining a minimum of 20% relative activity. The salt concentration range, shown in Figure 2A, spans from 0–900 mM NaCl. The temperature range (Figure 2B) is between

FIGURE 2

Relative activity of Saltonase under conditions of (A) high salt environment, (B) broad temperature range, (C) broad pH range, (D) ≥1 mM of magnesium.



15 °C and 55 °C, indicating that Saltonase performs effectively at room temperature. The pH range (Figure 2C) is between 6.8 and 9.3, and magnesium (Figure 2D) is indispensable for Saltonase activity. It is always recommended to add at least 1 mM of magnesium to ensure optimal enzyme performance. By adjusting one of these parameters, the enzyme's performance can be tailored to specific experimental conditions.

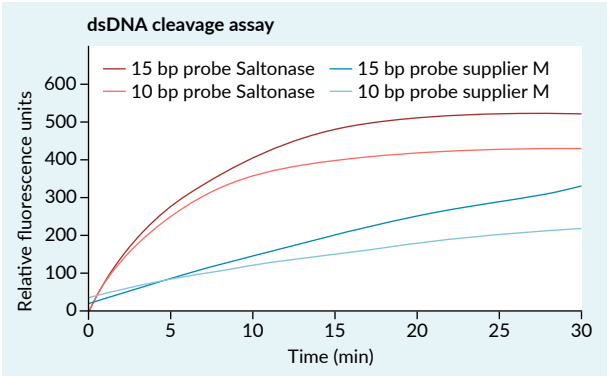
EXPERIMENTAL COMPARISON OF SALTONASE AND ANOTHER ENDONUCLEASE TO ASSESS ROBUST DNA CLEAVAGE

Safety is a key aspect of bioprocessing, making the robustness of digestion a critical feature for effective DNA depletion. According to enzyme manufacturers [1–3], most endonucleases can digest DNA into fragments smaller than 10 nucleotides. To assess Saltonase's efficiency in DNA digestion, a study was carried out to compare it against the endonuclease of another supplier. For this evaluation, a double-labeled, double-stranded DNA as a substrate was used, featuring 10- and 15-base pair probes with a quencher positioned in close proximity to a fluorophore.

The mechanism operates while the DNA remains intact, and the quencher absorbs energy from the fluorophore to prevent

FIGURE 3

DNA digestion of Saltonase compared to an endonuclease of another supplier.



fluorescence emission. Once the DNA is cleaved, the quencher can no longer suppress the fluorophore, resulting in an increase in the fluorescence signal. The relative fluorescence units (RFU) were measured by detecting the specific wavelength of the fluorophore at various time intervals. Higher RFU values indicated more effective DNA digestion. As shown in Figure 3, the higher RFU values suggest that Saltonase can digest DNA down to fragments of three to five nucleotides, making it a safer choice for applications requiring stringent DNA depletion.

EXPERIMENTAL COMPARISON OF SALTONASE AND BOVINE SERUM ALBUMIN TO ASSESS THERMAL STABILITY

The thermal stability of Saltonase was also tested using nanoDSF, a technique that measures change in fluorescence emission as the protein sample is gradually heated. As the temperature increased, the proteins began to unfold, altering their fluorescence emission. The emission spectra were recorded throughout the temperature ramp, with the onset temperature marking the beginning of unfolding and the inflection point, or melting temperature, indicated when the protein was half unfolded. This data was able to provide insights into the protein's thermal stability.

When tested and compared to bovine serum albumin (BSA), a highly stable protein, Saltonase demonstrated an inflection point >2 °C higher than BSA, as shown in Figure 4. This indicated that Saltonase was more thermally stable even than BSA, further highlighting its substantial stability.

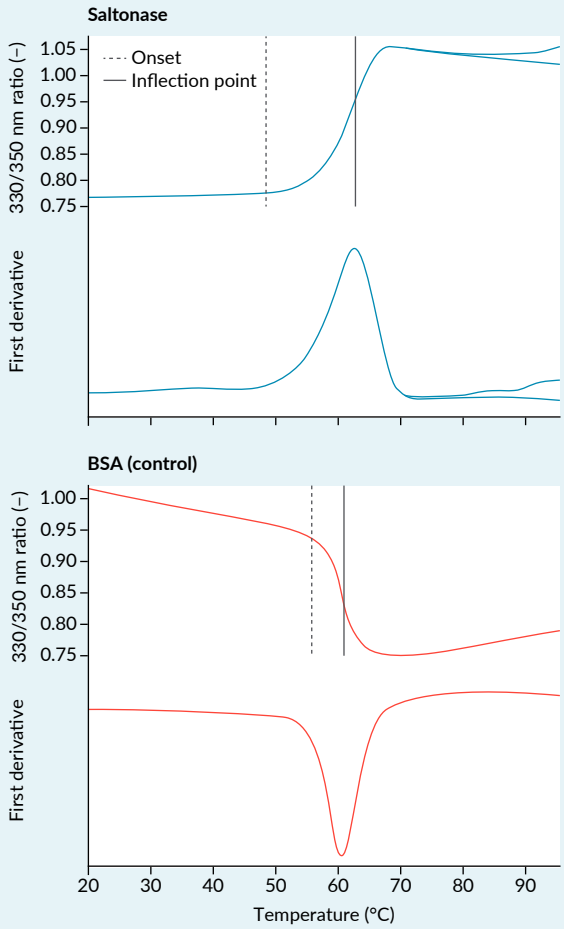
When selecting enzymes, including endonucleases, it is important to prioritize those that are not only stable at the low storage temperatures of −80 °C but also at reaction temperatures of 37 °C and often at room temperature. To further assess Saltonase's thermal stability, the activity of three different lots of the enzyme at 37 °C and 23 °C were tested over a period of 3.5 months. The results are depicted in Figure 5A and B, respectively.

The results indicated that Saltonase retained 100% of its activity for up to 6 weeks at both temperatures, while further retaining at least 80% of its activity for up to 3 months at 37 °C, and at least 90% at 23 °C.

An additional important aspect in determining the thermal stability of the enzyme was its resistance to the temperature fluctuations

FIGURE 4

Experimental comparison of Saltonase (top) and BSA (bottom) to assess the thermal stability of the enzyme.



Sample	Concentration (mg/mL)	Onset (°C)	Inflection point (°C)	CV%
Saltonase	0.866	48.73	62.93	0.07
BSA	2.000	55.67	60.83	0.08

BSA: bovine serum albumin.

caused by the freeze-thaw cycles. To test this, Saltonase activity under various conditions was measured across 21 freeze-thaw cycles, including transitions from −80 °C to 4 °C, −80 °C to room temperature, −20 °C to 4 °C, and −20 °C to room temperature. A lower activity threshold of 250 U/µL was established, and the results (illustrated in Figure 6) demonstrated that there was no observed loss of activity under any of the conditions tested after 21 cycles.

FIGURE 5

Three lots of Saltonase kept at temperatures of (A) 37 °C and (B) 23 °C, over a period of 3.5 months.

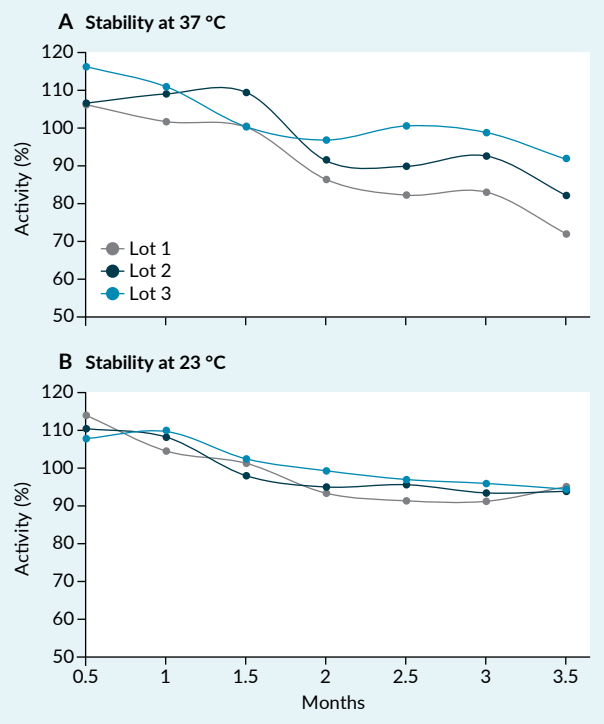
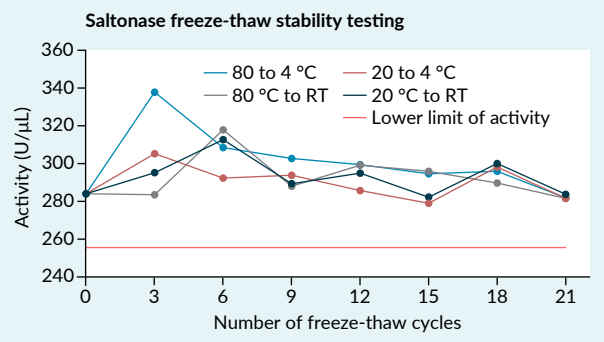


FIGURE 6

Thermal stability of Saltonase under fluctuating temperatures.



ACTIVITY OF SALTONASE ACROSS VARIOUS NaCl AND pH LEVELS

Saltonase's performance was compared with two other endonucleases: one which was salt intolerant, depicted as gray in Figure 7, and the other which was salt tolerant, shown in dark blue. The comparison was conducted at three NaCl concentrations—0, 150, and 500 mM—across three pH levels—6.8, 7.4, and 8.5—and at two reaction temperatures, 37 °C and room temperature.

As shown in both temperature panels of Figure 7, Saltonase consistently outperformed the other salt-tolerant endonuclease under all tested salt and pH conditions. When compared to the salt-intolerant endonuclease at physiological pH (7.4) and pH 8.5, Saltonase demonstrated greater performance at salt concentrations from 200 mM and above. At the lower pH of 6.8, it showed better performance at salt concentrations exceeding 300 mM.

DEMONSTRATING SALTONASE USE IN ADENOVIRUS BIOPROCESS OPTIMIZATION: HOST CELL DNA REMOVAL

The efficiency of Saltonase in industry-related applications has been demonstrated in several recent publications [4,5], particularly in the purification processes of adenovirus and AAVs. In one study [4], Saltonase, at a final concentration of 100 U/mL, was incorporated into the lysate mixture at a salt concentration of 500 mM. The mixture was incubated at room temperature for 3 hours with continuous mixing, allowing Saltonase to effectively degrade nucleic acids and assist in the clarification process prior to the depth filtration and tangential flow filtration (TFF) purification steps.

Although measuring complex samples such as clarified lysates can be challenging, the total reduction in DNA highlights the robustness

of this process in achieving high final product purity. It was found that utilizing Saltonase for pre-chromatography impurity removal led to a 99% reduction of host cell DNA by the end of the process. This emphasizes the importance of effective upstream strategies and pre-chromatographic steps.

Another example of Saltonase being tested under less optimal and more challenging conditions was demonstrated via its application in lentiviral (LV) and virus-like particle (VLP) purification. For LV purification, as shown in Figure 8A, Saltonase was used at the concentration of 50 U/mL with a lower salt concentration of 150 mM NaCL, at pH 7.0, and a temperature of 8 °C for 12 hours. As shown in Figure 8A, this led to a 99.8% reduction in host cell DNA.

Saltonase was further tested during VLP purification under more challenging conditions. The digestion conditions remained the same at 50 U/mL. However, the digestion time was significantly shorter at 45 minutes. Additionally, the salt concentration was higher at 500 mM NaCl, with the pH lowered to 6.5 and the temperature set at 23 °C. Despite the more stringent conditions, Figure 8B demonstrates that Saltonase

FIGURE 7

Activity (U/µL) of Saltonase conducted at three NaCl concentrations across three pH levels at 37 °C and 23 °C.

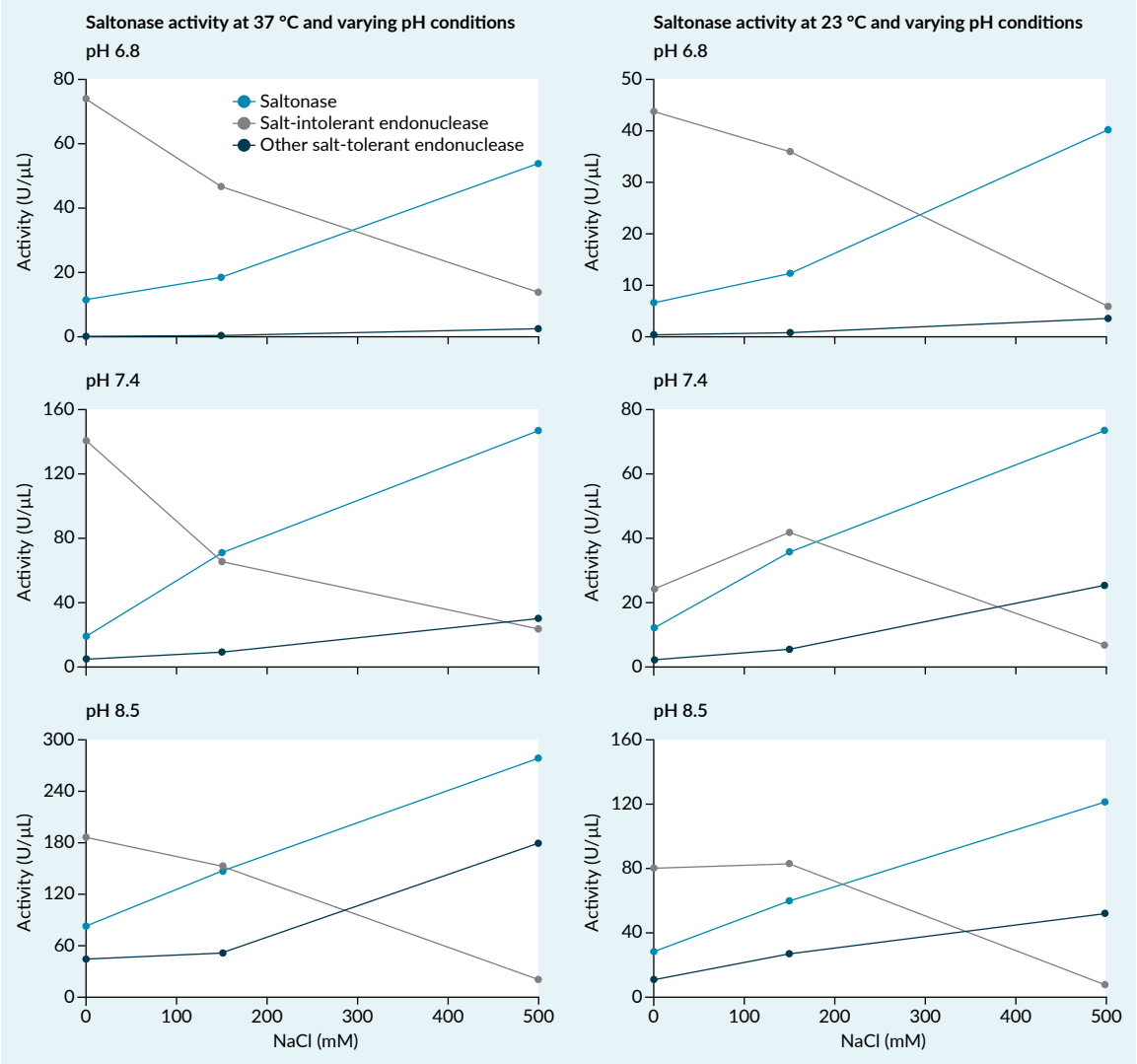
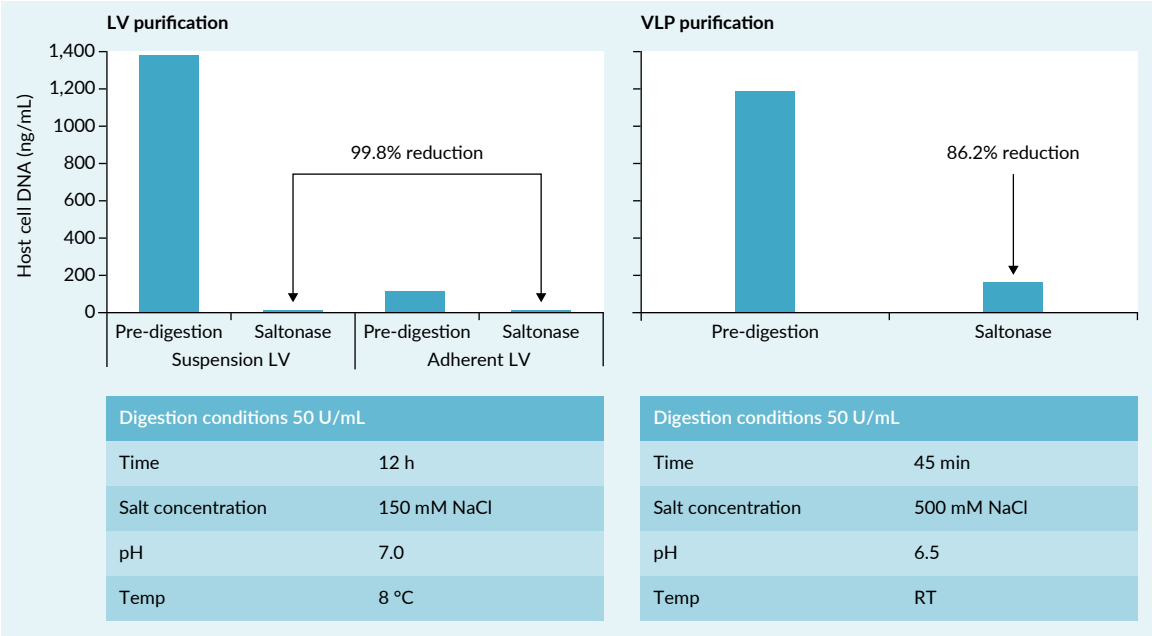


FIGURE 8

Efficiency of Saltonase in host cell DNA removal in (A) lentiviral purification and (B) virus-like particle purification.



Digestion conditions 50 U/mL	
Time	12 h
Salt concentration	150 mM NaCl
pH	7.0
Temp	8 °C

Digestion conditions 50 U/mL	
Time	45 min
Salt concentration	500 mM NaCl
pH	6.5
Temp	RT

achieved >86% digestion of host DNA, further showcasing its versatility and efficiency.

An important aspect to address is how to detect Saltonase levels after its removal. Saltonase can be removed through several downstream processes, including depth filtration for clarification, diafiltration, and chromatography. Successful removal can be verified using QIAGEN's newly developed ELISA, which is highly accurate due to the use of monoclonal antibodies and offers a broad detection range from 30–1,000 picograms/mL.

SUMMARY

Effective cell lysis and DNA removal require enzymes to be highly pure, free from contaminants, and consistently active across batches. High salt concentrations during lysis aid DNA removal, minimizing risks such as oncogenicity and viscosity issues. Saltonase, a salt-active endonuclease, addresses these challenges by performing well under diverse conditions. It demonstrates superior DNA digestion compared to other endonucleases and exhibits excellent thermal stability. Saltonase has proven effective in purifying adenoviruses, LVs, and VLPs, highlighting that it is a suitable choice for simplifying workflows in bioprocessing.

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Q&A

Eleonora Turco and Joanna Niska-Blakie

Q If the magnesium is depleted, does this mean complete inactivation of the Saltonase?

JNB: Magnesium is a crucial co-factor for Saltonase. The presence of chelating agents such as EDTA, which binds to and chelates magnesium, effectively inhibits Saltonase activity. The extent of this inhibition depends on both the concentration of Saltonase and the EDTA. To verify the extent of inhibition, it is advisable to conduct an enzyme activity assay. It is important to note that this type of inhibition is rapid but temporary, as it is reversible. The recovery of Saltonase activity depends on the subsequent processes. If EDTA is absent, Saltonase activity should resume.

In such cases, it is recommended not only to inhibit but also remove Saltonase using chromatography methods combined with TFF. Finally, it is essential to verify that no residual Saltonase remains by employing an ELISA specifically designed for Saltonase detection.

Q Does QIAGEN offer immobilized Saltonase?

JNB: Immobilized Saltonase has been considered, but it has not been implemented. There is currently no endonuclease on the market that is immobilized on solid support through covalent forces, such as magnetic beads.

The potential benefits of this approach are substantial. Primarily, an immobilized system would offer reusability, which is particularly advantageous given the high cost of enzymes in large-scale industrial applications. While some enzyme activity may be lost during the

immobilization, the process would also facilitate the removal of the enzyme, as it can be removed along with the support material.

Q Has Saltonase performance been tested in different detergents?

ET: Saltonase is typically used in lysis buffers, which often contain detergent. However, the studies performed at QIAGEN in formulation buffer did not contain detergents. We have received feedback from clients who tested Saltonase activity in the presence of various detergents, and they have not shown any significant differences in enzyme activity.

Q Why is Saltonase's onset temperature on nanoDSF lower than BSA when its melting temperature is higher?

ET: When analyzing the curves generated by the nanoDSF, fluorescent emission changes with temperature have been detected. The curve's slope indicates how quickly the protein unfolds as temperature increases.

The onset temperature is where the slope of the curve begins, signaling the start of protein unfolding. A gentle slope means that the unfolding process is slow. For Saltonase, although the onset temperature of unfolding is relatively low, the unfolding occurs gradually. As a result, Saltonase reaches 50% unfolding, which corresponds to its melting temperature, later than BSA. This gradual unfolding is reflected in the melting temperature being higher for Saltonase compared to BSA.

Q What is the shelf life of Saltonase?

JNB: The current shelf life of Saltonase is 2 years. However, real-time tests are being conducted as well as accelerated stability tests under various conditions. Preliminary results suggest that the shelf life may be able to be extended in the future. For now, Saltonase is guaranteed to remain stable for a minimum of 2 years.

BIOGRAPHIES

ELEONORA TURCO earned her PhD from Max Perutz Labs Vienna, Vienna, Austria in 2015, where she specialized in histone ubiquitynation. She then was a postdoctoral fellow at the University of Vienna, Vienna, Austria, conducting research on autophagy. Following her postdoc, Eleonora became a Senior Principal Scientist and Functional Lead at Novartis, where she led the Manufacturing Science and Technology Downstream Development Lab. She is currently the Director of R&D Enzymes at QIAGEN, Gdańsk, Poland. With a deep expertise in molecular and cellular biology, Eleonora brings extensive experience to the field of bioprocess purification.

JOANNA NISKA-BLAKIE completed her Master's research study on telomeres at École Normale Supérieure de Lyon, Lyon, France and earned a PhD in cancer research from the FIRC Institute for Molecular Oncology, Milan, Italy in 2013. Later, during her postdoctoral work at A*STAR in Singapore, she focused on oncology and the gut microbiome. Currently, Joanna strategically manages the Biopharma Enzymes portfolio at QIAGEN, Gdańsk, Poland.

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

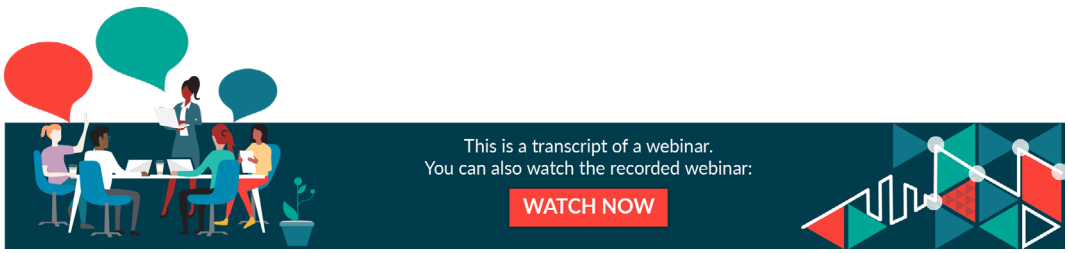
Acknowledgements: None.

Disclosure and potential conflicts of interest: The authors are QIAGEN employees and hold Saltonase® IP patents.

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

Article source: This article was based on a webinar, which can be found here.

Webinar conducted: Aug 22, 2024; **Revised manuscript received:** Oct 8, 2024;
Publication date: Oct 30, 2024.



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INNOVATOR INSIGHT

How will process development and analytical innovation drive growth for the future of cell and gene therapies?

Mark Santos

We are at a critical inflection point for commercialization as more cell and gene therapies (CGTs) enter clinical trials. However, developing CGTs presents unique complexities, such as variable production methods and analytical challenges. This article explores strategies for addressing these challenges through standardization and scalability. These include platforms that reduce hands-on time and cost of goods (COGs) in cell therapy, as well as 3D suspension-based approaches and perfusion technologies for improving viral vector and exosome production. These innovations aim to streamline manufacturing and support the broader adoption of advanced therapeutics.

Cell & Gene Therapy Insights 2024; 10(9), 1055–1065

DOI: 10.18609/cgti.2024.120

As demonstrated through other biologic modalities success standardization and scalability are pillars of manufacturing innovation success. The cost of goods (COGs) challenge still remains a problem today, as the field must scale up manufacturing to drive down the COGs and ensure accessibility to patients across the world. In the past, large-scale production was co-invested with Lonza and Genentech to help decrease COGs and share production across multiple programs at large scale to make them affordable to patients.

In the cell and gene therapy (CGT) journey, we are now making remarkable progress, but the question of how to create scalable solutions remains. There are multiple modalities and scalability challenges to consider,

particularly in the cell therapy space. CGTs are different from other biologics in terms of demand, standardization, analytical methods, and scalability. As cell therapies are living systems, the way they are produced differs from product to product and their analytical methods are complex. There are limitations to what can be tested for within a living system compared to one that is stable and not living.

MANUFACTURING INNOVATION FOR CGT

Manufacturing remains a key challenge across the industry. After Phase 3, CGTs experience nearly five times more discontinuation or significant delays due to CMC issues than monoclonal antibodies. The first consideration is many of the treatments being pursued are for unmet medical needs, meaning there are expedited approval pathways, and thus reduced development timelines. Being ready to support those timelines is key, ensuring teams are agile and future-focused.

Scaling up versus scaling out is another consideration, as CGT manufacture is complex and cannot be ramped up linearly. Gene therapies may hold the potential to be scaled up in a similar way to other biologics modalities, but for autologous programs, scaling out may be the solution. Ensuring that the field has scale-out solutions that are as viable as scale-up is challenging.

The cost of development and manufacturing is another challenge. For some of these products, costs can exceed $500,000 per dose. We need to find solutions to reduce the COGs early so that later, economies of scale can reduce costs for patients.

Product quality, defining CQAs, and analytical method development are fundamental. Analytical methods and CQAs are required to justify to regulatory agencies that a process is under control, and that robust quality systems demonstrate the product is safe, consistent, and accessible to everyone in the world. The regulatory landscape is rapidly evolving in this space differently to other, more mature modalities. New regulations, approaches, and solutions are being considered, but experience and stability matter.

A healthy pipeline of programs will be approved over the next 10 years, as shown in Figure 1. The percentage of programs in Phase 3 and commercial today demonstrates that a breakthrough period is coming wherein more programs will flood the commercial late-phase market, enabling a tremendous amount of learning within the industry. The market has gone through a large evolution, beginning with early excitement, and a 60% year-over-year increase in new programs coming onto the market. New ideas and approaches to process development and patient treatment in this new space have been tried. Over time, there has been some uncertainty regarding having the data to prove these therapies are viable and can reach patients with the required scalability. As this breakthrough period happens, and as these learnings are solidified, we will enter the rational growth period other modalities have experienced. We anticipate a ~22% rational growth period of new programs coming in between 2025 and 2033.

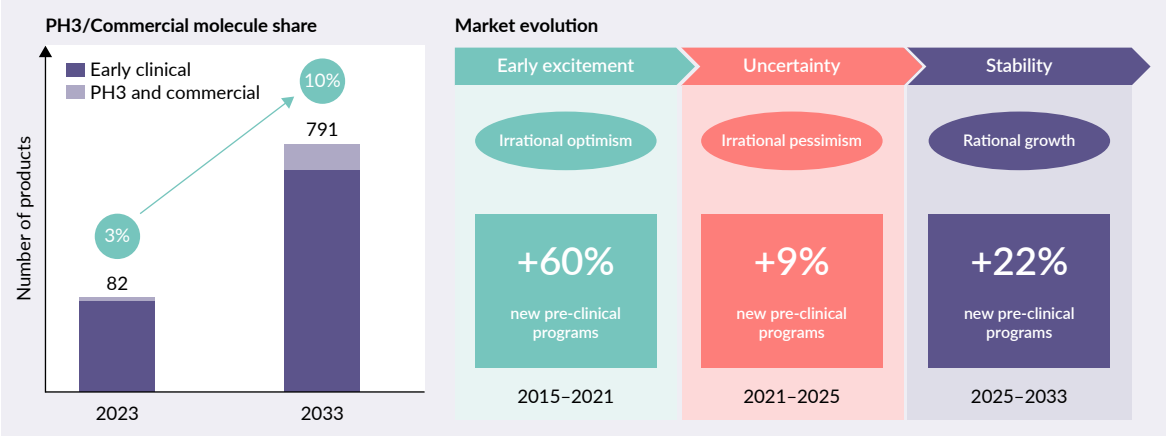
STEP-BY-STEP APPROACH TO DEVELOP A COMMERCIALLY VIABLE PROCESS

To ensure these programs make it through commercialization, process development (PD) is foundational. For successful therapy commercialization, we need solutions to convert PD ideas into clinically and commercially viable processes. Lonza aims to work with customers and partners to ensure robust processes and analytics are in place so that batches can be manufactured safely and of high quality so that ultimately, medicines reach patients.

To do so, developing commercially viable processes is key. Our approach is to start with baseline processes and observe the gaps that will be present in the future that must be closed before treating patients in Phase 1.

FIGURE 1

Maturation in the market will drive stability.



In designing that process, the CQAs must be considered to ensure a process can be developed for industrialization. To confirm a process has been appropriately developed, it is transferred to our GMP suites, which involves executing pilot, training, clinical, and BLA runs.

Within CGTs, there is diversity within this baseline process journey. This diversity can be exemplified by autologous programs. The steps in producing an autologous product may seem similar across every program from washing, elution, transfection, transduction, to fill-finish and eventually shipment.

However, a key challenge surrounds the technologies and materials used within each product. In some cases, starting materials may require homogenization, which is labor-intensive and requires skilled scientists, which can be difficult to scale to hundreds of batches in a controlled manner. Another critical element is closed processing, as open processes introduce a quality risk around contamination. As a CDMO, standardized raw materials would be advantageous, as every customized product has a unique bill of materials, so economies of scale can be challenging to achieve.

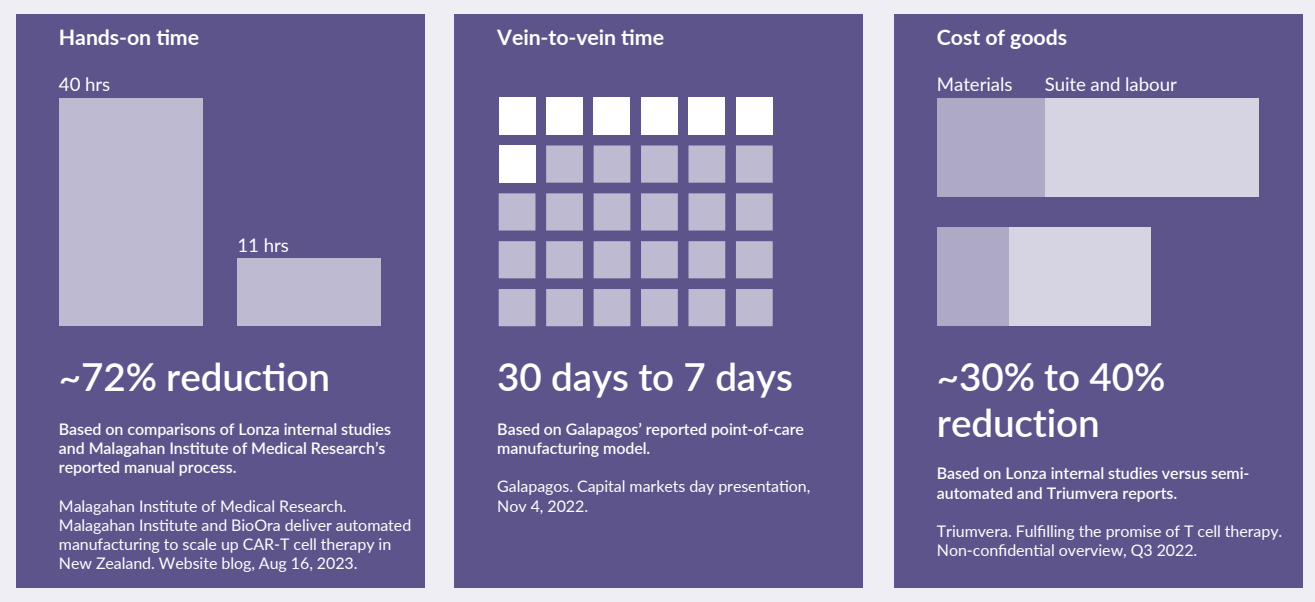
A final processing challenge is particulate management. As part of the fill-finish steps, it is important to understand the kinds of particulates you may see in a process. Qualifying those and ensuring the release process is ready to manage those particulates is a critical step early in the development lifecycle.

On top of the processing challenges, there are also treatment challenges. Within CAR-T treatments, the quality of the cells varies, as cells are manufactured for each patient. It is not possible to produce a completely standardized product. In the JULIET study where Kymriah® was used to treat relapsed refractory diffuse large B cell lymphoma patients, 13% of patients never received the CAR-T cell product due to disease progression. That is a difficult challenge for us to deal with in the space. It is not always possible to harvest and manufacture adequate lymphocyte numbers, especially with patients whose lymphatic systems are affected by their disease or previous chemotherapy treatments.

These challenges are things that manufacturers cannot necessarily control, but they can impact the output of what is produced. One innovation we are pursuing in the CGT space at Lonza is the Cocoon® Platform. The Cocoon Platform's vision is to reduce hands-on time, reduce vein-to-vein time, and reduce the overall COGs, as presented in Figure 2. This vision is backed by our bench-to-IND platform process solutions, which look to find a standardized approach to process development and bill of materials so that

FIGURE 2

Cocoon performance in the lab and field.



customers can use an approach that has been tried and true and can ultimately accelerate their journey toward patients.

As a specific case study, we supported a small biotech company that was dealing with a request for critical data from the US FDA before they could proceed with developing a treatment for a debilitating ocular disorder in Phase 2. As part of the solution, we addressed their current cell banking strategy. Within that, it was clear the testing and the process development around that cell bank needed to be more robust and viable for commercialization. We worked with our customers to help develop that strategy and ensure that it was accepted by the FDA. Ultimately, this success story was facilitated by teams coming together with agility in creative thinking and problem-solving.

VECTOR AND EXOSOME PRODUCTION PROCESSES

In viral vector production, transfection yield and purity are some of the key challenge statements. Some of the unit operations underpinning viral vector production look similar to a mammalian biologic. There are critical differences though which must be addressed. For example, how to achieve full virus instead of partially full and empty capsids. Then, most importantly, how do you ensure that virus production is purified with high yield?

For transient transfection, the purification step is critical and development around this step can make or break COGs decisions later. The difference between a 50% yield and purity and 60% full virus and a 20% yield and a 30% purity is large—as cumulative factors can drastically impact COGs. The challenge statement will exponentially increase when trying to treat thousands if not millions of patients globally.

The top challenges for viral vector manufacturing surround transfection, yield, and purity. To solve the closed processing and manual operation challenges across the industry, the pursuit of platform processes that allow the standardization of unit operations, raw materials, and process parameters across multiple programs is essential. This will greatly accelerate our ability to develop processes faster and get products to patients for an IND quicker.

As a scalability solution, Lonza's transient transfection platform is designed to overcome

these challenges. Moving away from an adherent 2D approach to a 3D suspension-based approach drives up the potential COGs later. We are developing a producer cell line, a solution that will mean we no longer need the transient transfection step. This will improve the quality, scalability, and overall predictability of these programs in the future.

When considering the upcoming exosome space, key challenges surround managing the complexity and the potential. Mammalian technologies can help with the exosomes themselves, but there is a great deal of diversity within the cell lines. Each cell line may need to be immortalized, and immortalization technology could become an important tool. Key considerations surround how to engineer exosomes and attach payloads to them. Different solutions will result in a wide range of product types. Finding the right combination of attachments and loading technologies to produce multiple products in a similar process will enable consistency and scalability across multiple programs. Perfusion technologies will drive efficiencies in exosome production, particularly in scalability and COGs benefits down the line. If dosing quantities and demand becomes high, there may be limitations to the perfusion technologies and engineering solutions may be required. Platform processes and analytical approaches will help tremendously here.

ADDRESSING KEY CELL THERAPY CHALLENGES

In the cell therapy space, yield, criticality, scalability, and speed are paramount. Ultimately, the diversity and technologies of the unit operations make it difficult to have closed processing and less manual operations. By standardizing as much as possible and developing a plug-and-play approach across each of the unit operations and technologies, alongside the supporting analytics, we will begin to see efficiencies delivering results.

For autologous therapies, we must consider scale-out in terms of the suites, the setup, the procedures, and the quality systems, and how these can be consistent across all sites. For allogeneic therapies with a scale-up approach, we want to move towards a 3D approach as quickly as possible and before we patients begin treatment to ensure a viable, scalable path forward that will drive down COGs for later phase production.

A standardized bill of materials will help across all programs to reduce complexity and drive down COGs. Innovation within the quality control methods and leveraging inline analytics and quality by design for process analytical technologies are the solutions that could drive the key needs in this space.

Managing multi-product suites for products to be delivered within short timelines for delivery does drive some efficiency questions. When efficiency is key to driving COGs and keeping high utilization of personnel, this challenge must be thought through in facility design and planning.

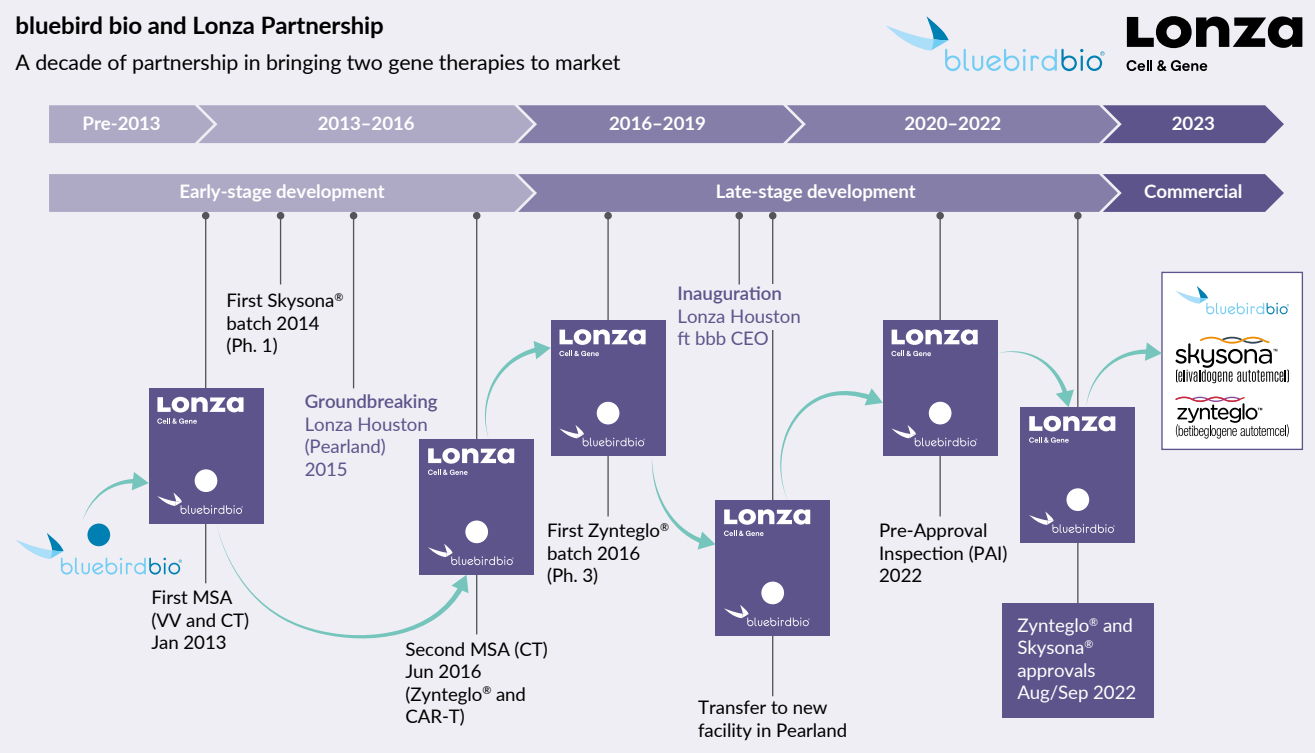
Quality systems are the cornerstone of any CGT-related product, and the pillar for Lonza to ensure that our customers get through Biologics Licensing Applications (BLAs) right first time. The quality system spans the production system, facilities, and equipment, laboratory controls, material systems, packaging, and labeling. From 2007–2020, there were 35 CGT facility inspections with the following findings:

- 89 observations on quality
- 66 observations on facilities and equipment
- 60 observations on production
- 43 observations on materials
- 34 observations on laboratories
- 20 observations on packaging and labeling

These types of challenges can result in delays. It is important to have a partner with

FIGURE 3

Lonza and Bluebird bio: an innovative, decade-long partnership.



a robust quality system that has been used across multiple modalities and tested with confidence so that you can ensure the safety of the products you are delivering to patients.

A PROVEN INNOVATION PARTNER

Lonza is proud of our partnership with Bluebird bio (Figure 3). This innovative partnership approach spanned from January 2013. 2022 marked the pre-approval inspection in a brand-new facility, in addition to two commercially approved products. This timeline was fast for multiple reasons, including that the facility was dedicated and available for use and that the investment required was made early.

Further successes publicly supported by Lonza include Bristol Myres Squibb's commercial product Breyanzi® in 2021, and in 2023, Vertex and Lonza coming together to build a dedicated facility for a potentially curative treatment for type 1 diabetes.

To industrialize manufacturing processes from concept to patient, Lonza's key solutions lie within our global footprint and flexible capacity, offering innovation and the ability to develop solutions for scalable and robust manufacturing of CGT. Our expertise in various cell vector types, automation, and scalable technologies enables our customers to get their products to the market.

SUMMARY AND KEY MESSAGES

We are at a critical inflection point in the commercialization of CGTs. Currently, 75% of existing programs are in Phase 1 or preclinical phases and 75% of the companies supporting these products are small biotechs or academic spin-offs. From 2024, we expect the number of CGTs to reach commercialization in one year to be higher than the total number of CGTs launched since 2017.

For us, delivering the future of these treatments is within our grasp. It is critical to invest early in process development; reduce

open and manual processes with technology and automation; standardize approaches and processes for scalable manufacturing; standardize analytics and consumables wherever possible; and know the importance of quality systems and people. Delivering standardization, innovation, and technology remains the key.

Q&A

Mark Santos

Q What do you see as the most important innovations that will drive COGs impacts in each of the technologies?

MS: We need to take the innovations that we have had in the CGT space and try to standardize where we need to. How do we develop a process that can be leveraged across multiple programs so that supporting raw materials and analytics can ultimately drive better COGs? There will be many scientific discoveries that will help us, but from a manufacturer's point of view, the only area in which we can influence COGs is in standardizing and streamlining as much as possible. If we can do that across multiple programs, across each of these technologies, that will make a difference.

Q What would be your key pieces of advice for a scientist to consider when developing their processes?

MS: Reach out for help. Often, the best thing to do is to talk to folks who have taken a product through Phase 1 or a program through a BLA. It is also important to understand each unique program may have specific challenges. There could be barriers or unique issues that you need to deal with, for example, to promote cell growth or to solve particulate management. As a CGT community, we can all help each other. Companies like Lonza are here to help provide guidance and we have seen many solutions and ideas implemented. Hopefully, we can help you accelerate those things where possible.

Q Should researchers and early-stage companies invest in their own CGT CGMP capabilities?

MS: For CGTs, there is the potential to consider building your own clean room for about $20 million and thus produce and support your own supply chain. The main challenge is not only the capital hurdle, but also the regulatory set-up and the quality systems that must be in place and compliant with GMP. Regulators will look at your historical processes, any previous challenges, and the number of past challenges you may have had. With that lens, you may have the right program or pipeline that justifies the demand for that capacity, flexibility, and control. This is a program and product-specific decision.

Ideally, starting outsourcing and insourcing later tends to be a more viable strategy. A blended approach with an outsourcing partner and an insourcing partner will help you manage the upfront investment risks, which could distract you from delivering programs to patients or supporting clinical trials.

Q What are the biggest lessons that you have learned and can take away from working with your customer Bluebird in the commercialization of their products?

MS: A strong partnership early was the most important for us. We understood the patient's needs and we were proud to be part of that journey. Scalability is one of the biggest challenges in most autologous treatments. In this case, thinking about how to grow from a clinical program treating 10–15 patients, to potentially thousands at multiple clinical sites with a fast turnaround from patient back to patient was about getting the right setup.

We thought carefully with Bluebird about how the facility should be designed. We considered the analytical methods, and the supply chain from the hospital through shipping to our sites, and shipping back to the clinical sites. We needed to ensure that the supporting infrastructure was there throughout the whole process. For us, the key lessons were about planning and thinking upfront with the customer about their program and how it will impact their patients.

Q Customization versus standardization: how do we trade off and can you give any examples?

MS: Regarding customization, the most critical thing is to ensure there is enough material for the patient dose. When testing media or a process to fine-tune ways to maximize cell growth, customization is required. You may need to screen many different unit operations and raw materials and if you can make a change that will truly impact the potential ability to dose a patient it should be done.

In standardization, the idea is to start with something that could be used and is scalable first. Customizing around the standard can save a lot of headaches later. The advice we give is to start with the known as much as you can rather than starting with a complete custom solution and trying to build from scratch. It may not be clear until later how decisions around

raw materials, equipment, and unit operations, will impact you begin to treat patients and move into GMP.

BIOGRAPHY

MARK SANTOS is the commercial and marketing lead for Lonza's CGT Business Unit, Portsmouth, NH, USA. His responsibilities include promoting and designing Lonza's CGT offerings and capabilities. This includes strategic relationships, investment prioritization and offerings within Lonza's CGT business to result in high value partnerships. Mark has over 15 years of experience in the biopharmaceutical space. He currently lives in the USA. Previously he was in Switzerland to help build the strategic and innovation roadmap for Lonza's Bioconjugate Business Unit. He has led the development and delivery of multiple large investment projects across mammalian, bioconjugate, and vaccine technologies. He worked for PwC Pharma R&D Ops to help transform business and operational planning for large pharmaceutical organizations. His career started in mammalian and microbial manufacturing. Mark has an MBA and a microbial degree from the University of New Hampshire, Durham, NH, USA.

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Contributions: The named author takes responsibility for the integrity of the work as a whole, and has given their approval for this version to be published.

Acknowledgements: None.

Disclosure and potential conflicts of interest: The author has no conflicts of interest.

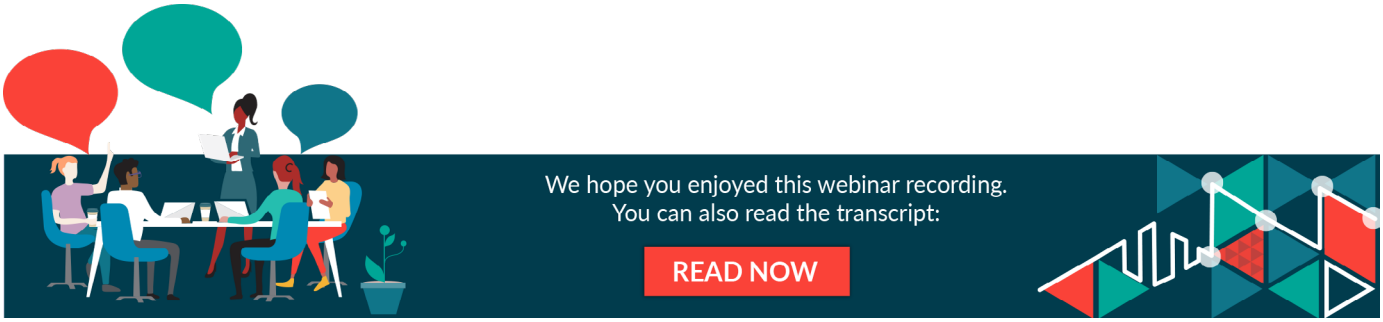
Funding declaration: The author received no financial support for the research, authorship and/or publication of this article.

ARTICLE & COPYRIGHT INFORMATION

Article source: This article was based on a webinar, which can be found here.

Webinar conducted: Jun 13, 2024; **Revised manuscript received:** Aug 5, 2024;

Publication date: Oct 7, 2024.



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T-Cell

Trends in innovation for enhanced gene therapy downstream process intensification and product yield

Rachel Legmann PhD Senior Director of Technology, Gene Therapy, Repligen
Andrew Tustian PhD, Senior Director, Preclinical Manufacturing and Process Development, Regeneron Pharmaceuticals

The gene therapy industry has a pressing need for advancements in the downstream processing of viral vectors to better accommodate more complex and fragile advanced therapy medicinal products. Particularly with AAVs, downstream processing is crucial to ensure high yield and purity of the final product. This infographic highlights the steps and challenges associated with downstream processing, emphasizing the importance of novel innovations and process developments.

CHALLENGES AND OPTIMIZATION OF DOWNSTREAM PROCESSING OF VIRAL VECTORS

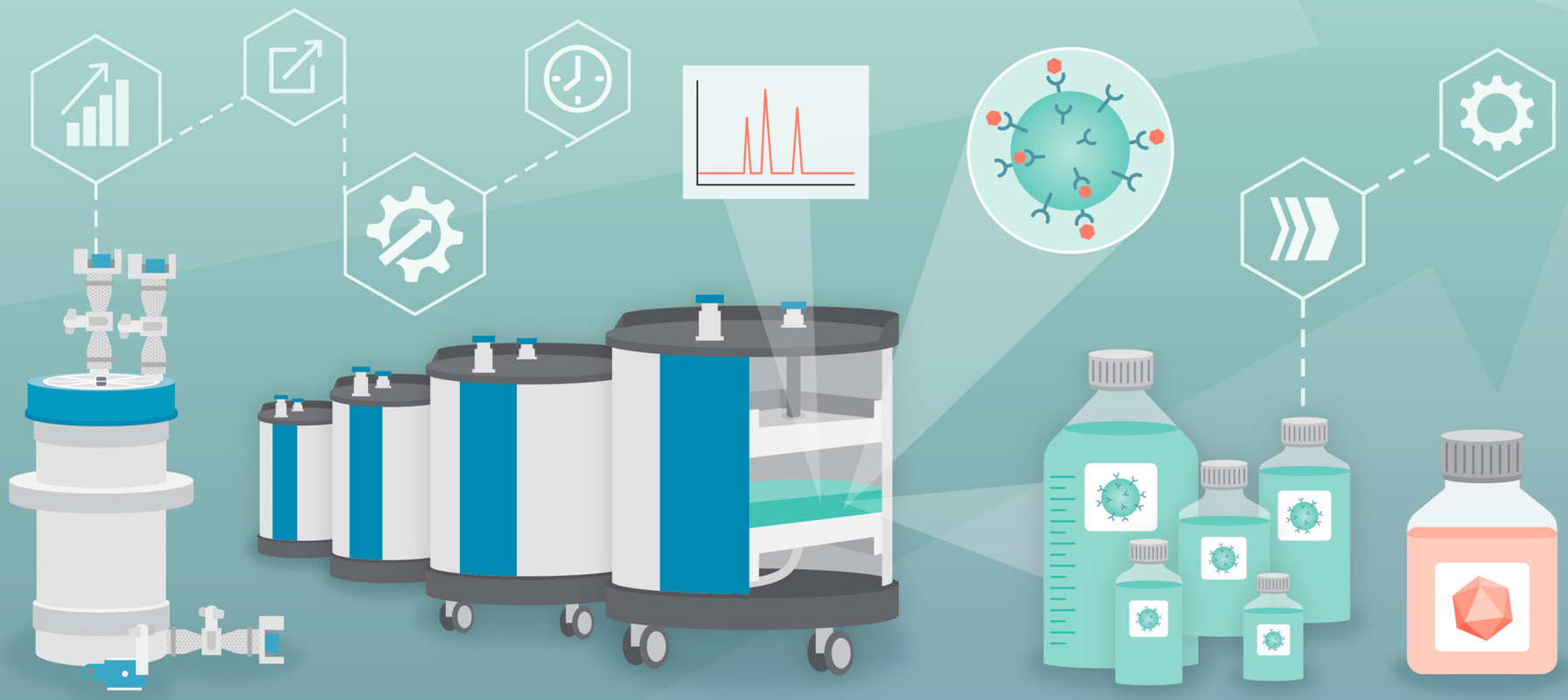
The downstream process of viral vectors begins with a lysis step to release the viral particles from within the production cells. This lysis is typically achieved using a detergent to break open the cells, thereby freeing the intracellular viruses. This step is crucial as AAVs can be found both inside and outside the cells, and complete lysis ensures maximum recovery of the viral particles.

Following lysis, a solid-liquid separation is used to remove cell debris. Given the often low concentration of viral particles in the solution, a concentration step using tangential flow filtration (TFF) is frequently employed. TFF helps to reduce the volume of the solution, thereby decreasing the load on the subsequent affinity capture step. This significantly enhances the efficiency of the process by concentrating the viral particles before further purification.

Affinity capture, typically using specific antibody-based ligands, selectively binds the viral particles. This capture step is highly specific and helps to isolate the viral vectors from other impurities present in the solution. Post-capture, further purification steps are required, including polishing chromatography to remove residual host cell proteins, DNA, and other contaminants. These steps ensure that the final product meets the required purity standards for therapeutic use.

One of the most significant challenges in AAV manufacturing is the separation of empty-full capsids. Full capsids contain the desired genetic material, and their presence can increase the efficacy and immunogenicity of the final product. The separation of the empty-full capsids remains difficult due to their similar physical properties. Advances in chromatography techniques, such as the use of monoliths and membranes, are being explored to achieve sharper separations.

More fragile viral vectors such as lentiviruses, are sensitive to shear stress, which can lead to low recovery yields. To address this, low-shear processes and innovations in single-use technologies and affinity chromatography are being developed. These advancements are improving recovery rates and reducing process times, which are critical for meeting the high demands of gene therapy markets.



SUMMARY

Downstream processing in viral vector manufacturing is a complex and evolving field. Techniques like TFF play a crucial role in concentration and purification, helping to enhance yield and purity. Ongoing innovations and advancements in this field promise to address existing challenges and improve efficiency and scalability, ultimately supporting the needs of gene therapy and other advanced therapeutic applications. As the demand for viral vectors continues to rise, these improvements will be crucial in ensuring the availability and quality of these products.

Watch the webinar here

Read the article here

CELL & GENE THERAPY INSIGHTS

Cell & Gene Therapy Insights 2024; 10(9), 1289; DOI: 10.18609/cgti.2024.146

IN PARTNERSHIP WITH REPLIGEN

WEBINAR DIGEST

Accelerating AAV process development with a PAT-driven TFF system

Teva Smith, Field Application Scientist, Repligen

Ultrafiltration/diafiltration (UF/DF) represents a crucial step in bioprocessing, but traditional methods can pose a range of challenges. This poster 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 demonstrates automated process control, exhibiting the system's efficacy across various final endpoints.

INTRODUCTION TO IN-LINE VARIABLE PATHLENGTH SPECTROSCOPY

The KrosFlo® KR2i Real-time Process Management (RPM™) is the first in the Repligen PAT-driven TFF System. The KrosFlo® KR2i TFF System is integrated with the CTech™ FlowVPX® in-line variable pathlength UV-Vis technology to provide UF/DF process monitoring and control through real-time concentration measurements and set points.

The FlowVPX System is the only in-line UV-Vis solution that utilizes Variable Pathlength Technology (VPT) to allow for real-time, continuous concentration measurement throughout the UF/DF process. VPT yields a broad dynamic pathlength range to automatically plot linear Slope regressions independent of concentration at the wavelengths of interest.

Figure 1. UF/DF AAV titer process monitoring using in-line VPT technology: AAV2 TFF run.

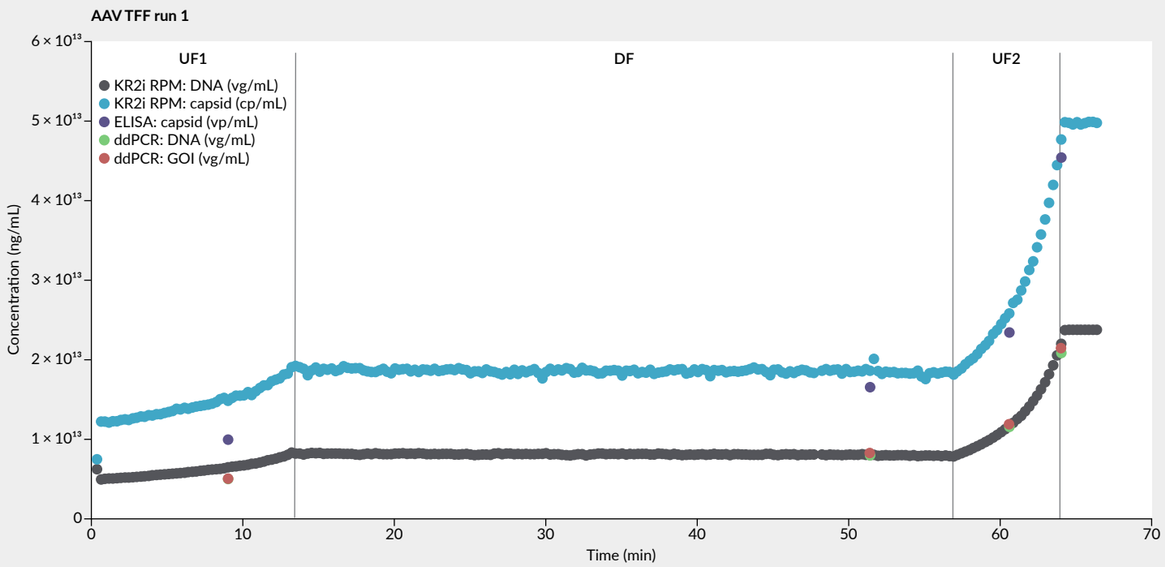


Table 1. UF/DF AAV2 titer process monitoring—KR2i RPM system results versus ELISA and ddPCR.

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

UF: ultrafiltration, DF: diafiltration.

VIRAL TITER DETERMINATION OF AAV2: A CASE STUDY

In this case study, an in-line variable pathlength spectroscopy method was evaluated for AAV viral titer monitoring for an AAV2 serotype. The assessment utilized the FlowVPX System to monitor the concentration of AAV2 during a UF/DF run **(Figure 1)**. 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 ddPCR and ELISA. 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 two-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 consistency during the process of collection with the FlowVPX.

SUMMARY

The KR2i RPM System seamlessly integrates the in-line FlowVPX instrument with the automated KR2i TFF System, strengthening process control and ensuring high-quality and 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.

Watch the webinar here

Read the full transcript here

CELL & GENE THERAPY INSIGHTS

Cell & Gene Therapy Insights 2024; 10(9), 1303 • DOI: 10.18609/cgti.2024.148

IN PARTNERSHIP WITH REPLIGEN INSPIRING ADVANCES IN BIOPROCESSING

WEBINAR DIGEST

Commercial-scale manufacture of lentivirus for *ex vivo* and *in vivo* therapies

Rachel Legmann, Senior Director of Technology, Gene Therapy, Repligen and **Michelle Yen Tran**, Process Development Scientist, McGill University

As the field of lentivirus (LV) progresses toward large-scale manufacturing to generate sufficient functional LVs for treating patients, scalability and consistency are important aspects to consider. This poster presents case study data to show how TFDF enables a scalable perfusion process that can provide sufficient LV doses for large patient populations.

A KrosFlo® Tangential Flow Depth Filtration (TFDF®) perfusion system that is adapted to the fragility of envelope viruses can be scaled to industrial-size production bioreactors where the LVs are harvested continuously and passed either onto the capture step of downstream purification or concentration/diafiltration step, greatly reducing process hold time and rendering less loss of functionality.

LENTIVIRUS UPSTREAM PROCESS INTENSIFICATION

In an internal study, a proof of concept for the development of a perfusion-intensified process for enveloped virus-based vector manufacturing to provide sufficient LVs for large patient populations is presented. In this study, the TFDF system is used both for growth and production while performing continuous clarification post-transfection.

Figure 1. LV upstream process intensification through cell growth and clarification.

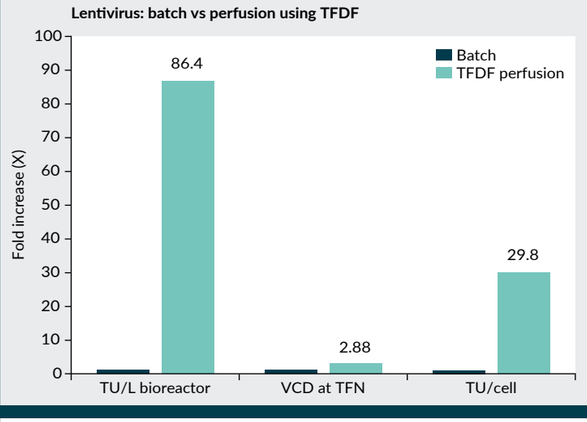


Figure 2. Intensified LV production: perfusion versus batch using stable cell line

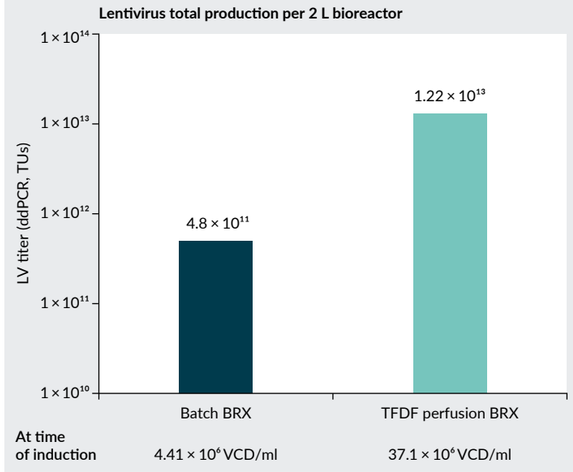
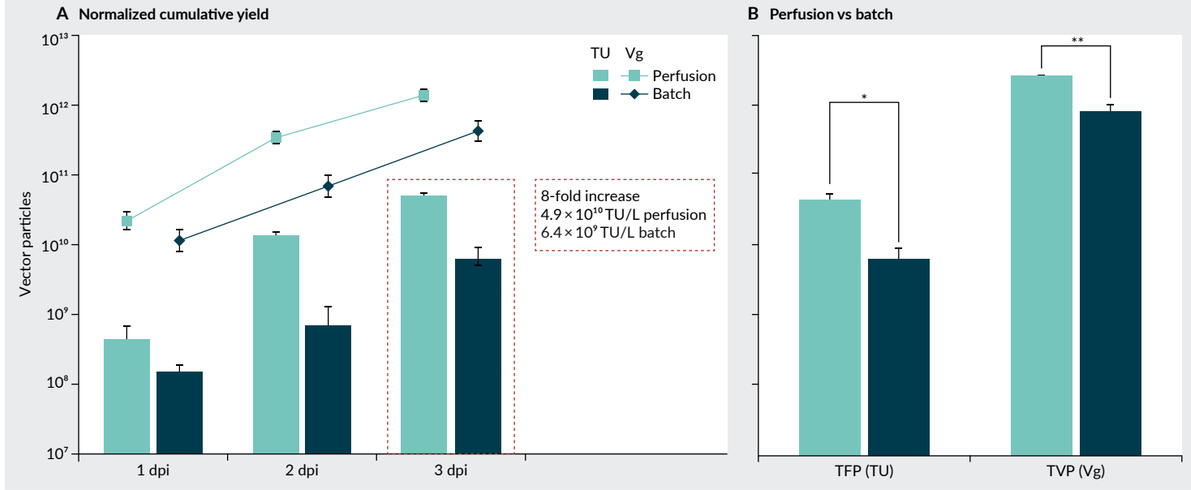


Figure 3. LV production in perfusion mode versus batch mode (normalized cumulative yield)



During the cell growth phase in perfusion mode, the cell density at the time of transfection is increased by almost 3X, as shown in **Figure 1**. By performing perfusion using TFDF after transfection, the specific productivity of the viral vector per cell is increased by almost 30X. Overall in this case study, LV production is enhanced by more than 80X using KrosFlo TFDF perfusion technology during the entire upstream process.

In a further case study, a proof of concept for the development of a perfusion-intensified process for enveloped virus-based vector manufacturing is presented. A stable cell line is used to produce sufficient LVs for a large patient population. **Figure 2** shows that during the growth phase, the cell density at the time of transfection increases from 4.4 million cells/mL in batch mode to 37 million cells/mL in perfusion mode. In this proof of concept, about 25X more potent LV doses are achieved per 2 L bioreactor post induction.

EVALUATION OF SCALABLE TFDF PERFUSION SYSTEM FOR LENTIVIRUS PRODUCTION USING STABLE CELL LINE

The TFDF device was explored in this case study as a perfusion device to support LV production in perfusion mode at a manufacturing scale, scalable up to a 2,000 L bioreactor.

The perfusion bioreactors were compared with batch bioreactors and the cumulative yields were normalized per 1 L of harvest. **Figure 3** shows that there is an 8-fold increase of functional lentivirus with perfusion bioreactors. An unpaired T-test was performed to compare the total functional particles and total vector particles attained in perfusion mode and batch mode. There was a significant increase in the total functional particles and total vector particles between perfusion mode and batch mode. Therefore, perfusion mode is worth implementing even for only 3 days to increase LV production.

Watch the webinar here

Read the full transcript here

CELL & GENE THERAPY INSIGHTS

Cell & Gene Therapy Insights 2024; 10(9), 1155 • DOI: 10.18609/cgti.2024.131

In partnership with: REPLIGEN INSPIRING ADVANCES IN BIOPROCESSING

FASTFACTS

High-throughput process development and scale-up to 2,000 L for rAAV production to address unmet patient needs

Pouria Motevalian, Director, Process & Analytical Development, Thermo Fisher Scientific

Recombinant (r)AAV-based gene therapies are poised to treat a variety of conditions impacting larger patient populations. However, multiple development and manufacturing challenges remain that may impact clinical effectiveness and patient access. This poster summary explores Thermo Fisher Scientific's methodology for rAAV scale-up to 2,000 L, including the use of high-throughput technologies for accelerated development.

rAAV is currently the most commonly used viral vector in gene therapy clinical trials worldwide. However, despite this widespread use, rAAV manufacturing remains problematic. Scale-up to clinical and commercial levels is an important and increasingly pressing challenge for the sector, given the need to maintain consistency and quality when transitioning from small-scale to larger commercial quantities needed to treat a broader patient demographic in an efficient and cost-effective manner. Other key challenges include a relatively high Cost of Goods (COGS)/dose compared to other modalities, and the need to accelerate process development in order to reduce time to market.

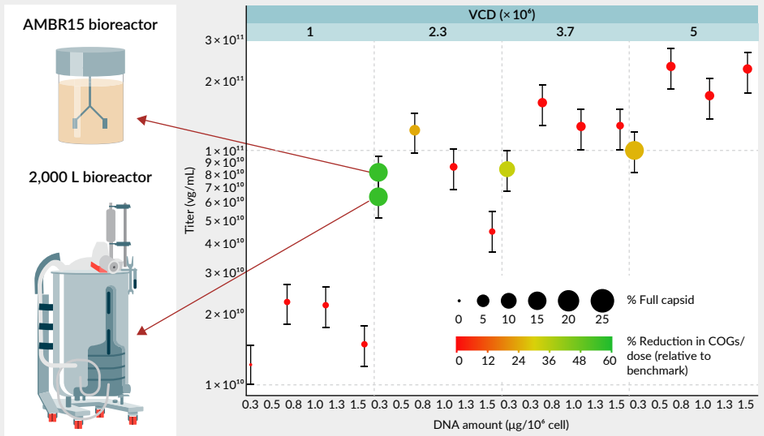
CASE STUDY: OPTIMIZING rAAV PRODUCTION USING A TRIPLE TRANSFECTION PROCESS

Process optimization—In a recent study, Thermo Fisher explored the capabilities of high-throughput technologies to maximize rAAV process productivity while minimizing process- and product-related impurities and COGS. One of the most commonly used rAAV serotypes was used for this study: rAAV9. In order to pressure-test the process optimization, a gene of interest (GOI) of ≈5 kbp was used, which is close to the packaging limit of AAV vectors. The Ambr® 15 Cell Culture Bioreactor System was employed. Following optimization, a titer of 8E10 vg/mL was achieved at harvest with ≈60% reduction in COGS relative to the current industry benchmark. Percentage full capsid was approximately 25%.

Scale-up—Next, the Ambr 15 process was directly scaled up to a 2,000 L bioreactor. As with the Ambr 15 process, VPC 2.0 cells and Thermo Fisher LV-Max production medium were employed. Cell expansion was performed in a shake flask and a Thermo Fisher 250L HyPerforma™ Single-Use Bioreactor (SUB). Production and pre-harvest treatments were performed in a 2,000 L HyPerforma™ bioreactor.

The results from the 2,000 L process compared to those from the Ambr 15 process are shown in **Figure 1**. The vg titer, percentage full capsid, and COGS reduction with the 2,000 L process all closely resemble those achieved by the optimized Ambr 15 process, demonstrating the success of this >70,000-fold scale-up approach.

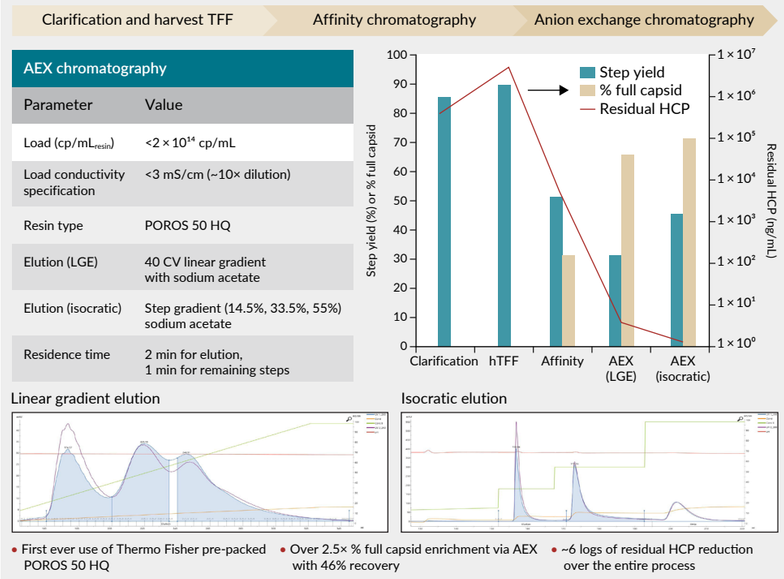
Figure 1. Results from scale-up of Ambr 15 process to 2,000 L HyPerforma bioreactor.



- Successful Right-First-Time scale up from AMBR 15 to 2,000 L (+70,000× scale up)
- AMBR15 and 2,000 L titer were 7.9×10^{10} vg/mL and 6.2×10^{10} vg/mL, respectively

Purification—The rAAV vector product was then purified through tangential flow filtration (TFF), followed by affinity chromatography using a pre-packed column of POROS™ AAV9 affinity resin. The affinity chromatography step achieved a ≈4 log reduction in host cell protein (HCP), achieving a ≈50% step yield, and the percentage full capsid in the neutralized affinity elution pool was ≈30%. Purification continued with an Anion Exchange Chromatography (AEX) polishing step, utilizing columns pre-packed with POROS 50 HQ resin. In **Figure 2**, both linear grade and isocratic elution chromatograms for the AEC polishing step are depicted, while in the graph to the top-right, the overall results of all three purification steps are shown. The AEX step resulted in a >2.5-fold enrichment in percentage full capsid, achieving >70% full capsid in AEX elution pool. Recovery was 46%, and ≈6 logs of residual HCP was removed over the entire process.

Figure 2. Results from AEC and overall 3-step downstream purification of AAV9.



- First ever use of Thermo Fisher pre-packed POROS 50 HQ
- Over 2.5× % full capsid enrichment via AEX with 46% recovery
- ~6 logs of residual HCP reduction over the entire process

SUMMARY

This study demonstrated a successful approach to scale-up of an optimized rAAV9 process from bench-scale to large-scale (2,000 L) production leveraging high-throughput technologies, with high purity and percentage full capsid achieved during downstream processing.

Cell & Gene Therapy Insights 2024; 10(9), 1255; DOI: 10.18609/cgti.2024.143

IN PARTNERSHIP WITH ThermoFisher SCIENTIFIC

FASTFACTS

Bag-to-bag consistency: homogeneous cell count distribution in biopharmaceutical aliquotation

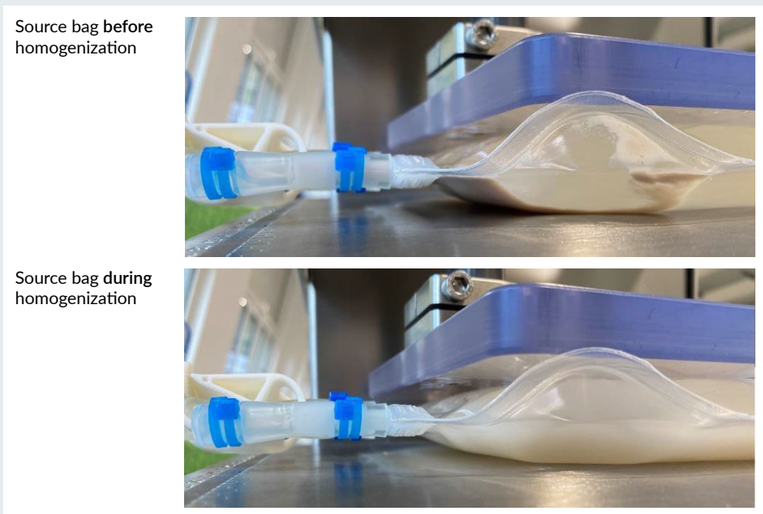
Alexander Fuchs, Head of Product Line Management, Single Use Support

The homogeneity of pharmaceuticals plays an important role in fluid management. Liquid drugs have varying properties, including viscosity and sedimentation rates, which make it difficult to standardize the aliquotation of liquids. Furthermore, manual homogenization of cell-based suspensions in 2D bags often leads to inconsistent drug and cell distribution. New study results demonstrate that fully automated and standardized homogenization processes can ensure uniform yeast cell distribution in single-use bioprocessing containers while reducing the risks of bag breakages and contamination, consequently improving patient safety.

AUTOMATED HOMOGENIZATION SYSTEM FOR IMPROVING THE ALIQUOTATION OF YEAST CELL SOLUTION

In a study conducted by Single Use Support in cooperation with the Institute of Hygiene and Medical Microbiology of the Medical University of Innsbruck, the RoSS.PADL system was used to homogenize *Saccharomyces cerevisiae* yeast cells stored in source bags. RoSS.PADL is a system designed for automated cooling and homogenization of single-use bags. The setup includes a human-machine interface for electrical control, an electrical cabinet, and a control unit. RoSS.PADL also has a temperature control unit for ensuring consistent cooling of single-use bags, and an actuator system that facilitates the homogenization process.

Figure 1. Homogenization of *Saccharomyces cerevisiae* yeast cells with the automated RoSS.PADL system.



The source bag that undergoes homogenization is connected via tubing to the RoSS.FILL Lab Scale platform, a filling system for small-volume single-use bags. In this experiment, RoSS.FILL Lab Scale was connected to 50 mL bags via tubing to ensure fast and accurate filling.

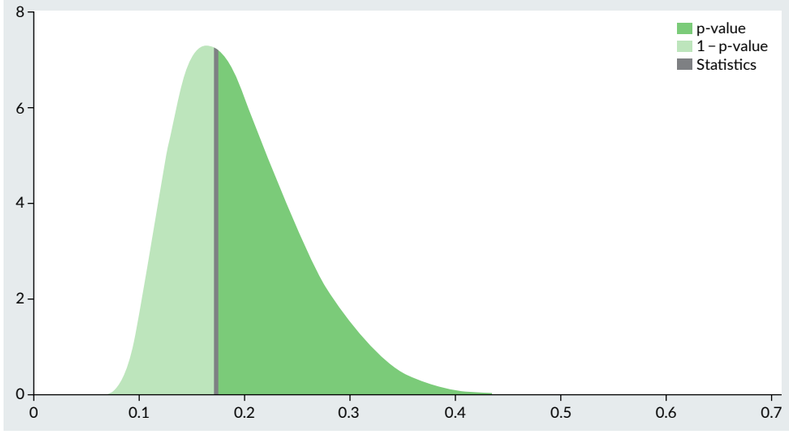
The source bag used in this process was a 2 L bag filled with 1.25 L of water-based yeast cell solution, containing approximately 10^8 yeast cells/mL. In total, six 50 mL bags were connected to the filling system, each filled with 15 mL of the solution at 5-minute intervals. After 15 minutes of homogenization, the first sample was taken with subsequent samples being taken every 5 minutes until all six bags were filled. Each single-use bag yields three samples, resulting in a total of 18 samples for the study.

As illustrated in **Figure 1**, the bottom of the source bag of yeast cells before the homogenization process had clear sedimentation. The homogenization process successfully eliminated sedimentation, as highlighted by homogeneous concentration throughout the bag in the images at the bottom of **Figure 1**.

ASSESSING VIABLE CELL COUNT CONSISTENCY IN HOMOGENIZED YEAST CELL SOLUTIONS

The results of the study were analyzed for normal distribution using the Kolmogorov-Smirnov (K-S) test **(Figure 2)**. The sample size consisted of 18 samples, with an average value of 80 and a median value of 85. By applying the null hypothesis (H_0), the Anderson-Darling (AD) value was calculated to be 0.173. Additionally, the p-value was calculated to be 0.594. Based on the K-S test results, it was concluded that there was no significant deviation of the data from a normal distribution. Therefore, there was no significant difference in the viable cell counts between 18 different bags containing yeast cell solution taken from the RoSS.PADL-homogenized liquid.

Figure 2. Kolmogorov-Smirnov test results illustrating a normal distribution of viable yeast cell counts.



SUMMARY

This study demonstrated that automated homogenization using the RoSS.PADL system effectively ensured uniform yeast cell distribution in single-use bioprocessing bags. Statistical analysis confirmed consistent viable cell counts across samples, highlighting the system's reliability in maintaining homogeneity and reducing contamination risks.

Cell & Gene Therapy Insights 2024; 10(9), 1253; DOI: 10.18609/cgti.2024.142