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CELL & GENE THERAPY INSIGHTS

SPOTLIGHT ON
Fulfiling the potential of gene editing: at the tipping point

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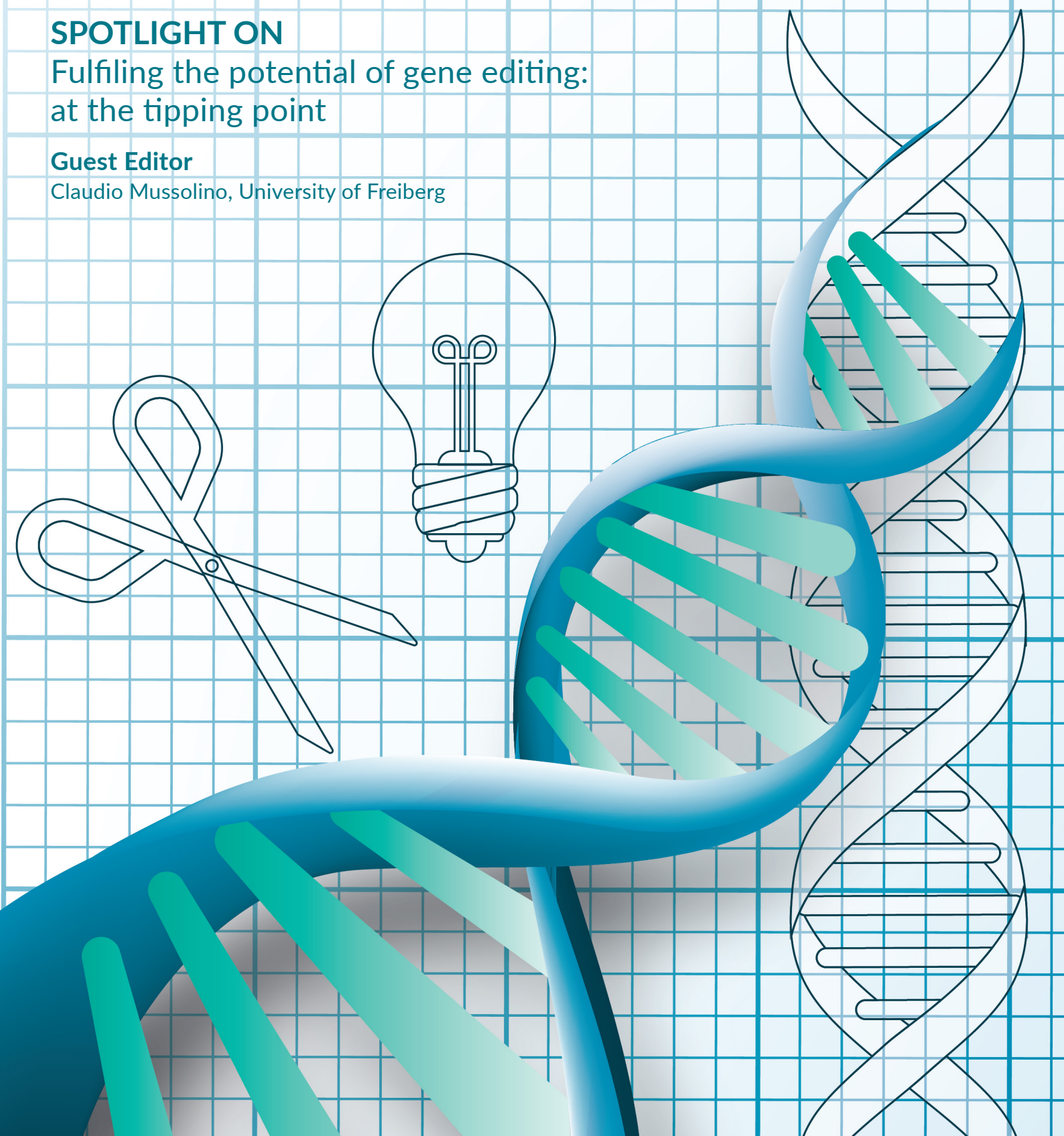

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FULFILLING THE POTENTIAL OF GENE EDITING: AT THE TIPPING POINT

SPOTLIGHT

EXPERT INSIGHT

Considerations for development of gene-edited PSC-based therapies

Brent Morse & Amanda Mack

PSCs provide a replenishable starting material for the consistent, scalable production of cell therapies with a broad range of therapeutic applications. The recent emergence of robust gene editing technologies offers the potential to significantly extend the reach and utility of PSCs by increasing potency and consistency of drug products derived from them. Full realization of the potential of this new platform will require careful consideration and planning with respect to the intended target products, approaches to process design, and the development of fit-for-purpose analytical control strategies.

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INTRODUCTION

Pluripotent stem cells (PSCs), which include human embryonic stem cells and induced pluripotent stem cells (iPSCs), provide an unlimited starting material with capacity to make any cell type with potential to produce thousands of patient doses in a single manufacturing lot. Preliminary evidence of their potential as a platform for clinical development is only now emerging despite their discovery over two decades ago [1,2].

Some of the earliest attempts at the production of PSC-derived cellular drug products resulted in material that lacked robust efficacy and/or displayed cell surface signatures recognized by the immune system as foreign, thereby marking it for rejection as

non-self [3,4]. Those trials and failures increased process understanding resulting in the development of more refined protocols that improved production through increased control. PSC-derived pancreatic β-islet cell replacement therapy for the treatment of type 1 diabetes mellitus is exemplary of this progression [5]. The earliest therapeutic approach for treatment relied on cadaveric tissues as primary starting material and, while promising, suffered from limited, random tissue supply, with significant donor-to-donor variability limiting broader application. PSCs represented a genuine solution to address supply challenges and, together with the development of additional process controls through iterative, data-driven process improvements, resulted in insulin-producing cells that appear more effective than stem cell derived products generated previously [6,7].

Developers have leaned on a variety of approaches to minimize the potential for immune rejection of transplanted, PSC-derived, pancreatic β-islet cells including long-term immunosuppression. However, the side effects associated with immunosuppression often confound the therapeutic benefit of the cell therapy [7]. Therefore, alternative approaches rely on encapsulation to shield cells from the immune system and facilitate transplantation. Encapsulation approaches come in the form of small delivery devices that can be filled with cells or microencapsulated where individual, or clusters of cells are coated. Advances in encapsulation approaches have identified vascularization as a key design feature to enhance gas and nutrient transfer. However, this has also resulted in the continued need for immunosuppressive drugs due to the contact between the cell therapy and circulating blood cells [8,9].

Gene editing approaches are increasingly being used in the field to protect cells from immune attack, thereby improving cell retention and potentially eliminating the need for long-term reliance on immunosuppressive drugs. While precedent exists for non-engineered cell products in development discussed elsewhere, this communication will focus on those contexts where gene editing is anticipated to be advantageous [10]. For example, hypoimmune pluripotent stem cells may potentially enable off-the-shelf therapies making them more accessible to a broader range of patients. Many developers are pursuing edits that inactivate genes expressing major histocompatibility class molecules that are responsible for triggering an immune response [11–13]. Furthermore, others are forcing overexpression of a common protein found on the outer membrane of many cells, CD47, that is responsible for preventing phagocytosis and is often referred to as the 'don't eat me' signal [14]. While still early in development, preliminary evidence in small animals and non-human primates demonstrates potential for long-term survival of transplanted hypoimmune cells in the absence of long-term immunosuppression [15,16].

Gene editing is also being explored as a potential means to enhance cellular potency and performance. A notable example is the case of CAR-T cell therapies, which have demonstrated clinical and commercial success in the durable treatment of hematological malignancies [17,18]. Further refinement is required to enhance potency, minimize off-target cytotoxicity, and increase access to highly immunosuppressive tumor microenvironments like those of solid tumors, which harbor signals that diminish T cell function and effect expansion, functional activity, and long-term durability [19,20]. Combinatorial PSC platform strategies utilizing gene editing to enhance intrinsic properties, enable dynamic engagement, and overcome the tumor microenvironment are already in clinical development, with further clinical readouts anticipated soon [21].

Aside from providing an unlimited, consistent, and scalable supply of starting material, PSCs are also amenable to a wide range of vector delivery systems and gene editing approaches. Among the main challenges of developing a gene edited PSC-derived drug product is achieving maximal on-target

editing and minimal off-target editing while maintaining operational efficiency. Early approaches to gene editing, including meganucleases, zinc finger nucleases, transcription activator-like effector nucleases, are accurate [22] but require significant re-engineering with each new target [23]. This can be especially burdensome for product lines with multiple edits. More recently, the use of CRISPR/Cas9 has become increasingly common due to its versatility and efficiency [24]. However, use of the CRISPR/Cas9 system to perform gene-editing in PSCs is not without challenges, as some of the earliest attempts demonstrated p53-meditated cytotoxicity [25]. Inhibition of p53 resulted in significant improvements in both editing efficiency as well as viability post-editing, enabling the opportunity to explore the potential of iPSCs more freely [26]. Transient inhibition through small molecule inhibitors, for example, were demonstrated to be sufficient reducing the risk of tumor formation more long-term suppression might impart. Thus, careful consideration must be given to the manufacturing process and the analytical control strategy to efficiently produce a gene edited PSC-derived drug product displaying high on-target editing and minimal off-target editing.

Ultimately, the ability to perform edits to the PSC genome introduces control further upstream in product development creating opportunity for intervention earlier to manage risk, reduce variability, and improve manufacturing costs in the long-term. A single edited bank of PSCs can support development of multiple product pipelines, increasing optionality and enabling the potential to address key regulatory concerns across programs more efficiently. For example, a PSC platform could be established with a set of initial edits anticipated to be common across products, making cellular starting material available to support multiple pipelines distinguished by subsequent, product-specific edits as depicted in Figure 1. PSC clones with product-specific edits can then be scaled to produce master cell banks and working cell banks to support clinical development at a scale and consistency not afforded by non-PSC-based therapies.

DEVELOPMENT & MANUFACTURING CONSIDERATIONS

Consistent manufacture of a gene edited, PSC-derived product is highly dependent on the quality of the starting materials and critical reagents, including the cellular source tissue, reagents for reprogramming, gene editing, and other manufacturing components (e.g., media, growth factors, and other reagents). As with any gene modified cell therapy, sufficient attention should be paid to the quality of these components, as this can have a profound impact on manufacturing consistency and product safety [27,28]. Xenobiotic free material of the highest grade should be used whenever possible, and manufacture and release of these materials should be in accordance with cGMP or other relevant guidance or regulation. In addition, particular care should be paid to donor eligibility requirements. Substantial regulatory guidance exists to assist sponsors in this regard [29–31], with a particular focus on appropriate screening for adventitious agents.

The manufacturing process needed to support production of a gene edited PSC platform will depend on many factors including the number and type of edits and the design of the gene editing components. A prospective design strategy that considers efficiency and potential for off-target editing is strongly advised since product complexity increases as the number of edits increases. As noted previously, a range of gene editing tools are available including, but not limited to, zinc finger nucleases, transcription activator-like effector nucleases, piggybac transposons and the CRISPR/Cas9 system. Each approach should be assessed individually to determine efficiency and potential for off-target editing events. Poor design choices for gene editing components (e.g., low efficiency, or high propensity for off-target editing) can

directly impact both efficiency and potential for off-target mutations. While this can be addressed during clone screening, the overall efficiency of the screening process may be negatively impacted.

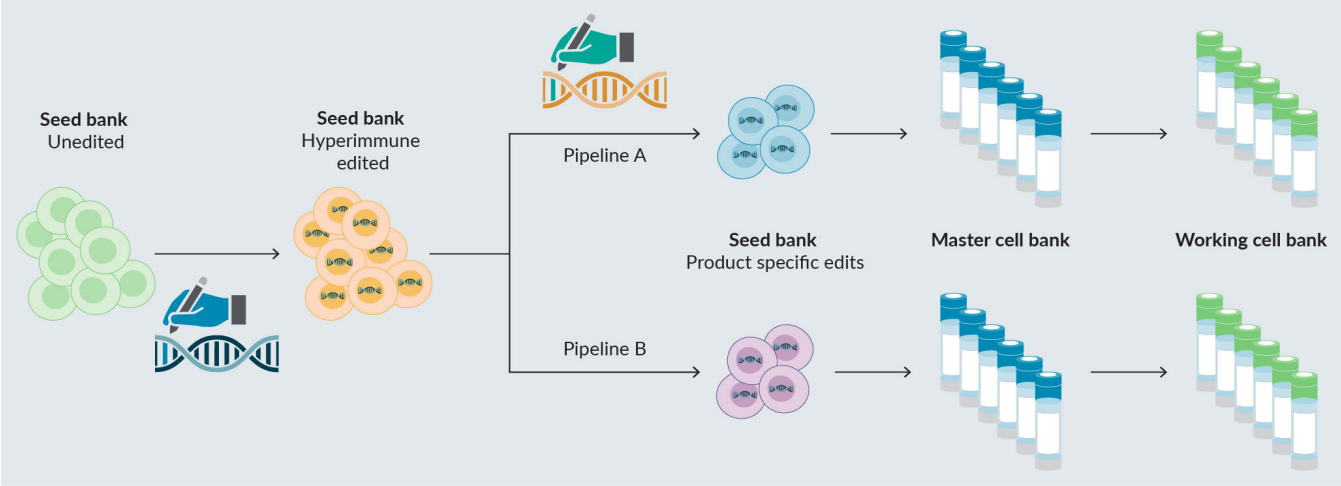
A baseline workflow for a single edit includes delivery of the gene editing components via approaches such as electroporation, lipofection, and/or viral transduction. Typically, the pooled, transfected cells are distributed across a multi-well format by limiting dilution to facilitate clonal expansion and are screened at low resolution (e.g., via Sanger sequencing of PCR amplified targets) to quickly identify those clones that contain the desired edit and filter them from those that do not. All or a subset of the desired clones are further expanded for more extensive screening to identify off-target events (e.g., using a targeted method such as amplicon–NGS or a broad approach such as whole genome sequencing), transgene copy number following a targeted knock-in (e.g, droplet digital PCR or equivalent), in addition to quality control tests commonly applied to PSCs (see discussion in 'Analytical considerations' section).

An added advantage of the PSC platform is the opportunity to pursue clonal populations. The need for, extent of, and when to pursue clonality during the process is primarily driven by the complexity of the editing process and intended therapeutic outcome of the final drug product. Ultimately, balancing the level of control with drug product performance and risk is important when considering the strategy for product development.

In more complex scenarios where multiple edits are needed, developers pursue a stepwise or multiplexed approach. A stepwise approach entails iterative rounds of gene editing and high throughput, low-resolution screening. Positive clones identified in each iteration are then expanded to undergo the next round. Some developers are engineering cells with selection tools (e.g., antibiotics and fluorescent tags) to streamline enrichment of target clones [32]. However, a risk assessment should be applied to mitigate potential regulatory considerations for clinical development, depending on the nature and likelihood of persistence of the selection tool employed. Others are leveraging automation and capitalizing on the advantages of machine learning to tighten process controls and improve the consistency and quality of the resulting material [33,34].

FIGURE 1

Potential banking strategy to leverage gene edited PSC platforms to support multiple, allogeneic development pipelines.



Begins with an unedited, PSC line deemed suitable for clinical development followed by gene editing to confer resistance to immune detection, for example. This hypoimmune bank becomes the source material used to develop multiple products that may differ in antigen specificity in the final drug product.

A multiplexed gene editing strategy could be selected to both consolidate the number of process steps and reduce manufacturing time. However, increasing the number of edits performed simultaneously increases the potential to negatively impact the relative efficiency of the desired edit and can heighten the potential for off-target mutation or translocations. For example, the ability for endonucleases to cleave DNA can be target strand, locus, and half-life dependent. In some scenarios, increasing the number of guide RNAs could compete for a limited pool of endonucleases with declining activity leading to reduced editing efficiencies [35]. Furthermore, improvements in Cas design to lower off-target activity can result in a decreased affinity for its target sequence and reduced mutagenesis rate, reinforcing the barrier to better efficiency [36].

Variability in editing efficiency often increases the manufacturing burden due to the requirement for a larger number of PSC clones to be screened to identify those with the desired edits [37]. This often means several clones to manage in parallel requiring additional resources and technical expertise. While the industrialization of manufacturing processes for PSCs is ongoing, broad standardization is yet to be consolidated. Until this occurs, the burden of carrying multiple PSC cultures requiring manual intervention and qualitative assessment will remain, with the potential to result in clone variability that can impact downstream performance.

Furthermore, the stress associated with repeated rounds of gene editing, extended culturing periods, and repeated freeze/thaw cycles increases the risk of genomic instability. Gene editing extends PSC culture duration, thereby increasing the potential for mutations that could impart a selective advantage leading to preferential expansion of less desirable clones compromising product quality and safety. Gene editing components like CRISPR/Cas9 that create site-specific double stranded breaks have been previously shown to trigger genomic instability in iPSCs [38–40]. Therefore, an informed approach that considers the design strategy for each gene editing component and the overall time in culture is strongly recommended to minimize the potential for genomic instability. While the potential benefits of gene editing far outweigh the potential disadvantages, numerous challenges remain.

ANALYTICAL CONSIDERATIONS

A phase appropriate control strategy for gene-edited, PSC-derived drug products should account for control of starting materials, critical reagents, and in-process and release testing of both cell banks and drug product. Historically, cellular starting material has been considered a significant source of product variability and potential safety risk for many cell and gene therapies. In the PSC context, the variability can be partially mitigated through the strategy of establishing a well-characterized set of cell banks from which all batches of the drug product will be generated. Likewise, the risk to safety can be partially mitigated through exhaustive viral testing of the cell banks [41] and the application of appropriate procedural controls prior to and during collection of donor material [31].

Ideally, a staged testing approach is implemented by which comprehensive testing of the master cell bank may allow for limited testing of the working cell bank and/or drug product and may be applicable to attributes such as viral testing or on-target and off-target editing for gene-edited products. If utilized, such an approach should be supported by appropriate development data and be consistent with both the quality target product profile and relevant regulatory guidance.

The quality of gene modification reagents, which may include viral vector, gene editing enzymes, and single guide RNA are critical to efficacy and safety of the drug product and, therefore, should be appropriately controlled and characterized. Minimally, the release and characterization data package should support the safety and activity of these reagents. This

includes standard safety testing (sterility, mycoplasma, endotoxin, and replication competent virus, as appropriate) as well as testing to confirm the activity and specificity of these reagents. Of note are single guide RNA sequence purity assays, which may inform potential safety risks due to off target editing.

Table 1, below, provides a listing of the key attributes that should be considered for release testing of PSC master and working cell banks. This list is not comprehensive and may not apply to all products but covers most of the types of testing needed for most gene-edited iPSC product classes. Suggested specifications are also provided, but in most cases, these should be defined by the process and product knowledge. Discussion of selected attributes follow.

Establishing the safety of the cell bank is among the top priorities of the analytical control strategy. Safety assays, including compendial safety assays (sterility, mycoplasma, and endotoxin), viral testing assays, and adventitious agent testing should be performed on both the master and working cell banks. Where appropriate, the implementation of rapid safety testing can be considered. For iPSCs, clearance of the vectors used for reprogramming (e.g., plasmids) to derive iPSCs should be demonstrated at release and should also be supported by in-process data.

Several approaches can be utilized to establish PSC identity, including verification of PSC morphology, confirmation of genetic identity [42] and expression of PSC markers. Morphology, along with viability, can also be indicative of appropriate culturing. Genetic identity is established relative to the cellular starting material, typically via analysis of short tandem repeats [43], and for gene-modified

TABLE 1

Typical cell bank release testing panel.

Category	Attribute	Specification
Safety	Sterility	Negative
	Mycoplasma	Not detected
	Endotoxin	Typically, 0.5–1.0 EU/ml[1]
	Viral testing	No virus detected
	Adventitious agents	Not detected
	Vector clearance	Not detected
Content	Cell count and viability	Minimal cell number per vial Viability typically >70%[2]
Identity	Morphology	Characteristic colony morphology[3]
	PSC marker expression	Positive for defined PSC markers[4]
	Genetic identity	Genetically identical to starting material Positive for desired genomic modifications
Purity/impurity	PSC marker expression	Minimal expression of defined PSC markers[4] Expression of defined impurities below threshold
	% on-target editing	>Threshold for on-target editing for each gene
Genomic integrity	Sequence integrity	<Limit of unexpected mutations or abnormalities
	Chromosomal integrity	Matches source material
	% off-target editing	<Threshold for off-target editing for each gene
Pluripotency	Differentiation	Protein expression representative of each germ layer mRNA expression representative of each germ layer

[1]Endotoxin limits should be justified based on both product and patient risk.
[2]A minimum viability threshold should be justified based on development data.
[3]Characteristic morphology can be defined based on literature and/or process history.
[4]See text for references containing commonly used PSC markers.
EU: Endotoxin units; PSC: Pluripotent stem cell.

PSCs confirmation of genetic identity may also include the introduced genetic modifications (i.e., gene insertions and/or edits). For analysis of gene editing, the clonal nature of the cell bank can make this relatively straightforward, as gene editing could potentially be confirmed via Sanger sequence analysis of a PCR amplicon containing the targeted gene, rather than more elaborate methods that would be required to analyze a population of cells containing a mixture of edits [44]. The panel of PSC markers to be implemented will depend on the method used to establish pluripotency, and many common markers are already well described in the literature [45,46]. Multiple orthogonal identity methods may be implemented within a single release panel.

For a gene-modified PSC, the minimal consideration for analysis of purities and impurities should include measurement of the PSC markers, selected cellular contaminants, and on-target gene-editing. PSC marker and cellular contaminant limits should be established based on the process capability and biology. A key consideration of the biology would include confirmation that the proposed minimal PSC and maximal contaminant thresholds are consistent with a pluripotent cell bank capable of generating a differentiated, functional drug product with minimal contaminant cells. A threshold for on-target editing of each target gene is also necessary to ensure manufacture of an efficacious drug product.

A regulatory expectation for gene-edited products includes a thorough and comprehensive assessment of off-target editing. This should typically include an off-target discovery phase employing both biased and unbiased methods [47] to establish the candidate list of potential off-target sites, followed by confirmation of candidate off-target sites using targeted cell-based approaches of sufficient sensitivity [48–50]. The potential for translocations also needs to be considered, particularly for products with multiple edits. The risk of each confirmed translocation or off-target editing site should be assessed, including the biology of the location (e.g., whether the site is associated with oncogenic or tumor suppressor activities) and the magnitude and consistency of the translocation or off-target edit. Confirmed off-target and translocation sites may be routinely assayed for release or characterization. Conceptually, this approach also applies to gene-edited PSCs although there may be differences in implementation relative to other types of gene-edited products. One important difference is that clonal derivation of the banks will mean that genomic modifications should be clonal as well, and therefore present in approximately 50 or 100% of DNA for non-duplicated genes. Thus, the sensitivity requirements for establishing confirmation for candidate off-target sites may be less stringent for a cell bank than for a nonclonal cell population. Another key difference also lies in the patient risk/benefit calculation. Justification for any off-target edits or translocations must consider the possibility of simply selecting a 'clean' clone.

Analysis of genomic integrity is critical to support the safety assessment for gene-edited PSC-derived products. Existing PSC lines have previously been demonstrated to carry characteristic mutations and other genetic abnormalities and are also prone to acquiring certain genetic defects in culture [51–54]. Likewise, there is evidence in the literature that gene-editing methods may contribute to unwanted genetic effects [40,55,56]. Karyotyping and whole genome sequencing approaches can be used in tandem to confirm genomic integrity; both should be used throughout development to fully characterize baseline genomic integrity, as well as the potential for genomic instability. However, careful thought should be given to implementation of these or any other methods chosen to evaluate genetic variation, as each have distinct advantages and disadvantages [57]. For example, karyotyping approaches such as G-banding can suffer from lack of sensitivity [58], whereas whole genome sequencing depth and coverage can potentially limit the information provided by this assay [59,60].

DRUG PRODUCT TESTING STRATEGY

The drug product testing strategy should fulfill the requirements of the quality target product profile, but as noted previously may leverage cell bank testing data and other development data to streamline testing requirements. A typical testing scenario is presented in Table 2, followed by commentary on selected analytical topics.

Identity and potency parameters will be dependent, in part, on the differentiated cell type, including both intracellular and cell surface expression of specific markers, as well as one or more appropriate functional readouts. In addition, wherever possible the targeted gene modifications should be incorporated into identity and/or potency testing. For example, in cases of disruption of a cell surface gene, a component of identity could include confirmation of the loss of expression. Likewise, functional consequences of the deletion could be assessed as part of the potency matrix. Ideally, every gene modification should be accounted for within the battery of potency tests.

Assessment of drug product purity (and impurities) should account for both the differentiated cell type and the intended gene modification(s). Therefore, drug product should be assayed for both markers specific to the intended drug product cell type, and for markers that demonstrate each of the intended genetic modifications (e.g., loss or change in expression). Genomic assessment of on-target editing should be performed to confirm consistency with the results obtained for the cell bank, although if sufficient data exists to demonstrate stability of the editing percentage during differentiation there may be an opportunity to remove this testing for release. For impurity assessment, one of the most important assays will be measurement of residual PSCs, which present a significant risk of teratoma formation in patients [46], and many approaches have been described in the literature [61,62].

Genomic integrity, including karyotyping and off-target editing, should be performed to confirm that the results are consistent with the cell bank. For the latter, if sufficient data exists to demonstrate stability of the off target editing percentage during differentiation,

TABLE 2

Selected release testing attributes for gene-edited PSC derived drug product.

Category	Attribute	Specification
Identity	Protein and/or mRNA expression	Consistent with the specific cell type of the product Consistent with intended gene modification
Content	Cell count Cell viability	Consistent with needs of drug product ≥70%
Purity/impurity	Differentiation markers On-target editing Residual PSC	Consistent with the specific cell type of the product Consistent with cell bank results[1] Minimal level, e.g., <1/100,000[2]
Potency	Functional assay matrix	Consistent with the specific cell type of the product Consistent with intended gene modification
Genomic integrity	Karyotype Off-target editing	Consistent with cell bank results Consistent with cell bank results[1]
Safety	Sterility Mycoplasma Endotoxin Proliferative capacity	Negative via USP <71> Not detected via USP <63> Consistent with USP <85> No aberrant growth

[1]Testing for on-target and off-target editing could be removed pending availability of supporting data.
[2]Limit should be justified based on analytical capability and preclinical safety assessment.

there may be an opportunity to remove this testing for release. Likewise, thorough adventitious agent and viral testing in the cell banks, along with development data demonstrating minimal to no risk in the drug product, may provide a justification for the removal of testing of the drug product.

Finally, as with any gene-edited drug product, developers should consider appropriate assays to assess aberrant proliferative capacity [46]. The details of the assay will be dependent on the cell type of the drug product (e.g., a T cell or natural killer cell product might utilize a cytokine-independent growth assay).

TRANSLATIONAL INSIGHTS

The development pipeline for PSC-based therapeutic candidates is expected to grow, provided that preliminary evidence demonstrating robust efficacy for those therapies can be demonstrated reproducibly [63]. The preliminary data for Vertex's stem cell derived therapy to treat type 1 diabetes (VX-880) provides one such example [64], and a recent press release suggests that gene-edited hypoimmune versions are in development or soon will be [65]. In addition, several clinical read-outs are anticipated for other PSC-based therapies in the near future. Many of these approaches leverage gene editing to progress and/or demonstrate potential for PSC-based therapies and include work by organizations including Sana Therapeutics [66], Fate Therapeutics [67], and Century Therapeutics [68]. The technological capability that gene editing affords is likely to catalyze the field and pave the way for a robust and enduring pipeline of activity in this space.

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CELL & GENE THERAPY INSIGHTS

FULFILLING THE POTENTIAL OF GENE EDITING: AT THE TIPPING POINT

SPOTLIGHT

EXPERT INSIGHT

‘Soft’ genome editing using CRISPR nickases as a potential source of safer cell products

Zhen Li & Manuel AFV Gonçalves

The integration of the gene and cell therapy fields through the application of genome editing principles permits generating *ex vivo* transplantable grafts from stem cells or from their differentiated progenies (e.g., T and NK cells) with novel genetically-engineered function(s). As such, these technologies are offering new therapeutic avenues to previously intractable inherited and acquired disorders (e.g., malignant and infectious diseases). In this article, we discuss the main characteristics, advantages and limitations of genome editing involving the targeted chromosomal insertion of transgenes upon site-specific double-stranded DNA break (DSB) formation by programmable nucleases, namely, RNA-programmable CRISPR nucleases. Subsequently, building on this information and recent findings, we put forward the view that targeted transgene insertion strategies based on CRISPR nickases, as opposed to nucleases, address important limitations of conventional DSB-dependent genome editing approaches. In particular, the cytotoxicity and high genotoxicity resulting from DSBs especially in cell types highly sensitive to DNA damage, including pluripotent and hematopoietic stem cells.

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Genome editing or genome engineering is a fast-evolving field with growing impact on basic science, biotechnology, and medicine [1]. Particularly versatile genome editing strategies consist of inserting exogenous donor DNA constructs into specific genomic loci (knock-in) subjected to double-stranded DNA breaks (DSBs) made by

engineered nucleases derived from class 2 type II CRISPR systems consisting of single guide RNA (gRNA) and Cas9 ribonucleoprotein complexes (CRISPR-Cas9 nucleases) [2]. This versatility stems from the robust activity and straightforward designing of these RNA-programmable nucleases and the amenability of gene knock-in strategies to genomic modifications spanning entire transgenes, including those encoding chimeric antigen receptors (CARs) and T-cell receptors (TCRs) alone or together with auxiliary factors, such as positive-selection markers and safety genetic switches [3,4]. Indeed, notwithstanding the growing mining for and adaption of CRISPR and CRISPR-like systems for genome editing purposes, engineered CRISPR-Cas9 nucleases based on the prototypic *Streptococcus pyogenes* CRISPR system and their molecularly evolved or structurally-guided designed variants (e.g., high-specificity and targeting range-expanded variants), remain leading tools for a wide variety of genome engineering applications [5,6].

MAIN ATTRIBUTES OF CRISPR NUCLEASE-ASSISTED GENOME EDITING

Chromosomal gene knock-in procedures often entail the delivery of CRISPR-Cas9 nucleases together with donor DNA constructs designed as substrates for either homology-independent or homology-dependent repair (HDR) pathways [7]. Generally, HDR-mediated transgene knock-ins are more precise than those resulting from homology-independent processes in that they are naturally inserted at the chromosomal target site in a predefined orientation and present neither multiple-copy insertions nor imprecise 'footprints' at the junctions between genomic and exogenous DNA sequences [8,9]. Importantly, as HDR takes place during the late G2 and S phases of the cell cycle, therapeutically relevant dividing cell types, such as induced pluripotent stem cells (iPSCs), natural killer (NK) cells and T lymphocytes, are amenable to precise HDR-mediated genome editing. For instance, in what valuable target cells is concerned, genetically engineered CAR-T cells, serving as personalized 'living drugs', are yielding impressive results in terms of treating CD19-positive hematological malignancies [3,10]. This is so despite their high costs that stem in part from the difficulties in generating the large amounts of the respective engineered cell products. Since 2017, a growing number of these CAR-T cell products have in fact started to be approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) [11]. Building on the resulting CD19-targeted cancer therapy datasets, over 500 CAR-T cell therapies directed at different antigens in liquid and solid tumors are currently undergoing clinical testing worldwide [11,12]. Significantly, CAR-NK cells are also entering the adoptive immunotherapy field as a potential alternative to CAR-T cells owing to their intrinsic tumor-cell killing activity and fewer adverse effects in patients [13]. Yet, regardless of the target cell type, in what the genetic modification procedures are concerned, the adoptive immunotherapy field is moving from randomly integrating retroviral vector and DNA transposon systems to targeted transgene insertion approaches using programmable nucleases [3,10]. In contrast to unpredictable *CAR* or *TCR* donor construct integration, programmable nuclease-assisted genome editing assures stable and homogeneous transgene expression while minimizing insertional mutagenesis risks inherent to randomly integrating vehicles. In fact, in contrast to random, targeted TCR transgene insertion leads to predictable T-cell function *in vivo* [14]. In this context, genomic loci generically dubbed 'safe harbor' are particularly appealing endogenous landing pads for *CAR* and *TCR* transgenes as insertions at these sites minimize the chances for gene silencing or variegated transgene expression while preserving the endogenous transcriptome of engineered cells [15,16].

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MAIN LIMITATIONS OF CRISPR NUCLEASE-ASSISTED GENOME EDITING

As aforementioned, programmable nucleases and HDR-tailored donor DNA constructs yield precise gene knock-ins. However, a major limitation regarding the use of programmable nucleases is the fact that, in mammalian cells, DSBs are prevalently repaired via mutagenic non-homologous end joining (NHEJ) or microhomology-mediated end joining (MMEJ) processes instead of accurate HDR [17,18]. Moreover, in contrast to HDR, end-joining processes take place throughout the cell cycle. As a result, amongst cells exposed to donor constructs and programmable nucleases, the vast majority contains one or both target alleles disrupted by NHEJ- or MMEJ-derived small insertions and deletions (indels). This mutagenic burden, in the form of indel 'footprints', can lead to target protein imbalances and cell fitness losses [19]. In addition, on-target DSB formation can also yield translocations and gross chromosomal rearrangements [19–22]. Recent studies have further uncovered that on-target DSBs are capable of triggering extensive chromosome fragmentation followed by haphazard reassembly (chromothripsis) [23,24] and the partial or entire loss of chromosomes (aneuploidy) [25]. Notably, the chromothripsis and aneuploidy phenomena were readily detected in T cells and hematopoietic progenitor cells subjected to CRISPR-Cas9 nuclease reagents used in clinical trials [23,25]. Notwithstanding these phenomena, recent findings are more reassuring in that, contingent upon gRNA target site selection, chromosomal losses in particular can be substantially minimized by inducing DSB formation before, as opposed to after, the activation/stimulation of the primary T-cell populations [26].

Finally, on-target DSBs trigger P53-dependent cell cycle arrest and apoptosis which limits the efficacy of HDR-mediated genome editing in regular P53-positive cells [27,28], and creates selective pressure for the emergence of mutations associated with tumorigenesis. Related to the latter matter, during sub-culturing, pluripotent stem cells can acquire 'spontaneous' tumor-associated P53 mutations in a recurrent fashion [29] which, by virtue of being more resistant to DSBs, are in principle more prone to expansion than their wild-type counterparts once exposed to programmable nucleases. Indeed, CRISPR-Cas9 nuclease activation of certain signaling pathways can lead to the selection of cells with potentially harmful loss-of-function or dominant-negative mutations in the tumor-suppressor P53 transcription factor or gain-of-function mutations in the KRAS oncoprotein [27,30]. Furthermore, recent mouse modelling experiments indicate that p53 mutant cells, rather than proceeding to malignancy via an haphazard route, are instead subjected to an unexpectedly more deterministic set of genetic instability events [31]. Together, these cytotoxic and genotoxic effects raise tangible concerns on the use of programmable nucleases for the optimal generation of autologous genetically-corrected cell products.

RATIONALE FOR 'SOFT' GENOME EDITING BASED ON CRISPR NICKASES

Although emerging high-specificity programmable nucleases can greatly minimize off-target DNA cleavage, e.g., eSpCas9(1.1) [32] and Cas9-HF1 [33], they are inherently incapable of eliminating the potentially deleterious effects resulting from on-target DSB formation. Therefore, the substantial genotoxicity and cytotoxicity profiles associated with conventional nuclease-assisted genome editing create a pressing need for the development of alternative genetic engineering systems that reliably generate safer and functionally robust cell products. Indeed, DSB-dependent genome editing is expected to be particularly risky in the context to cell therapies based on the transplantation of populations of genetically engineered pluripotent stem cells, T lymphocytes and

NK cells. The reasons are twofold. Firstly, in the context of extensive *ex vivo* cell amplification protocols underpinning the generation of these cell transplantation products, DSB-derived mutations and/or chromosomal rearrangements can cooperate in cell transformation and clonal expansion. Secondly, in instances where targeting multiple genes is needed for achieving a robust anti-tumor effect, e.g., via combinatorial exogenous *CAR* transgene knock-ins and endogenous *TCR* or programmed cell death protein 1 (*PD1*) gene knockouts, simultaneous induction of the attendant DSBs at different genomic positions is expected to exacerbate the levels of undesirable genome editing by-products in the form of translocations and chromosomal rearrangements. In this context, investigations exploring alternative HDR-mediated gene knock-in approaches that rely on sequence- and strand-specific nucleases ('nickases') are valuable in that the resulting single-stranded DNA breaks (SSBs), or nicks, are substrates for neither NHEJ nor MMEJ. As a corollary, the balance between precise HDR to undesired end-joining events are dramatically biased towards the former. Moreover, although genomic SSBs are, *per se*, poor HDR stimuli, earlier experiments from our laboratory using the native adeno-associated virus Rep68/78 nickases demonstrated that concomitant SSB formation at acceptor sequences and donor DNA constructs fosters HDR-mediated gene knock-in at an endogenous human locus, namely, the prototypic safe harbour locus *AAVS1* [34]. The application of this generic in trans paired nicking (ITPN) principle was subsequently expanded to other genomic sequences through the use of more versatile RNA-programmable CRISPR-Cas9 nickases [35,36] that are simply obtained through site-directed mutagenesis of one of the two nucleases domains of the parental Cas9 protein (i.e., HNH or RuvC) (Figure 1) [37]. Indeed, by stimulating otherwise inefficient SSB-dependent HDR, ITPN approaches based on the delivery of nicking CRISPR-Cas9 complexes and matched nickase-susceptible HDR donor constructs, are valuable for seamless and scarless chromosomal editing, including at multiple-copy or essential genomic tracts [19,38]. Additional examples regarding the application of ITPN methodologies in various mammalian cell types, e.g., iPSCs, keratinocytes and organoids featuring normal or cancer traits, encompass:

- Repairing or installing predefined gene mutations [35,38–41];
- Maximizing the integrity of unmodified alleles during allele-specific gene editing [42,43], and
- Streamlining one-step biallelic gene editing or one-step multiplexing gene knock-in or tagging [35,44,45].

It is equally worth mentioning that, in contrast to regular and high-specificity CRISPR-Cas9 nucleases, CRISPR-Cas9 nickases constitute poor P53-dependent signalling triggers in human cells, including in DNA damage-sensitive iPSCs [38,40]. Hence, it is expected that the aforementioned growing mining for CRISPR systems buried in large genomic and metagenomic databases, will start unearthing enzymes that, via either their intrinsic or engineered nicking activities, enlarge the toolset for DSB-free genome editing. Examples include the HNH-negative IsrB nickase derived from the ancestral CRISPR-like system OMEGA and the RuvC-only CRISPR class 2 type V Cas12i nuclease that nick and preferentially nicks, respectively, double-stranded DNA substrates [46–48]. Moreover, often, newly discovered CRISPR systems also yield genome editing components whose small sizes renders them more fitting for delivery through commonly used adeno-associated viral (AAV) vectors [49].

Finally, another recent 'soft' HDR-mediated genome editing concept that might be particularly suited for the repair of heterozygous or dominant mutations involves

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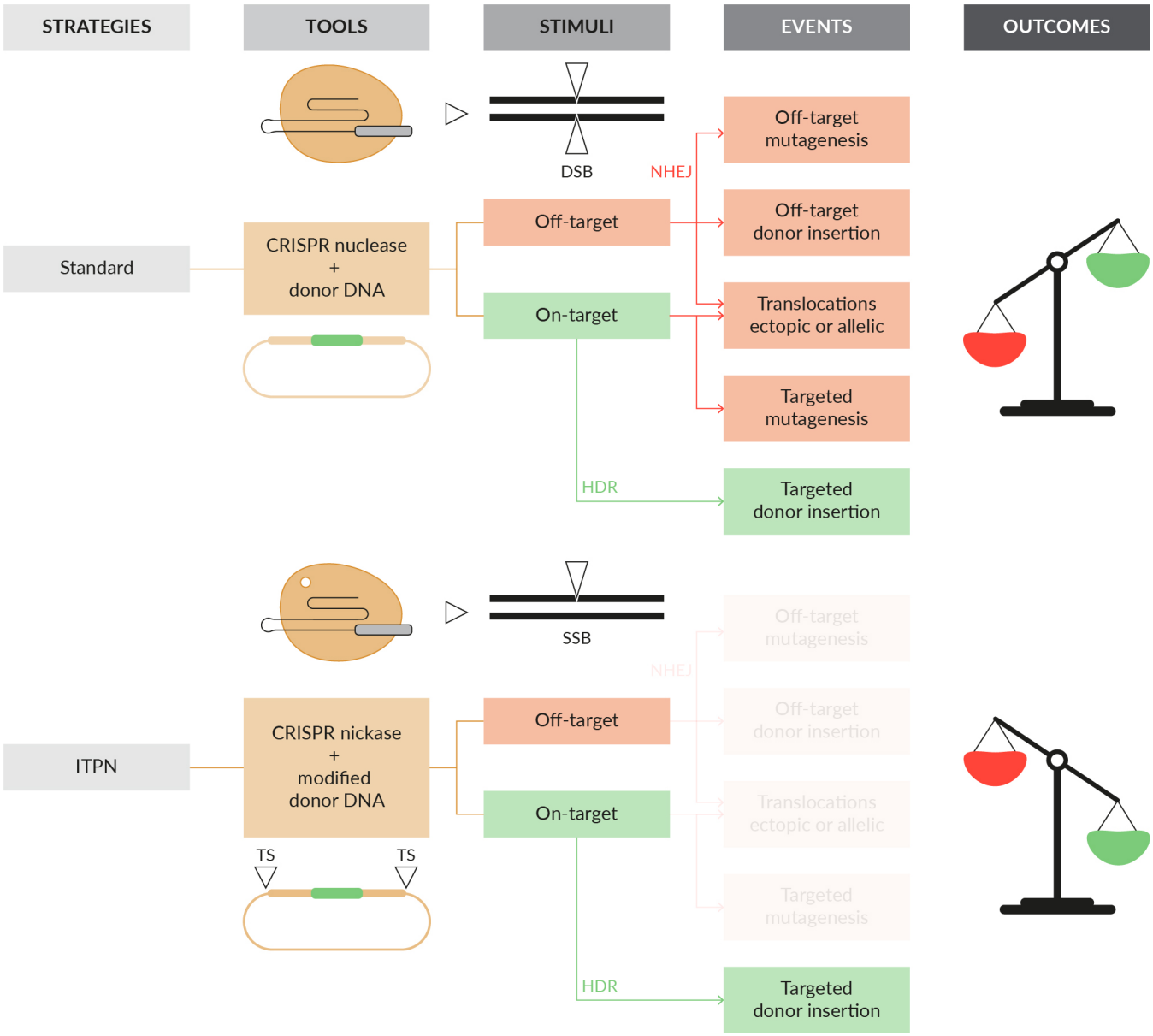
FIGURE 1

Standard versus *in trans* paired nicking genome editing.

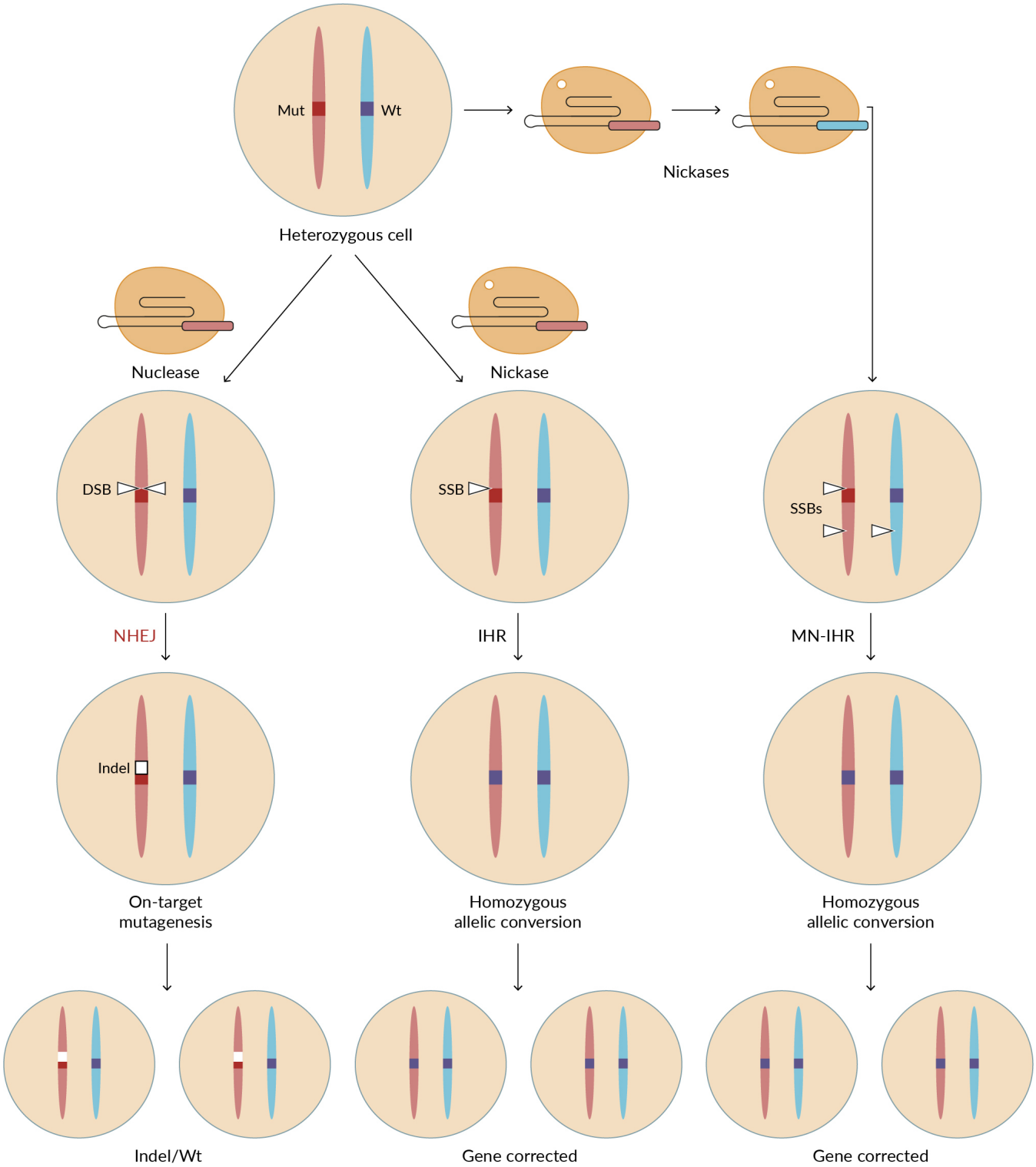


The relative weights of desired and undesired genome editing outcomes derived from, respectively, homology-directed repair (HDR) and imprecise events caused by competing end-joining DNA repair pathways, e.g., non-homologous end joining (NHEJ), are illustrated. DSB and SSB, double-stranded and single-stranded chromosomal breaks, respectively; 'nickases', sequence- and strand-specific nucleases. In contrast to standard donor constructs, modified donor constructs have nickase-susceptible target sites (TS) framing their targeting modules consisting of exogenous DNA (green bar) flanked by sequences homologous to the genomic target region ('homology arms').

allele-specific chromosome nicking for the stimulation of interhomolog recombination (IHR) in somatic cells [50,51]. Through this process of allelic conversion, a pathogenic mutation in one allele can, in principle, be corrected using as donor template the endogenous 'healthy' allele (Figure 2). This elegant concept of using CRISPR-Cas9 nickases and endogenous homologous chromosomal DNA as repairing templates has been demonstrated in *Drosophila* models [51] and human cell lines [50,52]. Regarding the application of such exogenous donor DNA-free genome editing principles in human cells,

FIGURE 2

Gene correction via interhomolog recombination between heterozygous allelic sequences.



Interhomolog recombination (IHR) characteristic of meiosis in germ cells can be fostered in somatic cells subjected to allele-specific double-stranded DNA breaks (DSB), yet the major products are on-target mutagenesis in the form of NHEJ-derived small insertions and deletions (indels). In contrast, allele-specific single-stranded DNA breaks (SSB) can equally foster IHR in somatic cells especially when using multiplexing CRISPR-Cas9 nickases for in trans multiple nicking IHR (MN-IHR). In somatic cells with heterozygous mutations or compound heterozygous mutations (not shown) underlying genetic disorders, CRISPR-Cas9 nickase-induced IHR offers the prospect for new genetic therapy interventions via wild-type allele-templated gene repair.

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recent investigations argue for multiplexing approaches in which primary allelic-specific gRNAs act in concert with secondary gRNAs to direct in trans paired nicking of homologous chromosomes and ensuing allelic conversion via IHR (Figure 2) [52]. Further research will be instrumental to advance CRISPR-Cas9 nickase-induced IHR from enticing proof-of-concept studies in cell lines to its application in human stem and progenitor cells.

TRANSLATION INSIGHT & OUTLOOK

There is a pressing need for investigating and validating alternative DSB-free and precise genome editing tools and strategies in various stem and progenitor cell types, e.g., *bona fide* T and NK cells as well as precursor iPSCs from which different effector cells can be differentiated from, including immunotherapeutic T and NK cell candidates. Genome engineering strategies covering targeted and precise chromosomal incorporation of genetic payloads with varying sizes will become ever-more relevant. In this regard, CRISPR nickases per se and fused to reverse transcriptases offer a complementary toolbox for 'soft' genome editing involving HDR and prime editing, respectively. Contrary to HDR, prime editing does not require the transfer of donor DNA substrates and allows for genomic insertion of up to ~44-bp of foreign DNA despite the need for substantial optimization of extended prime-editing gRNAs (pegRNAs) [53]. Moreover, in contrast to HDR-based genome editing, prime editing can take place in post-mitotic cells albeit to lower efficiencies than in cycling cells [54]. Recent prime editing developments include the combinatorial use of dual pegRNAs and site-specific recombinases designed for replacing genomic sequences with up 250-bp of foreign DNA and inserting entire transgenes at prime editor-defined recombinase target sites, respectively [53]. Despite powerful and versatile, such combinatorial strategies require the delivery of large and multicomponent reagents into target cells. An aspect warranting attention when considering multiplexing approaches concerns the importance of introducing balanced amounts of the attendant individual components to maximize the performance and precision of genome editing interventions [55]. In addition, prime editing involving the delivery of dual pegRNAs is not compatible with large edits whereas sequential prime editing and site-specific recombination is not amenable to subtle genomic edits underlying endogenous gene repair due to discontinuous 'footprint' installation in the form of recombinase target sites.

In conclusion, considering the herein discussed findings and matters, one can submit that cell therapy products derived from the use of RNA-programmable nickases as such or with heterologous domains, will offer a complementary set of 'soft' genome engineering options whose safety profiles are potentially higher than those associated with the exposure of cells to programmable nucleases.

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CELL & GENE THERAPY INSIGHTS

GENE EDITING

SPOTLIGHT

INTERVIEW

Bringing the *Sleeping Beauty* transposon system to the clinic

The range of technology options for nucleic acid delivery and genome engineering continues to expand, with a number of promising platforms now entering the clinic. **David McCall**, Senior Editor, Cell & Gene Therapy Insights, speaks with **Zoltan Ivics**, Head of Research Centre, Division of Hematology, Gene and Cell Therapy at the Paul-Ehrlich-Institut, about recent progress and next steps for one such example—the *Sleeping Beauty* transposon system.

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Q What are you working on right now?

ZI: I am working on the *Sleeping Beauty* transposon system. There are two components of a transposon system—a DNA component and a protein component. We are trying to modify these components by genetically engineering biochemical aspects of transposition, thereby changing how the transposon works so that it is more fine-tuned towards medically relevant applications.

I am also working to push the system into medical applications through collaboration; teaming up with medical doctors and clinical scientists, showing them how promising the system is so that they can bring it into a clinically relevant environment.

The very first European clinical trial using this system, which was based on the most highly developed *Sleeping Beauty* components using a CAR-T application, was completed just

two months ago. This study was conducted by a Horizon 2020-funded research consortium with the goal of utilizing *Sleeping Beauty* technology in multiple myeloma patients.

That was a really exciting development for the field, and the next clinical trials are already being planned. Eventually, this technology will be brought into the context of a drug product that a doctor can prescribe to a cancer patient, or indeed, any type of patient.

Q When we last spoke at the beginning of 2021 [1], we discussed some of the challenges in developing *Sleeping Beauty* transposons and applying them in human medicines—specifically, efficiency, toxicity, and avoiding a negative impact on the cell from the integration of the *Sleeping Beauty* transposon into the genome. Can you update us on what recent progress you have seen in these areas?

ZI: We have made very good progress in increasing both the efficiency and the safety of adding genes to cells. We have modified the transposase polypeptide (the *Sleeping Beauty* transposase) dramatically. We were able to switch a single amino acid in that polypeptide, shifting the genomic integration pattern of the transposon from a pattern that we had annotated and described in different cell types (including human cells).

We identified that *Sleeping Beauty* integrates relatively randomly in the human genome. Random integration is actually not too bad, considering that some viral vectors, including lentiviruses and retroviruses, do not have random distribution in the genome, but rather tend to favor transcriptionally active regions, transcription units, gene bodies, and transcriptionally relevant regulatory regions of genes for integration. These regions carry a certain risk in the context of a gene therapy application. By comparison, the random transposition of *Sleeping Beauty* is relatively safe, and we made it even safer by simply shifting around that single amino acid in the catalytic core domain of the transposase. We believe that the biochemical explanation for this is that after this change, the transposase can neither bend target DNA nor can it maintain a bent structure.

Bending DNA is an important structural feature of DNA integration machineries, including those employed by retroviruses and transposons. The inability of the generated mutant to undergo a bend reaction or to stabilize a bent structure is a defect, so to speak, in the transposase and therefore, it seeks out sites in the genome that are already highly bendable. These sites happen to have a biased base composition in that they have adenine-thymine (A-T)-rich DNA. These A-T repeated base pair sequences do not occur very frequently in exons and they are also typically depleted in the transcriptional regulatory regions of genes. The outcome of this is that when we implement *Sleeping Beauty* transposition in human cells with this particular transposase mutant, the integration sites tend to avoid coding sequences represented by exons, and also regulatory promoter

"We are trying to modify these components by genetically engineering biochemical aspects of transposition, thereby changing how the transposon works so that it is more fine-tuned towards medically relevant applications."

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elements and enhancer elements, which are probably the riskiest parts of the genome for foreign gene integration [2].

This is very significant. By using this mutant transposase, gene delivery becomes safer because a lower percentage of the integrations will end up in these potentially dangerous sites of the genome. Again, a single amino acid change can do this.

In terms of improving efficiency, we again shifted a single amino acid in the *Sleeping Beauty* transposase, which allows for hyperactivity in the transposition reaction. This highlights that there is still potential in this polypeptide composed of 340 amino acids for further improvement from both safety and efficiency perspectives.

Finally, we have shown that we can combine these two features and make a double mutant transposase, shifting transposition sites away from the dangerous sites in the human genome and providing hyperactivity. By fusing these two features, we can make polypeptides that are highly efficient in moving genes into genomes, and at the same time reducing the relative risk of adverse genetic engineering in human cells.

Q How has the field progressed in terms of the capability of nucleic acid delivery technologies such as lipid nanoparticles (LNPs) to efficiently deliver naked nucleic acids into primary human cells?

ZI: The LNP field is blooming, mainly due to the COVID-19 pandemic and the successful application of LNPs for mRNA delivery in some of the vaccines. This provided a tremendous boost to the field in the context of using LNPs as nucleic acid carriers not only for vaccination, but also for gene therapy or for other therapeutic purposes that require moving nucleic acids to cells. Today, we are seeing LNP applied as a delivery technology across the nucleic acid space, whether it be mRNA, small interfering (si)RNA, or, in the context of CRISPR-Cas9 engineering, single guide RNAs. There is an array of new developments, new findings, and new technologies based on LNP-mediated RNA delivery in the context of gene therapy applications.

Despite this success, there are some limiting factors to LNPs that need to be addressed. For one thing, we are still finding it difficult to move DNA (particularly plasmid-sized DNA) molecules into cells. The chemical composition of an LNP plays a fundamental role in the fate of the nucleic acid. In order for DNA to be expressed, it needs to be localized in the nucleus of the cell where it can undergo transcription. This is quite different from simply moving RNA molecules into the cells. However, I am beginning to see abstracts at conferences and titles of presentations that promise that researchers may be breaking through these boundaries and tapping into the potential of using LNP technology with DNA.

I am really excited about this because it will allow us to fully explore the potential of the *Sleeping Beauty* transposon with non-viral technology, especially for *in vivo* applications. LNP technologies represent a highly promising option in this context because they have the potential to be tagged by different targeting proteins that may interact with certain antigens, or with other proteins either exposed on the cell surface or in the cell. In the future, a fully non-viral and synthetic *in vivo* gene therapy application that carries a certain nucleic acid into a specific type of cell or organ in the human body could be achieved using this method.

Q What are the pros and cons of transposon systems in the context of the full range of genome editing tool options that is currently available to advanced therapy developers?

ZI: Genome engineering provides many different opportunities to treat genetic diseases, allowing investigators and medical doctors to convert a disease phenotype. There is now an entire spectrum of genetic engineering tools that are available to accomplish this.

For example, if a single base change can fix a disease, one may tap into base editing. If just a few base pairs need to be edited, prime editing can be applied. If longer pieces of genetic information need to be added to a cell, one may consider using either a viral-based system or a transposon-based system because of the robustness of these particular gene vectors in inserting larger pieces of DNA.

That is the niche of the transposon: to efficiently move larger pieces of DNA into a target cell genome. Nothing else can do this as well as a transposon. Prime editing is suitable for inserting up to 200 base pairs into the genome, but that is the rough limitation of that particular reaction. Transposons circumvent that limitation since they typically utilize longer pieces of genetic information for their own propagation.

As we have already discussed, transposons do this integration relatively randomly, which, in this context, may present a disadvantage compared to other systems. Targeted gene editing systems such as CRISPR-Cas9 systems, base editing systems, and prime editing systems are site-specific, which allows them to very precisely introduce a genetic change in the genome. Transposons cannot be this precise currently, but we are working to engineer transposon systems to overcome this disadvantage so that they become safer for human applications.

Q As someone who works at the Paul-Ehrlich-Institut (PEI), an organization that represents the interface between science and regulation, what is your view of recent regulatory guidance development relevant to this space? And what might we expect to see in the way of further guidance moving forward?

ZI: In terms of recent regulatory guidance, German advanced therapy stakeholders and politicians recently launched a national strategy for gene and cell therapy. This came out of the realization that the pipeline for translating gene and cell therapy preclinical research findings into the clinical and, subsequently, into full drug development, was inefficient. This new strategy is specifically working toward helping sponsors and clinical trial applicants through regulatory procedures, expediting dialogue between regulators and developers, and standardizing regulatory procedures on a national level.

Right now, every sponsor and every applicant is communicating with the PEI on an individual basis. This will not change, however, what can change is that through an enhanced level of input and communication, it will be much simpler to spell out standardized expectations and requirements in order to troubleshoot an application.

Additionally, since the beginning of 2023, there has been a new European system that does not necessarily change regulatory guidance with respect to how gene and cell therapies need to go through clinical trials, but rather introduces a new web-based portal where clinical trial applications need to be submitted. This means that everything goes through a

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> "...since the beginning of 2023, there has been a new European system that does not necessarily change regulatory guidance with respect to how gene and cell therapies need to go through clinical trials, but rather introduces a new web-based portal where clinical trial applications need to be submitted."

central portal, which will hopefully expedite the process of a developer gaining regulatory approval for a clinical trial application.

Looking to the future, transposon technology has gone through clinical tests and trials very quickly, and it will now be expected to turn this into a drug that is available to patients. However, regulatory guidance and the actual tests that need to be documented and carried out in the dossier that an investigator submits to the regulatory authorities in a clinical trial are still evolving alongside the science. For example, because of the potential risks that off-target CRISPR-Cas9 genome cleavage can introduce to a certain drug product, regulatory guidance for that application was based on annotating the sites where cleavage occurred. Of course, guide RNA design has drastically improved over the last couple of years and regulators have followed what that innovation produced with respect to assays and bioinformatics tools.

What is still missing, though, from the perspective of a regulator, is a sophisticated tool to predict potential risks associated with genetic engineering technologies. This concern is not necessarily limited to a CRISPR-Cas9, or any other gene editing system for that matter, but encompasses any kind of genetic change that we introduce into a cell including, of course, *Sleeping Beauty* transposition. We need to think about how to put together cell-based assays that a sponsor can employ preclinically with the actual components of genetic engineering (e.g., CRISPR-Cas9, base editing, prime editing, or a *Sleeping Beauty* transposon), and with a certain level of output (bioinformatic output, transcriptional output, or cell phenotype-based output), deliver strong data with respect to the safety of that particular genetic engineering step. Of course, this kind of tool would be mostly applicable to *ex vivo* genetic engineering and not an in vivo therapy, but if ex vivo applications were associated with a certain level of risk, that would help regulators and developers alike.

Q Can you highlight some of the key future applications and areas of development for transposon platforms that you expect to see moving forward?

ZI: In the coming years, transposon technology will be translationally applied to the genetic engineering of immune cells. That leads us to the engineering of T cells with either CARs or with a T cell receptor. This is a relatively straightforward and efficient genetic engineering technology mainly for *ex vivo* applications where T cells, for example, can be electroporated with plasmid constructs and mRNA encoding the transposase.

There are clear advantages to using *Sleeping Beauty* or another transposon (as opposed to using viral technology) in the genetic engineering of immune cells. *Sleeping Beauty*-engineered CAR-T cells are, biologically speaking, as potent as lentiviral-engineered CAR-T cells, but the actual application is easier. Regulatory approval is also more streamlined because there

"*Sleeping Beauty*-engineered CAR-T cells are, biologically speaking, as potent as lentiviral-engineered CAR-T cells, but the actual application is easier."

is no environmental risk assessment needed for a transposon vector, which is just a nucleic acid. There is no potential release of an infectious viral particle to the investigator or to other human beings. Furthermore, the costs associated with manufacturing nucleic acids at the GMP level are lower than those for viral engineering technology.

There are currently quite a number of CAR-T cells engineered with transposons in the early stages of clinical development, and I believe that number will continue to grow. Another area of application that we will likely soon see is *Sleeping Beauty*-engineered natural killer cells. Furthermore, I am hopeful that we will see additional immune system applications in the future, using cell types like macrophages, to treat not just cancer but potentially other human pathologies, too.

Another application that I am currently working on relates to tapping into hematopoietic stem cells (HSCs). This is really a different level of application, being not so focused on the cancer field but rather on the area of inherited monogenic diseases that affect one component of the blood system – either the immune system or other components of the blood. We are using electroporation techniques with *Sleeping Beauty* components, but instead of a human T cell, we are using HSCs. By resupplying or converting a missing factor in these HSCs throughout their differentiation into blood cells, we can phenotypically correct the cells to be without disease. This is not as advanced as the CAR-T cell applications – with the *Sleeping Beauty* transposon system, we are still at the level of preclinical research. However, the preclinical data looks very promising. We are in a situation now where we have robust technologies to genetically modify both mouse and human HSCs.

We are working on this while simultaneously pushing forward with the CAR-T cell applications (mainly in the context of cancer therapy) in the second wave of development. My expectation is that in the next wave of clinically relevant applications (hopefully within the next 2 years), the first clinical trials with *Sleeping Beauty* vectors will be initiated, widening the horizon, and allowing us to work on treating not only cancer but also rare genetic diseases with this particular non-viral genetic engineering technology.

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BIOGRAPHY

ZOLTÁN IVICS received his PhD in Molecular Biology in 1994. After postdoctoral studies at the University of Minnesota in the USA and the Netherlands Cancer Institute, he was appointed as a Research Group Leader at the Max Delbrück Center for Molecular Medicine in Berlin, Germany. He was appointed as Head of Division at the Paul Ehrlich Institute in Langen, Germany, in 2011, where he is currently heading the Research Centre embedded in the Division of Hematology, Gene and Cell Therapy. Professor Ivics' major scientific achievement is the molecular reconstruction of the *Sleeping Beauty* transposon and development of technologies based on *Sleeping Beauty* gene transfer for a wide array of applications involving genetic engineering of cells. Prof. Ivics has published >150 papers in peer reviewed journals, with a total Impact Factor of >1200 and >8000 citations (h-index: 64 by Google Scholar), and is co-inventor on 12 issued patents. Since 2000, his research efforts were supported by 25 research grants from the German Research Foundation, the German Ministry of Education and Research, the European Commission, the Volkswagen Foundation and the German Consortium for Translational Cancer Research. He received recognition of the 'Molecule of the Year' in 2009 for developing a hyperactive *Sleeping Beauty* transposase that opened the door for clinical applications. He served as a member of the Board of the European Society of Gene and Cell Therapy (ESGCT) between 2012 and 2022. He is a current member of the Board of the German Society of Gene Therapy and member of the committee for "Clinical trials and regulatory affairs" of the German Stem Cell Network. Professor Ivics organized several international conferences, including the Annual Congress of the ESGCT in Berlin in 2017. He is an Elected Member of the Academia Europaea and the Hungarian Academy of Sciences. His current interests focus on establishing clinically relevant methods and protocols for non-viral gene therapy.

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CELL & GENE THERAPY INSIGHTS

FULFILLING THE POTENTIAL OF GENE EDITING: AT THE TIPPING POINT

SPOTLIGHT

COMMENTARY

Unravelling immunogenicity induced by Cas9 in gene therapy: a comprehensive commentary of current understanding and implications

Anna Lina Cavallo

Gene therapy holds immense potential for treating genetic disorders by manipulating the patient's genetic material. The application of CRISPR-Cas9, a revolutionary gene editing system, has paved the way for precise and efficient genetic modifications. However, understanding the Immunogenicity induced by Cas9 is crucial for optimizing its use in gene therapy. This comprehensive commentary aims to explore the current knowledge on Immunogenicity associated with Cas9 in the context of gene therapy. By elucidating the immunological responses triggered by Cas9, this work seeks to enhance our understanding and address potential implications for successful clinical implementation.

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The CRISPR-Cas9 system is a revolutionary gene editing tool that has transformed the field of gene therapy. CRISPR-Cas9 utilizes a guide RNA molecule to target specific DNA sequences and the Cas9 enzyme to cut the DNA at the targeted site [1].

The CRISPR-Cas9 system offers several advantages over previous gene editing

techniques. It is highly efficient, cost-effective, and relatively easy to use. The ability to precisely edit the genome has opened up new possibilities for studying gene function, developing disease models, and potentially treating genetic disorders [2,3].

Indeed, in addition to its applications in research, the CRISPR-Cas9 system has garnered significant attention for its application in gene therapy. Extensive preclinical studies have demonstrated its capability to precisely modify DNA sequences, offering the prospect of correcting disease-causing mutations at their genetic source [4–8]. Actually, this technology has progressed beyond the confines of laboratories and entered the realm of clinical trials, marking a significant step towards its potential real-world application [9,10].

In the clinical domain, the transformative power of CRISPR-Cas9 has been exemplified through trials targeting a range of genetic disorders. Notably, diseases like sickle cell disease and beta-thalassemia, which are characterized by faulty hemoglobin production and resultant blood-related issues, have been at the forefront of these trials. Researchers are investigating the feasibility of using CRISPR-Cas9 to edit the relevant genes responsible for these conditions, with the ultimate aim of restoring normal hemoglobin function and ameliorating the symptoms experienced by patients [11].

CRISPR-Cas9 has also been tested in clinical trials for specific types of cancer. By harnessing the technology's precision and the ability to modify genes associated with cancer development, scientists are exploring ways to disrupt cancerous processes at a genetic level [12]. CRISPR plays a pivotal role in the advancement of T-cell therapy, which includes engineered TCR therapy and chimeric antigen receptor T (CAR-T cell therapy) [13,14]. These approaches leverage gene editing to precisely modify T cells to improve cancer cells' targeting and elimination. A further approach includes the immune checkpoint blockade by disrupting immunological 'brakes' like PD-1 to boost the overall immune response against cancer [15–17]. This personalized approach holds immense potential for revolutionizing cancer treatment by creating a highly targeted and potent immune response against tumors.

Beyond oncology, CRISPR-Cas9's potential shines in addressing other intricate medical challenges. For instance, in the case of cystic fibrosis, a genetic disorder affecting the respiratory and digestive systems, CRISPR's potential lies in its capacity to revert the underlying genetic anomaly [18]. Meanwhile, for Duchenne muscular dystrophy (DMD), a progressively debilitating muscle disorder, researchers are employing CRISPR to precisely edit the dystrophin gene, which holds responsibility for the disease [19–22]. Furthermore, the extraordinary capabilities of CRISPR extend to the correction of mutations responsible for hearing and vision impairments, presenting a ground-breaking avenue for addressing genetic disorders that lead to sensory loss [23–25].

It is important to note that while CRISPR-Cas9 has shown promise in preclinical studies and early-stage clinical trials for several diseases, these efforts are still largely in the research phase. Challenges mainly related to safety and toxicity, must be addressed before widespread clinical application beyond rare diseases.

Besides the prominent concern of off-target effects and the subsequent potential repercussions on genomic stability [26–28], another important aspect that has drawn attention is the apprehension surrounding the potential Immunogenicity of CRISPR-Cas9 [29].

Immunogenicity refers to the ability of a substance to induce an immune response [30–32]. In the context of CRISPR-Cas9 gene therapy, it refers to the potential of the CRISPR-Cas9 components to trigger an immune response in the patient [33]. This immune response can range from mild inflammation to severe immune reactions, which, on one hand may limit the effectiveness of the therapy, while on another it may cause severe unwanted effects. Recent studies have demonstrated an innate and adaptive cellular

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immune response to Cas9 in mouse models and the presence of anti-Cas9 antibodies and T cells in human plasma [34,35].

This prompts the necessity for a comprehensive exploration of Cas9 immunogenicity, including an investigation into its mechanisms, factors influencing its extent, and strategies to mitigate its effects.

Several factors can influence the immunogenicity of CRISPR-Cas9, including the specific components used, the delivery system employed, the route of administration, and the patient's individual immune profile.

The interaction between Cas9 and the immune system is complex and important for the success and safety of Cas9-based treatments. The immune system has two main components: innate immunity, which responds quickly to foreign substances, and adaptive immunity, which develops a memory of previous encounters.

Innate immunity is the first line of defense against foreign invaders, including bacteria expressing the CRISPR-Cas9 system. When Cas9 enters the body, cells of the innate immune system, such as macrophages and dendritic cells, recognize it as foreign and activate an immune response. These cells have receptors that can detect specific molecular patterns associated with pathogens or foreign substances [36].

Upon recognition of Cas9, the innate immune cells release cytokines and chemokines that act as alarm signals, priming other immune cells and then triggering an inflammatory response.

The inflammatory response initiated by innate immunity sets the stage for further immune reactions. It creates an environment that can either promote or dampen immune responses. The balance between pro-inflammatory and anti-inflammatory signals determines the overall outcome of the immune response to Cas9.

Simultaneously, adaptive immunity comes into play. Adaptive immunity is a more specialized and long-lasting immune response that develops over time. It involves the activation of T and B cells, which are key players in the adaptive immune system.

CD8 cytotoxic T cells, a pivotal element of the immune system, have drawn significant attention in conjunction with the integration of CRISPR-Cas9 technology into gene therapy applications. These specialized immune cells assume a critical role in the identification and eradication of infected or aberrant cells within the body [37,38].

When cells undergo engineering and express Cas9, whether for genome editing or other purposes, they acquire a distinctive molecular fingerprint that possesses the potential for recognition by various components of the immune system, notably including cytotoxic CD8 T cells. Within these cells, the Cas9 protein takes on the role of an antigen, effectively signaling the immune system to the presence of modified or engineered cells.

Upon the identification of Cas9-expressing cells by cytotoxic CD8 T cells, a multifaceted immune response unfolds. These cells are internalized by antigen-presenting cells, which process the antigens originating from Cas9 and subsequently display them on their surfaces through major histocompatibility complex (MHC) molecules. Surveying the body for cells showcasing foreign antigens, cytotoxic CD8 T cells are prompted to initiate an activation process when encountering cells presenting Cas9-derived antigens. Subsequently, these activated CD8 T cells undergo proliferation and differentiation, maturing into effector cytotoxic T cells. These specialized effector cells are equipped to engage and eliminate cells that exhibit the identified antigens—specifically, the Cas9-expressing cells. The effector cytotoxic T cells deploy cytotoxic agents such as perforin and granzymes, initiating a cascade that ultimately induces apoptosis in the targeted Cas9-expressing cells. This orchestrated immune response is directed at eradicating potentially modified or abnormal cells.

In parallel, when Cas9 is encountered by the adaptive immune system, helper T cells recognize and interact with Cas9 fragments

presented on the surface of antigen-presenting cells. This interaction triggers the activation of B cells, which are responsible for producing antibodies [39].

These antibodies, known as anti-Cas9 antibodies, can neutralize Cas9 or mark it for destruction by other immune cells. The production of anti-Cas9 antibodies is a hallmark of the adaptive immune response and demonstrates the interaction between Cas9 and the adaptive immune system.

Importantly, the adaptive immune response also involves the development of memory cells. Memory cells 'remember' previous encounters with Cas9, allowing for a faster and more robust immune response upon subsequent exposures. This memory response is the basis for long-term immunity and provides protection against future Cas9 exposures.

Overall, the interaction between Cas9 and the immune system is a dynamic and intricate process. Innate immunity recognizes Cas9 as foreign and initiates an inflammatory response, while adaptive immunity creates a memory of Cas9 and produces specific antibodies. Understanding this interaction is crucial for optimizing the efficacy and safety of Cas9-based interventions (Figure 1). Moreover, pre-existing immunity against therapeutic Cas9 delivery could decrease its efficacy *in vivo* and thus pose significant safety issues [40].

When a patient has antibodies against Cas9, it can have implications potentially hindering the effectiveness of gene therapy. If a patient has pre-existing antibodies against Cas9, the antibodies can neutralize or inactivate the Cas9 enzyme before it can perform its gene-editing function. This means their immune system recognizes Cas9 as a foreign substance and mounts an immune response against it. In some cases, the immune response triggered by the antibodies against Cas9 can also lead to adverse effects. The immune system may mount an inflammatory response, causing inflammation and tissue damage at the site of Cas9 delivery. This can further impede the success of gene therapy and potentially pose risks to the patient's health [41].

Balancing the advantages of Cas9-mediated gene editing with potential immune responses requires careful consideration. Researchers are exploring strategies to minimize unwanted immune reactions while optimizing the therapeutic effects of Cas9-expressing cells. This includes techniques to modulate the immune response, tailor Cas9 variants to reduce immunogenicity, and employ delivery methods that enhance the cells' acceptance by the immune system.

The two most commonly utilized forms of Cas9, which originate from *Staphylococcus aureus* (SaCas9) or *Streptococcus pyogenes* (SpCas9), are of notable mention. These bacteria are frequently found within the human microbiota and have the potential to induce disease, as exemplified by strep throat [42–44]. While the existence of a responsive immune reaction to Cas9 might not pose a primary hurdle for *ex vivo* treatments—where cells are manipulated in an immune-deficient environment and transplanted after complete Cas9 protein degradation—it presents a significant challenge for the progress of *in vivo* therapies [45].

Significantly, a substantial proportion of healthy individuals (around 80%) exhibit immune responses against both *S. aureus* and *S. pyogenes*, involving both humoral (antibody-mediated) and cellular (T-cell-mediated) immunity. These responses are predominantly directed against proteins that these bacteria secrete and those present on their cellular membranes—easily accessible targets for the immune system. It's important to note that while administering a single dose of CRISPR-Cas9 could be tolerated by patients lacking pre-existing anti-Cas9 immunity, substantial immune responses could arise after initial exposure. Once an immune response is triggered, subsequent applications of CRISPR-Cas9 in treatments would be restricted, a constraint with specific relevance to certain medical conditions [46,47]. To overcome these obstacles, the development of

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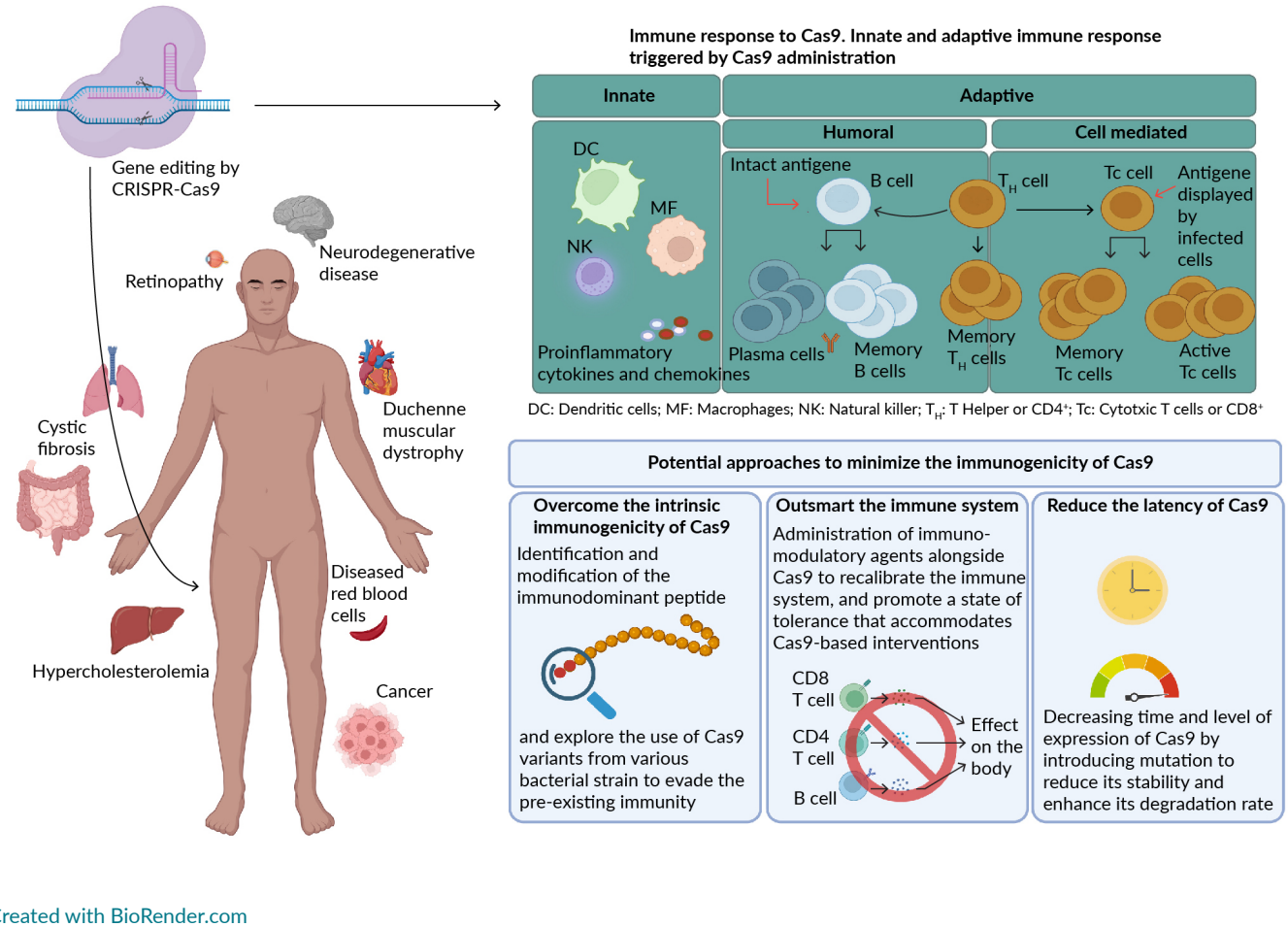
strategies is crucial, aiming to avoid the provocation of a Cas9-targeted immune response. When using Cas9 for gene editing, it is crucial to deliver the enzyme into the target cells efficiently and safely. CRISPR cargo can manifest in three primary forms: DNA, mRNA, and recombinant protein, each with distinctive characteristics regarding the immune system's recognition [48]. When Cas9 is administered as mRNA, it could evoke a milder immune response, primarily due to the transient nature of mRNA, which does not persist within cells for an extended period. However, if gRNA and mRNA molecules are co-delivered, there may be a risk of gRNA degradation before the mRNA's translation, potentially leading to suboptimal editing efficiency. Conversely, when CRISPR-Cas9 is introduced using a plasmid or viral vector, it offers greater stability but could also provoke a more pronounced immune response compared to mRNA. This heightened immune response has the potential to impede the genome editing process.

In the context of purified protein delivery, it typically tends to elicit a weaker immune response in comparison to DNA or mRNA. However, it's essential to note that the Cas9 protein's bacterial origin can confer antigenic potential, making it potentially immunogenic.

The delivery of CRISPR-Cas9 entails the transfer of CRISPR cargo into target cells, a process involving both the genetic material and the delivery vehicle. The selection of a delivery method relies on different factors, such

FIGURE 1

Graphical abstract.



Created with BioRender.com

as the size of the cargo, the type of target cells, the duration of gene expression, and safety-related considerations. Various delivery systems have been developed to facilitate the delivery of Cas9, including viral vectors, lipid-based nanoparticles, and physical methods such as electroporation [49].

Viral vectors, such as adeno-associated viruses (AAVs) and lentiviruses, have been widely used for Cas9 delivery. These vectors can efficiently deliver Cas9 and the guide RNA into target cells, allowing for precise gene editing. However, one concern with viral vectors is their potential immunogenicity. Viral vectors can trigger an immune response in the host, leading to the production of neutralizing antibodies against the vector. Indeed, immunogenicity directed against the AAV capsid represents a pivotal immunity-related concern within the vector system. AAV, as a non-enveloped vector, is highly susceptible to antibody responses by host immune cells. This could potentially lead to adverse outcomes, including impediments to effective delivery or immune-driven elimination of edited cells [50,51].

To overcome the immunogenicity associated with viral vectors, researchers have been exploring non-viral delivery systems [52]. Lipid-based nanoparticles (LNPs) are anon-viral delivery system that has shown promise for Cas9 delivery [53]. LNPs can encapsulate Cas9 and gRNA, protecting them from degradation and facilitating their uptake by target cells. LNPs have been shown to have lower Immunogenicity compared to viral vectors, making them an attractive alternative for Cas9 delivery. In *ex vivo* gene therapy, the immune system's exposure to CRISPR components is limited because the editing process takes place outside the patient's body before the modified cells are reintroduced. Physical methods such as electroporation are commonly employed to introduce the CRISPR components into these cells. This approach minimizes the potential risks associated with viral vectors or LNPs. However, it's worth noting that this approach is applicable to a limited range of diseases [54].

As the field of gene editing advances with the CRISPR-Cas9 system, the interplay between innovation and immunogenicity becomes a critical focus. In this work, we delve into the current landscape of strategies aimed at mitigating or eliminating the immune responses triggered by Cas9. A wide array of techniques has emerged, all with the common goal of harmonizing Cas9-mediated interventions with the immune system. These strategies encompass various approaches, ranging from molecular engineering to targeted immunomodulation. One approach involves identifying and subsequently modifying immunodominant peptides to evade the immune response directed against the Cas9 protein while maintaining its editing capabilities. These modified variants can be designed to minimize or eliminate potential immunogenic epitopes, effectively evading detection by the immune system [55,56].

In addition to molecular engineering, sophisticated delivery systems have been developed to shield Cas9 from immune surveillance during its journey to the target cells. Nanoparticles and viral vectors are examples of such delivery systems that can camouflage Cas9 by providing a protective shield, allowing it to pass through the immune system undetected. One approach involves refining the delivery methods used to introduce Cas9 into target cells. Reducing the immunogenicity of adeno-associated viruses is a crucial endeavor in the field of gene therapy. Researchers are continually developing strategies to mitigate the Immunogenicity of AAV vectors. These efforts encompass modifying the AAV capsid, leveraging tissue-specific promoters to restrict transgene expression, and exploring novel vector designs [57–62].

Furthermore, the administration of immunomodulatory agents alongside Cas9 presents another avenue for managing immune responses. These agents can be used to recalibrate the immune system, promoting a state of tolerance that accommodates Cas9-based

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interventions. By modulating the immune response, these agents help create an environment that is more conducive to the success of Cas9-mediated treatments [63].

In the context of Cas9, the longer it remains active within the system, the higher the likelihood of the immune system recognizing it as a foreign entity and mounting immune reactions against it. Reducing the persistency of Cas9, an essential step in genome editing, holds a crucial role in mitigating its Immunogenicity and enhancing its viability for clinical applications. Indeed, researchers are exploring strategies to limit the presence of Cas9 in the body, thereby minimizing potential immune responses.

Additionally, by exploiting the cell's natural protein disposal machinery, researchers can enhance the transient nature of Cas9's activity. Engineering Cas9 variants with altered properties can aid in modulating its persistence. Fine-tuning the stability of Cas9 or introducing modifications that enhance its degradation rate can help expedite its clearance from the system once it has executed its genome-editing function [64,65]. This would limit the potential for sustained immune reactions against the Cas9 protein.

Another strategy involves coupling Cas9 with molecular tags that mark it for rapid degradation within cells. This targeted degradation mechanism ensures that Cas9's presence is short-lived, thus reducing the likelihood of prolonged immune responses [66,67].

As genome editing moves toward tailoring treatments to individual patients, the immune responses to Cas9 become more intricate. Different individuals may exhibit varying levels of immunogenicity, influenced by factors such as genetic predisposition, prior exposure to Cas9, and overall health. This diversity in immune responses necessitates a personalized approach to treatment planning, wherein the potential for immune reactions against Cas9 needs to be carefully considered when designing interventions.

In summary, the immunogenicity of Cas9 profoundly influences its applications in clinical translation and personalized medicine. Effective management of immune responses is essential to maximize the benefits of Cas9-mediated therapies while minimizing potential setbacks. The immunogenicity associated with Cas9 presence can be curtailed by strategically reducing the persistency of Cas9 through innovative delivery techniques, engineered variants, targeted degradation mechanisms, and immune-suppressive strategies. These efforts pave the way for more successful and safer clinical translation of Cas9-based therapies, ensuring that the transformative potential of genome editing can be harnessed without triggering undesirable immune reactions. Addressing these immunogenicity implications ensures that the transformative potential of genome editing can be realized in a safe, efficacious, and ethically responsible manner.

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CELL & GENE THERAPY INSIGHTS

FULFILLING THE POTENTIAL OF GENE EDITING: AT THE TIPPING POINT

SPOTLIGHT

COMMENTARY

An update on prime editing: recent advances and applications

Karl Petri and Julian Grünewald

CRISPR PE is a substantial advancement of earlier CRISPR technologies, enabling the RNA-encoded installation of all substitution edits, small insertions, and small deletions without requiring neither DNA double-strand breaks nor DNA donor templates. The development of new PE technologies occurs rapidly, and recent work enhanced the PE platform by improving informatic prediction of PE outcomes, developing PEs with new capabilities, optimizing PE delivery, and introducing new PE off-target detection methods. PE is a groundbreaking technology that will continue to evolve in the coming years regarding technological capability and its deployment to the clinic. Here, we summarize the most recent progress involving PE technology and provide a perspective on its future development.

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INTRODUCTION

Since its introduction in 2019 [1] the gene editing field has broadly adopted CRISPR prime editing (PE). PE potentially enables the double-strand break-free installation of all substitution edits, small insertions, and small deletions into DNA. The PE system consists of a catalytically impaired version of Cas9 inducing a DNA single-strand break (SpCas9-H840A nickase, or nCas9 in short), a reverse transcriptase (RT) usually tethered to nCas9, and a PE guide RNA (pegRNA) to program both the editing location in the genome and the edit itself. Since the original description of PE, multiple landmark studies

have improved the PE platform, including studies demonstrating that mismatch repair inhibition [2] and pegRNA exonuclease protection [3] can increase PE efficiency. The developments in the first three years following the initial PE publication were exhaustively reviewed previously [4]. This commentary summarizes the most recent results and updates concerning PE technology.

NEW PE TECHNOLOGY

An important new development for PE was the introduction of dual pegRNA (or 'dual flap') designs, instead of combining a pegRNA and a nicking gRNA for classic PE3(b) approaches. Dual flap approaches such as Prime-Del, PEDAR or Twin-PE allow for large deletions [5,6] as well as larger insertions and more extended recoding [7].

A recent report demonstrated that PE with pairs of pegRNAs enables the installation of tandem duplications in the mammalian genome, potentially allowing the modeling of disease-relevant tandem duplications [8].

Zheng *et al.* developed a PE-based technique called template-jumping PE, allowing the insertion of larger DNA fragments through a putative mechanism akin to the retrotransposon integration mechanism. Using template-jumping PE, the authors inserted a 200 bp sequence with up to 50% efficiency [9], complementing previous approaches for the PE-mediated installation of larger insertions without requiring DNA donor templates [10].

Moreover, two groups reported PE-mediated genomic installation of much larger, gene-sized DNA fragments employing PE in combination with DNA recombinases and a DNA template [7,11]. Recently, another study reported that this approach enabled the insertion of up to 11.1 kilobases of DNA into the genome of plants [12]. The authors used this system, which they call PrimeRoot, to insert resistance genes in rice.

In another report, the authors showed that they can use PE to induce graded and targeted downregulation of gene translation in plants. The authors do this by PE-mediated generation or extension of upstream ORFs in the 5'UTR of target genes. Upstream ORFs generally inhibit the protein expression of the primary ORF. Thereby, the authors provide a novel approach for the gene editing-mediated targeted downregulation of genes [13].

To increase the purity of PE outcomes, Lee *et al.* engineered nCas9 [14]. The authors observed that nCas9 (H840A) retains DNA double-strand cleavage activity, causing unwanted PE insertion and/or deletion (indel) byproducts. The authors engineered nCas9 to decrease its ability to induce DNA double-strand breaks and demonstrated that PE systems employing the engineered nCas9 exhibited lower indel frequencies. In another study, the authors increased PE efficiencies by recruiting the transcription factor P65 [15] thereby increasing chromatin accessibility.

Two recent papers highlighted that interactions between pegRNA spacer and extension can negatively impact PE efficiency [16,17]. These papers showed that sequence complementarity between the spacer and extension sequence of a pegRNA can cause decreased PE efficiency by auto-inhibition via reduction of pegRNA binding efficiency and target recognition. One study reported that PE efficiency can be rescued by optimizing the pegRNAs extension annealing temperature and that a transient cold shock treatment can increase PE efficiency [17]. A recent study described that pegRNA spacer-scaffold interactions can also be overcome by a refolding procedure or the inclusion of additional mutations destabilizing inhibitory pegRNA secondary structure [18]. Similarly, recent work has shown that editing efficiencies can be enhanced by modifying pegRNA structure and/or sequences [19].

PE nuclease is an enzyme variant of prime editor protein that retains the ability to cleave double-stranded DNA. Previous studies employed PE nuclease to increase PE efficiencies [20] and introduce large deletions [6]. In recent work, another group improved the

precision of PE nuclease-mediated editing by co-introducing a 53PP1-inhibitory ubiquitin variant inhibiting nonhomologous end joining [21]. Notably, one previous study also used PE nucleases in combination with small molecule-mediated NHEJ inhibition [20]. Furthermore, the PE nuclease approach has been used to extend the repertoire of nucleases for PE to type V systems, such as Cas12a [22].

Regarding the PE architecture, Split-PE variants with untethered RT domains [23, 24] or split pegRNAs [23] have been shown to work efficiently in human cells. Moreover, RTs other than MMLV-RT have been tested, engineered, and/or evolved to increase editing efficiencies or to reduce the size of the PE construct (e.g., by truncating the RNAseH domain) [23–26]. A recent study demonstrated that phage-assisted evolution can be used to engineer prime editor proteins. The authors showed that the prime edit type determines the optimal choice of prime editor protein and successfully converted low activity reverse transcriptases into efficient ones using phage-assisted evolution [26].

Recent work demonstrated that PE can also be leveraged for the high-throughput characterization of genetic variants. The authors of the study assessed the impact of genetic variants on function of the *NPC1* gene and conclude that 410 of 706 induced missense mutations had deleterious effects on protein function [27].

PREDICTING PE OUTCOMES

The design of pegRNAs for PE is complex and involves optimizing multiple parameters, including pegRNA spacer and extension sequences. Our knowledge regarding the optimal design of pegRNAs is currently incomplete. Still, large-scale analyses of PE outcomes using high-throughput approaches represent a promising method to further our understanding of optimal pegRNA design. In early efforts, Kim *et al.* developed DeepPE [28] to predict the efficiency of pegRNAs by employing high-throughput molecular approaches and machine learning. Multiple second-generation prediction tools complementing this earlier work were recently introduced, including PRIDICT [29], DeepPrime [30], and MinsePIE [31], employing high-throughput data generation in various cell types and at an increased number of target sites. MinsePIE focuses on elucidating the impact of the insertion sequences on PE efficiencies [31]. Notably, many of these studies offer web tools through which users can leverage the developed prediction models for designing pegRNAs.

Another recent high-throughput study demonstrated that the cis-chromatin environment impacts PE efficiencies [32]. The authors also show that PE efficiency can be modulated by altering the chromatin state of target regions. Next-generation prediction tools such as ePRIDICT include chromatin context information to predict PE efficiencies [33].

PE DELIVERY & CLINICAL TARGETS

Despite much recent progress, delivery of PE is still challenging due to the large size of prime editors. Broadly, we can distinguish between *ex vivo* and *in vivo* delivery depending on whether we deliver PE to cells inside or outside a living organism. A recent study described that PE could correct the causative mutation of sickle cell disease in hematopoietic stem and progenitor cells from sickle cell patients. Transplanted cells persisted in immunodeficient mice expressing the corrected hemoglobin allele after 17 weeks, with an average of 42% of erythroblasts expressing the corrected allele exceeding predicted thresholds for therapeutic benefit [34]. Another related study reported that PE can correct the sickle cell mutation in a murine sickle cell disease model [35].

Prime editors are large fusion proteins generally exceeding the packaging capacity of AAV vectors (~4.7 kb), complicating

in vivo delivery. A common approach to overcome challenges with the delivery of large proteins via AAV is splitting the protein, delivering protein fragments as separate AAV vectors, and reconstituting full-length protein in the cell of interest via a split intein system. Two recent studies demonstrated delivery of prime editors using split AAV systems [36,37]. One of these reports determined that a dual AAV system can correct a causative mutation of Leber's congenital amaurosis in mice with up to 16 % editing efficiency [37]. Another study showed relevant *in vivo* editing efficiencies in the mouse liver using dual-AAV (intein-split) as well as unsplit adenoviral delivery of PE [25]. Another recent study performed an extensive optimization of dual AAV *in vivo* delivery of PE and achieved 42, 46 & 11% PE in the mouse brain, liver, and heart, respectively [36]. Another study demonstrated that a causative mutation for familial dilated cardiomyopathy in the *RBM20* gene could be corrected using PE in human induced pluripotent stem cells (hiPSCs) with 40% PE efficiency [38]. PE was also tested directly in hiPSC-derived cardiomyocytes with intact and Split-PE architectures with efficiencies of up to approximately 15% [24]. In another report, researchers introduced Cre-inducible PE into the mouse germline, facilitating the rapid engineering of genetic variants of interest, such as the installation of *Kras* and *Trp53* mutations [39].

PE OFF-TARGETS

Considering gene editor off-target activity is important before deploying them to the clinic. Two methods for identifying PE off-targets have been introduced recently—TAPE-seq [40] and PE-tag [41], complementing previous work [42]. Both methods employ pegRNAs that install a defined DNA tag sequence in the genome. The DNA tag is installed at on- and potential off-target loci. The defined and known sequence of the tag then functions as a molecular handle, enabling amplification of the genomic regions surrounding the sites of tag integration. These PCR libraries can then be prepared for next-generation sequencing, and informatic analyses reveal PE off-target candidates. Notably, PE-tag is usable in both an *in vitro* and an *in vivo* format.

Interestingly, one study also leveraged DNA double-strand break-inducing PE nuclease to analyze Cas9 off-targets using a technique called PEAC-seq [43]. Like TAPE-seq and PE-tag, PEAC-seq installs a defined DNA tag sequence at sites of PE nuclease activity. Using PEAC-seq, the authors were able to detect Cas9-induced off-targets and translocations. These recent studies generally support the notion that the PE system is highly specific but provide critical new tools to assess the accuracy of prime editors. A recent study reported the finding that CRISPR base editors and PE can induce genotoxic effects including detrimental transcriptional responses and genotoxic byproducts in hematopoietic stem cells [44]. These effects were most prominent for cytosine base editors. Evaluating putative off-target activity is an essential requirement before clinically translating PE technology.

TRANSLATION INSIGHTS

Summary of progress

Overall, the gene editing field has broadly adopted the PE platform. Progress continues to be quick, encompassing advances in PE technology, high-throughput prediction of PE efficiency, PE delivery, and PE specificity evaluation. Importantly, early data from non-human primate studies suggest that lipid nanoparticle/mRNA delivery to the liver can yield up to approximately 50% *in vivo* PE [45]. However, to this date, there is no clinical trial employing PE, which will be an essential next technological milestone. So far, PE technology productively co-exists with earlier-generation CRISPR tools, such as CRISPR nucleases or CRISPR base

editing, with each platform contributing a unique set of advantages and disadvantages depending on the indication and potential use cases.

Open questions/areas of improvement

Even though the PE platform constantly improves, PE efficiencies can still substantially vary between cell types and target sites. Advances in predicting PE efficiency using machine learning and computational approaches and further engineering of PE proteins (and pegRNAs) will likely increase the robustness of PE across different cell types, tissues, and organisms. The PE-mediated integration of large DNA sequences is an active area of research in which we will probably see further improvements. PE-employing CRISPR screens will enable assessing the impact of genetic variants on cell phenotype at large scale. The application of PE in clinical trials is a logical next step, given the unique capabilities and advantages that PE shows when compared to previous CRISPR technologies.

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CELL & GENE THERAPY INSIGHTS

SPOTLIGHT

INTERVIEW

Exploring the convergence of gene editing and RNAi in iPSC-derived allogeneic cell therapy

Within cell and gene therapy, there is a growing reliance on innovation in genome editing technology. **David McCall** (Senior Editor, BioInsights), speaks to **Vlad Seitan**, (Chief Scientific Officer, Laverock Therapeutics) about enhancing advanced therapies through a novel approach that combines the power of gene editing with the RNA interference (RNAi) pathway.

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Q What are you working on right now?

VS: Currently, I am working with the rest of the team at Laverock Therapeutics to determine how a cell's microRNAs (miRNAs) can be rewired to engineer improved cell and gene therapies. The rewiring of miRNAs forms the foundation of our novel gene silencing technology, called Gene Editing-induced Gene Silencing (GEiGS®). GEiGS harnesses the advantages of both gene editing and the RNA interference (RNAi) pathway to silence one or multiple desired target genes.

miRNAs are small non-coding RNAs that are integral to the physiological gene regulation at post-transcriptional level. After genes are transcribed, the persistence of messenger RNA (mRNA) in the cell and its translational efficiency are pivotal steps in gene regulation, as they ultimately determine the level of gene expression. miRNAs exert their influence by binding to their target mRNAs through sequence complementarity, and recruiting components of the RNAi machinery that mediate translation inhibition and mRNA degradation, thereby reducing expression of the gene. This mechanism is leveraged by RNAi gene silencing approaches such as small interfering RNA (siRNA) and short hairpin RNA (shRNA).

While existing RNAi approaches work by introducing exogenous synthetic short RNAs (siRNA) or transgenically expressed precursors (shRNA) into the cell, Laverock Therapeutics' novel approach—GEiGS—uses endogenous miRNA genes as vectors of expression for new silencing RNAs. GEiGS centers on identifying endogenous miRNA genes that are already expressed in the cell type of interest, and editing their sequence to redirect their activity toward new targets **(Figure 1)**. To avoid potential adverse effects caused by losing the physiological activity of the redirected miRNA, GEiGS is exploiting redundancies in the miRNA network. Many miRNA genes belong to families of related miRNAs that regulate the same target genes, which allows editing individual miRNAs within these families without significantly disrupting the expression of their physiological cognate targets.

In contrast to siRNA and shRNA which use only part of the RNAi pathway, GEiGS leverages the physiological RNAi pathway in its entirety, and this gives it a unique combination of benefits, namely the ability to induce stable, tunable and programmable gene silencing with minimal off-target effects.

Q As an expert in the field, can you outline the underpinning rationale for, and give us a potted history of, gene editing applied specifically in the RNAi research area?

VS: Initially, gene editing and RNAi had a competitive relationship. This relationship can be traced back to the emergence of gene editing tools, including TALENs and Zinc-finger nucleases, with the most significant transformation occurring with CRISPR technology, of course. CRISPR enabled the efficient generation of gene knockouts, leading to a noticeable shift from RNAi. Prior to the advent of gene editing tools, RNAi was the preferred method for studying gene function, as well as the focus of many R&D programs trying to develop gene silencing for therapeutic applications.

The introduction of gene editing technologies prompted a shift in focus from RNAi to gene editing. Several factors contributed to this shift, including how RNAi was initially applied, primarily through transfection of siRNA oligos and overexpression of shRNA hairpins. These applications were associated with significant challenges, including difficulties in delivering the reagents to the right tissue and off-target effects caused by supra-physiological doses. In fact, around 2010, these challenges lead to a considerable decrease in interest and investment in the RNAi field. As genome editing technologies became established, skepticism in RNAi was already growing, so this accelerated the switch in focus from one technology to the other.

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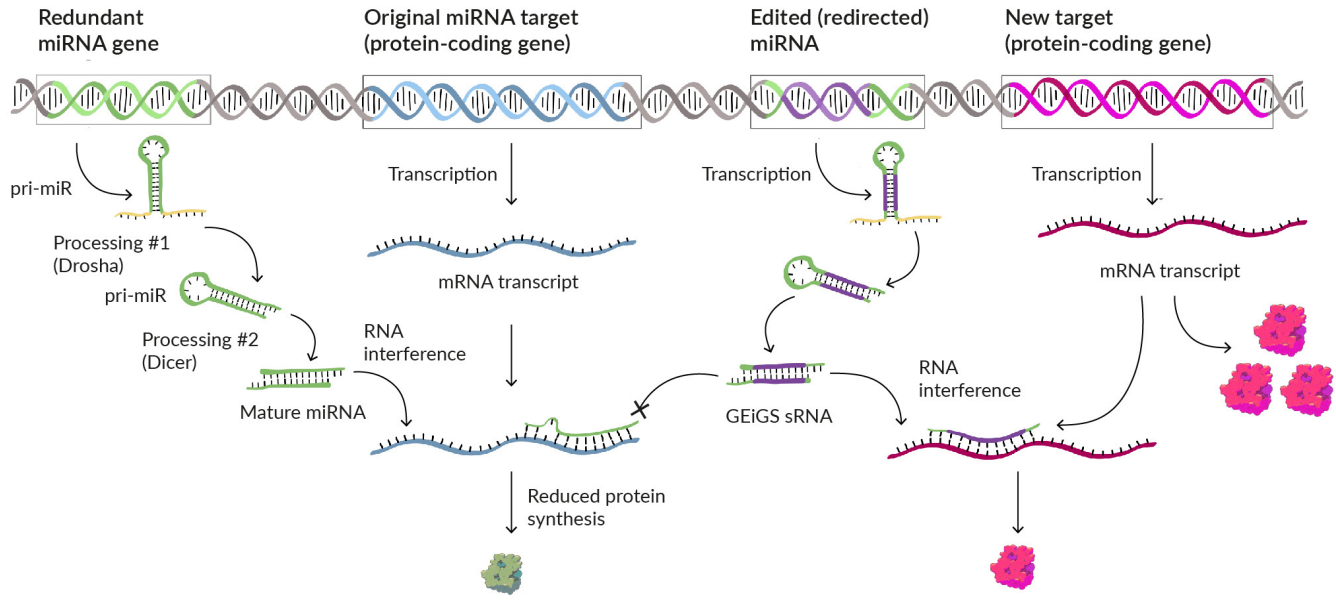
FIGURE 1

Gene Editing-induced Gene Silencing (GEiGS).



While gene editing initially contributed to the decline of RNAi technologies, it quickly became an important tool for studying the RNAi pathway and miRNA functions, and eventually helped improve our understanding in this field. Before the widespread use of gene editing, functional studies in miRNA biology were heavily reliant on synthetic miRNA mimics, which were problematic due to off-target effects, frequent incorporation of mutations, and ultimately did not function the same as endogenous miRNAs. Consequently, published results were sometimes difficult to reproduce, fueling ambiguity around certain aspects of miRNA biology.

As gene editing became more widespread, researchers gained the capability to easily knockout miRNA genes and mutate putative miRNA binding sites within target transcripts, enabling them to study loss-of-function scenarios and better understand the roles of miRNAs. These genetic approaches also illustrated the redundancy exhibited by the miRNA network, as knocking out individual members of miRNA families was often insufficient to cause a distinct phenotype. (GEiGS is exploiting these redundancies by redirecting specific miRNA genes to silence desired targets without otherwise disrupting the normal physiology of the cell.)

In the last few years, RNAi approaches have been experiencing a resurgence. Improvements in specificity and delivery have seen the first siRNA drugs receiving FDA approval, while the development of miRNA-based shRNA cassettes has enabled the *ex vivo* transgenic implementation of RNAi in the clinic with positive outcomes.

The development of GEiGS is now bringing gene editing and RNAi together, drawing advantages from both technologies. While the silencing engine in GEiGS is the RNAi pathway, the implementation is based on gene editing. So, we can say that gene editing is now expanding the applications of RNAi through this novel approach that maximizes the benefits of RNAi while minimizing its off-target effects.

Q Tell us more about Laverock Therapeutics, and your specific gene silencing platform and approach to allogeneic cell therapy development—what differentiates it?

VS: As mentioned, GEiGS represents a novel way of harnessing the RNAi pathway. Unlike previous gene silencing methods, which rely on either synthetic oligos or transgenes, Laverock Therapeutics' approach involves editing endogenous miRNA genes already expressed in the cell type of interest so that—instead of the original miRNA—they express a new silencing RNA directed at a target of interest. The target can be endogenous such as mRNAs, or foreign such as viral genomes.

GEiGS does not involve the addition of exogenous regulatory elements, but relies entirely on the physiological expression of the endogenous locus. This offers it several distinct advantages, namely the ability to induce gene silencing in a stable, tunable, and, most importantly, programmable manner. Moreover, GEiGS can achieve this with a much more favorable off-target profile than existing gene silencing technologies.

If you think of existing RNAi approaches, siRNA is intrinsically transient, requiring continuous re-dosing, while shRNA transgenes are frequently inactivated through epigenetic mechanisms, leading to variegated expression and loss of silencing activity. By contrast, because GEiGS silencing RNAs are encoded within endogenous loci, their expression is protected from epigenetic inactivation, so it remains stable. Moreover, because the silencing activity is dependent on the expression level of the edited miRNA locus, it can be tuned by choosing miRNAs with the right level of abundance—if complete silencing is desired, highly expressed miRNAs can be edited, while an intermediate level of silencing can be achieved by editing less abundant miRNAs.

A major challenge with RNAi approaches has been the potential for off-target effects. These are to a large extent dosage dependent, and the problem with siRNA and shRNA applications is that they lack an intrinsic mechanism to ensure silencing RNAs are administered within the limits of what cells can physiologically tolerate. Consequently, these technologies can often deliver supra-physiological concentrations (through high doses of synthetic oligos or by using very strong promoters). At high concentrations exogenous silencing RNAs are much more likely to bind and silence unintended targets, or to sequester the RNAi machinery required for endogenous miRNAs to function, which leads to toxicity. By contrast, in GEiGS, the abundance of silencing RNAs always remains within physiological limits because it follows the expression program of an endogenous miRNA gene. Thus, GEiGS does not disrupt the normal miRNA-based gene regulation and it is less likely to silence unintended targets. In fact, so far, our experiments

"GEiGS does not disrupt the normal miRNA-based gene regulation and it is less likely to silence unintended targets. In fact, so far, our experiments have shown that GEiGS can very effectively and specifically silence targets in the absence of detectable off-target effects."

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have shown that GEiGS can very effectively and specifically silence targets in the absence of detectable off-target effects.

Perhaps the most differentiating aspect of our technology is its ability to program when the gene silencing activity is induced. Gene knockout methods such as CRISPR KO result in constitutive gene silencing, meaning the target gene remains inactive regardless of the cell's state or external stimuli. By contrast, GEiGS leverages the variability of miRNA expression patterns, which can significantly differ not only between various cell types but also across different cellular states. Consequently, cells can be edited to activate gene silencing only when they reach specific developmental stages or encounter particular stimuli. We can therefore program gene silencing to occur precisely within the right cell type, at the right location, and at the right moment.

To illustrate the applicability of programmable GEiGS, consider edited induced pluripotent stem cells (iPSC) for allogeneic cell therapy. We can modify the iPSC bank, ensuring gene silencing activity is triggered exclusively when these iPSCs differentiate into the mature cell type used for therapy, or when the therapeutic cells become exposed to specific disease environments. Similarly, in cellular immunotherapies we can use GEiGS to program cells to respond to their entry in the tumor microenvironment, maximizing the anti-tumor activity while minimizing the risk of immune adverse effects. This represents a significant enhancement over existing gene silencing methods, providing a refined and highly adaptable approach.

Q Can you discuss the long-standing challenges and limitations that have faced gene edited iPSC-derived therapies, and how Laverock's approach addresses them?

VS: Some of the challenges we encounter are common to all iPSC-derived therapies, but certain considerations are more specific to gene editing methodologies. One crucial aspect is carefully selecting the appropriate iPSC cell lines, recognizing that not all are identical. Many iPSC lines exhibit lineage bias, so it is important to select a line that can efficiently differentiate into the cell type you are looking to deploy therapeutically.

Chromosomal instability can occur during the iPSC cloning and expansion phase, so genome integrity needs to be carefully monitored both before and after cell banking. Additionally, while gene editing techniques are powerful, they can cause off-target effects, so following the introduction of gene edits, it is imperative to conduct rigorous QC assessments to detect any potential undesired mutations.

A safety concern with iPSC-derived therapies is that following the *in vitro* differentiation of therapeutic cells, some undifferentiated iPSCs may remain in the product, and following transplantation these could proliferate and form teratomas. Another concern is that *in vitro* derived therapeutic cells are not as robustly differentiated as primary cells, and may lose their cell identity post-transplantation, facilitating oncogenic transformation. These risks are significantly amplified in allogeneic approaches because a major goal in the development of these therapies is to avoid rejection by the host immune system, so the cells are genetically engineered to silence major alloantigens. Currently, the engineering approaches are based on

constitutive inactivation of immunogenic factors through gene KO. This means that not only the therapeutic cells are hypoimmunogenic, but also the iPSCs used to generate them and any intermediate progenitors. Therefore, if iPSCs persist in the final product, or if the therapeutic cells de-differentiate, the risk of generating tumors would be greatly amplified by the fact these cells are equipped to evade immune surveillance.

Laverock Therapeutics' technology enables the editing of iPSCs in a manner that programs the silencing of immunogenic factors to occur exclusively in the fully differentiated cell types. This means that only the mature therapeutic cells are hypoimmunogenic, while any traces of iPSCs in the product or de-differentiated cells will maintain full expression of alloantigens and be rejected by the patient's immune system. We think this programmable approach, which we call conditional hypoimmunogenicity, offers a notable safety advantage.

Q What specific capabilities does the bioinformatics component of Laverock Therapeutics' platform offer?

VS: The GEiGS technology relies on a proprietary computational pipeline known as BioCompute. This tool has been meticulously fine-tuned to integrate data from miRNA expression profiles, genome annotations and target sequences. Its primary function is to swiftly provide insights into which specific miRNAs should be edited, and how these edits should be executed to effectively silence our desired target genes. In addition to the computational pipeline, we have established a workflow for rapidly validating these proposed solutions experimentally. This process takes us from the initial design phase to creating edited cells as efficiently as possible. BioCompute has been constructed based on extensive experience, and we continuously integrate our real-world laboratory results into its algorithm. This iterative approach allows us to refine and enhance the pipeline throughout our research and development process.

Q What is your perspective on recent trends and breakthroughs in terms of emerging genome editing platforms in general, and how could they potentially be harnessed to further advance and extend the reach of Laverock's therapeutic platform moving forward?

VS: A strong advantage of GEiGS is that it is not dependent on a particular gene editing technology, so any one of the existing or future editing approaches can be used. This means that breakthroughs in genome editing technologies can be readily incorporated in the GEiGS workflow to advance our platform. This can be improvements in an existing technology we are already using, but equally, if a better genome editing approach emerges that benefits the application of GEiGS, this can be swapped in.

Currently, a prominent concern in gene editing approaches revolves around the issue of off-target effects. Recent systematic studies have revealed that off-targets are not uncommon and pose a considerable health risk, particularly those involving approaches based on introducing double-strand DNA breaks—such as CRISPR/Cas9—as these can lead to genomic instability. Substantial efforts are being invested in minimizing off-targets and developing screening

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"Prime editing has also the potential to broaden the applicability of our technology, which centers on introducing specific gene edits into the genome."

methods to detect them. In the context of iPSC-derived cell therapies, one advantage is that once cells are edited, extensive QC can be performed on clonal populations to meticulously screen for potential off-target effects before clearing them for therapeutic use. However, cloning homogenous populations is not an option when working with donor-derived cells, or with *in vivo* approaches, so ultimately the emphasis has to be on minimizing off-targets rather than screening. One of the most promising advancements is the emergence of base editing and prime editing. These techniques reduce the risk of genomic instability since they do not rely on inducing DNA double-strand breaks.

Prime editing has also the potential to broaden the applicability of our technology, which centers on introducing specific gene edits into the genome. Presently, the preferred approach for introducing specific gene edits is CRISPR knock-in. However, this method is mostly efficient in proliferating cells, as homology-directed repair (HDR) is predominantly active in such cells. In non-proliferating cells, CRISPR is efficient at introducing insertions and deletions (indels), primarily suitable for gene knockout strategies, but inadequate for specific gene edits. By not relying on HDR, prime editing opens the door to introducing precise gene edits into non-proliferating cells, which is the state that most of the cells in the human body are in. This development therefore has the potential to facilitate the application of our technology across a broader spectrum of cell types and expand its utility for *in vivo* approaches. In the latter context, gene edits could be introduced directly into a patient's cells, transcending the challenges associated with cell therapy manufacturing and delivery.

Q Where next for innovation in genome editing—how will tomorrow's platforms and components improve upon today's?

VS: Genome editing approaches need to enhance their specificity and broaden their applicability to enable the introduction of specific edits in a more diverse range of cell types.

CRISPR/Cas9-based gene editing has been successfully applied in a number of cell types, but, as mentioned, has shown itself to be mostly efficient at introducing indels, and this is reflected in the ways this technology is currently used in clinical trials. For these applications, the priority now is increasing the specificity to reduce or eliminate off-target effects. By comparison, the ability of CRISPR/Cas9 to introduce specific edits has been lagging behind in terms of efficiency and applicability, currently limited to proliferating cells. Alternative technologies such as base and prime editing—which rely on different editing mechanisms and DNA repair pathways—promise to deliver higher efficiencies in non-proliferating cells, including many primary cell types. However, these newer approaches have their own challenges. Base editing,

while being able to work efficiently, it is still limited to very simple edits. Prime editing on the other hand can enable more complex edits, but its efficiency is still comparatively low. It is encouraging to see that a lot of effort is currently put into improving the efficiency of prime editing and that notable progress is being made.

Ultimately, to expand their therapeutic utility, genome editing platforms will have to be able to introduce specific edits of reasonable complexity with very high efficiency, and in as many cell types as possible. This would enable the development of treatments for genetic diseases where gene knockouts are not suitable as a therapeutic approach, and would also expand the applicability of GEiGS for therapeutic approaches where target inactivation needs to be induced in a programmable manner (for example in response to disease environments).

Another area of development for gene editing technologies is improving their applicability to *in vivo* approaches. Currently, most clinical trials involving gene editing are based on *ex vivo* approaches. This is a sensible approach given that the specificity and safety profile of these technologies are still being scrutinized, as it allows for a thorough assessment of potential off-targets prior to administration. For regenerative medicine treatments, where there is a need to replace lost cells, this approach is ideal. However, for other treatments it may be preferable to edit the patient's cells directly *in vivo*. This is because cell therapies, while transformative, can also be challenging to produce and implement. They rely heavily on highly specialized and complex manufacturing processes, making them very costly and therefore less accessible, and can encounter barriers in the form of immune rejection and lack of functional engraftment. *In vivo* approaches, where genome editing is applied directly in the patient's body, circumvent these challenges, especially if the necessary reagents can be delivered through non-viral nanoparticles. While some level of manufacturing is still required, it does not entail the same degree of complexity associated with cell therapy manufacturing. Such an approach could potentially streamline therapeutic applications, making them more cost-effective and accessible to patients.

Q Finally, can you sum up one or two key goals and priorities for Laverock Therapeutics over the coming 12–24 months?

VS: Over the past year and a half, our primary focus has been on establishing our platform technology. We have dedicated significant time to this and are now confident in its capabilities. Our extensive testing has demonstrated its effectiveness in gene silencing and its exceptional specificity. We have specifically addressed concerns regarding potential off-target effects, such as unintended gene silencing or loss of the edited miRNA function. We are highly satisfied with the efficiency and precision it offers.

Moving forward, our key priorities for the next couple of years involve demonstrating the practical application of our technology in therapeutics. We are actively engaged in a series of proof-of-concept studies to illustrate how this technology can be harnessed to create cell therapies. Additionally, as I alluded to earlier, we are exploring avenues for applying our technology directly *in vivo*. This would enable us to introduce specific gene edits within the patient's cells to silence target genes without needing cell delivery. While these goals are ambitious, they hold

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great promise. We are enthusiastic about the potential of our technology, and our primary focus is now on showcasing its practical applications in the field of human therapeutics.

BIOGRAPHY

VLAD SEITAN is the Chief Scientific Officer and Co-Founder of Laverock Therapeutics. Previously he was a Principal Investigator and MRC Fellow at King's College London, in the Department of Medical and Molecular Genetics. He has a PhD in Human Genetics and Biomedical Sciences from Newcastle University and a BSc from the University of Edinburgh. A molecular biologist by training, he spent most of his career researching how the mammalian genome is organized and how it functions. His contributions were important for understanding the mechanisms by which gene expression is regulated in human development, and how their disruption leads to disease.

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AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

FULFILLING THE POTENTIAL OF GENE EDITING:
AT THE TIPPING POINT

SPOTLIGHT

INTERVIEW

Developing a multiplex gene editing approach targeting viral infection

David McCall, Senior Editor, *Cell & Gene Therapy Insights*, speaks to **TJ Cradick**, Chief Scientific Officer, Excision BioTherapeutics, reflecting on his 20-year career in gene editing and discussing the cutting edge in multiplexing with CRISPR.

Cell & Gene Therapy Insights 2023; 9(9), 1287–1292

DOI: 10.18609/cgti.2023.165

Q What are you working on right now?

TJC: At Excision, we are developing dual guide RNA (gRNA), CRISPR-based gene therapies to cure chronic viral infectious diseases. We are excited about our first-in-human phase 1/2 clinical trials in HIV for our lead candidate, EBT-101, which is currently enrolling and dosing additional participants. The trial is designed to evaluate safety and biodistribution. We are also undertaking exploratory assay development to help us measure both the gene editing itself and changes in the HIV viral reservoir. The field of HIV assays is an area in which many groups, including our own, are developing cutting-edge technologies to understand the low levels of viruses that are present, and whether or not they are full-length and functional.

Excision is using a multiplex gene editing approach for EBT-101. This unique approach gives us three target sites, which means three chances to cleave and therefore, three different possible large viral excisions. Any of these three excisions could completely inactivate HIV. This means three shots on goal, which is a huge advantage compared to a single guide. These large excisions made by EBT-101, are approximately 1000, 8000, and 9000 nucleotides in size, which are vastly larger than the small number of nucleotides that are deleted when using a single gRNA. Using multiple guides is important to increase viral inactivation and it greatly reduces any chance of viral escape and rebound. We have performed bioinformatic modeling of how the cutting works to understand the best target sites. This software has been applied to EBT-101 to target HIV, in addition to hepatitis B virus (HBV) and herpes simplex virus (HSV).

The R&D team is working on HBV and HSV programs, built upon the technologies demonstrated with HIV. It is hoped that the process can be expedited to other indications moving forward. There are two primary areas of active research: developing guides and nucleases to target HBV and HSV, and employing animal models to characterize the safety and efficacy of these therapeutic strategies.

Q Having amassed two decades of experience at the cutting edge of genome editing, what are your high-level reflections on the field today?

TJC: Excision is one of several organizations developing and clinically testing *in vivo* editing strategies. Early research in the field focused on gene editing cells *ex vivo* and providing them as adoptive cell therapy.

The field has taken decades to evolve from the seminal work of establishing and understanding zinc finger nucleases (ZFNs). When transcription activator-like effector nucleases (TALENs) subsequently came out, numerous labs that provided key reagents allowed the field to expand towards a democratic way of working, so that researchers without expertise in ZFNs could get involved. Those building blocks established a great network of people ready to work on gene editing. Then, when CRISPR came along, the field exploded in size, and we all had the opportunity to capitalize on the wide array of relevant information that helped propel the field forward.

As we have improved our ability to edit precisely, we also have identified a range of other editing options, including different nucleases, base editors, prime editing, and other systems. Through decades of work, we have also established the assays and sequencing methods that will enable those emerging platforms to move forward. Meanwhile, delivery technologies continue to improve. A group hoping to tackle a given disease can look at advancements in editing and choose an applicable system.

Manufacturing remains a challenge that the field continues to address. However, assays and other complementary technologies continue to evolve to improve the technologies. Excision remains committed to developing more active and specific nucleases and assays that will enable us to characterize these advances and measure decreases in viral load.

Q Having been instrumental in the development of several of the foremost genome editing platforms currently used in clinical

application, what made Excision's platform and approach really stand out to you?

TJC: As I mentioned, Excision is using a multi-guide approach. Data initially from the Khalili lab, which has been repeated by many others in subsequent years, has demonstrated an increase in viral inactivation from using multiple nuclease target sites. This is important for activity and to create large deletions between the cut sites. These large deletions between the different target sites excise nucleotide fragments from the HIV genome, effectively preventing the chance for viral escape and rebound.

When we started targeting viruses, we used ZFNs to target HBV. Several papers have described the difficulty in doing this, but we succeeded in creating a pair of ZFNs that bind with correct orientation spacing and cut at a single site. As we showed in our first publication, this led to a single nucleotide but often up to four nucleotides changing. However, people who studied the data later realized that there was a chance for viral escape with error-prone polymerases. This is one of the problems, amongst others, in designing and delivering ZFNs.

One thing to consider is whether using more guides increases the possibility of unintended edits. We take care to pick viral DNA target sites that are vastly different from the human genome. With our HIV multiplexing, we see very few sites nominated by the bioinformatics even when using a greater number of mismatches. As we look towards other viruses, we are using software for sequencing, aligning, selecting the target sites, and modeling the multiplex cutting. This allows us to take this platform and apply it to other viruses.

Q Can you tell us more about the Excision pipeline and the rationale behind the diseases you are currently targeting?

TJC: At Excision, all of our programs target viruses. In addition to HIV, Excision is targeting HBV and HSV. Demonstrating the safety and tolerability of a potential CRISPR-based cure for HIV signals the promise of multiplex *in vivo* gene editing as a potential cure for other viral infectious diseases such as HBV and HSV, which both have large patient populations and represent a significant unmet need.

Ongoing advancements in editing technologies and viral and non-viral delivery, plus a better understanding of the editing and DNA repair mechanisms, have the potential to accelerate more effective therapeutics and potentially cure chronic viral infectious diseases.

Q What might be some future application areas of interest?

TJC: The bioinformatics and strategies demonstrated with HIV, HBV, and HSV can be similarly applied to a range of other viral targets, which is an exciting aspect of this technology. Establishing that we can do this effectively and safely allows us to think about what we can do to similar or even completely different viruses with small or large patient populations. We are actively discussing additional targets but have yet to disclose these internal or partnered projects.

Q The range of ***in vivo*** delivery platform options is growing for genome editing therapeutic developers—how would you profile this technology space currently?

TJC: Advances are coming both in viral delivery and non-viral delivery that permit effective targeting of the necessary tissues and cells.

The central idea is that we are developing better manufacturing and better targeting. Manufacturing advances in the wider field are useful, such as how advances in AAV can allow us to achieve higher, more homogeneous titer. Similarly, there have been many improvements in the development of lipid nanoparticles. A challenge we have had as a field is understanding how to harness these advancements to get to the relevant tissues for our viral targets. We successfully demonstrated that we get to the reservoirs for HIV in a recent publication looking at rhesus macaques.

Q In an area where regulatory guidance and opinion are still being formed, what are the keys to ensuring industry best practices align with regulators' expectations?

TJC: We want to weigh up the risk–benefit balance and make the safest but most effective medicines that we can. Just as the editing technologies are quickly advancing, so are the sequencing and detection methods. It remains important to evaluate the new developments and employ them once they are characterized and established. Advances in sequencing have permitted assaying for rarer events than in past years. We also know more about the noise and possible artifacts that might be present around these low levels. It is important, then, to weigh the output of these assays as part of the bigger risk–benefit calculation to ensure that we are providing an effective therapeutic strategy.

We are continuing to have discussions with the regulators and the wider field about learning from previous studies to enable us to move through this process quicker. While we all appreciate the opportunity to deliver for patients faster, we do not want to take any shortcuts in the safety regard, so understanding the process is important.

Q Where next for innovation in gene editing platforms?

TJC: The development of assays is key as once we can measure things, we know how to improve them. We are also continuing to embrace our ability to computationally model in order to help us design our targeting strategies.

Other groups that are editing a range of disease-causing mutations face a challenging task, but it is exciting to see several different technologies being developed that offer alternative means to correct the range of mutations causing some of these diseases. As these technologies develop, they will enable the field to target new indications or allow more effective targeting of diseases currently being investigated.

Similarly, advances are coming both in viral delivery and non-viral delivery that permit effective targeting of the necessary tissues and cells. Decades of work on AAV have produced a

range of improvements in the technology, including novel serotypes with improved specificity and manufacturing scalability. Similarly, the field of non-viral delivery has seen dramatic improvements in recent years, best demonstrated by lipid nanoparticles in their application with COVID-19 vaccines.

Q What for you are the most pressing next steps for the field in making genome editing-based therapeutics more 'commercializable'?

TJC: There are several challenges in making these therapies more accessible to patients. As mentioned earlier, one of the biggest priorities for the field is addressing manufacturing challenges. As we have more gene editing indications taken to the clinic and we get going on manufacturing, this will lead to improvements, and each improvement will drive down costs.

To expedite the regulatory process and get the data to the agencies quicker, we need better assays and manufacturing. This will lead to an increase in both the number of approved products and the speed to commercialization.

We are excited about how quickly we are approaching commercialization with this technology. We have learned a lot already that can be applied to help the whole field of *in vivo* gene editing. The more people doing this, the easier it is for others to follow along, file clinical trials, and drive towards commercialization.

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

TJC: Firstly, our team at Excision is excited to continue to enroll for the first-in-human EBT-101 clinical study for HIV, continuing the ascending dose trial. Secondly, we are excited about our advances in targeting HSV and HBV and evaluating the efficacy of our approach in animal models. Our goal is to advance both forward.

It has been exciting to see a number of platforms we have worked on, such as ZFNs, TALENs, and CRISPR, moving forward now with great data. However, there are also technologies coming along that will be more applicable to other disease indications, which will be exciting to see. Having been in the field for a while, it is amazing to watch the advancements in disease targets we have been chasing for decades.

BIOGRAPHY

TJ CRADICK has more than two decades of experience in gene editing, working on therapeutics, bioinformatics and assays as the field progressed from Zinc Finger Nucleases (ZFNs) and TAL Effector Nucleases (TALENs) to CRISPR-Cas9. This research included academic work at the University of Iowa and as faculty at the Georgia Institute of Technology. Cradick has held positions at Sangamo Therapeutics and as the Head of Genome Editing at CRISPR Therapeutics. He is Chief Scientific Officer at Excision BioTherapeutics, where he leads the company's research and development functions, including gene editing, bioinformatics, development of the viral-targeting platform and collaborations with academic and industry partners.

Cradick led the first work on the use of engineered nucleases as a therapeutic strategy for targeting virus, ZFNs that specifically cleaved hepatitis B virus DNAs, and co-authored the first publication on the topic. He has co-authored manuscripts on ZFNs, TALENs and CRISPR and on developing bioinformatics web tools, including ZFN-Site, PROGNOS, SAPTA, and COSMID. At the Georgia Institute of Technology, Cradick was a member of the faculty and director of the protein engineering facility, where his research included developing assays and bioinformatics for CRISPR/Cas9 specificity, which have been applied across a range of gene therapy targets.

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AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

FULFILLING THE POTENTIAL OF GENE EDITING: AT THE TIPPING POINT

SPOTLIGHT

INTERVIEW

Advancing multiplex base editing into the clinic

David McCall, Senior Editor, BioInsights, speaks to **Gopi Shanker**, Chief Scientific Officer, Beam Therapeutics, about the company's mission to realize the full potential of base editing for patient treatment. They discuss the stand-out advantages of base editing, as well as the current state of the art in the area of multiplex gene editing.

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Q What are you working on right now?

GS: Beam Therapeutics is interested in bringing the full potential of base editing into clinical programs, to generate value for patients. We have three pillars of focus: efforts in hematology, primarily focused on sickle cell disease; efforts in the immuno-oncology space with CAR-T; and finally, efforts in genetic disease with a focus on *in vivo* editing. The immunology and hematology franchises are currently *ex vivo* focused.

Our current goal is to advance those three pillars. We currently have two ongoing clinical-stage programs in our R&D pipeline, one in sickle cell disease and one in oncology. In clinical trials, we dosed our first patient this year (to my knowledge, the first ever clinical trial patient in the USA dosed with a base-editing therapy) and have two *in vivo* gene editing

programs in the liver, with which we are working towards the regulatory milestones required to initiate clinical trials.

Q Can you expand on the latest progress, both at Beam Therapeutics and within the gene therapy field at large, towards bringing the benefits of base editing to bear in the clinical setting?

GS: After speaking to a variety of leaders in the field to gain an understanding of where the field is moving, it is clear there are many different gene editing technologies now coming into place. The first generation was nucleases and CRISPR-Cas9-based efforts such as those by Intellia Therapeutics and others. Then along came base editing, which is designed to be more nuanced and to allow precise single nucleotide changes without the risk of double-stranded DNA breakage. That provides a lot of flexibility and precision in how we want to modulate biology at large.

Efforts by Beam Therapeutics and others have helped to rapidly improve the efficiency with which we can get base editing to work. This is a critical advantage and differentiating factor for base editing as a platform. But there are many other exciting technologies emerging such as prime editing and RNA writing and editing; I see them all as different tools in the umbrella toolbox of gene editing.

In addition to our program, Verve Therapeutics, our collaborator, has a base editing program now in the clinic. We have both begun to dose our first clinical trial patients with investigational base editing therapies.

Q Tell us more about Beam Tx's specific approach and platform—what differentiates it in what is an increasingly competitive space?

GS: Among the critical potential advantages of this approach are the high efficiency and the lack of unwanted effects from double-stranded DNA breaks. This is designed for predictable editing outcomes with a single nucleotide resolution, which makes it a highly versatile editing platform.

Nucleases, which are further along in the clinic, can typically only knock out protein expression. Base editing can also do that but potentially with greater precision than nucleases. Due to that precision, there is potentially more control, and the possibility to achieve many more editing outcomes. With base editing, we can subtly modulate gene function—we can inactivate or repress it, for example. Our lead development program in sickle cell disease is designed to directly activate fetal hemoglobin gene expression by making changes in the regulatory regions. That would be difficult for a nuclease to do.

We can modulate protein function in subtle ways. We have an exciting development program called ESCAPE (Stem Cell Antibody Paired Evasion), where we are introducing an edit into the cell on a protein called c-KIT. We do this in a way that does not modulate the function of the c-KIT protein but does ensure it can no longer be recognized by a bespoke antibody that we can use for conditioning.

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"Base editing is designed to do complex multiplexing without the risk of double-stranded breaks and the consequent increased risk for genomic rearrangements."

Typically for *ex vivo* cell therapy for sickle cell disease patients, they firstly have to go through a regimen of chemotherapy to clear the niche, which has many adverse effects. If our ESCAPE program proves to be successful in the clinic, the dream is that we can use the antibody to do the conditioning to clear the niche and thereby avoid the chemotherapty step. Again, if we are successful in the clinic, this means that the unedited cells would be removed, and the edited cells, which have both the fetal hemoglobin expression turned on and a second edit to escape recognition by the antibody, would survive. These cells are designed to have a therapeutic effect. So, not only is this structured to introduce a therapeutic edit but to also allow patients to not have to undergo the harmful chemotherapy regimen, thus giving them a better probability of success and we believe a better clinical outcome overall.

There is also the potential to multiplex. Nucleases can do multiplexing as well, but you can only knock out a series of genes and significantly increase the potential for unwanted genomic rearrangements when you have more than two edits. With base editing, we can do combinations. We can knock out one gene, upregulate another gene, and silently change the function of another protein. Base editing is designed to do complex multiplexing without the risk of double-stranded breaks and the consequent increased risk for genomic rearrangements. We have the potential to precisely understand the consequences of those single nucleotide changes performed in a multiplex fashion. That combinatory power also becomes extremely important for us.

We have also invested in delivery technology. A while ago, we acquired a company called Guide Therapeutics and gained access to their lipid nanoparticle (LNP) library. We are also working on other innovations relating to payloads such as in guide RNA chemistry and mRNA manufacturing. We have invested in complex manufacturing expertise because this is a big challenge for many genetic medicine companies. By concentrating not just on technology payload development, but on delivery and manufacturing, too, we are striving to position Beam Therapeutics not only as a leader in gene editing technology, but also as an end-to-end leader in genetic medicine development.

Q What is the current state of the art in the area of multiplex gene editing as you see it? And what are some of the key current challenges in the space?

GS: The more edits that you make in a cell, the more you increase the potential for unwanted genomic rearrangements and the associated risk. The resolution with which you can understand the impact of the changes made and your ability to characterize what exactly is going on in the cells becomes important.

"...the more we want to do this work at scale, the faster we want to go, and the more we wish to apply this technology to different indications will all necessitate a continued expansion of the capabilities and capacities of the analytical toolkit."

This is where base editing and the high efficiency with which we can edit can differentiate us, because with nucleases, you introduce many double-stranded breaks and indels. While you might be able to knock out a given gene or a set of genes with high efficiency, when you characterize those cells, you get a heterogeneous mix due to the different kinds of indel patterns created and, with two or more simultaneous edits, potentially completely novel chromosomes due to the incorrect joining of the different chromosomal pieces. With base editing technology, even if we are doing three, four, or five edits in a cell, we have the potential to know with high precision and resolution what edits are happening, and to characterize the effects of that.

As I mentioned earlier, due to the single-nucleotide resolution, not only do we have the potential to knock out a gene, but to also perform highly complex upregulations or maskings of gene functions.

In terms of challenges, delivery remains a challenge for the entire genetic medicines field. We are investing in LNP technology amongst others. Before coming to Beam Therapeutics, I spent time in the AAV field. AAV, unfortunately, is not an ideal delivery vehicle for gene editing in general. This is because the payloads are too large to be efficiently packed into an AAV capsid, and also due to concerns around immunoreactivity with Cas9. With gene editing, you do not want the chronic expression of the nuclease or the editor—you want transient expression. With AAV, that becomes a challenge as well.

I believe the whole field has to rally together to find an optimal delivery solution.

Q How is the analytical toolbox shaping up? What are some key recent breakthroughs there, and what needs remain?

GS: The analytical toolbox is shaping up well, thanks to the ever-increasing precision and sizable repertoire of sequencing technologies, which helps us to understand the genome at an ever-decreasing cost. This has aided us greatly in figuring out how to characterize the multiplex edits in everything we do with great depth and precision.

In terms of what still needs to shape up, the more we want to do this work at scale, the faster we want to go, and the more we wish to apply this technology to different indications will all necessitate a continued expansion of the capabilities and capacities of the analytical toolkit. This is especially the case for cell-based therapies.

In general, for the genetic medicine space, the CRO/CDMO ecosystem has not quite kept pace with the progress of the technologies that we have seen. Consequently, we have reached a capacity crunch. Unfortunately, it is challenging to do everything in-house and not every

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company can manage this. Even for Beam Therapeutics, doing everything we want to do in-house is not a trivial task. The field has seen a mind-boggling pace of progress for a novel modality; base editing as a technology was only discovered in 2016 and here we are today, already in the clinic.

Q What will be the key next steps for the field towards a future of successful commercial multiplex gene edited advanced therapies?

GS: As we work to bring these products through the clinic and to commercialization, we need strategies to better diagnose and recruit patients, to help our therapies reach the patients who need them the most. This is personalized genomic medicine, so the more genomic diagnoses and the greater the understanding of the genomic landscape that leads to disease that there is, the better we can match our therapies to the patients who will most benefit from them.

On the commercial front, given the nascency of the field, the reimbursement strategies and the cost of developing these therapies remain challenging. The ecosystem at large needs to think about optimizing reimbursement strategies and making these therapies available to more patients worldwide, so that there is an incentive for companies to continue to invest and develop therapies. At the same time, these therapies need to reach the patients for whom we are developing them in an affordable and accessible way.

Q Finally, can you sum up one or two key goals and priorities, both for yourself in your role and Beam Therapeutics as a whole, over the coming 12–24 months?

GS: For Beam Therapeutics, the goal is to continue to execute our clinical programs with excellence, including advancing our *in vivo* gene editing programs into the clinic and starting to dose patients. Ultimately, advancing these programs towards providing access for patients is a key priority.

In my role as CSO, my goal is to build the future pipeline. I am always thinking about how I can actualize the immense potential of base editing. How can I maximize the number of patients who can benefit from the incredible potential of this technology? What additional disease indications could be targeted? What are some creative ways in which we can leverage the power of base editing to address more unmet needs? My focus lies in adding more high-value, transformative programs into our pipeline and bringing them to patients.

BIOGRAPHY

GOPI SHANKER is the Chief Scientific Officer of Beam Therapeutics. Dr Shanker has more than two decades of broad drug discovery experience at Novartis, Amgen, and Regeneron ranging from developing target concepts to advancing molecules through clinical development. He has contributed to the development of several clinical candidates and to the migraine drug, Aimovig®. With a strong background across multiple drug modalities including small molecules, biologics and gene therapies, he brings great experience and leadership to the R&D teams. Dr Shanker has a proven ability in successfully building, coaching,

and developing cross-functional drug discovery teams as well as creating purpose-driven, highly productive research organizations. He is passionate about mentoring individuals and teams towards having high impact. Dr Shanker joined Beam Therapeutics after leading Tevard Biosciences and, prior to that, he was with Novartis Institute of Biomedical Research and during his tenure at Novartis, he held multiple key leadership roles including Head of Neuroscience Disease Area, Head of Psychiatry and Neurodevelopmental Disorders, and Head, Ion Channels and Receptors. Dr Shanker holds a PhD in Biochemistry from the Indian Institute of Science, Bangalore, India, an MSc in Biotechnology from Madurai Kamaraj University, India, and a BSc in Microbiology from Osmania University, India.

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CELL & GENE THERAPY INSIGHTS

DOWNSTREAM BIOPROCESSING

INNOVATOR INSIGHT

A scalable single-use two-step plasmid purification process

Peter Guterstam, Hans Blom, Simon Aberg, Linda Hagman & József Vasi

The current global demand for biopharmaceuticals is over US$300 billion, with an estimated growth of at least 12% annually. One of the fastest growing areas of biopharmaceuticals are mRNA and viral vectors—a fundamental prerequisite for both of these is a reliable source of high-quality plasmid DNA (pDNA). For both gene therapy and DNA vaccine applications, clinical grade pDNA with a percentage of supercoiled pDNA above 80% is usually required. Plasmids used in bioprocess applications are commonly in the range of 5–20 kb. Here we present a scalable single-use two-step pDNA purification process using a 7.3 kb model plasmid. In a high level comparison with an existing three-step purification process, it can be concluded that the two-step process provides a significant reduction in both process time and buffer consumption. In combination, this gives advantages from a sustainability perspective.

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pDNA is an important genetic engineering tool used to clone and amplify or express genes for biotechnology applications. pDNA of good manufacturing practice (GMP)-grade has many applications including DNA vaccines and gene therapy, with the production of viral vectors and mRNA being dependent on the production of pDNA [1].

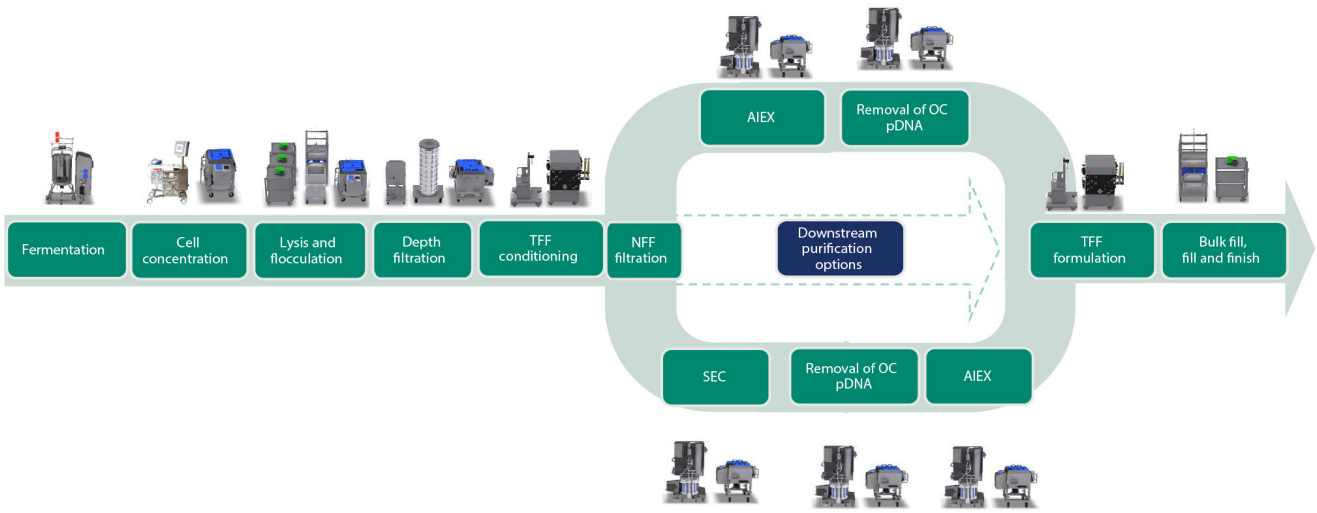
The main aim with the presented work was to design a process with a higher productivity compared to the three-step purification process [2]. An overview of the process that was developed is shown in Figure 1 and includes the capture of pDNA using a Mustang™ Q XT single-use membrane adsorber, followed by purification of supercoiled (sc) pDNA with Capto™ PlasmidSelect resin. The benefit of

using the Mustang Q XT membrane adsorber is a significantly higher binding capacity to comparable resins with the same ligand. This is mainly due to the size the pDNA, which allows for very limited access to internal volume of the resin beads. Consequently, a large proportion of the ligands derivatized on resin beads are not accessible for binding pDNA, which severely impacts the binding capacity.

In addition to a higher binding capacity, the Mustang Q XT membrane adsorber allows convective flow, providing a significantly higher productivity. The described two-step chromatography process meets large-scale regulatory manufacturing requirements and is scalable up to at least a 50 L fermentation volume. Figure 2 provides a comparison to our previous three-step purification process.

FIGURE 1

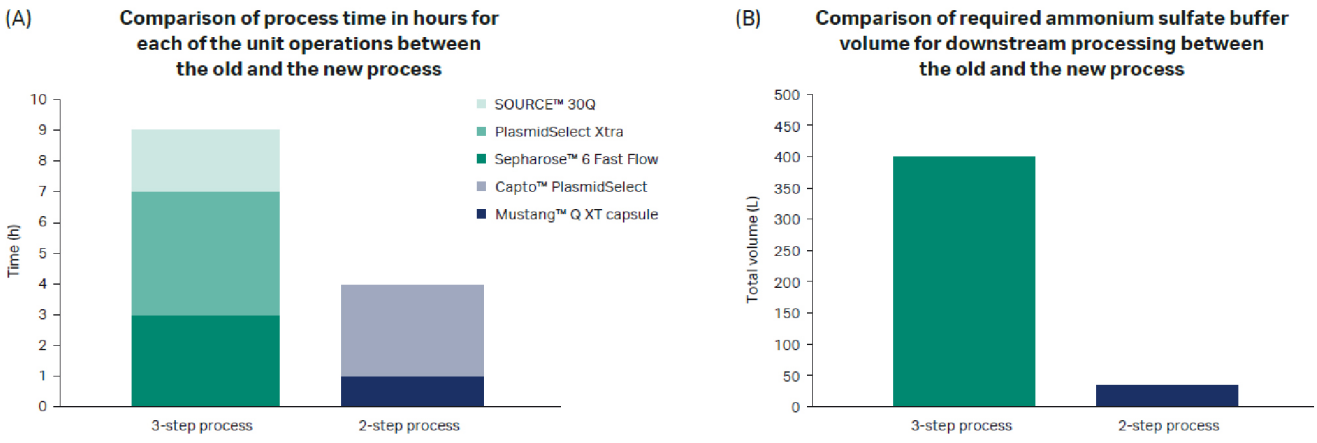
Process overview for pDNA.



The described two-step chromatography process is shown in the upper track. The lower track shows an earlier described three-step chromatography process.

FIGURE 2

Calculated benefits for pDNA downstream process at 50 L scale.



The described two-step process for plasmid purification can be performed quicker and with less buffer consumption than a three-step process that include size-exclusion chromatography and resin-based anion exchange chromatography.

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The economy calculations show that the new process provides a significant reduction in process time and improved sustainability.

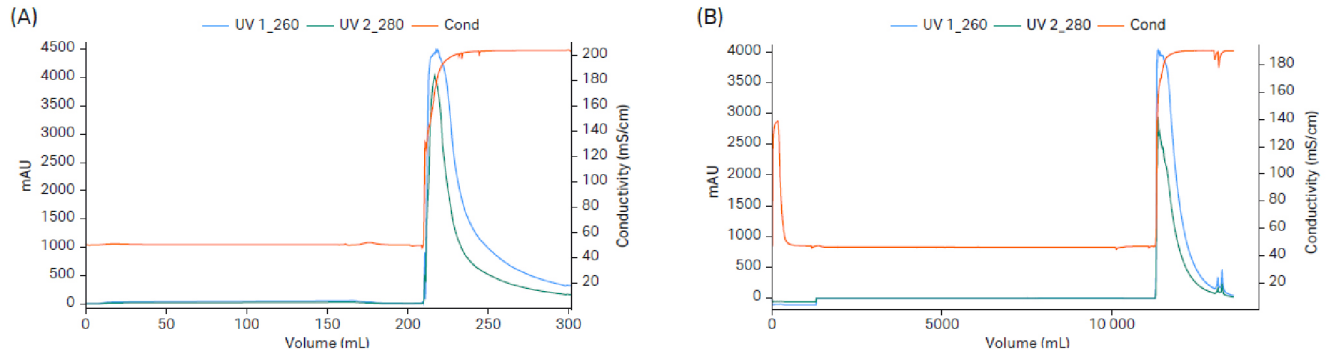
UPSTREAM & MIDSTREAM CONSIDERATIONS

In order to meet process goals, it is important to consider the whole process from fermentation to final filtration. For example, excessive addition of antifoam or an excessive feed profile resulting in glycerol accumulation can influence the ratio of open circular (oc) to sc pDNA during fermentation. In addition, lysis parameters are critical to maximize the process efficiency, both to maximize the yield and to maintain the stability of the pDNA and to reduce the levels of host cell proteins (HCP), endotoxins and genomic DNA (gDNA), where special care must be taken to avoid fragmentation of gDNA. The addition of a $CaCl_2$ precipitation step following lysis but prior to the flocculation lift with ammonium hydrogen carbonate will efficiently reduce the levels of RNA. All together, these steps are crucial to set up the downstream process for success.

More information on scaling up an *E. coli* upstream process for pDNA production can be found on the Cytiva website [3].

FIGURE 3

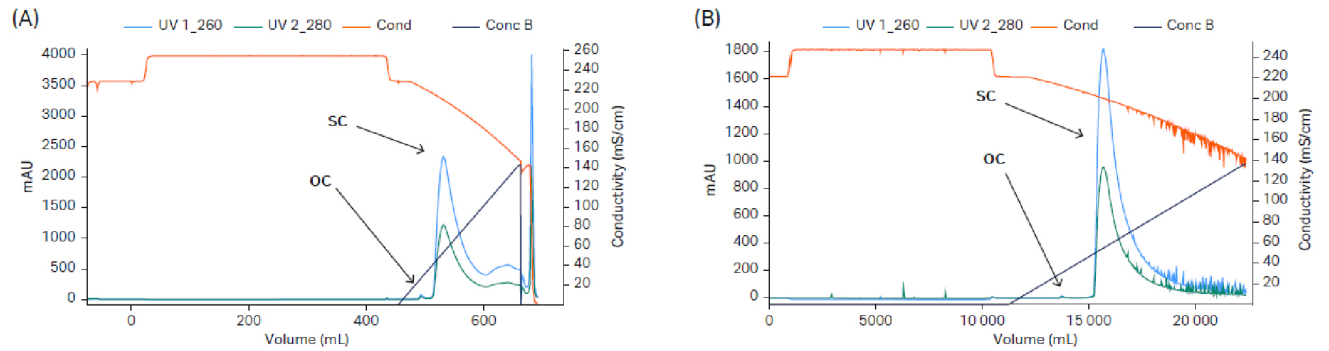
High-capacity quick chromatography cycle with Mustang Q XT adsorber.



(A) Lab scale Mustang Q XT5 with a load of 12 mg/MV (membrane volume) and step yield of 58%. **(B)** Production scale Mustang Q XT140 with a load of 8.95 mg/MV and step yield of 67%.

FIGURE 4

Scalable enrichment of SC pDNA using Capto™ PlasmidSelect.



(A) Lab scale Capto™ PlasmidSelect 18 mL with a load of 2.8 g/L, 220 cm/h sample application, 120 cm/h elution, and step yield of 77%. **(B)** Production scale Capto™ PlasmidSelect 1 L in a ReadyToProcess™ (RTP) prepacked column with a load of 0.85 g/L, 179 cm/h sample application, 90 cm/h elution, and step yield of 72%.

RESULTS & DISCUSSION

The presented process was first developed using a 15 L stainless steel fermentation reactor, and was later scaled up to 50 L using an XDR50 single-use reactor. The downstream process included capture of pDNA using Mustang Q XT140 membrane adsorber (Figure 3). This was followed by removal of oc pDNA using Capto PlasmidSelect (Figure 4). The process step yields are shown in Table 1.

ANALYTICS

A comprehensive analytical package was applied to samples throughout the process. The single-use two-step process meets FDA guidelines (Table 2). Thiophilic aromatic adsorption chromatography using Capto PlasmidSelect can also be used for analysis of process samples [4] and despite not being an orthogonal technique, it performs equivalently to capillary gel electrophoresis (CGE), as shown in Table 3.

TABLE 1

Process performance.

Fraction	Approximate fraction weight (g)	Concentration (mg/mL)	pDNA (mg)	Step yield (%)	Total yield (%)
Lysate after upstream harvest	~120,000	0.01	1411	N/A	N/A
UF/DF HF, 500 kDa, 1.15 m^2	~9400	0.14	1303	92	92
NFF (0.5 µm, Load 2018 L/m^2	~9300	0.14	1277	98	90
Mustang Q XT140 capsule eluate	~2000	0.40	854	67	60
Capto PlasmidSelect eluate	~4000	0.15	618	72	44
Final UF/DF HF, 500 kDa, 290 cm^2	~500	1.04	482	78	34
Final 0.2 µm sterile filtration	~400	1.04	442	92	31

DF: Diafiltration; HF: Hollow fiber; NFF: Normal flow flitration; UF: Ultrafiltration.

TABLE 2

The production scale pDNA process meets US FDA guidelines.

Fraction	sc pDNA (%)	*E. coli* DNA (µg DNA/mg pDNA)	*E. coli* HCP (µg HCP/mg pDNA)	Endotoxin (EU/mg pDNA)
Lysate	91	16.4	2789	2,581,641
UF/DF	98	1.0	7.7	122
NFF	97	1.7	5.1	87
Mustang Q XT140 eluate	97	1.4	<1*	4
Capto PlasmidSelect eluate	99	0.5	<1*	<7*
Final UF/DF	99	0.6	<1*	<1*
Final filtration 0.2 µm	100	0.6	<1*	<1*
Acceptance criteria Cytiva	>95	<2.0	<1.0*	<10
Acceptance criteria US FDA†	>80	<10	<1.0*	<40
Attribute	pDNA quality	*E. coli* residual DNA	*E. coli* HCP	Endotoxin
Method	Capto PlasmidSelect	ddPCR	Gyrolab assay	LAL test

*Results for HCP and endotoxin were below LOQ, but were set to LOQ to be able to calculate result/mg pDNA. †Considerations for Plasmid DNA Vaccines for Infectious Disease Indications 2005D-0047. DF: Difiltration; HCP: Host cell proteins; LOQ: Limit of quantification; UF: Ultrafiltration.

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Analysis by agarose gel electrophoresis (AGE) shows almost undetectable levels of oc DNA after UF/DF and demonstrates the efficient reduction of RNA by employing a $CaCl_2$ precipitation step following lysis (Figure 5).

PROCESS SELECTION

The scale and quantity of plasmids required for different pDNA applications varies. However, the purification process can be optimized to meet the desired purity and concentration requirements. Such optimization may include many different parameters and considerations, such as process time, scalability, flexibility, process control, batch cost (OpEx), footprint, environmental impact and waste handling. Another important parameter for choice of downstream process is the upstream feed material. The feed material used in this study already had over 90% scDNA content after the midstream step. In this case, the Capto PlasmidSelect step to enrich scDNA may rather act as a method to remove endotoxin. In other cases where the initial feed material has another composition, enrichment of scDNA may be crucial to meet the desired percentage of scDNA. In essence, the parameters and considerations are usually different for each process. Figure 6 provides guidance on selecting a process for your specific plasmid DNA requirements. For example, if scalability, endotoxin reduction, enrichment of scDNA and low buffer consumption is important, the two-step chromatography process with Mustang Q XT membrane adsorber and Capto PlasmidSelect resin to purify pDNA is advantageous.

TABLE 3

Rapid analysis of process samples for oc/sc pDNA ratio determination can be performed with analytical mode thiophilic aromatic adsorption chromatography using Capto PlasmidSelect.

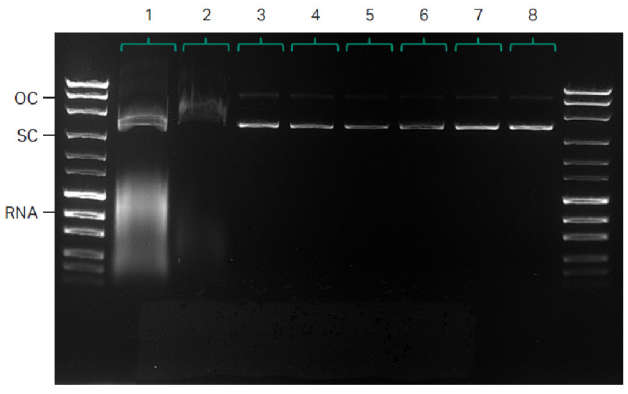
Fraction	sc (%) by Capto PlasmidSelect	sc (%) by CGE
Mustang Q XT140	97	97.9
Capto PlasmidSelect	99	98.9
Final filtration	100	98.8

CGE: Capillary gel electrophoresis.

FIGURE 5

Quality control by agarose gel electrophoresis.

1. Lysate
2. NFF (Depth filter, 174 L/m^2)
3. UF/DF
4. NFF
5. Mustang Q XT140
6. Capto PlasmidSelect
7. Final UF/DF
8. 0.2 µm filtration



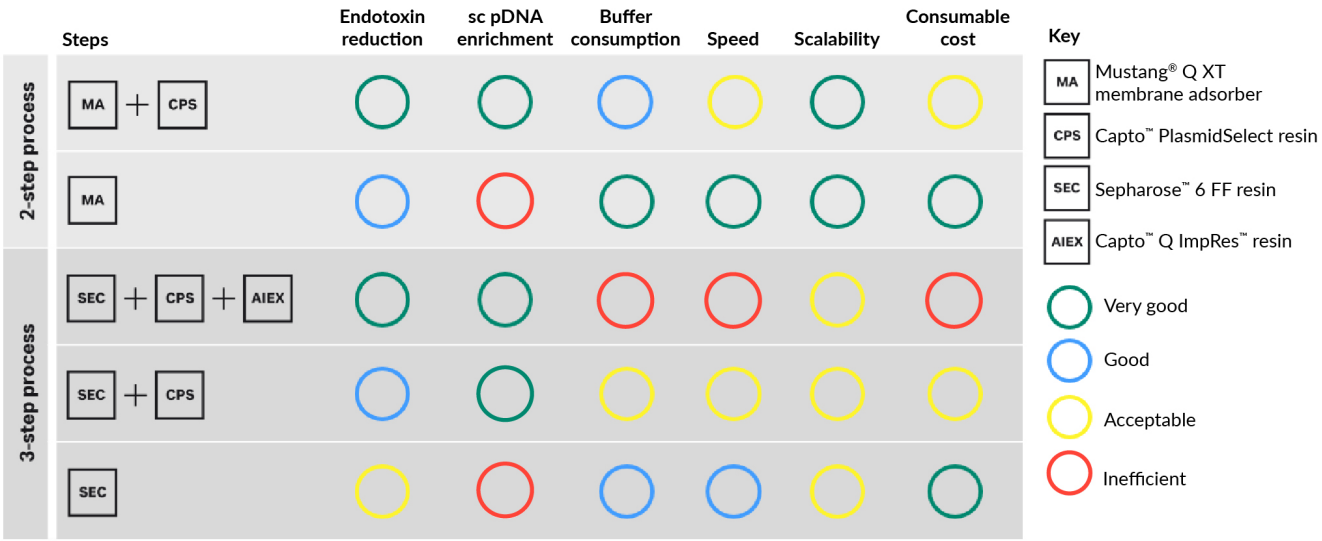
CONCLUSIONS

- The new two-step plasmid process is scalable and meets FDA guidelines, and achieved:
- Efficient RNA reduction by $CaCl_2$ precipitation following lysis.
- Rapid capture of pDNA with Mustang Q XT membrane, followed by selective purification of sc pDNA by Capto PlasmidSelect resin.
- Significant reduction in production time for downstream and improved sustainability with reduction in ammonium sulfate consumption **(Figure 2)**.

More information on bioprocessing strategies for current and new biotheraputics can be found on the Cytiva website [5].

FIGURE 6

Selection guide for two- and three-step downstream plasmid purification processes.



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AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

DOWNSTREAM BIOPROCESSING

INNOVATOR INSIGHT

Considerations for affinity capture in an AAV platform downstream process

Jett Appel

In the rapidly evolving field of gene therapy, AAV vectors have shown increasing therapeutic promise. In recent years, a variety of different therapies using various AAV serotypes have received regulatory approval, and hundreds more are in clinical trials. From discovery to the clinic, as the quest to produce new, high-quality AAV molecules intensifies, an efficient and scalable platform purification process for multiple AAV serotypes is advantageous for both drug developers and CDMOs. This article explores the considerations when evaluating a platform affinity capture step for the purification of AAV vectors. Specificity, binding capacity, purity, yield, scalability, and reusability parameters will be addressed.

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AAV DOWNSTREAM PROCESS

The typical AAV downstream process begins with cell lysis and endonuclease treatment. After this step, the vector material is typically clarified, which can be done via centrifugation or depth filtration. This clarified material can then be loaded onto an affinity resin. After affinity capture, the material can then be further polished for the enrichment of full capsids. This purified material is then concentrated and finally, formulated into a bulk drug substance.

There are some challenges that one can face when working with AAV. To obtain a high yield of AAV, the cells must be lysed, which increases the impurity burden with residual host cell proteins or DNA. In addition, AAV gene therapy manufacturers are commonly working with a large range of serotypes,

including native and engineered capsids. Having an affinity step that can recognize multiple serotypes can therefore be beneficial.

Depending on the indication and dosages, recovery is an important factor. Good recoveries for the affinity capture step improve process economics. Scalability can pose another challenge, especially for customers working with ultracentrifugation techniques with long processing times. The enrichment of full capsids is a further challenge due to the similar physical-chemical properties of empty and full capsids. Lastly, one should demonstrate the clearance of adventitious viruses.

Thermo Fisher offers the high performance POROS™ CaptureSelect™ AAV affinity resins to enable platform approaches for AAV affinity capture.

CONSIDERATIONS FOR PLATFORM AAV AFFINITY CAPTURE

Considerations for developing an AAV affinity platform capture step include the ability to have broad specificity to different AAV serotypes. A resin that can recognize both native and engineered capsids is ideal. Having a high dynamic binding capacity is key to reducing column size requirements and maximizing productivity. High purity and recovery after an affinity step are desirable and can be further improved by wash and elution optimization. Ensuring consistent performance upon scale-up is another important consideration. Lastly, reusability helps to reduce cost of goods as well as the risk of carryover.

Broad specificity binding

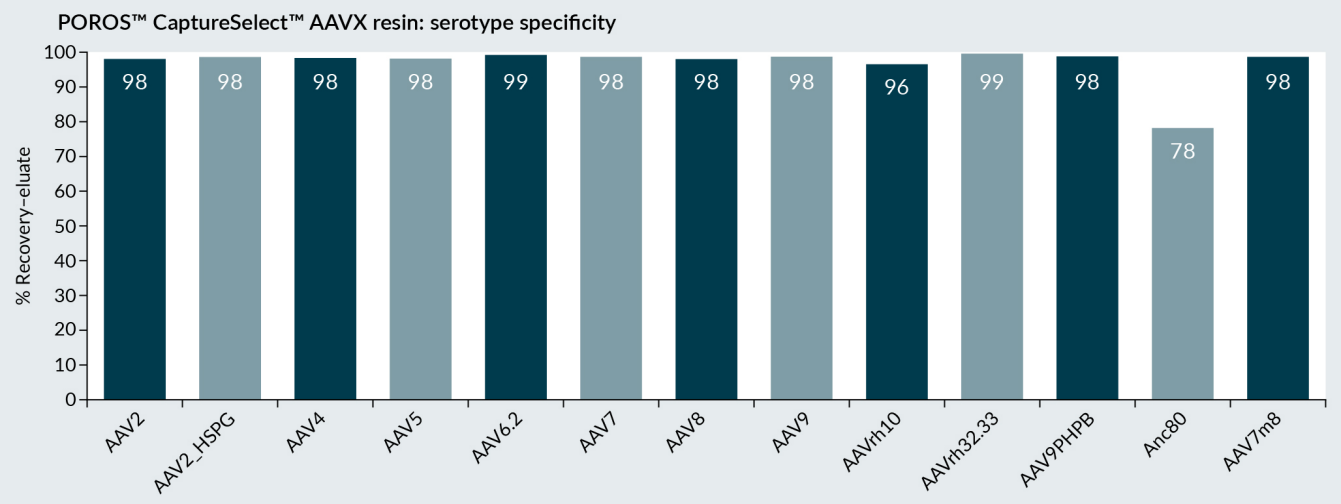
Data presented in Figure 1 was generated by researchers from Massachusetts Eye and Ear using POROS CaptureSelect AAVX resin. A static binding experiment was performed with a mix of AAV serotypes with AAVX resin. The AAV was quantified by qPCR. All serotypes tested were able to demonstrate binding to the AAVX resin.

Dynamic binding capacity

Dynamic binding capacity data collected from various sources demonstrates that POROS CaptureSelect AAVX has high dynamic binding capacity for multiple serotypes, including at short residence times (Figure 2). Dynamic binding capacity for AAVX was also found to be four-to-six times higher than for other commercially available AAV affinity resins.

FIGURE 1

POROS CaptureSelect AAVX resin: broad specificity binding data.



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Elution optimization

An internal elution optimization study was conducted using the AAVX resin. This began with high-throughput screening in a 96-well plate using various elution buffers. Purified AAV6 was used as the load material, with a column load density of 1×10^{14} capsids/mL. A high salt wash was included. For the elution, different pH values (2.0, 2.5, and 3.0) and different excipients (arginine, magnesium chloride, and propylene glycol) were tested. The eluate was quantified by absorbance at 280 nm and the results are given in Figure 3.

A pH of ≤2.5 increased recoveries to 80–90%. For this capsid, the inclusion of arginine was helpful for recovery. In line with the high-throughput data, magnesium chloride was not beneficial to the recovery in this case. The data show that buffer composition (pH and excipients) can be optimized to maximize AAV recovery, specific to each molecule and process.

Wash optimization

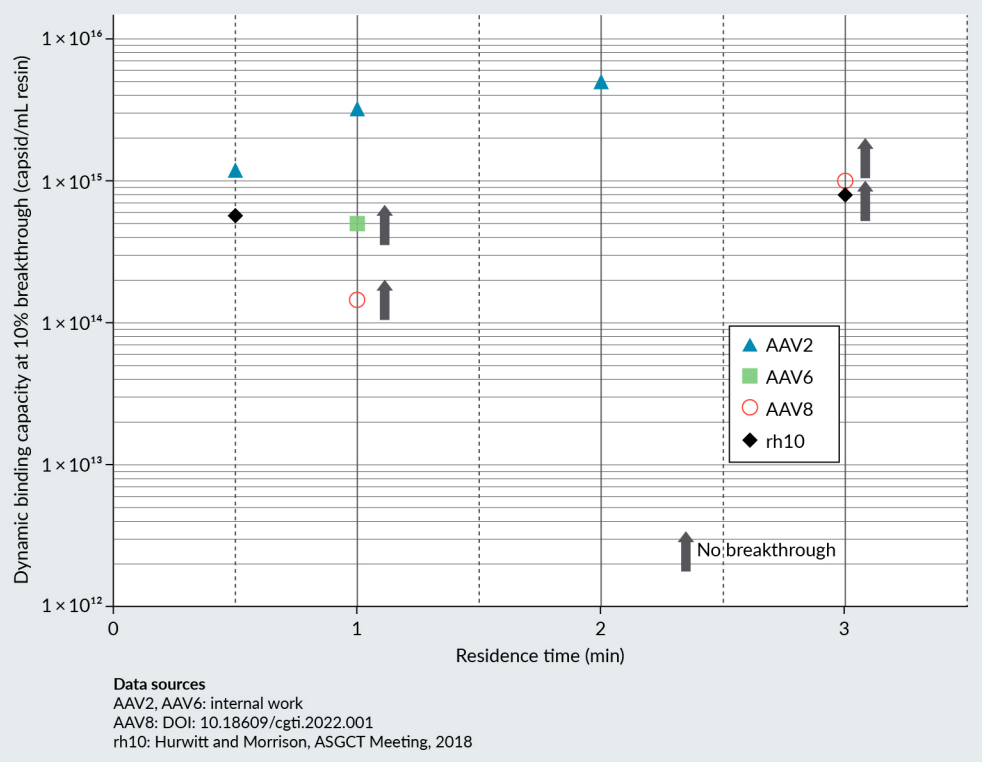
A wash optimization study for AAV6 was performed, using wash buffers with variable salt concentrations, pH, and inclusion of arginine. The samples were clarified with diatomaceous earth and filtered with a 0.22 µm PES vacuum filter. 1 mL POROS GoPure™ AAVX Pre-packed columns were used. The sample concentration was 1.0×10^{11} vg/mL and the column loading density was 1.0×10^{13} vg/mL. The impurity clearance results are shown in Figure 4. Intermediate wash optimization results in improved clearance of process-related impurities. Regardless of the wash conditions evaluated, 80% recovery was achieved for all the conditions tested.

Scalability considerations

Data presented at the American Chemical Society earlier this year looked at productivity optimization and process calculations for the AAVX resin. Findings from

FIGURE 2

POROS CaptureSelect AAVX resin: dynamic binding capacity data.



this data included that binding capacities of over 1×10^{15} capsids/mL at residence times ≥0.5 min for AAV2 can be achieved with POROS CaptureSelect AAVX resin. Productivity was found to be maximized at load residence times ≤0.5 min depending on titer, but hardware and/or system considerations limited operation closer to 1 min. Process calculations suggested that for large bioreactor volumes (e.g. 2,000 L) and high titers (e.g. 6×10^{11} vg/mL), columns 20–30 cm in diameter meet the typical processing limits while maximizing resin utilization.

Additional scalability data for the AAVX resin was generated at Brammer Bio (Figure 5). Vector recovery was measured for multiple serotypes at multiple scales, ranging from 2 L to 500–1,000 L. Consistent AAV recoveries were found across scales and serotypes with POROS CaptureSelect AAVX resin.

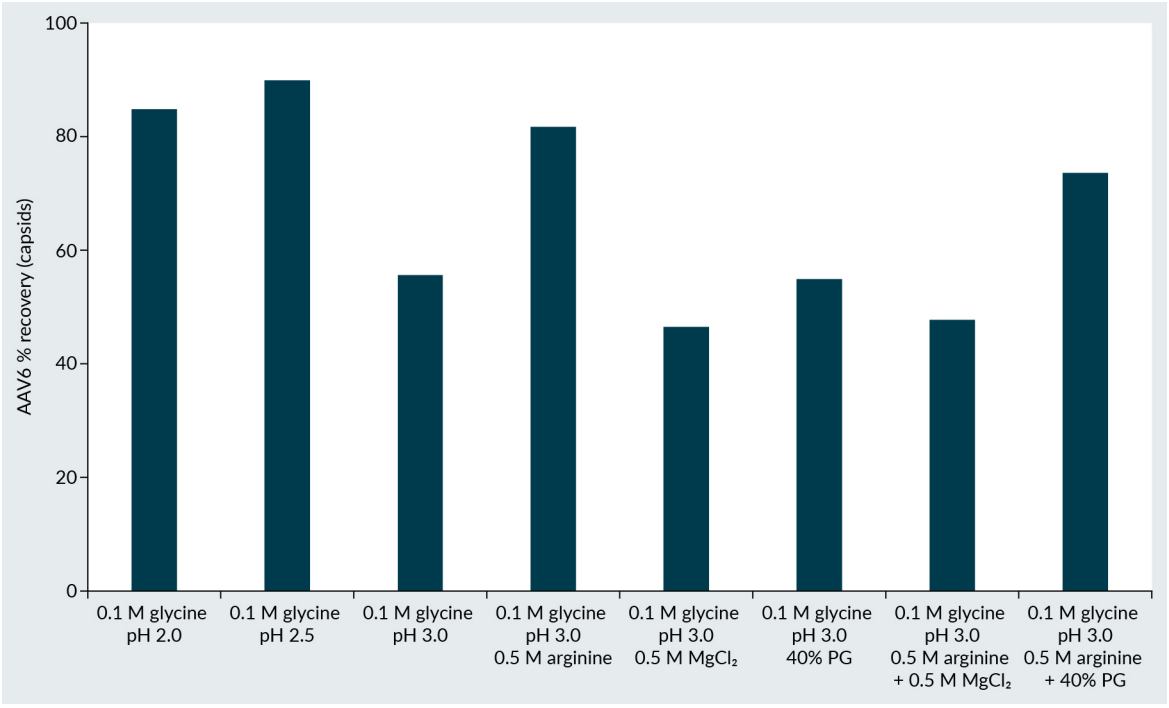
Reuse and carryover

Another important consideration is the reuse of the resin. Carryover was measured over a set number of cycles. For the study, the steps were conducted at a 1 min residence time for an AAV6-GFP serotype on a 1 mL column, loaded to 2×10^{13} capsids/mL. The column was regenerated using 0.1 M phosphoric acid and further cleaned using 6 M guanidine hydrochloride. A blank run was performed after 14 cycles, and then a further blank run was executed for clean resin. Compared to the new resin, the aged resin had a signal only slightly above the limit of detection, demonstrating little to no carryover. This was measured using a total capsid ELISA.

In addition, consistent chromatographic performance and yield were seen over 35 reuse cycles using clarified AAV2 lysate. The load was prepared by concentration and buffer exchange and loaded on the 1 mL AAVX column with 6.6×10^{14} capsids/mL. For the cleaning, 0.2 M phosphoric acid was used with a 15 min contact time, followed by 6 M guanidine hydrochloride. Consistent recoveries were seen throughout the 35-reuse cycle lifetime of the study, with an

FIGURE 3

POROS CaptureSelect AAVX: elution optimization data.



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average recovery of around 86%. This data demonstrates good cyclability of the resin. However, it should be noted that this can vary based on load material and clarification procedures.

Viral clearance considerations

Viral clearance data for an AAV8 serotype was also generated for the AAVX affinity resin in collaboration with REGENXBIO, Texcell NA, MockV Solutions, and Thermo Fisher. The study included multiple model viruses, including XMuLV and MVN.

In addition to a manufacturing center point process, a worst-case scenario was investigated with a defined target load density and target residence time. For this worst-case scenario, a 33% higher load density and a 70% increase in residence time represented a non-ideal situation to potentially co-purify these model viruses. For all the model viruses, contributing or effective clearance was seen with the AAVX affinity resin. The data demonstrated that good log reduction value can be achieved for these model viruses, demonstrating viral clearance capabilities. This can vary depending on serotype, load material, or process conditions. For example, additional wash optimization can help improve viral clearance.

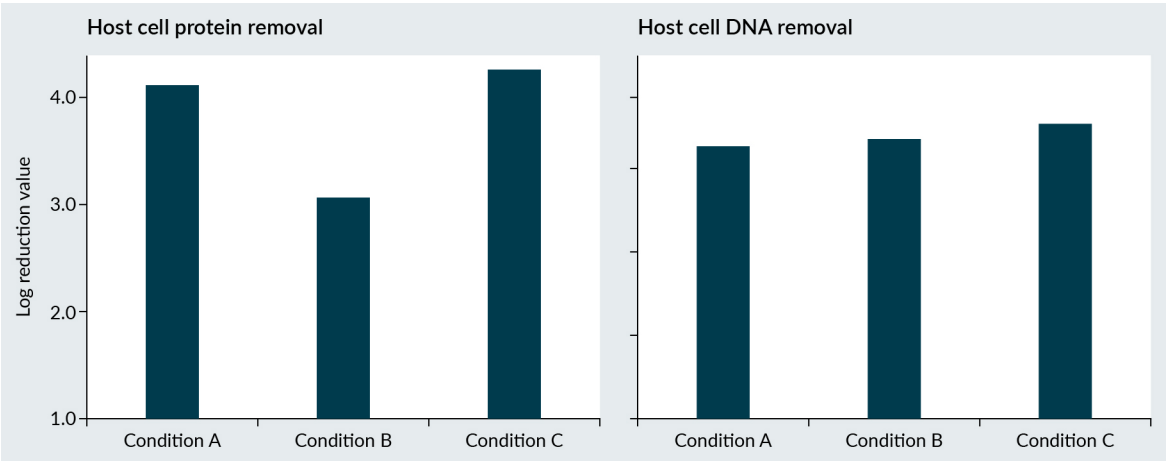
TECHNOLOGY BEHIND MANUFACTURING-READY, PLATFORM-ABLE AAV PURIFICATION SOLUTIONS

Thermo Fisher offers several platform-able affinity solutions to support AAV purification that employ powerful base–bead and ligand technologies The POROS CaptureSelect AAV8, the POROS CaptureSelect AAV9, and a broad affinity POROS CaptureSelect AAVX resin bind a range of serotypes, including native non-human and synthetic serotypes. Each resin comes with full regulatory support enabling usage in commercial manufacturing.

CaptureSelect ligands are single-domain antibodies derived from the unique structure

FIGURE 4

POROS CaptureSelect AAVX resin: impurity clearance data.



Condition	Intermediate washes
A	1: 50 mM Tris + 1.5 M sodium chloride, pH 9.0 2: 50 mM Tris pH 7.5
B	1: 50 mM Tris + 1.5 M sodium chloride, pH 9.0 2: 50 mM citrate + 0.25 M arginine pH 5.5
C	1: 50 mM Tris + 1.5 M sodium chloride, pH 9.0 2: 50 mM Tris pH 9.0

→ 80% recovery achieved independent of wash conditions

of Camelid Igs (Figure 6). These Igs are heavy chain only and lack a CH1 domain. The VHH fragment is screened for parameters such as specificity, elution properties, and stability. The small size of the ligands allows them to easily access epitopes and the compact structure provides general chemical stability. VHH affinity ligands are produced in yeast in an animal origin-free, GMP-friendly production process.

The unique pore structure of the POROS bead allows for efficient purification of large molecules such as viral vectors. The bead's polystyrene-divinylbenzene backbone provides rigid, linear, and scalable performance in addition to easy handling and high chemical stability. The large throughpores help to reduce mass transfer resistance and maintain capacity and resolution. This allows for relatively high capacities and fast flow rates. The

FIGURE 5

POROS CaptureSelect AAVX resin: scalability data.

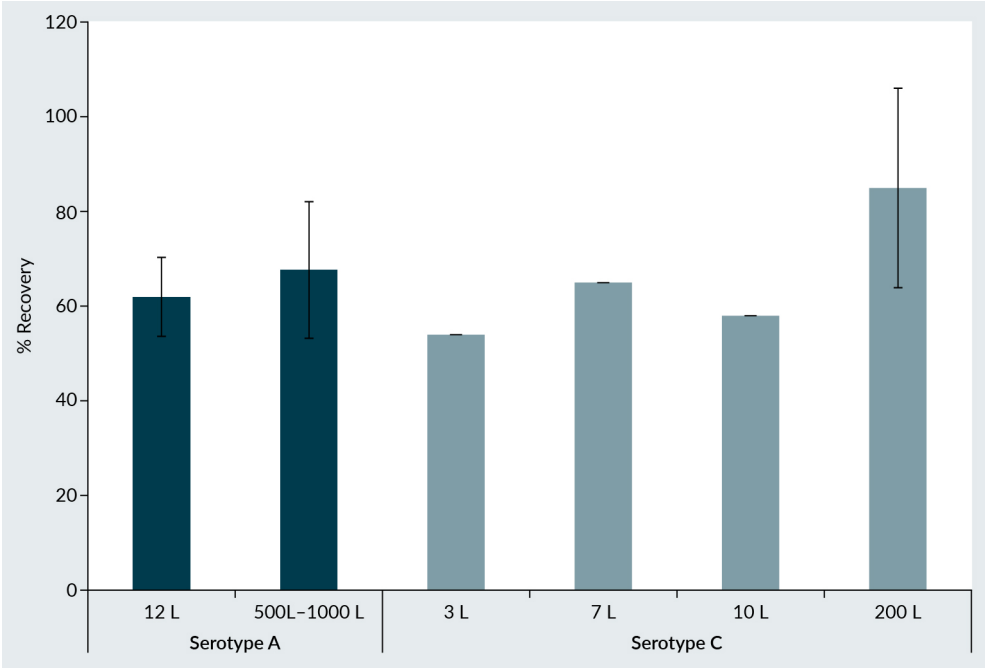
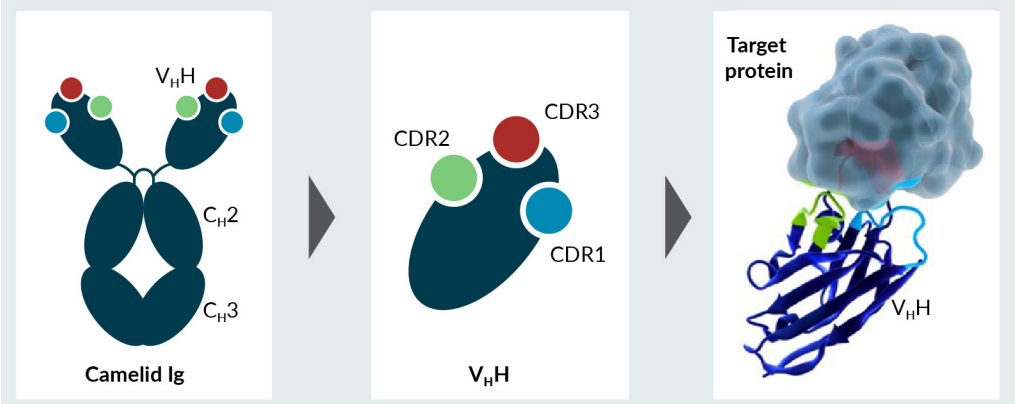


FIGURE 6

Affinity chromatography through antibody-based selectivity.



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50 µm particle size improves capacity resolution and due to the rigidity of the beads, provides good pressure-flow characteristics.

CONCLUSIONS & SUMMARY

The POROS CaptureSelect AAVX resin is a platform resin for the purification of multiple AAV serotypes including native and engineered capsids. Data starting from clarified lysate shows the ligand provides high purity and recovery. The AAVX resin shows high capacity and elution recovery independent of flow rate, which is advantageous for scalability. Due to the broad specificity, the scalability advantages of the POROS bead mean it provides a good platform and scalable solution for GMP processes. Data also showed effective clearance of model viruses for the AAVX resin.

Q&A

Jett Appel

Q Does the residence time for the loading impact recovery?

JA: This depends. It is possible that if you are operating at a very fast flow rate and the column is overloaded, your recovery can be impacted by AAV not binding to the resin and being present in the flow through. However, that is typically not what we observe in the field. Recoveries are usually impacted by other factors, such as optimization of clarification techniques, elution conditions, and various other factors.

Q Are the wash and elution conditions described in the presentation translatable to other AAV serotypes?

JA: Yes, they are translatable. This will differ slightly with every serotype and the set of impurity profiles present in your starting material. We recommend the use of an orthogonal method. High salt disrupts ionically bound impurities, and low salt disrupts hydrophobic, nonspecific bound impurities. As there are many impurities present in the lysate, some of these

may form complexes with the capsid, so it is important to use an orthogonal approach and evaluate it for your specific process.

Q Were there any additional salts in the glycine elution buffers?

JA: For the elution study, for the conditions that did not contain the excipients, all the elution buffers contained pluronic to help prevent any non-specific absorption loss of AAV. As this was AAV6, we typically do not see issues with having low salt. However, if you are working with other serotypes such as AAV2 or AAV5, low conductivity can cause potential precipitation of your AAV.

We do recommend some inclusion of salt of around 50 mM. The presence of sodium chloride could have an impact on your elution recovery. From our internal and collaborative data, we see there may be a hydrophobic aspect to the affinity binding. Having higher salt can potentially lower your recoveries, but the inclusion of a small amount could be beneficial for some capsids.

Q Why are the recoveries using affinity resins not closer to 100% like with monoclonal antibodies (mAbs)? From mass balance, where is the rest of the 20–40% AAV lost?

JA: AAV has its own challenges relative to mAbs. The variabilities are not as wide as we see in mAbs because typically, people are using PCR-based assays. That is why we recommend performing mass balances. For those getting low recovery, it is important to measure your flow through, strip, and elution, or it may point to potential optimization needed in the analytics.

If a lot of AAV is present in the flow through, more clarification optimization may be needed to help improve the binding to the resin. Other customers have shown that even with well-optimized analytics, it is common to see variabilities up to 20% or greater. Our cycling data is mainly consistent, but occasionally recoveries can be a lot higher or lower.

Q For your wash study, are you concerned with capsid stability if using a pH 9.0 wash?

JA: Typically, since it is slightly alkaline, we are not too concerned about the alkaline pH 9.0 condition because AAV is part of the Parvoviridae family, which is stable.

However, we tend to be more cautious about an intermediate pH of around 5.0 because that emulates the late end of activity when AAV is taken up by the cell. Researchers from the University of Florida demonstrated that over time, they lose stability at that pH more than at a higher pH, and even at a very low pH of below 3.0.

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CaptureSelect ligands and resins: for research use or further manufacturing; not for diagnostic use or direct administration in humans or animals.

BIOGRAPHY

JETT APPEL has been a Purification Field Applications Scientist at Thermo Fisher Scientific since 2021, supporting purification development and scale-up for monoclonal antibodies, viral vectors, nucleic acids, and recombinant proteins. Prior to joining Thermo Fisher, Jett worked at Avid Bioservices, a contract development manufacturing organization (CDMO) based in Orange County, California. During his 5 years of working at Avid, Jett first worked in process development and eventually transitioned to a senior engineer role in the Manufacturing Sciences and Technology (MSAT) team, where he supported purification development and technology transfers for monoclonal IgGs, IgMs, bispecific antibodies, scFvs, and recombinant enzymes up to 2000 L bioreactor scale. Jett received a Bachelor's degree in Chemical and Biomolecular Engineering from UCLA in 2016. While at UCLA, he was involved in epigenetics research at the Steve Jacobsen Lab, which included studying the pathways involved in RNA-directed DNA methylation in *Arabidopsis thaliana*.

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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 his approval for this version to be published.

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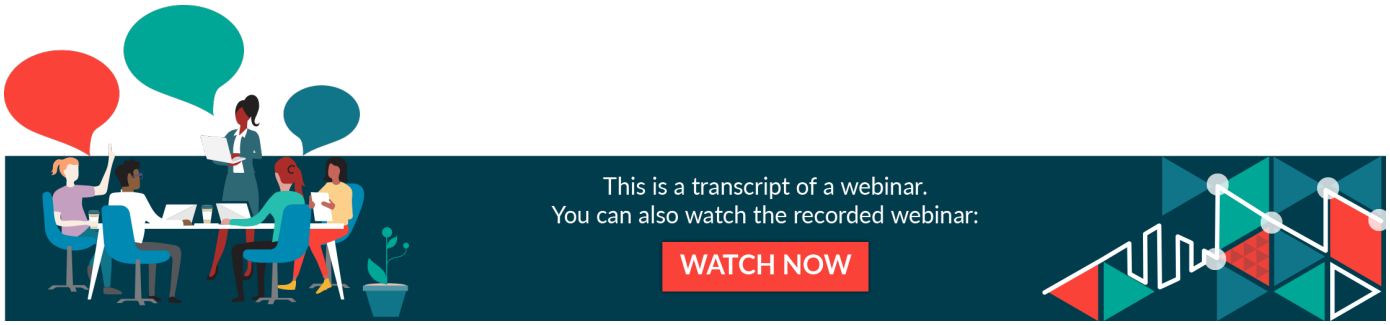

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INTERVIEW

Navigating lentiviral vector downstream bioprocessing: an engineering perspective

Abi Pinchbeck (Editor, BioInsights) speaks to **Andrea Rayat** (Associate Professor, Bioseparations and Downstream Processing, Department of Biochemical Engineering, UCL).
They discuss the pressing innovation gaps in the viral vector processing field, in addition to how to solve them through scale-down modeling, unit operation engineering, partnerships and collaborations.

Cell & Gene Therapy Insights 2023; 9(9), 1117–1124

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Q Can you tell me about your path to work in the viral vector bioprocessing field?

AR: I started working in viral vector processing in 2018. Before that, I worked on antibody-based products such as monoclonal antibodies (mAbs) and antibody fragments (Fab). I have also been working on recombinant enzymes using *E. coli*. I work on the recovery and purification of various bioproducts using different expression systems. I see my background as a strength, that has powered my ability to bring in experience from other areas of biotechnology. I find analogies, parallels, or general patterns and connect my knowledge and expertise in these different product-systems to apply, reframe, or repurpose things that I have learned to create a new framework or concept to better understand viral vector processing.

"...vectors were an up-and-coming class of products without many downstream bioprocess studies at that time, specifically on membranes. [...] New analytical techniques that can help us evaluate the state of the viral vectors at each stage of the process. [...] We need to know exactly what is going on; explain why we get certain results which can then help accelerate the growth in innovation in this space."

Q What are you working on right now?

AR: We recently published a paper on viral vector processing, where we have shown, among others, that the impact of shear on viral vectors is not as severe as we thought [1], and I am completing a few more similar papers. I have been working on these for three or four years, and it has taken a long time and a lot of effort to reach this point. We gained a real depth of knowledge which enabled us to develop new methodologies. There is a lot of careful consideration in our approach to experimental design and how we conduct studies since they are, in fact, the first of their kind for viral vector applications.

Q What is your assessment of the current technological state of the art in lentiviral vector downstream bioprocessing—where have the most valuable recent advances been?

AR: In my experience, the study of viral vector processing, specifically lentiviral vectors (LVs), is thin on the ground. I became interested in moving into viral vectors because of my work on membrane processing; I realized that vectors were an up-and-coming class of products without many downstream bioprocess studies reported at that time, specifically on membrane filtration. Moving into the field, I realized that it takes a long time to establish yourself and to develop a track record of robust studies to add to the knowledge pool in this area.

In terms of the state-of-the-art, I found that most of the focus for LVs, and viral vectors in general, is on the first part of the production process itself. There is limited focus on downstream process studies. I am glad to see that we are moving towards working on understanding the process to produce and recover higher titers, including the applications of stable cell lines.

There are only a few stable cell lines out there, mostly inducible ones. Studies on the applications of these stable cell lines, including our work, show that they can be used to produce LVs. I am still seeing very little on the downstream processing side. Recently, I have seen several papers that are working on affinity chromatography for LVs, working to understand the kinetics of adsorption during anion exchange for viral vector production, and looking

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at producing data with ion exchange chromatography studies. All of these, however, are focused on one unit operation only.

Q What are the most pressing remaining fit-for-purpose innovation gaps?

AR: The gaps that we see surround creating materials for downstream processing specifically for LVs. However, we understand that this is a big task. Innovation in terms of creating such materials may come a bit later on. New analytical techniques that can help us evaluate the state of the viral vectors at each stage of the process are essential for process and product insights.

The other part that can help, at least for now, is publishing studies to broaden and deepen our understanding of viral vector processing. We need to know exactly what is going on; explain why we get certain results which can then help accelerate the growth in innovation in this space. We are now seeing this more, specifically in chromatography. More studies on membrane processes would also be useful, as well as the interaction between these unit operations, process materials, and equipment. I recently attended the ECI Single-use Technologies conference in Boston, MA. There are many plastic devices and assemblies in the manufacture of viral vectors, and in cell and gene therapy (CGT) applications in general. The pressing need to better understand the impact of single-use technologies in this emerging field was highlighted in that meeting. These include understanding or determining material characterization, supply chain, and regulatory requirements.

Q What are the impacts on/considerations for downstream processing of the various expression systems and cell lines currently utilized in LV production?

AR: One consideration is finding a more suitable cell line, or making the current cell lines we are using more suitable, for GMP manufacturing. Right now, we see that most people use HEK cells for producing viral vectors. Perhaps there might be other cell lines that are useful to produce LVs. We need to consider the things that are needed to produce such vectors because we know that sometimes their production is detrimental to cells. We are asking a lot from these cells to produce our viral vector products, but we know that the vectors may cause cytotoxicity later on.

The other consideration is seeing how cell engineering, and even engineering biology, can improve production. My background in biochemical and chemical engineering allows me to evaluate each unit operation in the manufacturing process and think about how they could be improved. Sometimes there are ways that we can improve unit operations by looking at the type of cells that we are using, or the type of other materials used in production such as reagents or buffers. Looking at the interactions between cell line expression systems and the engineering environment when producing such materials is important.

I am also an experimentalist. Although I appreciate the benefit of using artificial intelligence (AI) or models, at the moment, in some areas we do not have enough process data for such tools to be effective in guiding our process decisions. I am an advocate for using

"...ultra scale-down methodologies including devices...can only help people if vendor technology companies supporting the CGT manufacturing space also develop commercial products for scale-down methodologies."

scale-down experimentation to produce data under different conditions, with different cell lines, and different operating environments. This helps process understanding by generating a lot of data while using mL-scale materials. Our time is now mainly used to focus on the interpretation and analysis of these data, and here we can leverage the power of modelling and even AI.

Q What are the contributing factors to the loss of LVs during the scale-up of membrane processes for cell and gene therapy manufacturing?

AR: There are many factors. From my understanding, we do not know the compounding effects of the materials used in the production, for example the buffer chosen, the beads used in chromatography, or the membrane filters together with viral vectors. There is also not a lot of information on the impurities that the cells produce together with the vectors or even information on the different variants of viruses that are being produced by the cells.

In a recent study, it is hypothesized that these variants are causing low recovery. They may be becoming bound to the chromatography beads compared to other variants that can be reversibly unbound from the beads. We do not yet have enough understanding of those aspects. Still, we can infer that the interactions between the vectors and the bioprocess material (the beads, the membrane filters, and the buffers) contribute to the losses. Our recent paper [1] shows some of these negative factor interactions between process shear and other process conditions or operating parameters during membrane filtration, indicating the need for precise selection of filtration conditions and their operation.

The other thing we do not know yet is the effect of localized processing conditions. As an analogy, let's say you have a small wound or burn. You might think that as only a small part of your body is affected, it is not a big problem. However, it may cause a lot of issues later if there is an infection, for example. It is easy to see those spots on our skin, but these viral vectors are so tiny, and within a complex fluid, that it is difficult to study any small deformation on them which may cause huge problems in processing, including binding to columns.

An example of these localized effects would be a mismatch in the pH of the buffer and the type of membranes we are using. There are conditions that although good for the vector as a whole (e.g., choice of buffer pH), might cause some localized impact if there are negative effects due to factor-interactions (e.g., buffer pH may affect the fouling profile of the membrane filter as it changes membrane properties). These local factors could be one of the reasons for losses. Much of these is driven by the engineering environment, which has not yet been fully studied in many areas of viral vector processing.

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Q Where do you see opportunities for improvement in the scalability of lentiviral processes during early development and clinical production?

AR: The opportunities for improvement would come with gaining knowledge from experts studying viral vector processing, especially LVs, and publishing and sharing data so we can see which types of conditions work for each specific purpose. Then, there would be the opportunity for AI-driven analysis to see whether there are links within these conditions.

Other opportunities for improvement could lie in scale-down processing. In our group at UCL, I develop ultra scale-down methodologies including devices, but this can only help people if vendor technology companies supporting the CGT manufacturing space also develop commercial products for scale-down methodologies.

The creation of specific biomaterials is key. We need membrane filters, adsorbents, and beads that are designed specifically for viral vector manufacturing. We need scale-down or miniaturized versions of these materials to be available during early development. There are a lot of larger-scale materials readily available, but for those who are just starting out, given the cost of production and the uncertainty of scale-up, it is risky to go straight to large scale. Scale-down tools and materials being commercially available would enable the early development of clinical production, especially moving toward full-scale manufacturing aligned with the materials used in early development stages. This means developing manufacturing processes from the start rather than reworking processes down the line.

Q What is your key advice to those looking to achieve high quality, high concentration LV products from robust and scalable processes?

AR: In terms of scalability, three key pieces of advice would be to look at using scale-down methodologies, question the perceptions and preconceptions of vector processing that apply in certain situations but may not apply to your product, and partner with an equipment vendor, or form industry–academic collaborations, to help evaluate and scale up processes. Given that many may not be familiar with the unit operations, vendor partnerships or industry–academia relationships can be valuable when designing early process and product development studies. Do not shy away from looking at the basics of the unit operations.

Testing scale-down versions is important, as is understanding what the scale-down version can offer in terms of process insights. These scale-down versions may be different from the GMP version or may be physically different from the larger scale equipment, such as our ultra scale-down devices. However, knowing what these scale-down versions offer could mean that you gain more insights earlier by appropriately applying these in process studies, early in the development stage. You can go to full scale trial with more confidence in certain aspects, having less guesswork at these crucial late stages of development. In our recent paper, we have shown that many preconceptions of viral vector processing are true in certain aspects but may not necessarily be true for all products or cell lines. Each scale-down process model needs testing. We have shown that shear, which is a factor generally considered to be the main reason for loss of recovery, does not necessarily cause losses. For membrane

processing specifically, we need a relatively high shear, to help clean the membrane with the sweeping action of the process fluid parallel to the membrane. This helps the vectors to not adsorb onto the membrane.

In a relatively low-shear environment, which is often used in current processes, we experience losses and very low recovery. While it might be true that for certain vectors or certain cell lines high shear can lead to high losses, shear is not always the reason. Therefore, experimentally testing various membrane processing conditions for your own products and cell lines would be beneficial to optimize the process. Testing combinations of process conditions and materials with scale-down methods allows you to de-risk the cost of experimentation, and save time, as these scale-down experiments are quick and only require small volumes.

"...training our future bioprocess engineers and scientists in viral vector production, and CGT in general, is key."

Q Finally, can you sum up your major goals and priorities for your work over the coming 12–24 months?

AR: In the next few years, I will be focusing on scale-down membrane processing for viral and non-viral vector applications, and remain active in other areas of recovery and downstream processing of biotech products. I continue to seek collaborations as this is the only way to unravel complex bioprocesses. Although I am very much an engineer working with the design and performance analysis of unit operations, I collaborate with other experts in virology, engineering biology and cell engineering, among others, to see how changes in the design of vectors, cells, or cell components can affect downstream purification. At UCL, we have a Centre for Doctoral Training (CDT) funded by the Engineering and Physical Sciences Research Council (EPSRC) where we collaborate with industry partners to address bioprocessing challenges. Some of my current projects with the CDT are with the Cytiva and Astra Zeneca Centres of Excellence (CoE) at UCL. We are investigating cell retention devices for perfusion and novel unit operations for process intensification. The insights that we obtain from this area may be applied to viral vectors.

We are completing our Biotechnology and Biological Sciences Research Council (BBSRC) project on LV production through novel engineering biology solutions. In this collaborative project with the UK's MHRA, we separated the viral particle production from the envelope production and performed the assembly of the envelope and the viral particle outside the cell. I am excited about this project because this can also help us understand whether we can produce vectors in this way at a manufacturing scale, as this has already been performed in the lab.

Since we have separated the production of the envelope from the viral particle, we hope to use these two particles to develop an understanding of how LVs interact with the different process materials I mentioned earlier.

Finally, and most importantly, to accomplish all these, training our future bioprocess engineers and scientists in viral vector production, and in the CGT area, in general, is key. Educating students in our biochemical engineering degree programs and providing leadership, supervision and mentoring to the researchers in my research group are very important

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aspects of my role. I look forward to helping my final year students establish their publication record and complete their doctoral studies. I am excited to start three new doctoral projects this year, all on LV production and downstream processing. The projects are funded by the EPSRC-CDT with Cytiva CoE, the BBSRC-CTP (Collaborative Training Partnerships) with Oxford Biomedica, and Autolus through an Industrial Fellowship with the Royal Commission for the Exhibition of 1851. Although not without its challenges, seeing the progression of these students and researchers as contributors to the field is very satisfying, personally, and for the whole CGT industry, this is very much needed.

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BIOGRAPHY

ANDREA RAYAT is an Associate Professor in Bioseparations and DSP at University College London (UCL). She holds Biochemical and Chemical Engineering degrees from UCL (PhD), TU Delft (MS), and UPLB (BS). Her main research aim is to contribute to the understanding of bioseparations of novel, biologically-derived products and to reveal the science and engineering that underpin recovery and purification operation, and their scale-up. Her group achieves this through the design and application of ultra scale-down and millifluidic devices, and other high throughput techniques. Together with scale-down studies, they apply modelling and multi-variate data analysis (and in the future, AI) to accelerate the robust manufacture of these novel biotech products. Her work on ultra scale-down as applied to industrial enzyme production has been recognised by the IChemE Global Awards (2020) and by the InnovateUK KTP awards (2021). In the area of LV downstream processing, her research group is one of the very few groups that study membrane filtration and other membrane processing steps for LV production. Their review paper [2], has comprehensively reviewed unit operations employed in LV production, outlining the critical process parameters and conditions for LV recovery. Their latest research paper demystifies the impact of process shear on LVs [1]. In the autumn of 2023, she is to start several projects in collaboration with cell and gene therapy companies which demonstrate the rapid expansion of her work in viral vector recovery and purification.

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AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

DOWNSTREAM BIOPROCESSING

INNOVATOR INSIGHT

Disruptive bench-scale purification of lentivirus using affinity-liquid phase separation technology

Nicole L Votaw, Melissa Callander, Torie Broer, Alyssa Wheeler, Michael Dzuricky & Kelli Luginbuhl

Lentiviral vectors are widely used in cell and gene therapy, but their high production costs hamper early-stage research efforts. Furthermore, many concentration and purification methods available to early-stage researchers today are difficult to scale or lack specificity for lentiviral purification, hindering preclinical research and translation to clinical and commercial scales. We have developed a research-scale lentiviral reagent with the potential to transform lentiviral purification workflows. The reagent is quick and easy to use, yields high titer lentiviral vectors, and, because of its specificity, is more effective at contaminant removal than other LV purification products designed for research use. By specifically attaching to the viral envelope in solution and then forming liquid droplets around the lentivirus, IsoTag™ LV protects viruses from aggregation and degradation while dramatically increasing the effective size and density of the LV, thus facilitating easy separation from other smaller and less dense contaminants (i.e., host cell proteins). The actual process of capture is very simple and can be implemented by any user, at any skill level, with low-cost lab equipment. The design and development of a small-scale centrifugation process and novel reagent detailed herein lays the foundation for additional development of the IsoTag™ LV for larger scale processing. IsoTag™ LV is poised to democratize LV production and purification in the research world and accelerate development of new cell and gene therapies.

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INTRODUCTION

Lentiviral vectors (LVs) are widely used viral vectors necessary for the production of several commercial cell and gene therapies [1]. However, their innate fragility and cytotoxicity, due to their lipid bilayer and VSVG (vesicular stomatitis virus envelope glycoprotein) pseudotype, respectively, means that the manufacturing cost of these vectors is extremely high [2–5]. Traditional downstream purification strategies are being commercially adapted for large and complex viruses [3,6,7]. However, these methods lack standardization, require significant optimization, and have suboptimal product recovery, thereby contributing to the high development costs for lentivirus-based gene therapies. As such, potentially curative treatments and therapies are often prohibitively expensive, in some cases costing over one million USD per dose [8]. Early-stage research on these therapies typically begins in academic laboratories working at small scale [9,10] here defined as 1 L or less and termed 'research-scale'. Research-scale production typically relies on ultracentrifugation [11] or low-speed centrifugation using PEGprecipitation. Little has been done to improve upon the concentration, purity, or scalability of these methods, despite their criticality to the early-stage development of advanced therapies. Standardized downstream purification of LV at the research-scale, especially with methods improving high-throughput and quality, would improve the reliability of the vector, increase the speed of preclinical research, and ease the translation of new technologies to clinical and commercial scales.

Research-scale LV is most commonly produced using a triple transfection protocol [11] in an adherent or suspension cell line with a customized cocktail of additives. Following harvest, researchers concentrate and purify their LV through an assortment of methods that vary in cost, efficacy, scalability, and ease of operation. Unfortunately, no single method or product provides both rapid concentration and contaminant removal, but common methods are outlined herein. Standard concentration and purification methods available to researchers involve ultracentrifugation, low speed centrifugation, and filtration [6,12]. Briefly, ultracentrifugation involves layering crude LV harvest material over a sucrose gradient and using ultracentrifugation to separate LV particles from the viral supernatant. This method is technically challenging, requires costly equipment, and it is not scalable. Many researchers choose simply to centrifuge their harvest material at low speed to pellet and remove large cellular debris, but this method does not concentrate the LV, nor does it remove smaller contaminants and dsDNA that can interfere with downstream applications [6,13]. As the importance of creating a high-quality LV product that can be reproducibly used for experiments has recently been emphasized [6,12], it is important to consider contaminant removal even at small scales.

To create quality LV, one group proposed mimicking commercial scale approaches to LV manufacturing [12] involving a complex set of steps requiring equipment similar to that used in clinical-scale manufacturing suites. With this method, they were able to reduce DNA contaminants by 1log with total LV recoveries of around 20–40% [12]. Unfortunately, this protocol requires expensive equipment and highly skilled operators better suited for a vector core than an individual researcher or laboratory, and thus is inaccessible to many laboratory-scale LV producers. Other commercially available options provided in kit formats that employ common laboratory equipment are more accessible to individual researchers or research groups. Many of these kit-type offerings rely on PEG precipitation to concentrate LV from harvest material, leaving the PEG reagent in the final LV product and creating large pellets that limit final concentration. While these reagents are non-toxic and may protect the virus during freeze-thaw [14] it has been demonstrated that PEG monomer contaminants and breakdown products can potentially be cytotoxic

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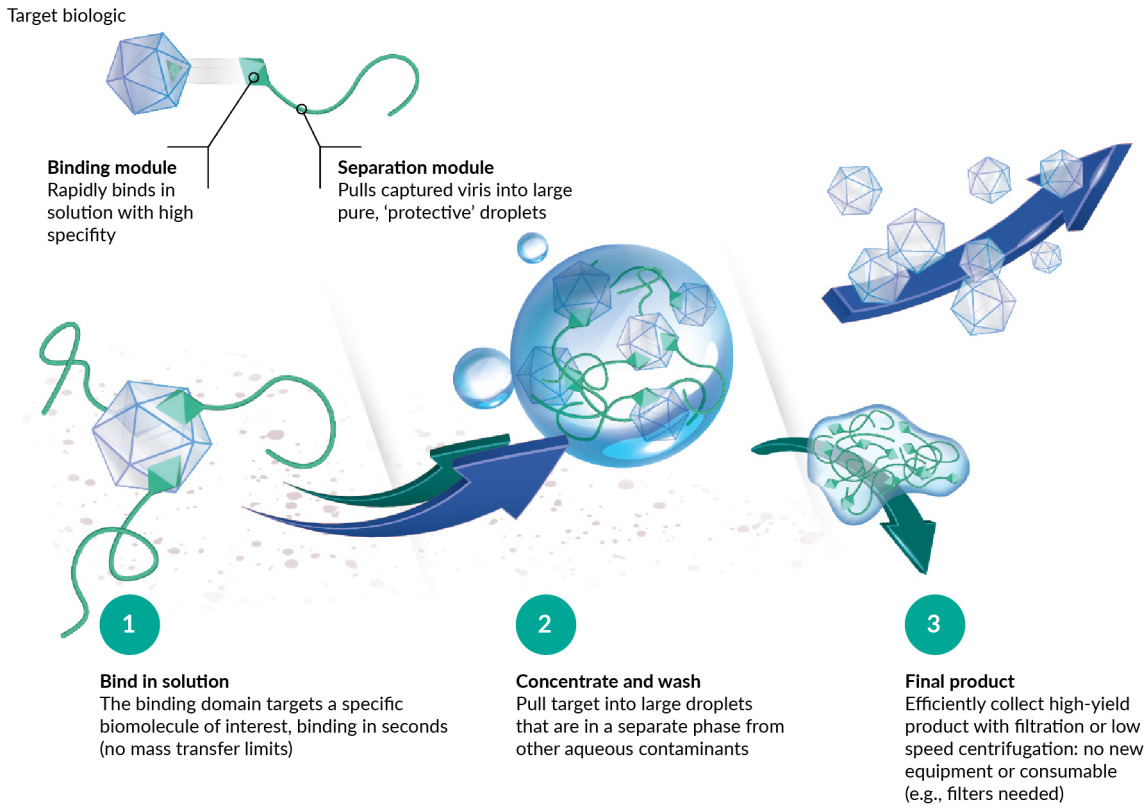
or limit *in vivo* use due to their non-biodegradability and antigenicity [15–17]. Other options include magnetic bead sorting, but use of this reagent requires the purchase of accessories such as a magnetic tube rack specific to each tube size and cannot be easily scaled up. Kits offer specific benefits to research scale LV production including simplicity, speed, protection during storage, and removal of the concentrating reagent. However, no single method provides a combination of all these benefits in an ultimate, easy-to-use product for research scale users. We sought to design a product that offered maximum flexibility to the user by demonstrating compatibility across broad feed streams and wide titer ranges with a protocol that is rapid and reproducibly yields high-quality LV.

To that end, we designed and demonstrated proof-of-concept on a research-scale LV purification reagent. The LVspecific reagent, termed IsoTag™ LV, is a recombinant protein comprising an LVspecific affinity ligand combined with Isolere's proprietary bioinspired biopolymer that provides tuneable and reversible liquid–liquid phase separation behavior [18]. The tuneable phase separation behavior arises from the stimulus-responsive biopolymer, whose phase separation is triggered by an external environmental cue, such as shift in temperature, pH, or conductivity [19]. The IsoTag™ LV process described herein was designed to undergo reversible phase transition with the modulation of sodium chloride at ambient temperature. However, temperature manipulation could be used in place of conductivity shift. The process is illustrated in the Figure 1 schematic: IsoTag™ binds in solution to a vector, pulls the vector into a droplet upon the addition of the environmental trigger, and then releases the vector under elution conditions. Vector purification occurs in the

FIGURE 1

IsoTag™ process schematic.



Visual representation of droplet formation and vector sequestering with the IsoTag™ LV reagent.

droplet phase—the droplets are much denser than the remaining particles and thus can be separated by low-speed centrifugation. The droplets could also be separated on the basis of size with microfiltration. While a scalable microfiltration method is outside the scope of this publication, it is under development and will be the subject of future publications. Of note, the cartoons in Figure 1 are not drawn to scale, IsoTag™ proteins are roughly 50 kD in size in their soluble state, so much smaller than the vector they are shown to capture. Furthermore, the droplets are 1–10 µm in size, much larger relative to the vector than shown in Figure 1. Lastly, Figure 1 serves to illustrate the general concept of purification using IsoTag™ reagents, herein we describe a process to capture and concentrate LV without removing the IsoTag™ LV reagent, emphasizing simplicity and speed for small-scale researchers.

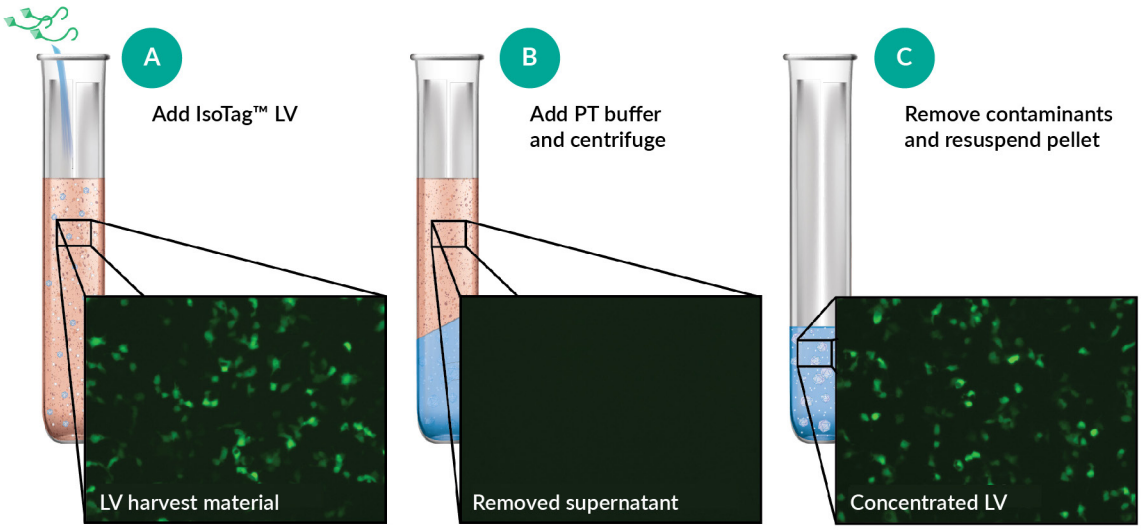
The IsoTag™ LV reagent process delivers highly selective capture of LV particles by employing a *de novo*-designed affinity binding domain that interacts with the VSVGs. Because of this, the IsoTag™ LV reagent could likely bind to and purify any VSVG pseudotyped particle, however, the focus of our development was on VSVG pseudotyped lentiviral purification. Combining the affinity capture and phase-separating phenomena, this purification method is herein referred to as affinity liquid phase separation via centrifugation (ALPS-CF). Unlike the non-specific capture methods available today, the high specificity of our reagent enables exquisitely selective capture of the target vectors. This results in higher yields and higher purity with a robust reagent agnostic to virus titer and feed material.

RESULTS

The IsoTag™ LV reagent is simple to use: a small volume of reagent from a concentrated stock is mixed into harvest LV material (Figure 2A) before adding a phase transition buffer to pull LV particles into protective, phase-separated, virus-containing droplets (Figure 2B) that are isolated by centrifuging at low speed. The viral supernatant is then removed, and the virus-containing pellet is

FIGURE 2

One step concentration and purification of crude lentiviral harvest material.



Schematic illustrating LV purification by **(A)** the addition of IsoTag LV to crude harvest material, **(B)** droplet sequestering of LV with the addition of phase transition (PT) buffer, and **(C)** contaminant removal. Representative images of CMVLVeGFP transducing HEK293T cells at different stages of the purification process, diluted to identical volumes are shown in the pop-outs.

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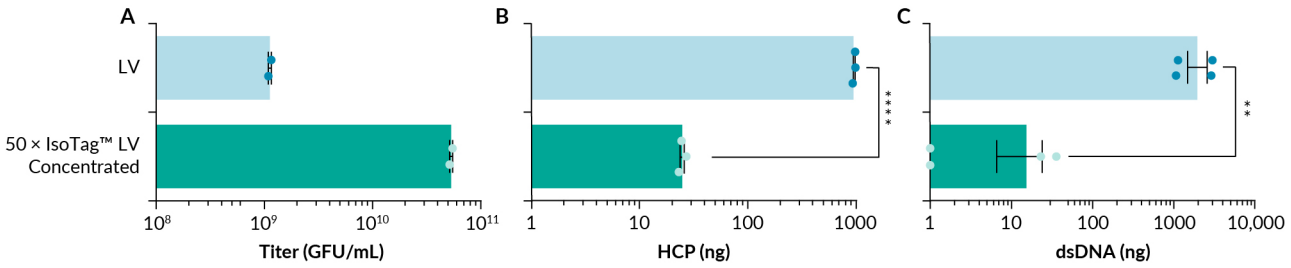
resuspended in the user's buffer and volume of choice (Figure 2C). This process results in over 95% LV recovery, as visualized by the GFP+ infected cells (Figure 2). Because the IsoTag™ LV reagent is VSVG-specific, the concentrated virus has greatly reduced levels of contaminants even without a dedicated washing step.

Initial work involved evaluation of the IsoTag™ LV reagent's ability to bind and concentrate LV, while simultaneously removing host cell proteins and other contaminants. As the ALPS-CF process is performed on a volumetric basis, the protocol is fixed regardless of the starting titer. In these first experiments, the 50 mL of removed supernatant waste was replaced with 1 mL of PBS easily. The pellet, resuspended in this 1 mL of PBS, yields purified LV that is concentrated 50-fold. When the IsoTag™ LV reagent was used to concentrate crude LV from adherent HEK293T cells (with no additional clarification or nuclease treatment steps), we were able to concentrate 50-fold to a functional titer of 5.3e10 GFU/mL (Figure 3A). Functional recoveries of over 98% were observed (Figure S1D). This indicated the IsoTag™ LV reagent bound and captured nearly all the crude LV in solution. Without the need to add a dedicated wash step in the protocol, concentrating crude LV using the IsoTag™ LV reagent also resulted in a 1.5-log reduction of the contaminating host cell proteins to 25 ng (Figure 3B), and a 2-log reduction in dsDNA contaminants to 15.2 ng, with two of the replicates below the detection limit (Figure 3C). This is because the IsoTag™ LV reagent contains an affinity ligand to bind specifically to LV particles, thus avoiding precipitation of other unwanted contaminants and impurities. Similar to other reagents on the market today, the IsoTag™ LV reagent remains in the final product with the concentrated LV.

As some of these competing products claim to provide additional storage benefits during freeze–thaw [14] we investigated the extent to which the IsoTag™ LV reagent could provide thermostabilizing properties to the LV particles. This effect is of particular interest to researchers because it creates a more reliable final product and easier storage and development workflows. Over the various conditions we tested, crude LV recoveries improved by 50–90% when the IsoTag™ LV reagent was present as an additive (Figure 4). From a starting titer of 1.6e7 GFU/mL, a functional recovery of 60% was obtained when storing crude LV at 4–8 °C with IsoTag™ LV reagent for one month, compared to 30% recovery for LV alone in the same storage conditions without IsoTag™ LV reagent (Figure 4A, Figure S2A). This data demonstrates a two-fold improvement in LV stability, and the trend is consistent across weekly timepoints. Furthermore, crude LV samples stored at −80 °C containing IsoTag™ LV additive had significant

FIGURE 3

IsoTag™ LV is a successful LV concentration reagent with excellent contaminant removal.



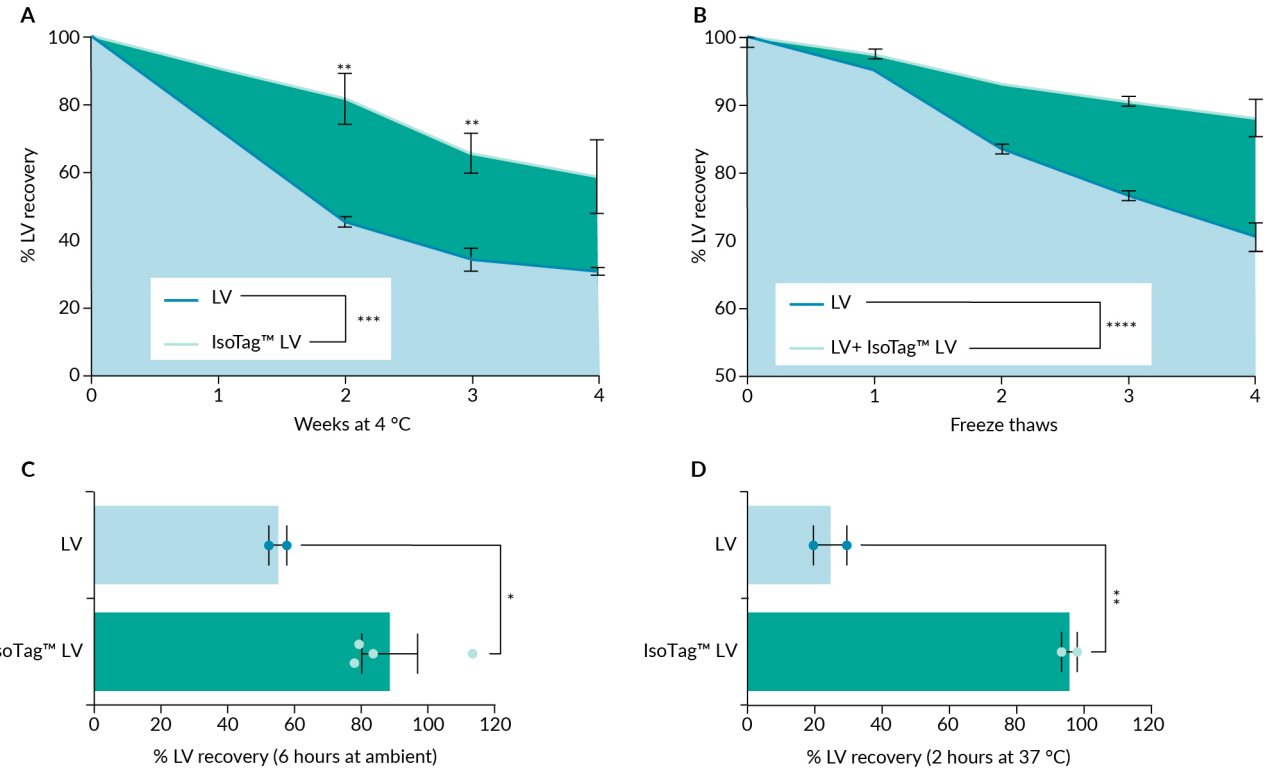
(A) IsoTag™ LV was able to concentrate LV 50-fold to 5.3e10 GFU/mL and concentration resulted in a **(B)** 1.5-log reduction in host cell protein (HCP) to 24.9 ng and a **(C)** 2-log reduction in dsDNA contaminants to 15.2 ng. Undetectable dsDNA samples were given a value of 1. n=2–6, error bars represent the standard error of the mean. Statistical significance calculated using a **(A & B)** one-way ANOVA or **(C)** student t-test. ns=not significantly different. **p<0.01; ****p<0.0001.

improvement in recoveries of 88% functional recovery after three or more freeze-thaw cycles when compared to LV alone (Figure 4B & Figure S2D). When left at ambient temperature (20–24 °C) for 6 h, a significant improvement in functional LV recovery of 88.6% was observed in IsoTag™ LV-containing samples compared to LV alone (Figure 4C & Figure S2B). Pushing this phenomenon even further, the IsoTag™ LV reagent improved functional LV recoveries over 4 × after a 2-h incubation at 37 °C with 95.7% functional recovery (Figure 4D, Figure S2C). Interestingly, when IsoTag™ LV reagent was mixed with the LV sample before the time 0 data point, an increase in the starting titer was noted (Figure S2). This was accounted for in all analyses by including a time 0 sample containing an LV and IsoTag™ LV reagent mixture to directly compare LV recoveries with an appropriate baseline. Taken together, these results highlight additional workflow advantages provided by the IsoTag™ LV reagent and its ability to stabilize these labile viruses in a variety of conditions.

Next, to explore the broad utility of the ALPS-CF process, the IsoTag™ LV reagent binding performance was evaluated across a variety of conditions. Throughout these experimental conditions, over 98% of the functional crude LV was retained on average (Figure 5). First, crude LV was concentrated from a variety of starting volumes (1–80 mL) and the total volume did not alter or interfere with the ALPS-CF process (Figure 5). 80 mL was the largest volume tested because it required the use of two 50 mL conical tubes, and it was assumed

FIGURE 4

IsoTag™ LV addition significantly improves LV stability.



The presence of IsoTag™ LV reagent resulted in a significant improvement in functional LV when stored at **(A)** 4 °C for 4 weeks (60% functional recovery), **(B)** after 4 freeze-thaw cycles (88% functional recovery), **(C)** after 6 h at ambient temperature (20–25 °C) (88.6% functional recovery), and **(D)** after 2 h at 37 °C (95.7% functional recovery). n=2–3, error bars represent the standard error of the mean. Statistical significance calculated using a **(A & B)** two-way ANOVA with Fishers LSD post hoc test or **(C & D)** students t-test. *p<0.05; **p<0.01.

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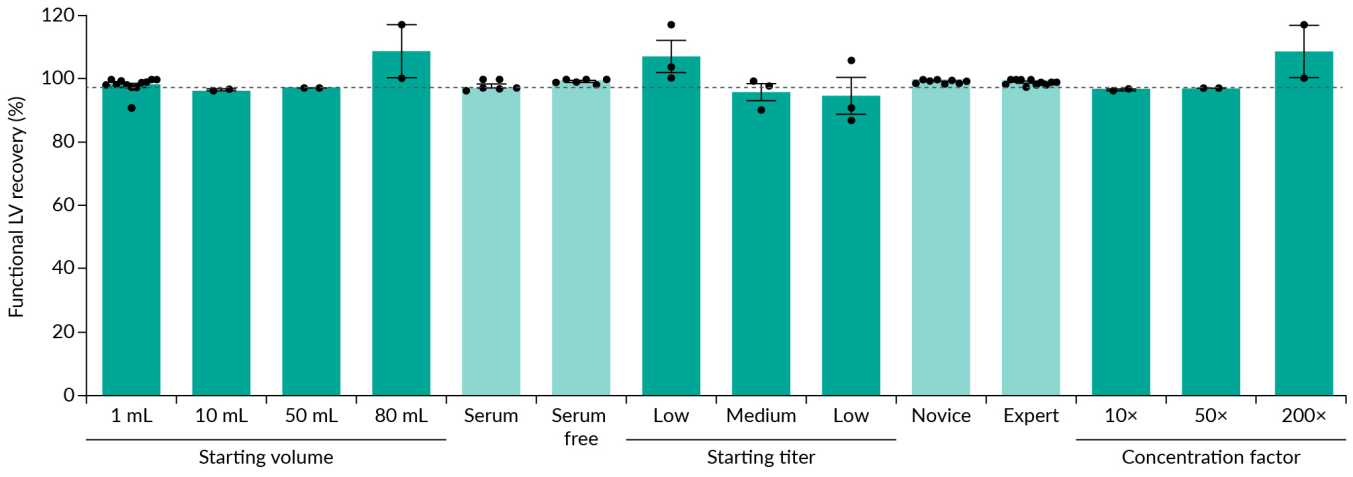
any larger volumes would be processed in a similar manner and thus have similar functional recoveries. IsoTag™ LV reagent binding performance was further examined with different feed streams, adherent production containing serum and serum-free suspension production, as they are known to have different contaminant profiles and binding efficiencies with other purification technologies. Again, the IsoTag™ LV reagent bound and concentrated over 98% of the LV regardless of feed stream (Figure 5). To ensure ALPS-CF is compatible across a wide range of LV harvest titers, binding with IsoTag™ LV reagent was examined with material titer from 1e6 to 1e8 GFU/mL. Titers following upstream production can be highly variable based on the size and complexity of the inserted gene [20] and starting titer can have a dramatic impact on processing time and functional recoveries with other industry standard methods [3,6]. When the IsoTag™ LV reagent was mixed with high (1e8 GFU/mL), medium (1e7 GFU/mL), and low (1e6 GFU/mL) starting LV titers and used to capture LV, functional recovery titers were again above 97% (Figure 5). Furthermore, processing times were greatly reduced with the streamlined ALPS-CF process. While comparable kits take anywhere from 2–12 h to process LV material, using the IsoTag™ LV reagent took just 30 min.

The ALPS-CF process is easy to implement in any laboratory outfitted with equipment basics. Six first-time users following the protocol without any additional prior training or guidance were able to recover greater than 98% of their crude LV using the ALPS-CF protocol. LV recoveries achieved by first-time users were indistinguishable from experienced users (Figure 5 & Figure S1E). New users will be able to readily adopt this ALPS-CF process for concentrating and purifying their crude LV vectors with consistent results for each lot on the first attempt. Last, the final concentration factor had little impact on the overall LV recoveries, again consistently meeting the 98% average when concentrating anywhere in the range of 10–200× (Figure 5). Taken together, these data validate a robust and reproducible new method for LV recovery, concentration, and purification with IsoTag™ LV reagent.

Given the robust and simple implementation, we next sought to compare the IsoTag™ LV reagent with similar research grade,

FIGURE 5

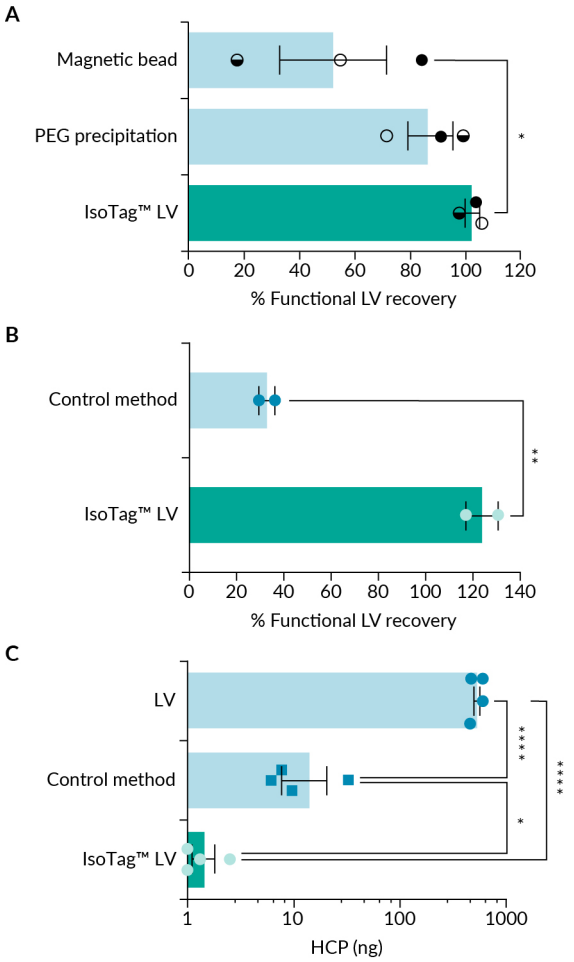
IsoTag™ LV is a robust LV concentration reagent.



IsoTag™ LV reagent was used to concentrate LV under various conditions with an average functional recovery of 98% (gray line). High LV recovery was seen when using the IsoTag™ LV reagent regardless of starting volume, feed material, starting titer, experience level, or concentration factor. High, medium, and low, refer to functional starting titers of 1e8, 1e7, and 1e6 GFU/mL, respectively. n=2–12, error bars represent the standard error of the mean.

FIGURE 6

External validation using the IsoTag™ LV reagent.



(A) Using high (closed circle), medium (half-filled circle), and low (open circle) starting titers, the IsoTag LV concentration reagent was compared to two competing products, PEGit™ and Mag4CLV. **(B)** LV recovery following concentration using IsoTag™ LV or the user's standard PEGprecipitation method referred to here as control method. **(C)** Host cell protein removal using the two concentration methods compared to the starting material. Undetectable HCP samples were given a value of 1. n=2–4, error bars represent the standard error of the mean. Statistical significance calculated using a **(B)** students t-test or **(A & C)** one-way ANOVA with Fishers LSD *post hoc* test. *p<0.05; **p<0.01; ****p<0.0001.

commercially available reagents. This testing was done both internally at Isolere's laboratories and with an external partner, for further validation that the method can be successfully implemented without extensive experience with the underlying phase separation technology. When compared to a reagent that concentrates via PEG precipitation or magnetic bead separation, the IsoTag™ LV reagent produced more consistent recoveries across all starting titers and was the only reagent to consistently achieve 95% recovery (Figure 6A & Figure S1A). These results were confirmed with an academic partner at Duke University who produces their own LV for *ex vivo* research use. Briefly, cells were transfected according to JetPrime protocol, [21,22] and crude LV harvest material was collected, mixed, and then split equally for concentration with either their standard PEGbased overnight precipitation protocol, [22,23] or with the IsoTag™ ALPS-CF method. The research group received a paper protocol for the IsoTag™ ALPS-CF method and no additional technical support or training. Samples from both concentration methods were analyzed and compared for functional titer (via flow cytometry) and contaminant removal (via host cell protein ELISA) (Figure 6B & C & Figure S3). Comparable to data collected at Isolere, the research group was able to concentrate their crude LV 200× with complete LV recovery using the IsoTag™ LV reagent to a titer of 1.5e10 GFU/mL (Figure 6B & Figure S3). The control method, which concentrates overnight using PEGprecipitation, recovered only 40% of the starting material when concentrated 200×. Concentration using IsoTag™ LV reagent was 2.5-fold more effective at recovering functional LV vectors than the control method. Furthermore, while both methods removed host cell protein contaminants from the concentrated LV product, at least 10× more HCP was removed using the IsoTag™ LV reagent compared to the control protocol (Figure 6C). Only 1.4 ng of HCP remained in samples concentrated using the IsoTag™ LV reagent (Figure 6C), with two samples reaching undetectable levels. This is attributed to the specificity of the ALPS-CF purification process, while PEG works through non-specific precipitation by acting as a crowding agent. Of note, the IsoTag™ LV reagent did not appear to induce any cytotoxic effects in the HEK293T cells (Figure S4). Taken together, these data validate the claims made by Isolere regarding LV recovery, contaminant removal, and ease of use by the target customer of the IsoTag™ LV reagent.

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DISCUSSION

The IsoTag™ technology has been described and evaluated here for its value to the field as a research-use LV purification reagent offering faster, more effective workflows that can be implemented in a high-throughput manner with simple equipment. Pure, highly concentrated LVs are of critical importance to researchers to ensure accurate and reproducible results. Furthermore, using a purification technique that can be quickly transferred to scaled-up manufacturing processes improves clinical translation of potentially lifesaving new discoveries. We sought to create a quick and easy-to-use purification reagent for researchers that repeatably and reliably concentrates LV from variable feed streams while also providing researchers with a high titer final product. LV was concentrated using the IsoTag™ LV reagent to 10×, 50×, and even 200× with over 95% recovery with titers as high as 1e10 GFU/mL. Recoveries were consistent regardless of starting titer or the presence or absence of serum, highlighting the robustness of the IsoTag™ LV reagent. The concentrated LV product had superior contaminant removal, important for final product quality and infectivity [12]. It is worth noting that the majority of experiments evaluating contaminant removal were performed using LV from adherent cultures, which contain fewer contaminants than LV produced from suspension cultures. Initial work has indicated similar contaminant removal profiles for LV produced from suspension cultures, but additional work remains to appropriately qualify contaminant removal with various feed streams. This will be a focus of future publications. These high recoveries and low contaminant profiles were due to the affinity-based ligand component of IsoTag™ LV reagent as well as the ALPS-CF volume-based process design. By binding only LV particles and pulling them into droplets, LV was efficiently concentrated with low-speed centrifugation regardless of starting titers or starting volumes.

Throughout the course of this evaluation, IsoTag™ LV was also found to improve LV stability and transduction. When stored at 4–8 °C, ambient temperature, and even 37 °C, LV recoveries were significantly higher in the presence of IsoTag™ LV. Reducing the sensitivity of LV to temperature variations will provide greater flexibility for researchers regarding how they process and store their LV samples. For example, a researcher can store LV at 4–8 °C for 2 weeks and have the functional titer drop only 20% compared to a titer loss of 55% when stored without IsoTag™ LV. This reduces the burden on −80 °C storage and allows the researcher easier access to their LV stocks. Additionally, protection of LV during freeze-thaw provides the user greater flexibility when using their LV stocks across multiple experiments. We hypothesize that this thermoprotection is due to IsoTag™ LV coating the LV particles, insulating them, and reducing aggregation. Further experiments will help clarify and improve upon the mechanistic understanding of this observation. The transduction enhancing properties discovered through the course of this work also warrant further exploration. We hypothesize that the IsoTag™ LV proteins could be weighing down the individual LV particles, thus bringing the particles in more direct contact with the cells, similar to spinfection [24]. This phenomenon could prove quite valuable to those with low titers or hard to transduce cell lines, but work will need to be conducted in cell lines that are more difficult to transduce than HEK293T cells. Additional analysis of physical titer would also provide insight into transduction efficiency per physical particle.

Before beginning this project, a research group at Duke University was interviewed to determine their needs and interests. Final titer (and by virtue, recovery percentage) and speed were identified as two of their top priorities. Using the IsoTag™ LV reagent, they were able to concentrate their LV 200× with virtually no loss of vector in under 4 h. Comparatively, their standard method required

18 h due to the overnight incubation and resulted in only a 40% recovery. In addition to the greater processing times required, the resulting PEGconcentrated material was highly viscous and separated during −80 °C storage. Finally, the PEGconcentrated pellet was not as compact as the IsoTag™ LVconcentrated pellet, so the researchers were unable to concentrate and purify their LV to the same extent. It has been shown that contaminant removal is an important step to produce LV vectors able to generate reproducible results [12]. The research group at Duke University was able to remove 99% of the contaminating host cell proteins using the IsoTag™ LV reagent, a strong indicator that IsoTag™ LV was able to precisely bind the vector target using the ALPS-CF process. In summary, the IsoTag™ LV reagent effectively concentrates and purifies crude LV harvest material for research use rapidly, reliably, and robustly.

Future work on this novel reagent will include investigating the IsoTag™ LV reagent for use with other VSVG pseudotyped vectors or nanoparticles, performing studies at larger scales, and developing an efficient elution process to separate the IsoTag™ LV reagent from the highly pure LV. The IsoTag™ LV reagent binds to VSVG, so this process is not compatible with LV pseudotyped with other surface proteins but should be compatible with other VSVG containing particles. Furthermore, the mechanics of concentrating dense particles remain consistent, so larger volume processing should result in similar functional recoveries and contaminant removal. However, this remains to be tested. Last, adding an elution step to remove the IsoTag™ LV reagent will be necessary to adapt this protocol for clinical-grade LV production. While the IsoTag™ LV reagent demonstrated stabilizing properties, its toxicity *in vivo* has yet to be reported, although studies are ongoing.

Of note, the IsoTag™ technology is also under development for adeno-associated viral vectors and the ALPS process produces results comparable to the centrifugation-based process described herein for LV [16]. We envision that the centrifugation-based concentration method outlined here (ALPS-CF) would be used for volumes up to 1L, after which it would be advantageous to use TFF to quickly concentrate and purify the droplets based on droplet size rather than density and with a filtration process that is highly scalable (ALPS-TFF). Development of the ALPS-TFF process is under development and we look forward to detailing this work in follow-on publications. The combination of ALPS-CF, described herein, with an ALPS-TFF process, would enable this platform technology to bridge a significant gap between vector material quality and purification processes utilized at different manufacturing scales and accelerate the translation of innovative medicines into clinical and commercial use.

In summary, IsoTag™ LV is a lentivirus purification reagent without compromise. It is much faster, higher yielding, and more effective than other LV purification products designed for research use because of its specificity and unique stabilizing effect on LVs. Moreover, it can be implemented by any user, at any skill level, who has access to basic lab-scale centrifugation equipment.

MATERIALS & METHODS

Cell culture

HEK293T cells (ATCC) were cultured in T75 flasks in DMEM (Cytiva Cat#: SH30243.FS) supplemented with 10% FBS (SigmaAldrich Cat#: F0926500ML), 1 × NEAA (Gibco Cat#: 11140050), 1 × penicillin/streptomycin (SigmaAldrich Cat#: P4333100ML). Cells were grown in a water jacketed incubator at 37 °C, 5% CO_2, and passaged once reaching 80% confluency. Cells were discarded after reaching passage number 25.

Adherent lentivirus production

500 mL of 15e7 GFU/mL VSVG pseudotyped lentivirus harvest material with a GFP

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reporter was purchased from the Viral Vector Core at Duke University and was produced following the STAR protocol as published [5]. In brief, the protocol outlines crude lentiviral harvest produced from adherent HEK293T cells using a calcium phosphate-based transfection. The production method recommends using a second-generation packaging system which requires three separate plasmids for transfection: one for the gene of interest, one plasmid responsible for providing necessary viral proteins, and a VSVG derived (pseudo) envelope plasmid.

LV was produced by the academic partner using the following method: when HEK293 cells were at approximately 80% confluency, media was changed (DMEM HG, 1× Pen Strep, 10% FBS). At least 30 min after media change (up to 8 h after) cells were transfected according to the JetPrime (Polyplus Cat#101000015) protocol [21]. Briefly, plasmid DNA for PAX2 (9 µg per plate), VSVG (3 µg per plate), and the vector of interest (PRRL backbone, 18 µg per plate) were mixed with Jet Prime buffer (1000 µL per plate). Solution was vortexed for 10 sec. 50 µL of JetPrime reagent was added to the solution. Solution was vortexed for 1 sec and incubated at room temperature for 10 min. The mixture was added dropwise to each plate. 16 h after transfection, the media was changed to remove transfection reagents. Crude harvest material was collected 24–48 h after the media change. The harvested material was then spun down to remove cell debris and the supernatant was filtered with a 0.45 µm bottle top filter.

Suspension lentivirus production

Gibco™ Viral Production Cells (Thermo Scientific Cat#: A35347) cultured in a 125 mL baffled flask with 30 mL LVMAX™ Production Medium (Thermo Scientific Cat#: A3583401) passaged once reaching a density of 5×10^6 cells/mL. Cells were grown in a water jacketed incubator at 37 °C, 8% CO_2, with an orbital shaker set to 125 rpm. Production followed the LVMAX™ Production System (Thermo Scientific Cat#: A35348) protocol. Cells were cultured in 250 mL baffled flasks with a starting volume of 50 mL and grown to 4.7×10^6 viable cells/mL for transfection. A total of 2.5 µg of plasmid DNA is required for every 1 mL of culture. In one conical, 1.5 µg Lentiviral packaging plasmid (Thermo Scientific Cat#: A43237) and 1 µg lentiviral transfer plasmid were diluted in OptiMEM™ I Medium (Thermo Scientific Cat#: 11058021) totaling 5% of the culture volume. In a separate conical, the LVMAX™ Transfection Reagent is also diluted to 6 mL/mL transfection culture in OptiMEM™ I Medium totaling 5% of the culture volume. The diluted plasmid DNA is added to the diluted LVMAX™ Transfection Reagent and incubated at room temperature for 10 min and then added to the culture flask immediately after the LVMAX™ Supplement (5% total volume). 5 h post transfection, the LVMAX™ Enhancer is added (4% total volume). Crude harvest material was collected 48 h post transfection. Harvest material was spun down and filtered with a 0.45 µm bottle top filter to remove cell debris.

IsoTag™ LV production

Recombinant production and purification of IsoTag™ LV reagent is comparable to the previously described production process for the IsoTag™AAV reagent [18]. 50× stock solutions of the reagent were made using precise weights of lyophilized protein powders.

IsoTag™ LV Reagent ALPS-CF Protocol

Crude LV was mixed with 1× IsoTag™ LV reagent and incubated on ice for 5 min. Afterward, phase transition buffer was added to 1× final concentration, and the solution was heated to 37 °C for 5 min. The reaction mixtures were transferred to a benchtop centrifuge and centrifuged at 1000× g for 10 min.

The supernatant was removed without disturbing the pellet and saved for analysis. Cold PBS was added to the desired concentration factor, and the samples were left on rotators at 4 °C for at least 1 h until the pellet had resuspended. The academic partner performed three separate 20–80 mL 200× concentrations using LV vectors from three independent LV productions.

Infectivity & functional titer assay

HEK293T cells were passaged into tissue culture treated 24-well plates at a density of 1×10^5 cells/mL with 0.5 mL per well. The cells were allowed to adhere overnight. The next day, lentivirus encoding GFP was added to the media at the dilution required to achieve 20–40% GFP positive cells. Cells were incubated at 37 °C, 5% CO_2 for 48 h following infection. After 48 h, with the use of the EVOS M5000 imaging system, samples were observed at 4× with a specialty GFP (470 nm/525 nm) light cube to identify infected cells. Then, the media was replaced with Trypsin to remove the cells from the plate. Cells were washed and analyzed by flow cytometry on a BD Accuri c6 to determine the percentage of GFP-containing cells. Functional titer was measured using Green Fluorescent Units (GFU) per mL and was calculated by the following formula with the total number of cells equalling 1×10^5.

Percent recovery was calculated by dividing the calculated titer for the experimental sample by the calculated titer for the control crude harvest material and multiplying by 100.

HEK293 HCP ELISA & QuantiT™ PicoGreen™ dsDNA Assay Kit

The HEK293 HCP ELISA quantification was determined using the HEK293 HCP ELISA Kit (Cygnus Technologies Item# F650S) according to manufacturer's instructions.

The dsDNA quantification was determined using the QuantiT™ PicoGreen™ dsDNA Assay Kit (Thermo Fisher Cat#: P7589) according to manufacturer's instructions.

Lentivirus-associated p24 ELISA, PEGit, Mag4C, & LentiX Kits

The lentivirus associated p24 determination was done using the QuickTiter™ Lentivirus Titer Kit (Cell Biolabs Cat#: VPK107) according to manufacturer's instructions.

The comparison products PEGit™ Virus Concentration Reagent (System Biosciences Cat#: LV810A1), Mag4CLV (Oz Biosciences Cat#: LKC11000), and LentiX Concentrator (Takara Cat#: 631232) were all tested according to manufacturer's instructions.

Stability testing

For harvest material samples, lentivirus was diluted 1:4 in PBS with 1× IsoTag™ LV reagent added. A control sample with diluted LV only was included. For purified virus samples, purified LV was diluted 1:100 in PBS with 1× IsoTag™ LV added. A control sample with no IsoTag™ LV was included. For storage at 4–8 °C, a 100 µL aliquot was taken from each sample each week for functional titering. For freeze/thaw testing, 1 mL sample aliquots were frozen at −80 °C for at least 1 h. Aliquots were thawed at room temperature, and a 100 µL sample was taken for functional titer analysis. This process was repeated for an additional four freeze/thaw cycles. For ambient storage, samples were left at room temperature (22 °C± 3 °C) for 6 h and a 100 µL aliquot was taken for functional titer analysis.

Data availability statement

All data supporting the findings of this study are available within the paper and its supplemental material. Should any raw data files be needed they are available from the corresponding author upon reasonable request.

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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 his approval for this version to be published.

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ANALYTICS CHANNEL

CHANNEL CONTENT

Designing cell therapy analytics for future success

Raymond Luke
Verismo Therapeutics

"Navigating the challenges of training, compliance, scalability, and facility design with strategic foresight will be the linchpin to realizing the potential of cell therapies."

VIEWPOINT

Cell & Gene Therapy Insights 2023; 9(9), 1107–1110
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Cell therapy is a rapidly evolving landscape that has emerged as a groundbreaking approach with immense potential to revolutionize patient treatment. As this field continues to expand and demonstrate its efficacy in treating a wide array of diseases, the need for compliant, scalable, and simple analytics becomes increasingly evident. The journey of a cell therapy from idea to commercial product is complex and often has vastly different requirements depending on the stage of therapeutic development. In light of this complexity, the design of effective cell therapy analytics holds the key to unlocking the full potential of these therapies.

When it comes to analytics, three primary areas must be considered to ensure that it is ready for future success: training burden, compliance and comparability issues, and scalability. Training burden is a hurdle in designing cell therapy analytics because many of the methods that are used rely on deep subject matter expertise to be performed properly. This can be an issue because even with well-developed procedures and rigorous training programs, the amount of training [1] necessary for late-stage and commercial products can overburden the system. Complex methods also require much more in-depth investigations if troubleshooting is required which may not be widely available with an inexperienced workforce.

Compliance and comparability issues can become significant [2,3] concerns as products reach later stages of development. Oftentimes emerging therapeutics like cell therapies are born out of an academic setting and are rapidly driven into the clinic. This speed to clinic is important to ensure that companies can quickly show proof-of-concept of their therapies allowing them to either raise money to continue clinical development or be acquired. While this speed is paramount to survival it can also mean that assays that are acceptable in an academic and first-in-human setting must be altered for later phase biotech products. This is compounded by the likely scenario that the FDA will request additional assays as more knowledge of the product's critical quality attributes is discovered. This can lead to a scenario in which a company is entering a pivotal trial with inadequate assays and no efficient way to 'upgrade' them.

The final aspect that is often most overlooked is analytics capacity [4,5]. Throughput is widely considered when designing production processes [6–8] and facilities even though autologous cell therapy requires a considerably different strategy than traditional biologics. These strategies often rely on scaling out and, increasingly, automation, but these same considerations are often overlooked for analytics. Autologous cell therapy analytics are impacted by the simple fact that scaling out production leads to an increase in the number of batches that must be tested. This is compounded by the highly complex nature of the drug which often necessitates an increased amount of in-process testing.

While these challenges are significant, they are not insurmountable and with proper forethought, a true competitive advantage can be obtained. The most important aspect of forward-looking planning that a company can take is to ensure there is enough archived material to allow for changes in the future. This simple yet powerful idea ensures that companies can move quickly into the clinic using existing analytics but will have the ability to generate the data needed to compliantly change the assay. Important points of consideration when generating a future-proof sampling plan are ensuring that the proper substrates are archived and that the stability plan is adequate for their future use. Proper planning can ensure that any assay changes and new assays that are developed can be done compliantly while also facilitating changes that can improve the simplicity and throughput of the assay.

The next step in ensuring future success in cell therapy analytics occurs during the design phase of the new or improved assays. When designing the next version of an assay it is important to consider how it will need to be used in the future. Generally, early-stage cell therapy trials are relatively small in nature and can utilize complex assays that require deep subject matter expertise. This changes as a therapy approaches commercialization and even more so if the planned indication will have many patients. When this happens, considerations must be made to ensure that the assays are as simple and scalable as possible which can often mean replacing many of the instruments that are currently standard in cell therapy. For example, flow cytometry is used widely throughout cell therapy analytics, but this method is known for its variability from operator to operator and site to site. Therefore, it is important for a company to either

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design the assay to have controls in place for this variability and a well thought out training plan or to identify alternative methods to replace them.

Lastly, the aspect that is most often overlooked when preparing for later phase production is the design of the facility itself. For many reasons, the primary focus when designing the concept for a new production facility is on the cGMP production space itself. While accounting for the capacity restraints in production spaces is important, of nearly equal importance is ensuring that there is proper capacity for the planned analytics. Similar to the differences in scaling-up production versus scaling-out, cell therapy analytics do not benefit as much from economies of scale as traditional biologics. This often means that relatively complex analytics must be performed far more frequently than they are with any other therapeutic modality. Increased throughput is also combined with the need for rapid testing results to ensure shorter vein-to-vein time for the patients. To plan for future success, it is important to consider additional testing space when expanding or planning for higher capacity.

Navigating the challenges of training, compliance, scalability, and facility design with strategic foresight will be the linchpin to realizing the potential of cell therapies. By cultivating adaptable analytics grounded in well-considered training approaches, maintaining compliance through stages of development, designing assays for simplicity and scalability, and ensuring ample testing capacity, the trajectory of cell therapies can be charted toward resounding future success. In these considerations, we find the blueprint to amplify the impact of cell therapy, bridging the gap between visionary ideas and a groundbreaking clinical reality.

BIOGRAPHY

RAYMOND LUKE is Director of Manufacturing Science and Technology at Verismo Therapeutics and has more than 10 years of experience in CMC in biotechs. Prior to joining Verismo Therapeutics, Mr Luke spent 6 years at Adaptimmune developing TCR-T and next generation cell therapies targeting NY-ESO-1, MAGE-A4, MAGE-A10 and AFP against solid tumor indications He held leadership roles within CMC and was responsible for GMP manufacturing and technical oversight at CDMOs, technology transfer (sending and receiving) into cGMP manufacturing and technical oversight of manufacturing operations. He also led teams responsible for developing automated processing solutions, analytical development of commercial-ready assays and late-stage process characterization for afamitresgene autoleucel. Raymond worked in a variety of roles at WuXi AppTec, University of Pennsylvania, Cancer Institute of New Jersey, LifeCell, and Chromocell. Mr. Luke holds a Bachelor of Arts Degree in Genetics from Rutgers University.

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CELL & GENE THERAPY INSIGHTS

VIEWPOINT

How to expedite clinical development of oncology agents: overcoming complexities of early-phase oncology trials for ATMPs

Dr Harriet Gray Stephens
Pharmaceutical Physician, Boyds

"Drug developers must develop ATMPs in a time- and resource-efficient manner to maximize their assets' potential. A close understanding of clinical development trends and regulatory opinions is central to this process..."

VIEWPOINT

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In this viewpoint article, Dr Harriet Gray Stephens MFPM, Pharmaceutical Physician at Boyds, reviews the key complexities and emerging trends in early-phase oncology clinical trials for ATMPs which promote efficient early clinical development of effective therapies.

Therapeutic options for the treatment of cancer are rapidly evolving. Increasingly, highly individualized advanced therapy medicinal products (ATMPs) including immuno-oncology agents are being developed. These therapies aim to provide superior patient outcomes both in terms of survival and treatment tolerability. Success for the developer and patients alike centers around time-efficient early clinical development and early identification of agents that will not provide significant patient benefit.

Unlike other therapeutic targets, early-phase oncology agents are generally investigated in patient populations. Investigators can gain early evidence of effectiveness, pharmacokinetics, and pharmacologic effects as well as safety and tolerability. Some new therapies have demonstrated incredible responses in early-phase trials [1], resulting in regulatory approval earlier on in clinical development than is traditionally seen. Regulatory authorities are providing additional support to these breakthrough ATMPs to assist with their streamlined development. As a result, some companies are choosing to focus more on earlier development. This shift introduces significant complexities in all aspects of clinical development.

APPROPRIATE DOSE SELECTION

Phase I oncology trials traditionally select dose levels by evaluating toxicity as part of dose ranging studies. They aim to estimate the maximum tolerated dose (MTD): the highest dose that can be administered with an acceptable level of toxicity and thus, the recommended dose for further studies. The classical rules-based 3+3 design remains the most widely used method: patients are recruited into cohorts with sequentially increasing doses, However, if a toxicity is seen, further patients are dosed at that dose level to extend the investigation of the drug's effects at that dose.

Rules-based study designs have significant limitations when applied to ATMPs because generally, fewer toxicities are expected. MTD-based studies rely on the assumption that toxicity and safety are directly correlated with anti-tumor activity- the higher the dose, the greater the biological effect but also the greater the toxicity. This makes evaluation based on MTD less relevant and more difficult owing to a lower incident frequency which can result in dose selection above an optimal therapeutic dose simply because toxicity has not occurred. Drug developers are increasingly shifting towards identification of optimal biologic dose (OBD) or minimum effective dose (MED) based on biological outcome data including biomarkers and efficacy endpoints.

If developers decide to still use MTD toxicity assessment, in combination with other endpoints for optimal dose selection, there are alternative clinical trial designs to 3+3 trials that are better able to detect MTD. These methods use models rather than rules, including "continual reassessment method" (CRM) and the "Bayesian optimal interval design" (BOIN). Better MTD determination occurs as a model is developed, which integrates a lot more information into the prior, potentially giving a more complete view of events occurring during the clinical trial. There is significant emerging evidence that the use of these novel designs of early-phase clinical trials can both reduce clinical development timescales and enhance the proportion of effective agents that have successful late-phase clinical trials [2].

BIOMARKERS & CLINICAL ACCEPTANCES

Establishing the OBD or MED can be problematic. Assays must have been developed

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and validated for use during the clinical trial if they are to be used to aid dose escalation decisions. Assay development is time- and resource-heavy but must be prioritized early on in clinical development.

Regulatory requirements for drug approval require substantial evidence of clinical benefit. Indeed, increased overall survival (OS) remains the gold standard in oncology. However, determining an improvement in OS is a time-consuming process. Accelerated approval increasingly relies on the use of surrogate endpoints such as tumor biomarkers. Thus, early biomarker development can assist with the data package for regulatory discussions even early in clinical development–in particular, by providing additional justification for the validity of these surrogate endpoints as an alternative to long-term clinical data. A good understanding of the regulatory requirements and incentives available for approval is helpful even at the early stages of clinical development in order to be able to adapt clinical development plans to meet the eligibility criteria for such regulatory incentives.

TIME CONSTRAINTS FOR CLINICAL ENDPOINTS

Dose escalation studies aim to minimize the time between dose escalation cohorts to enable time-efficient dose escalation and thus, dose selection for subsequent clinical trials. However, clinical outcomes data take additional time to be determined and therefore, may not be available during the dose escalation decision-making process. This is particularly important with ATMPs, where there is an increased incidence of delayed or late adverse reactions compared with traditional cytotoxic drugs. Careful consideration of the minimum data required and timescales for the development are needed to accurately determine OBD or MED. Clinical developers need to carefully consider timelines to avoid delays whilst maximizing the chance of optimal dose selection for late-phase development.

To overcome this, multiple expansion cohort trial designs are becoming more acceptable. These trials are intended to expedite OBD or MED dose selection by using concurrently accruing dose cohorts, rather than sequential cohorts. However, rapid enrolment generates the risk that many subjects are exposed to drugs with minimally characterized toxicity profiles including at higher doses than with a traditional 3+3 design.

To minimize this risk, it is important to carefully evaluate the benefit-risk of such designs in each investigational ATMP: the FDA has issued guidelines [3] to assist this process. The patient population should be a limited number of patients with serious oncologic disease with a strong rationale for investigation. Additionally, the characteristics of investigational drug products for suitability of such trial design should be evaluated: products with steep dose-toxicity curves and large interpatient variability should be avoided. If multiple expansion cohort design trials are to be used, the key is to have established a good infrastructure to streamline data collection and review, with plans to rapidly assess and disseminate. If toxicities occur, then the study should be put on clinical hold.

USE OF MULTIPLE DRUGS SIMULTANEOUSLY

Increasingly, early-phase oncology trials are incorporating either multiple drugs or multiple technologies where the dose of multiple agents may be varied. For example, cellular therapies may require conditioning chemotherapy regimens for lymphodepletion or cellular harvesting. Multiple different combinations of conditioning regimes exist, some with significant morbidity. This can make the determination of causes of toxicities difficult. Historically, the sponsor may select combinations with very well documented toxicities. However, this significantly limits the number of combinations that can be used and excludes the use of newer

therapies or combinations that may provide better toxicity profiles. Furthermore, this approach is not possible with some newer cellular therapies which require complex conditioning regimes.

Umbrella trials are useful to overcome these restrictions and evaluate combination treatments. Patients are recruited under a master protocol, which allows for testing multiple agents in parallel and may include specified modifications while the trial is ongoing. This can accelerate drug development and enable the evaluation of synergistic combinations. However, some studies have found that there is no enhanced patient benefit in terms of response or reduced toxicity when umbrella trials are used [4]. Where multiple agents are used, careful sponsor evaluation of the known characteristics of the individual and combination agents is required to optimize the design of umbrella trials for efficient early clinical development.

PATIENT RECRUITMENT

Increasingly, genomic sequencing is being undertaken to elucidate specific mutations present in multiple different cancer types as targets for ATMPs, irrespective of tumor type.

Clinical trial design is moving away from cancer type-specified trials to mutation-specific trials through the use of basket studies and seamless expansion models. Patients with multiple different types of cancer with the same molecular target of interest are recruited to the same protocol. This can facilitate more rapid recruitment and provide preliminary information of tumor types with improved response, which can be included in additional expansion cohorts as part of a seamless expansion model. Multiple hypotheses and patient subgroups can be evaluated in parallel.

Basket trials have their limitations, including higher cost, significant administrative and regulatory burden from multiple protocol amendments, and complex statistical design. The US FDA has published an editorial expressing its concerns over the rapid uptake of seamless expansion design trials, as it may increase the rate of therapies being given to large numbers of patients (equivalent to late-phase clinical trials) owing to inadequate review of early-phase data. The US FDA has suggested that additional safeguards should be in place to protect patients, including restricting this expedited, seamless expansion strategy to drugs that have been shown to have early clinical efficacy and thus, have been designated as breakthrough therapies [5].

PATIENT-CENTERED DRUG DEVELOPMENT

Traditional oncology agents, particularly cytotoxic agents, are associated with substantial toxicities that have a significant impact on quality of life. ATMPs may reduce the treatment burden on patients. This burden can be reported as patient-related outcomes (PROs) and are recommended to support new drug registrations, and this data is often collected during later-phase clinical development. However, PROs are notoriously difficult to report owing to a lack of standardization of data collection. Additionally, a significant overlap between disease and treatment symptoms makes it difficult to use this data.

Professional bodies have created value frameworks in order to provide standardized PROs that can be included in early-phase trials as key secondary endpoints [6]. More early-phase clinical trials are incorporating appropriate PROs during clinical trial design to generate this valuable data earlier in clinical development. This can be used to drive regulatory discussions surrounding significant patient benefit of the new ATMP therapy.

MANUFACTURING

ATMPs present additional manufacturing and distribution challenges for clinical trials. Patient-specific timescales must be accounted for in the clinical trial design to facilitate conditioning, harvesting, and manufacturing of patient-specific therapies.

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Achieving GMP-compliant ATMP manufacturing can present sponsors with significant hurdles: a limited number of manufacturing sites exist worldwide, with few sites being able to meet the complicated manufacturing requirements of cellular production processes. Sponsors need to be aware that the investigational therapy may be manufactured in a different continent! Careful individualized product tracking is required: sites must be compliant with regulations including good distribution practice. Compliant cold supply chain management is required to ensure high quality treatments are received and to prevent patient-product mismatch, which would have catastrophic consequences. Prior experience in both setting up manufacturing and managing the supply chain is key, owing to narrow treatment windows due to the complexity in preparing a patient for receiving treatment and fitting it around other treatments.

CONCLUSION

ATMPs provide significant advantages over conventional cytotoxic chemotherapies but are notoriously difficult to develop. The clinical development landscape is highly pressured. Drug developers must develop ATMPs in a time- and resource-efficient manner to maximize their assets' potential. A close understanding of clinical development trends and regulatory opinions is central to this process, including the trends discussed in this article. Cell and gene therapies are the future of personalized, optimal oncology care, meaning optimal clinical development is fundamental to maximizing patient benefits.

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CELL & GENE THERAPY INSIGHTS

COMMENTARY

A new era in medicine: unraveling the evolution and future of gene therapy

Mai Tanaka-Wakefield, Jonathan Ark, Yuvaraj Gambhir, Ishmael Qawiy & Geeta Vemuri

Over the last decade, gene therapy has rapidly evolved and has led to seven gene therapy approvals since 2017 by the US FDA. These gene therapies are potentially cures for previously incurable diseases. The field has gained tremendous interest from various stakeholders, including patients, providers, investors, and pharmaceutical companies. These therapies will continue to hold promise in the future with further innovation in durability of response, re-dosability and improvements in delivery methods. Regulatory agencies and payers are also seeking to support the growth of gene therapy commercialization through initiatives and innovative models. This commentary aims to highlight progresses made to-date and provide a window into next-generation approaches in discovery and development that may deliver meaningful advantages.

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INTRODUCTION

There are over 10,000 identified rare diseases [1]. These diseases are associated with particularly high unmet medical needs, due to lack of accurate diagnostic tools, inadequate access to newborn screening, and limited treatment options [1–3]. Gene therapy holds great promise as a curative therapy particularly for rare, monogenic diseases of which there are over 6,000 known diseases [4]. As of June 2023, 15 cellular and gene therapies have been approved by the US FDA, of which seven are gene therapies approved for rare diseases (Table 1). Currently, the FDA-approved therapies use viral delivery vehicles (Table 1),

TABLE 1

Seven FDA-approved gene therapies since 2017.

Asset/developer	Year FDA approved	Indication	Delivery method
Luxturna® Spark Therapeutics	2017	Patients with confirmed biallelic *RPE65* mutation-associated retinal dystrophy	AAV2
Zolgensma® Novartis Gene Therapies	2019	Pediatric patients less than 2 years of age with SMA, with bi-allelic mutations in the *SMNI* gene	AAV9
Hemgenix® UniQure	2022	Adults with hemophilia B (congenital Factor IX deficiency)	AAV5
Adstiladrin® Ferring Pharmaceuticals	2022	Adults with high-risk BCG-unresponsive NMIBC with CIS, with or without papillary tumors	Adenovirus
Vyjuvek™ Krystal Biotech	2023	Patients 6 months of age and older with dystrophic epidermolysis bullosa with mutation(s) in the collagen type VII alpha 1 chain (*COL7A1*) gene	HSV-1
Elevidys® Sarepta Therapeutics	2023	Ambulatory pediatric patients aged 4 through 5 years with DMD, with a confirmed mutation in the *DMD* gene	AAVrh74
Roctavian™ BioMarin	2023	Adults with severe hemophilia A (congenital factor VIII deficiency with factor VIII activity <1 IU/dL)	AAV5

BCG: Bacillus Calmette-Guérin; CIS: Carcinoma *in situ*; DMD: Duchenne muscular dystrophy; NMIBC: Non-muscle invasive bladder cancer; SMA: Spinal muscular atrophy.

including AAV, adenovirus, and herpes simplex virus (HSV). Here, we provide our perspectives on key elements and learnings from first generation gene therapies, as well as the outlook of the gene therapy market.

EVOLUTION OF FIRST-GENERATION GENE THERAPIES

Gene therapies are defined as the introduction or removal of genetic material or modification of gene expression to alter the biological function of an individual's genetic code with the aim to achieve a therapeutic benefit [6]. In general, types of gene therapies include: gene replacement, gene edition, gene inhibition, and gene editing. All of these approaches require the transfer of genetic materials into cells. As such, delivery method is one of the key aspects for gene therapy. Myriad of safety concerns can emerge depending on the delivery vehicle. For example, viral delivery methods, such as AAV, can elicit T cell responses that are sometimes toxic or immunogenic [6]. A pivotal, phase 3 study for uniQure's Hemgenix for hemophilia B was placed on clinical hold in December 2020 after a patient was diagnosed with hepatocellular carcinoma [7]. However, the hold was lifted in April 2021, and received FDA approval in November 2022, after concluding that the hepatocellular carcinoma was not likely related to Hemgenix treatment based on vector integration site analyses and whole genome sequencing [8]. Similarly, Pfizer's PF-06939926, an investigational, recombinant AAV9 gene therapy for Duchenne muscular dystrophy (DMD), experienced a clinical hold following three "severe adverse events of muscle weakness, two of which involved myocarditis (inflammation of the heart tissues)" according to Pfizer's press release [9]. Currently the field is using immunosuppressive corticosteroid regimens to mitigate such T cell responses [10], but the field will likely find virus types that will not require such immuno-suppressive agents.

Viral gene therapies have been a step forward in addressing the residual unmet needs for many rare diseases. Nevertheless, viral delivery methods can preclude significant proportions of the treatable population from receiving the therapy. Some patients cannot receive viral gene therapies due to their pre-existing humoral immunity [11]. To combat these issues, novel technologies are in development to mitigate these limitations. For example, a

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clinical trial is underway to assess the safety and efficacy of efgartigimod alfa-fcab (Vyvgart) to lower pre-existing AAV antibodies, which currently precludes some patients with DMD from receiving gene therapy [12]. Vyvgart is an approved drug currently used for the treatment of autoimmune diseases, and acts by blocking the recycling of IgG through the FcRN (neonatal Fc receptor), thereby reducing overall levels of circulating IgG antibodies [13]. Similarly, Sarepta is exploring to eliminate pre-existing neutralizing antibodies against the delivery vector for their recently approved DMD treatment, Elevidys, through pre-treatment with Hansa's IgG cleaving protease, imlifidase. Imlifidase has conditional marketing approval in Europe for the use prior to kidney transplantation in adults who are considered highly sensitized to diminish serum IgG [14]. If these studies yield positive results, it could potentially expand the eligible patient population that is currently not qualified and offer the possibility of administering the treatment multiple times.

Most of the approved gene therapies are administered once. Immune responses generated post-initial administration currently make it impossible for repeat dosing if the first dose proves insufficient or lacks durability of response. This is especially germane to pediatric patients who may need additional dosing as they grow into adulthood. For viral gene therapies, vector type and cellular turnover rate are relevant and key considerations of therapeutic durability. In tissues with high cell turnover, such as the liver, systemic, non-integrating gene therapies, like AAV, may result in significant vector dilution over time from tissue proliferation and organ growth [15]. Nonetheless, there is significant excitement in the field as Novartis announced that Zolgensma demonstrates sustained durability up to 7.5 years post-dosing from their two long-term follow-up studies [16]. Long-term follow-up studies from the other approved gene therapies will surely guide expectations for therapeutic durability, as well as the need for re-dosability, which are key considerations from cost perspectives to patient safety. From safety perspectives, a recently approved gene therapy, BioMarin's Roctavian for the treatment of severe hemophilia A, first received a complete response letter from FDA in August 2020 [17]. The complete response letter questioned the therapeutic durability, as the phase 3 data showed year-over-year decreases in average levels of factor VIII, suggesting this is a one-time therapy that is unlikely to last a lifetime. These concerns were addressed with an FDA approval of Roctavian in June 2023, after BioMarin demonstrated two additional years of phase 3 follow-up data [18].

From manufacturing perspectives, gene therapy manufacturing is complex, necessitating reproducible and scalable processes. For viral gene therapy manufacturing, the use of mammalian or insect producer cell lines may impact manufacturing efficiency, resulting in variations in virus production per batch [19]. Reliable reproducibility is also important to minimize batch-to-batch variation of these vectors. Indeed, advancements in viral vector purification techniques, such as suspension cell bioreactors, tangential flow filtration, and affinity/ion exchange chromatography, offer promising solutions to enhance vector yield and purity [20]. These analytical advancements are also supported by presence of CDMOs specializing in cell and gene therapies. These innovative technologies offer promising solutions and a step forward in overcoming manufacturing challenges and expanding accessibility to companies pursuing gene therapies. However, cell and gene therapy CDMO facilities have faced headwinds. Lonza, for example, reported lower margins than expected during their Capital Markets Day presentation in October 2023. Hence, it is worth monitoring these trends to understand commercial challenges faced by these opportunities.

REGULATORY & PATIENT ACCESS CONSIDERATIONS

Market sentiment has been influenced by the changing regulatory environment. At the 2023 Muscular Dystrophy Association Clinical and Scientific Conference, the FDA announced

plans to establish an Operation Warp Speed-like initiative for rare diseases to optimize access to gene therapies through the accelerated approval pathway. Operation Warp Speed was a federal effort that supported rapid the development of COVID-19 vaccine candidates [21]. Under this Operation Warp Speed for Rare Diseases, the FDA aims to have cell and gene therapies for rare diseases enter the market quickly without compromising their safety and efficacy. The FDA has begun using surrogate markers (e.g., biomarkers) as substitutes for other biological indicators in gene therapy trials as a part of the accelerated approval pathway [22]. Sarepta's Elevidys, a one-time gene therapy delivering micro-dystrophin, received an accelerated approval using expression of micro-dystrophin at 12 weeks as a surrogate endpoint [23]. Sarepta is completing its confirmatory trial, which is fully enrolled with topline results expected in late 2023. However, surrogate endpoints may pose questions around biological significance as was the case for Elevidys, where some of the FDA's Cellular, Tissue and Gene Therapies Advisory Committee (CTTAC) panelists raised concerns about the predictability of micro-dystrophin expression as a surrogate endpoint for clinical response.

Given the establishment of the Operation Warp Speed for Rare Diseases, there is an urgent need to effectively generate and interpret more robust clinical outcomes data so that decisions are based on compelling scientific evidence. For example, patient advocacy groups placed pressure on FDA for the recent accelerated approval of Elevidys to treat DMD patients. Leading up to the approval, the committee heard from seven parents of children who received Elevidys in a clinical trial and observed dramatic improvements in function and overall quality of life, but none from parents who saw little to no benefit. While this greenlight is celebrated by patient advocacy groups, the label from this accelerated approval is limited to DMD patients aged 4 through 5 years [24]. Hence, some patients and patient advocates believe that the approval is too narrow and excludes older, non-ambulatory patients. On the contrary, therapies like Hemgenix captured a broader label covering all adult hemophilia B patients compared to the clinical study population [25]. This adds to payer concerns on the clinical outcomes for the label population and broader financial implications.

Post-approval challenges include a successful commercial launch. For example, Bluebird Bio left the European Union (EU) market in 2021, after failing to reach agreements in Germany for Zynteglo, intended to treat beta thalassemia [26]. BioMarin has not disclosed whether a commercial patient has been dosed with Roctavian in the EU even though it was approved last year. In Germany, the national price for Roctavian has not yet been finalized, and the company is not pursuing outcomes-based contracts.

Pricing and patient access are key factors driving the slow commercial uptake, as gene therapies thus far have been associated with high one-time prices. An analysis by the Institute for Clinical and Economic Review (ICER) suggests that the average cost of a gene therapy is between US$1–2 million per dose [27]. BioMarin's Roctavian for the treatment of severe hemophilia A is priced at US$2.9 million, Hemgenix for hemophilia B costs US$3.5 million, Zolgensma for pediatric spinal muscular dystrophy costs US$2.1 million, and Luxturna for retinal dystrophy is priced at US$425,000 per eye (or US$850,000 for both eyes). These list prices have resulted in concerns about sufficient reimbursement and patient access. Although health economics and outcomes research support these prices by demonstrating long-term cost avoidance, there continue to be reimbursement and patient access issues. John Glasspool, a Venture Partner at Agent Capital, indicates that these one-time gene therapies are different from the "traditional 'pay-as-you-go' model, because there is an accrual of benefit. These benefits accrue over time, and this is associated with the acute cost."

To enable access to gene therapies, reimbursement contracts based on outcomes, risk, or predictability may help payers to manage these high-cost therapies. Various

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reimbursement models (e.g., subscription models, outcomes-based payments, outcomes-based rebates, outcomes-based annuities) are described elsewhere [28,29] and have also been incorporated into pricing strategies for gene therapies. For example, Zynteglo utilizes an outcomes-based rebates model, where Bluebird will refund up to 80% of the treatment's cost if a patient fails to achieve and maintain transfusion independence up to two years following infusion [30]. Separately, Spark has three payer programs for Luxturna to accommodate special circumstances [31]:

1. Outcomes-based rebate agreement if patients do not meet pre-specified thresholds at 30–90 days and at 2.5 years;
2. Buy-and-bill model where Spark sells directly to payers or payer's specialty pharmacies; and
3. Pilot subscription model (i.e., installment payments), proposed to Centers for Medicare and Medicaid Services (CMS)

These examples demonstrate a therapy can have one or more payment models, and that there is unlikely a single model that 'fits all'. It is crucial for companies at all stages to understand and evaluate payment models that align with specific constraints of each payer type. For instance, annuities or rebate models may be effective for some payers capable of making long-term commitments. However, these models may be less appropriate for Medicaid as individual states may struggle to accommodate such budgets [32].

To further support the growth of gene therapy innovation and access, the CMS and the FDA have enacted innovative models. CMS announced two new models: [33].

1. Cell and Gene Therapy Access Model, which seeks to create outcomes-based agreements with manufacturers; and
2. The Accelerating Clinical Evidence Model, which seeks to develop innovative payment methods for drugs approved under the accelerated approval pathway

These initiatives and models, along with the FDA's Prescription Drug User Fee Act (PDUFA) VII programs for real-world evidence, will potentially spark changes to the overall business strategies and ultimately bring value to patients and the healthcare system.

CONTINUED INNOVATION IN THE GENE THERAPY MARKET

From an investor perspective, viral vector-based therapies will continue to be a key area of interest, but there is an increased focus on other novel methods that can treat monogenic disorders. AAV delivery vectors have a limited packaging capacity that restricts the size and types of cargo, as well as the ability to redose, and patients targeted for treatment with these vectors often have pre-existing antibodies to them. Novel delivery modalities represent an active area of research by some academic labs and biotech companies, including other viral vectors, virus-like particle, lipid nanoparticles, among others. Carbon Biosciences, one of Agent Capital's portfolio companies, harnesses naturally occurring viruses that are phylogenetically similar to AAV, yet possess differentiated attributes including an improved immunogenicity profile and more generous gene packaging capacity stemming from their evolutionary variations. Carbon's lead program, for the treatment of cystic fibrosis, delivers the full-length cystic fibrosis transmembrane conductance regulator transgene to the lung. Joel Schneider, CEO of Carbon Biosciences, notes "Carbon's vector library is designed to tackle immunogenicity by not only developing vectors with enhanced tissue specificity, but by also creating vectors that are designed from parvoviruses native to a diverse range of mammalian species resulting in minimal pre-existing immunity for the majority of

patients." On top of that, Carbon Biosciences' vectors have on-target tissue tropism and reduced liver targeting, enhancing their immune evasive profile. Joel indicates that "the gene therapy field is going through a very critical transition period moving from first-generation technology to next-generation viral delivery approaches." These improvements and new discoveries will surely contribute to increasing patient eligibility, as well as long-term durability at reduced vector doses. As the field evolves, Joel postulates that "the most successful viral vector platforms will be the ones that can leverage the infrastructure and successes of the first generation but have the ability to go beyond where the earlier generation has left off".

Another area of exciting innovation includes the shift in the way viral gene therapies are being utilized. Agent Capital's portfolio company, Interius Biotherapeutics, repurposes lentiviruses to generate chimeric antigen receptors in vivo, helping to overcome the many issues facing cell therapies including manufacturing costs, patient risks by avoiding chemoablative conditioning regimens, and as such, increased patient accessibility. Interius does this by engineering the binding of lentiviruses so that they specifically transduce T cells. While the company focuses on oncology, Phil Johnson, CEO at Interius Biotherapeutics, notes that "this platform is applicable to many different indications". Phil hopes to engineer new cell types including the liver and beyond by swapping their T cell binder with other domains.

While all currently approved gene therapies employ viral delivery approaches, innovative new modalities are being aggressively pursued. In addition to non-viral gene therapy modalities, gene-modifying oligonucleotide therapeutics, for example, have seen thirteen approvals by the FDA since 2016. This class of therapies silences genes or alters the splicing of mutant genes to ameliorate disease. However, these are not without limitations including potential for innate immunogenicity, limited tropism, and durability. Gene modifying oligonucleotides are transient, which makes them less ideal for protein replacement strategies. However, new technologies that aim to extend the durability of expression are being explored using circular RNA (circRNA) constructs. Orbital Therapeutics is one of Agent Capital's portfolio companies that uses this novel technology to generate next-generation vaccines, immunotherapeutics and protein replacement therapies.

Not surprisingly, the promise of gene therapy as a rapidly growing area of innovation and a potentially curative intervention for a wide range of monogenic diseases has led to significant investments in this market over the last decade. The viral gene therapy market grew 625% from US$0.92 billion in private equity and venture capital financing in 2012 to over US$5.7 billion in 2020 [34]. The field has also gained tremendous interest from pharmaceutical companies, supplemented by an increase in the number of gene therapy mergers & acquisitions and partnership deals. However, big pharmaceutical companies have scaled back innovation in ultra-rare diseases, defined as diseases with prevalence less than one in 50,000 individuals [35]. This can have unfortunate consequences for innovation in the market, and considerations such as reimbursement and access of therapies to patients with ultra-rare diseases will play a part for commercial opportunity.

Patient attitudes toward gene therapy for rare monogenic diseases are largely optimistic, fueled by a hopeful vision for future advancements. This perspective is particularly prevalent among those affected by Duchenne Muscular Dystrophy (DMD). Agent Capital's summer intern, Yuvaraj (Yuva) Gambhir is a 21-year-old with DMD. He reflected on his treatment journey, stating: "When I was first diagnosed with Duchenne in 2003, there were virtually no treatment options ... Since then, I have witnessed the massive explosion of progress in the genetic therapy space."

Despite recent advancements, such as the approval of Elevidys, this individual

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emphasizes that, "there is still a significant unmet need for older patients with DMD and those who have antibodies to the viral vector."

This sentiment is echoed by another 23-year-old with DMD who, while acknowledging the benefits of current treatments like exon-skipping, remains hopeful for the potential of gene therapy: "I think that gene therapies will continue to become more effective and safer as time goes on."

Although these perspectives stem from their personal experiences with DMD, they reflect the broader sentiment of many patients suffering from a variety of rare monogenic diseases. The hope is that gene therapy, despite its current limitations, will evolve to provide transformative solutions for these devastating conditions.

Since Luxturna's approval in 2017, the gene therapy market has seen tremendous growth. Seven gene therapies have been approved by the FDA for non-redundant indications, and this innovative approach has been fueled by interest from investors, pharmaceutical companies, and patients. Although gene therapy holds tremendous promise, there are biological, clinical, manufacturing, and regulatory challenges. The field recognizes them and has begun to address many of these residual shortcomings through the next wave of gene therapies and regulatory strategies. As regulatory agencies evaluate and launch more gene therapies, companies will need to develop innovative go-to-market and patient access strategies to ensure equitable access to these novel therapies for patients.

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CELL & GENE THERAPY INSIGHTS

CONFERENCE REPORT

Current trends in ATMPs

Helena Strigård & Jonathan Royce

Having returned from a series of conferences staged in the spring of 2023, when barely any event missed the opportunity to highlight ATMPs through presentations and panel discussions, we felt that there is a general desire to grasp what this toolbox really means for those of us operating in life sciences. Consequently, we put together this overview of what's cooking in the space. The text that follows is based specifically on the talks and panel sessions held at the BIO International Convention 2023, with a 'sneak peek' forward to the upcoming Nordic Life Science Days, where ATMPs will again be at the top of the agenda.

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In the post-pandemic world where life science professionals are back to attending conferences, the big buzz has been around all of the possibilities that lies before us now that we have opened up the advanced therapeutic medicinal products (ATMP)/Cell & Gene Therapy toolbox.

As of June 30, 2023, there were 14 approved gene therapy products and 18 approved cell therapy products on the market, with the most recent approval being BioMarin's Roctavian for the treatment of hemophilia. But there are also approximately 500 ATMPs currently in clinical trials [1], and the technology is developing quickly. We set out to look at a few trends that are developing in the industry.

Having returned from a series of conferences staged in the spring of 2023, when barely any event missed the opportunity to highlight ATMPs through presentations and panel discussions, we felt that there is a general desire to grasp what this toolbox really means for those of us operating in life sciences. Consequently, we put together this overview of what's cooking in the space. The text that follows is based specifically on the talks and panel sessions held at the BIO International

Convention 2023, with a 'sneak peek' forward to the upcoming Nordic Life Science Days, where ATMPs will again be at the top of the agenda.

What are the recent developments from a scientific point of view? And what stands in the way of being able to move even faster into applications that benefit patients?

DIVERSIFICATION IN DELIVERY PLATFORMS

Gene therapies are normally delivered via a viral vector—vehicles which carry the gene payload into a patient's cell. The most common viral vector used in approved products and clinical projects is the recombinant adeno-associated viral (rAAV) vector. rAAV vectors are known for their single-dose gene payload delivery, high transduction rates, broad tissue specificity, low immunogenicity, and long-lasting therapeutic effects [2]. However, rAAVs are not without limitations, including immune tolerance, limited size of the gene payload, and relatively high manufacturing costs compared to those for other marketed therapeutics [3].

These limitations mean that researchers have also investigated a number of other viral vectors for gene payload delivery, including lentivirus (LV) and herpes simplex virus (HSV). Skysona® (Bluebird Bio) and Vyjuvek™ (Krystal Biotech) are examples of gene therapies that use LV and HSV, respectively, as the viral vector. In the case of LV, transduction is traditionally achieved via *ex vivo* LV gene transfer followed by autologous cell transplantation. However, recent studies have demonstrated that LV may also carry promise for *in vivo* applications [4]. At the same time, HSV overcomes the payload limitations of AAV by increasing the maximum packaging size from approximately 4.7–130 kB (although the practical limit of HSV may be closer to 12 kB based on current data [5]). Another advantage of HSV is that its genetics and molecular biology are very well understood.

Whilst viral vectors are the current preferred method of gene delivery, rapid advancements are being made in other areas. Synthetic nanoparticles have shown promise due to their tunable size, shape, surface, and biological behaviors, although challenges still exist—most prominently, biodegradation [6]. Liposomes, commonly used to package cytotoxins in chemotherapy, have already been demonstrated to be useful in the delivery of nucleic acid-based therapies such as siRNA and mRNA [7], as well as showing promise for the delivery of more traditional gene therapy payloads [8]. Finally, exosomes are cell-secreted nanoparticles, which hold promise for highly targeted deliveries of gene payloads [9]. However, the bankruptcy of Codiak Biosciences, Inc. earlier this year (a leader in the exosome space) may slow progress in the short term for this modality.

SPECIALIZATION OF MANUFACTURING ORGANIZATIONS & TECHNICAL COMPETENCY

The fact that manufacturing methods for ATMPs are still relatively immature compared to other therapeutics such as antibodies, coupled with the aforementioned diversity of modalities in the space, means that manufacturing teams must adopt a high degree of specialization. Many developers of ATMPs rely on CDMOs to provide manufacturing capacity. However, global manufacturing capacity for ATMPs continues to be a bottleneck, as recently noted by Center for Biologics Evaluation (CBER) Director, Dr Peter Marks, who pointed to batch sizes, batch consistency, and limited supply of overall manufacturing capacity as issues that the industry must address.

It is likely that increased levels of specialization at the CDMO level, and standardization at the industry level, will help to address some of these issues. But experience in ATMP manufacturing is lacking at a global level, and talent acquisition remains an issue for many

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organizations. The demand for talent far outstrips supply today, and as a young and innovative industry, it is especially difficult to recruit experienced and qualified staff. Talent shortages exist right across the industry and at every level of seniority, from technicians and engineers to manufacturers and executives. And with hundreds of start-ups popping up in this space, the talent pool remains thin.

GLOBALIZATION OF ATMPs

For the past decade, ATMP development has occurred primarily in the US and Europe, but the globalization of these therapies is happening at a staggering pace. The *20th Annual Report and Survey of Biopharmaceutical Manufacturing Capacity and Production* notes that in China, for example, there are 400 cell and gene therapy products currently in various phases of clinical trials, whereas 5 years ago there were essentially none. This year's report (2023) also notes that 63.7% of respondents report that their facility is involved in gene and/or cell therapies, up from 58.6% in 2022 and 54.3% in 2021.

Elsewhere, South Korea, already a powerhouse in the global production of monoclonal antibodies, should not be underestimated when it comes to ATMP production. SK Group, Korea's second largest conglomerate after Samsung, has invested heavily in ATMP manufacturing and currently has seven production facilities and five R&D centers in the USA, Europe, and Korea. India has lagged conspicuously behind some of the other global production hubs for biologics when it comes to ATMPs, but the local experience with viral vaccine production could be leveraged to expand rapidly into the ATMP space. In late 2022, it was noted that a raft of new biotech startups in the country were focused on this area.

AS A RAPIDLY DEVELOPING FIELD, CHOOSING THE CORRECT ATMP PARTNER IS IMPORTANT

The market for ATMPs is expanding rapidly. Technology development, diversification, and globalization are all trends that will impact the industry in unforeseen (and positive) ways. Most companies in the ATMP space are partnering with raw material suppliers, CDMOs, and even quality consultants to ensure that their clinical candidates are produced reliably and consistently. Choosing the right partner can be tricky, but the importance of this selection should not be overlooked. In many cases, partnerships between organizations of similar size with common interests can be a key to success. A 'right-sized' partner can help strike the right balance between credible technical competency and focused attention, which can be hard to achieve with partners that are either too small or too large.

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CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Exploring & overcoming the challenges of cell & gene-modified cell therapy CMC

Karen Doucette, Julia Sable & Larry Bellot

Cell and gene-modified cell therapies hold immense potential for transforming healthcare, but they come with unique complexities in chemistry, manufacturing, and controls (CMC) that can result in setbacks, such as manufacturing comparability failure, clinical holds, product approval delay, and even manufacturing issues during post-market surveillance. As more therapies are being developed, it is crucial to explore and overcome the CMC challenges that can impede program and market progress. This article will focus on addressing CMC issues for cell and gene therapy (CGT) products through a robust process and an analytical development program.

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THE CGT MARKET

With more cell and gene therapy (CGT) products reaching later-stage development and commercialization, the field has a great opportunity to learn how to avoid common bottlenecks. A McKinsey report published in *Nature* in 2022 compared the number of CGTs and monoclonal antibodies entering phase 3 trials over the last five years. There were a similar number of total programs for each modality—around 100—and each had a similar number of issues in efficacy at 17%. However, it was found that CGT products have about twice as many disruptions from safety issues, which can be addressed through better *in vivo* models and continued animal model improvement, and a fourfold increase in chemistry, manufacturing and control (CMC) issues, which will be the focus of this article.

Within the CGT market, *ex vivo* gene-modified cell therapies are the fastest-growing segment due to the regulatory precedent of several chimeric antigen receptor (CAR)-T cell therapy approvals beginning in 2017. *Ex vivo* genetic modification requires special considerations for not only cellular processing and

handling but also for the plasmids and viral vectors used to modify those cells.

VIRAL VECTOR PLATFORM DEVELOPMENT

In viral vector platform development, scale increases as progress is made toward commercialization. Research projects tend to be in-house for the ease of R&D work, and often occur in adherent platforms. It is important to consider the possibility of suspension work and moving into the final media choices as early as possible. In smaller biotech's, this is often left until after process development (PD), which slows down the path to commercialization. Larger pharma tends to go to market faster because they often begin with suspension and scale up on platforms. In the research phase, if work is in-house, many places do not have the technical capability to handle full-scale PD, so people look to viral vector platform development at a contract development and manufacturing organization (CDMO). After proof of concept and phase 2, people often choose to either bring PD back in-house or work with a commercial-ready CDMO.

In the R&D to CMC PD transition, key considerations surround the importance of establishing a robust supply chain for viral vector-based therapies. As a developer, it is important to consider phase-appropriate sourcing and quality management systems (QMS). Considering quality at the research stage will ensure that sustainable choices are made, and no later changes to aspects such as media selections are necessary. Representative analytical materials should be produced at a sufficient scale as early as possible. In the upstream, enhancers, gene of interest optimization, ratios, and stirred-tank reactor conditions using perfusion can be explored early, but they must be performed between the research and PD stages. Downstream, considerations around unit operations including sterile filtration, impurity removal, and final formulation studies must be made. Plasmid sourcing decisions made early can impact timelines long term. This includes the timing of master cell bank production and stability studies. If single-use plastics are selected, considerations surrounding machine design and ordering timelines will be needed.

In the PD to MSAT transition, the aim is to establish best practices for process optimization, technology transfer, and manufacturing scale-up to ensure successful commercialization while maintaining product quality, safety, and efficacy. When scaling up viral vector processes in particular, one must consider all the areas of possible failure within the process. Since experimental studies cannot be performed on a full commercial scale but only on scale-down models (SDM), the SDM needs to be qualified to ensure that it is representative of the full commercial-scale system. SDM qualification is commonly performed by comparing the results of the SDM with data on full scale but only under target conditions. Process performance qualification (PPQ) runs are often used for this purpose. However, to achieve statistical significance, phase 3 manufacturing runs are often included as well. Stirred-gap assessment and design of experiments (DoE) are key parts of this process characterization. Process characterization in a scale-down model leads to the technology transfer of the process in the final scale to MSAT. Within manufacturing and documentation, communication with the quality control and quality assurance teams is necessary. Finally, phase-appropriate embedding of the PD team with the MSAT team is needed to ensure that the handoff is complete and smooth.

CELL THERAPY PLATFORM DEVELOPMENT

There are many similarities between viral vector and cell therapy platform development, including starting in-house. Cell therapy platform development requires many early choices to be made in both 7 to 14-day processes and <7-day accelerated platforms,

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though process optimization and gap analysis are the same. Ultimately, the goal is to have a fully closed and preferably fully automated process in place.

The transition from research to PD typically involves the adoption of a platform that closes the process and can support good manufacturing practice (GMP) processing. The selection of a platform at this time allows for significant process performance data in phases 1 and 2 through pivotal that will inform process performance qualification limits and specifications. The appropriate platform, whether semi-automated or fully automated, minimizes manual manipulations and allows for formal process monitoring which both aid in improving the consistency of the process.

Additionally, the selection and sourcing of raw materials, which includes items such as cytokine, beads, and media, is also a consideration during development. Decisions pertaining to the final formulation of the drug product, which includes media and excipients, final product content, and whether final cryogenic storage is necessary, also need to be made. The considerations are generally made in line with the dosing and distribution logistics of the product.

For allogeneic processes, appropriate donor selection and establishing a donor pool to deliver apheresis materials also contributes significantly to building consistency into the process. As with the selection of a platform, understanding the characteristics of donor material that are critical for process end goals early in process development is key to building a consistent program.

Prior to commercialization, cold chain logistics, phase-appropriate sourcing, and manufacturing capacity must be figured out. The expectation is that the criticality of the raw materials sourced has been assessed and that the level of control in preparing the raw material is consistent with commercialization. Moreover, commitment to critical processes and analytical equipment also necessitates ensuring a consistent supply chain long-term due to the possibility of long lead times. On the upstream side, considerations include cytokines, different media formulations, and different reactor types for cell expansion. Downstream, a major decision is beads, bags, or no selection. Final formulation studies and final cryogenic storage are then considered.

Both traditional 7–14-day processes and those of less than 7 days are accelerated in support of the selected platforms whilst ensuring redundancy in equipment availability.

PLASMID & VIRAL VECTOR CMC

Phase-appropriate sourcing of materials used in manufacturing is critical because the time, cost, quantity, and quality requirements vary throughout the product development lifecycle. This is of particular interest for plasmids because of their critical nature as they carry the genetic payload of the final drug product. The general approach is to use research-grade material through the early R&D stages, and then scale up to a high-quality material for investigational new drug (IND)-enabling and early-phase clinical trials with GMP production in place in time for pivotal clinical trials and commercial application. In the CGT space particularly, pivotal trials happen much earlier in clinical development than for traditional small and large molecules. Getting to GMP as soon as possible is important to meet pivotal trial expectations.

Different groups have different approaches to and definitions of the high-quality material needed before full GMP. There is an evolving regulatory landscape around what is needed at the various stages, and there is limited guidance to date on the plasmids used as starting material for CGT products versus the final drug product. The Europeans Medicines Agency has issued a Q&A document summarizing when GMP principles should be applied to starting material. This document speaks to the importance of creating master and working cell banks for plasmids and viral vectors and applying GMP principles when these are used as starting material. Although high-quality material may not be produced in

a GMP facility, it will follow GMP principles such as using defined procedures, well-characterized analytics, and good documentation practices.

Along with cell banks, consistency in packaging plasmid production will also support quality grade changes. For example, in lentiviral production for CAR-T or other gene-modified cell therapy products, the four required plasmids are relatively universal envelope and helper plasmids, with only the gene of interest being custom for each product. In this case, having a phase-appropriate off-the-shelf option for these universal plasmids reduces time, cost, and risk significantly so developers can focus on the therapeutic gene of interest. This can be approached by testing the various grades of plasmids with the same methods, particularly for the packaging and helper plasmids. Acceptance criteria can be more relaxed for research-grade material.

The regulatory landscape for CGT, specifically for *ex vivo* gene-modified cell therapies, continues to evolve. Recommendations for this product type are spread throughout several key guidances, the first of which for potency tests was issued in 2011 and covers all CGT modalities focusing on the potency assay itself. The gene therapy CMC guidance from 2020 is also highly relevant since *ex vivo* gene modified cell therapies are technically gene therapies according to the FDA. The final relevant guidance is the 2022 draft guidance specific to CAR-T products which covers recommendations for both preclinical safety and efficacy requirements and the CMC development for CAR-T products in particular.

POTENCY ASSAY CONSIDERATIONS

Potency assays are covered in a specific guidance, speaking to the importance of this assay and the challenges that it presents. A potency assay must reflect the product's mechanism of action but also correlate to the safety and efficacy data generated in preclinical models and clinical studies. An orthogonal approach is critical because it is often unfeasible to demonstrate the mechanism of action for a complex biological product in a single assay. In the ideal situation, a potency assay will be developed early on to represent the mechanism of action based on various components: the capsid, the viral vector type, the gene of interest, and any armors or switches (for multi-cistronic vectors) that go into the cell product. A qualified potency assay would ideally be used to analyze all preclinical lots that are manufactured and verified prior to commercialization. The data can be correlated to *in vivo* activity to rationalize the use of this assay for functional potency.

Typically, an assay is developed through the preclinical proof of concept stage which is too variable for qualification. This is often the case for *in vivo* assays where the complete physiological state is represented in a mouse model, but the assay is too variable and needs to be adapted to an *in vitro* system, which can cause delays. It is therefore advisable to pursue multiple assays in parallel as developers go through the process of finding a potency assay that accurately represents the mechanism of action, is quantitative, and is able to be validated. Technically, in line with the FDA guidance, only one potency assay is needed for lot release and stability if it fits all of these parameters, but in reality, a combination of assays may be more appropriate.

For example, for a CAR-T product, functional activity requires demonstrating on-target tumor effect and the absence of off-target effects on both tumor and healthy tissues. While this readout can be used as an early screen for activity and specificity during early product development, the data can be used to build a case for lot-specific characterization and release tests in the future. Having this type of preclinical assay developed early for initial proof of concept and screening exercises adds value down the road.

A further key issue with potency assays is ensuring enough characterization data is available to support it. Significant data must

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be collected to support the critical quality attributes (CQAs) along the way, which requires having the assays in place early. Having a consistent supply and/or manufacturing stream of assay reference material is often overlooked.

RAW MATERIAL QUALIFICATION

In raw material qualification, there are four key considerations: identifying raw materials, qualifying raw material vendors, performing raw material testing, and performing sampling where necessary. The classification scheme as suggested by regulators is defined by criticality in the form of GMP level. Critical materials are not produced according to GMP requirements, so high levels of testing and sampling are needed to ensure that those materials are kept within the defined CQAs for their use. High-risk materials include *in vitro* diagnostics and research-use-only materials. Lower risk items are manufactured at a GMP facility and are highly qualified materials that are licensed or approved products. Classifying materials allows necessary controls to be placed around the higher risk and critical materials.

For the majority of allogeneic cell therapies, the key raw material is the leukopak, and consistency in sourcing this is key. It is necessary to ensure a defined and vetted donor stream that is varied enough to support a trial. The output of the donors should be assessed to ensure that the right material for the process is being collected. It is also important to ensure that the site has the necessary quality regulations and procedures in place.

Market donor variability and its management are demonstrated in the case study given in Figure 1. Donor management directly impacts consistency, and inherent donor-to-donor variability can negatively impact PD. Having the ability to recall specific donors can aid in reducing some of the initial variability. Robust donor management ensures consistent cell yields from the same donor over time.

EQUIPMENT AND INSTRUMENTATION COMPLIANCE

Having an understanding of the instrumentation being utilized for a process and its analytics is critical for compliance. To meet 21 CFR Part 11 compliance, it is necessary to limit system access to authorized individuals and use operational systems checks, authority checks, and device checks. The use of legacy systems can cause problems, such as in translation to GMP or in a lack of traceability. Following the installation protocols and having an Installation Qualification (IQ), Operational Qualification (OQ), and Performance Qualification (PQ) validation performed are necessary to ensure compliance of an instrument. NIST traceable standards should be used for calibrations, and monitoring systems should be in place for critical instruments. The necessity of redundancy in equipment and instrumentation is often overlooked. Building a process around a single instrument is extremely risky within the realm of GMP.

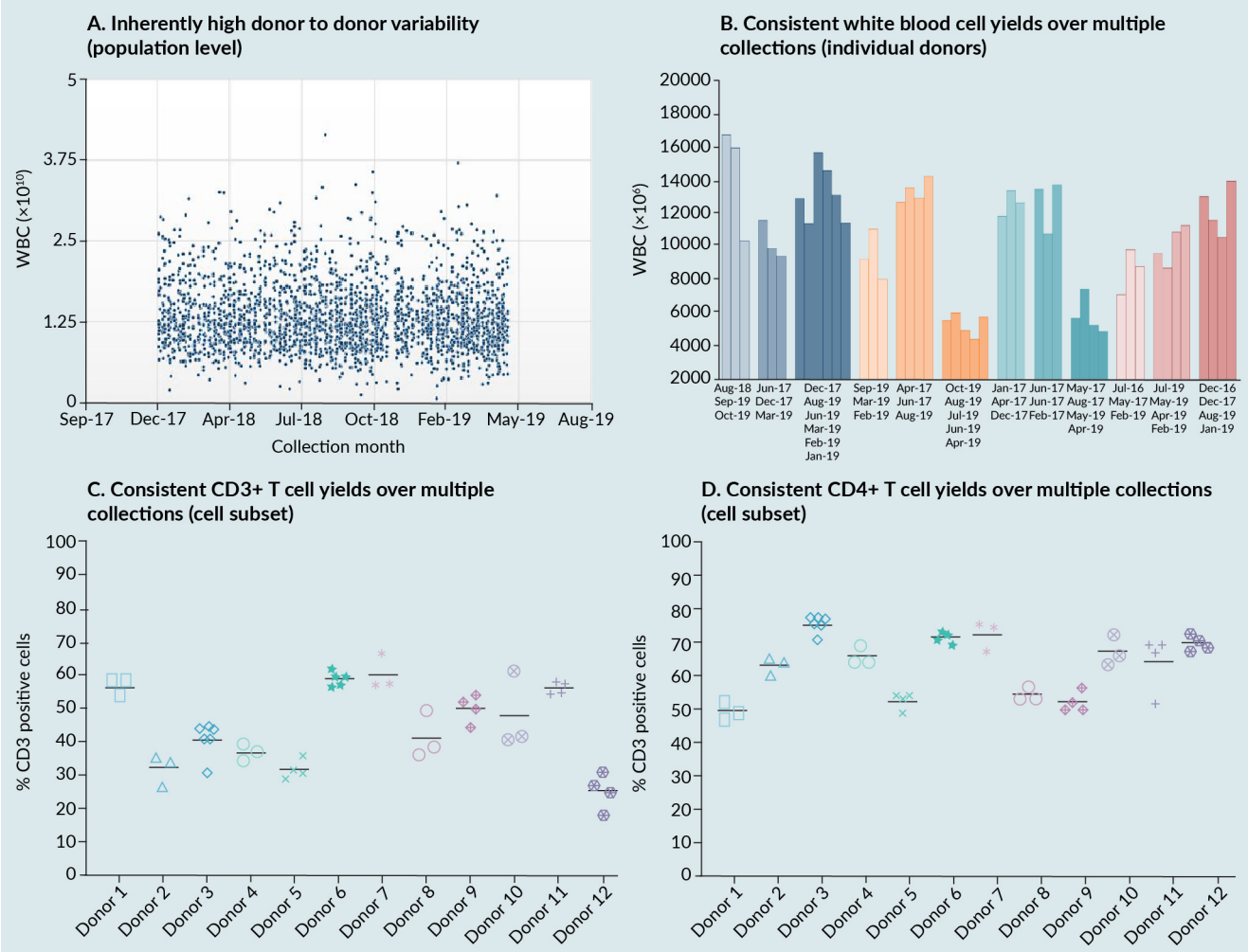
PD WITH THE END IN MIND: A SUMMARY

Charles River's development scientists de-risk scientifically complex cell therapies path to GMP readiness, through feasibility, optimization, and confirmation. This involves defining a development plan through process assessment and gap analysis, in addition to de-risking processes through process optimization and analytical assay development and qualification. Process robustness will be confirmed prior to transfer to GMP.

There is a push towards closed and automated processes in order to de-risk, in line with recent guidance from regulators. Process automation removes many risks associated with manual operator manipulations including the possibility of contamination, as does having closed processes. Ensuring process control and analytical technologies

FIGURE 1

Donor variability and consistent cell yields over multiple collections.



maintain control and monitoring of key process parameters allows the accumulation of process data for trending and better defining of critical process parameters.

The need for robust, efficient, and thoughtful analytical method development is critical to cell-based therapies. This can be accomplished through a well-defined project scope, being executed by a communicating, cross-functional team that is knowledgeable of process and analytics interactions. In terms of analytics, having a defined potency assay at the pivotal phase is key. This requires having an understandable and reproducible procedure, including clearly defined and characterized controls, and passing qualification and/or validation. Another frequently overlooked need is identifying and sourcing appropriate control/reference materials early in development. Inclusion of the control/reference materials in the method, including in fit-for-purpose testing and appropriate validation, is key to assay compliance. The whole assay suite for release must support the CQAs. This requires assays in place early on to allow sufficient characterization data to be gathered. To have an effective technology transfer, it is necessary to communicate all key points and positions to the manufacturing site to incorporate all the necessary updates, changes, equipment, raw materials, packages, and closure of the open process into that particular facility.

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Q&A



Karen Doucette, Julia Sable, and **Larry Bellot** (pictured left to right)

Q What comparability considerations do you suggest focusing on when changing to an automated system after phase 1?

KD: The FDA recently published draft guidance on comparability. The main consideration is to have a comparability plan from the beginning so that you know how many lots you will need to test pre- and post-change and to ensure your analytics are as robust as possible so that you can measure those quality attributes pre- and post-change.

If analytical methods are not yet qualified, having retains from previous lots so that you can go back and test them is critical. In the case of cell therapy products, particularly in the autologous space, doing comparability tests from the same donor is key. This involves gathering donor material and then splitting it in two to look at the pre- and post-change in parallel from the same starting material as that is one of the greatest sources of variability.

Q What are the biggest issues you have seen with critical raw materials?

JS: People often pick what they know early. This means they are not considering that the critical time between R&D and process development (PD) is the only opportunity to try as many options as possible. Something that looks the same on paper might not behave the same or may have other unintended side effects. This goes for both liquids and plastics. If the pandemic has taught us one thing, it is that supplier shortages can happen unexpectedly. Early in your PD work, try as many different things as you can.

Q Is there a common theme for where the most gaps are found in gap analysis for bench scale processes?

JS: Gap analysis is important but painful. There is no common theme because people make viral vectors under a lot of different manufacturing conditions. Gaps are highly dependent on your platform choices and your suppliers. They are also based on the culture of your company. Some companies are much more conservative, meaning risk assessments are more thorough and everything tends to take longer. Other, often smaller, companies can be nimbler and are able to go faster.

Q What is the most important consideration when choosing a CDMO for a successful tech transfer?

JS: This depends on what you are making. If you are making an autologous product, you want to find a place that has a lot of flexibility in terms of different manufacturing. Long term, looking for someone who has commercial capabilities early is probably the single best piece of advice I can give. It is very expensive in terms of time and cost to start over and move to someone else with those capabilities.

Q Where do you think digitization plays a role in improving the risk profile in cell therapy development and manufacturing?

LB: Digitization can affect the process of your batch production record (BPR) monitoring so that you can get a better understanding of your PD track. Digitization means you are not pushing paper around or having to move paper through the suites. Since many of these early processes are open, it de-risks the process overall.

The other thing that can be achieved with digitization is traceability. Even with your best efforts with paper in terms of having secondary signoffs, you are going to miss something. However, if you are digitally recording everything, it can make processes run a lot smoother, and have everybody be accountable. This speaks to the 21 CFR Part 11 traceability component.

Q What parameters for donor selection in the allogeneic setting may inform future CQA definitions? What readout is useful in guiding these CQA definitions at various stages of development?

LB: This is also dependent on what the product is. Fundamentally, there are certain drivers that you will discover during your R&D period as to what can give you the best outcome for the product being designed. While all that is predicated on how the final formulated product behaves in your research models, it is also dependent on the ratios that work best early on with the selected donors that you have chosen.

For example, when choosing NK cells, if there is a threshold level from your donor that works, you can select donors based on that principle alone. If you are dealing with a T cell

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harvest from your local bank, you can have more broad access, and choice is determined by what you get from that particular donor pool. CQAs are defined by the process, what your process outcomes are, and what gives you the best output during formulation.

Q Can you elaborate on the qualification and validation of potency assays?

KD: The main difference between qualification and validation is that for validation, you need to have predetermined acceptance criteria, which is not needed for qualification. You still need to assess the same ICH parameters like accuracy, precision, specificity, and sensitivity. For qualification, you are data gathering to identify the operating ranges of the assay. Then, you use the output from the qualification to set the specifications and the acceptance criteria for the validation. The assay setup, the number of lots, the number of runs, and the data analysis are often very similar, if not the same, between qualification and validation.

BIOGRAPHIES

KAREN DOUCETTE currently serves as Cell and Gene Therapy (CGT) Navigator at Charles River Laboratories. Karen plays a critical role in partnering with clients to map the development journey from discovery through commercial manufacturing, with a focus on providing process development guidance to avoid common, time-consuming pitfalls. Karen has over 20 years of experience with biopharmaceutical CROs and CDMOs, spanning pre-clinical and clinical research, development and manufacturing. She has been instrumental in collaboratively driving expansion of CGT service portfolios and aligning research innovation with customer need. Karen received her BSc in Animal Science from the University of Delaware and completed her MBA at Goldey-Beacom College.

JULIA SABLE is the Research and Process Development Lead for early and late phase Gene Therapy (University of Pennsylvania and Columbia) and Cell Therapy (Legend Biotech and Tmunity) and is Upstream and Downstream PD lead for Oncolytic Virus (OV) Therapies (Merck)—high throughput (HTP) virus characterization and potency assay development; analytical development for stability and release; end-to-end bioprocessing for lentivirus (LVV), retrovirus, adeno-associated virus (AAV); G-deleted rabies (RABVdG) platforms for preclinical to IND and BLA filings; viral vector purification, process optimization, process validation for adherent, fixed-bed, microcarrier, and suspension bioreactors, clarification (TFDF), chromatography, TFF, and sterile filtration. Expert in advanced microscopy (Confocal, TIRF, STED, 2-P, SLM and E-Phys setups); Lab Manager and Senior Research Scientist for Dr Michael P Sheetz (Lasker Winner); Dr Charles Rice (Lasker and Nobel Prize winner) and non-tenure-track faculty member at Columbia University as Director of Virology for the Zuckerman Institute.

LARRY BELLOT is a Scientific Advisor for the Charles River Cell and Gene Therapy CDMO business, having held previous positions as Analytical Sciences and Method Validation Manager and Senior Principal Scientist within the Manufacturing, Science & Technologies (MSAT) group. Prior to Cognate BioServices' acquisition by Charles River in 2021, Larry also gained experience as MS&T IV, QC Tech Transfer Scientist, and QC Analytical Scientist at the established cell therapy CDMO. A dedicated coach and mentor, Larry received his PhD with honors from The University of Texas Medical Branch at Galveston and has foundational degrees in Industrial Biotechnology and Microbiology and has a vast and varied industrial biotechnology experience.

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AUTHORSHIP & CONFLICT OF INTEREST

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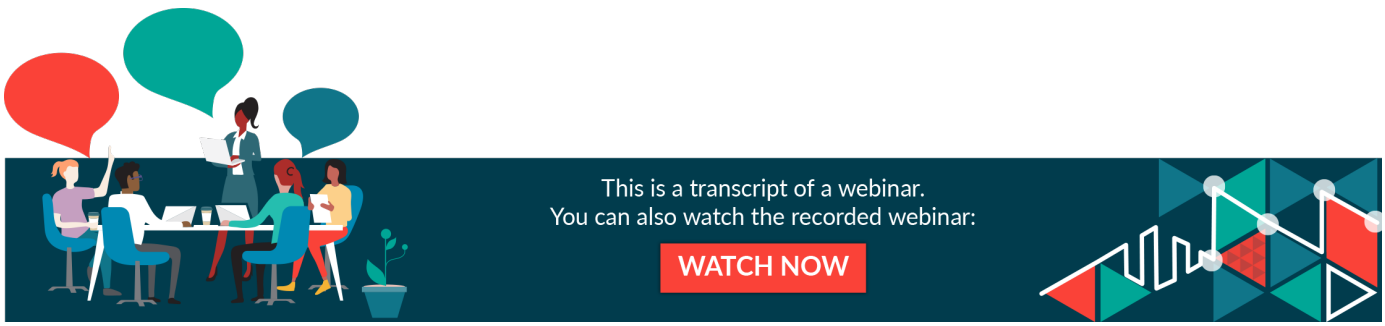

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CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Strategies to accelerate AAV chromatography process development

Kelley Kearns

Following rapid growth in the gene therapy sector, the viral vector manufacturing industry is currently facing the key challenge of enabling and optimizing large-scale AAV production. Cleanable, reusable affinity resins can help to reduce costs in downstream purification of AAV vectors. This article provides a technical overview of rapid process development for an AAV capture step, using AVIPure® AAV affinity resins in two case studies. A high-throughput design of experiments framework will be shared on how to effectively identify desirable conditions for AAV chromatography.

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THE CASE FOR THE DEVELOPMENT OF CAUSTIC STABLE AAV CAPTURE RESINS

Current dosages of AAV-driven gene therapies vary significantly depending on many factors, but primarily on how they are introduced. Targeted administration is more efficient, but systemic injection is generally preferred for ease of administration. Most systemically introduced dosages are in the range of 1×10^{14}–1×10^{16}. Given that a typical cell culture titer is around 3×10^{14} vg/L, processing of a 500 L bioreactor may be required for just a single dose.

Large-scale production of AAVs is one of the biggest challenges the viral vector manufacturing industry is currently facing. The growth of AAV therapies and indications requires the development of tools capable of enabling production of the first metric ton of capsids—only then will the AAV-driven gene therapy sector be prepared for a bright commercial future.

The utilization of cleanable, reusable affinity resins is one of the methods that can help achieve this goal. Downstream purification costs contribute to the overall economic burden for AAV manufacturing and the simplest

way to reduce these costs is with a resin that can be cycled many times over. The larger the production scale—for example, for systemic disease indications with large patient populations–the greater the cost reduction that is achieved from cycling resins.

AVIPURE AFFINITY LIGANDS

AVIPure affinity ligands exemplify biophysical characteristics required for use in bioprocessing applications. They are stable in NaOH and have proven robustness in extensive resin cycling. The ligands display high affinity and selectivity to eliminate host cell and product-related impurities, and are protein-based, animal origin-free, and manufacturable at large scale. AVIPure resins for AAV2, AAV5, AAV8, and AAV9 are currently available off the shelf with the AAV6 resin due to launch in late 2023.

The process of developing a new resin, either as a custom product for a single partner or a catalog resin, is broken into two stages: ligand discovery and resin development. The ligand discovery process is a 6-week endeavor starting with receiving and validating a target drug molecule from a partner and ending with the identification of a series of NaOH-stable high affinity hits. The resin development stage involves optimizing the bead type and the density of the ligand on the bead. The final deliverables from this stage are dynamic binding capacity versus residence time, a demonstration of resin cycling with a representative feedstream, delivery of a prototype resin, and the defined process for the affinity capture step, including the volumes and compositions of wash and elution buffers.

HIGH-THROUGHPUT PROCESS DEVELOPMENT OF AN AAV AFFINITY CAPTURE STEP

Overview: tools & methods

The line of OPUS® pre-packed columns supports product and process development at every stage of the product lifecycle. This line includes small OPUS RoboColumns® for high-throughput process development, OPUS MiniChrom® columns for further fine-tuning and process optimization on bench-scale chromatography systems, and large-scale OPUS columns for clinical and full-scale manufacturing.

In addition, pre-packed ValiChrom® columns are ideal for process validation studies, including viral clearance where reproducibility and scalable packing performance are critical. All OPUS products are packed according to standardized, validated methods, which enables scalable chromatography and efficient development workflows for downstream processing. Early process development work is supported by OPUS RoboColumns, which are miniaturized chromatography columns designed for automated, parallel chromatography. RoboColumns come in strips of eight columns each with a modular design that allows arrangement into an array of up to 96 columns. RoboColumns are well-established in the industry and can be operated in combination with robotic workstations, such as those offered by Tecan, to support early-stage downstream process development.

Case study 1: improving elution pH with AVIPure AAV9

This case study involves some early high-throughput process development work performed by Repligen for a partner using the AVIPure AAV9 resin. The AVIPure AAV resins can be operated with a slower (4 min) residence time to provide higher capacity with concentrated AAV feeds, or with a faster (1 min) residence time allowing for over 2×10^{14} viral particles (vp)/mL loading. The resin can be cleaned in place with 0.1 to 0.5 M NaOH and can be cycled over 100 times using 0.1 M NaOH and over 20 times using 0.5 M NaOH.

High, consistent yields are observed with elution at pH 2 along with high product purity with respect to host cell proteins (HCP) and DNA. However, one complaint received from customers regarding this resin is that the

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yield is impacted for elution at pH >2. In this study, the partner wished to elute at pH 3. The partner provided 3 L of clarified cell lysate containing 5×10^{14} total capsids of an engineered AAV9. The process development strategy was to start with a measurement of the dynamic binding capacity (DBC), determine optimal wash and elution conditions with buffer scouting experiments, and lastly to verify the determined load, wash, and elution conditions in a final column run.

Half of the available material (2.5×10^{14} vp) was used to measure the DBC at a 1 min residence time. The DBC measurement was performed using a 1 mL MiniChrom column. Due to the low titer of the lysate, a 1 min residence time was employed, where 1.5 L of clarified lysate were loaded onto the column over a period of 25 hours. The flow-through fractions were analyzed for total capsids by ELISA. The results showed that 10% breakthrough occurs at a loading of 2.6×10^{14} vp/mL resin. This result was in line with expectations based on the over 2×10^{14} vp/mL resin specification. The purified capsids in the elution pool were neutralized and retained for the elution scouting.

A high-throughput wash buffer screening was performed using 100 µL RoboColumns on a Tecan Freedom EVO system. Clarified lysate was loaded onto the RoboColumns below the determined DBC value at a 1 min residence time, washed with various buffer additives, and eluted at pH 2 according to the method details shown in Table 1. For the elution, a 4 min residence time is recommended to reduce rebinding events and minimize peak tailing to provide maximum yields. However, due to a limitation of the Tecan software, the longest residence time allowed by the predefined flow rates is 1.88 minutes or a linear velocity of 16 cm/hr. Nonetheless, more fractions were simply pooled from the elution collection plate. Eight wash buffers with a variety of different additives were chosen and the elution pools were neutralized. This is programmed easily with the Tecan method, where the neutralization buffer can be added directly to the collection plate immediately prior to the elution step. The neutralized elution pools were analyzed using a total capsid ELISA, HCP ELISA, and a PicoGreen assay for host cell DNA quantitation.

The results of the wash screening (Table 2) show that all eight buffers tested provide excellent purity, including the running buffer wash. The standard recommended wash buffer for AVIPure AAV9, which also provided

TABLE 1

Wash scouting AVIPure AAV9 RoboColumn method.

Step	Buffer	Column volume	Residence time (min)
Equilibrium	20 mM Tris, 200 mM sodium chloride, pH 7.5	10	1
Load	Clarified cell lysate	153	1
Chase	20 mM Tris, 200 mM sodium chloride, pH 7.5	5	1
Wash 1	Variable (see Wash Buffer table)	5	1
Wash 2	20 mM Tris, 200 mM sodium chloride, pH 7.5	5	1
Elution*	0.1 M glycine, 150 mM NaCl, pH 2	5	1.88
Strip**	0.1 M NaOH	5	1
Neutralize	1 M Tris, pH 7.5	5	1
Equilibration	20 mM Tris, 200 mM sodium chloride, pH 7.5	10	1

*Neutralized by adding 20% of fraction volume of 1M Tris, 150 mM sodium chloride, pH 9.
** Neutralized by adding 50% of fraction volume of 1M Tris, pH 7.5.

the best HCP clearance in this experiment, is 0.5 M urea with 50 mM of sodium octanoate. This buffer was selected for the process verification run.

Elution buffer scouting was performed similarly using the same 100 µL RoboColumns. For this experiment, columns were challenged with 1×10^{14} vp/mL resin using the purified capsids generated during the binding capacity experiment. Load and elution residence times were the same as for the wash scouting experiment. Sixteen elution buffers were tested–one at pH 2 as the reference control and the other 15 buffers at pH 3 with various additives. The neutralized elution pools were analyzed by total capsid ELISA to determine the yields. The results of the pH 3 elution screening are shown in Figure 1.

Condition 1 is the pH 2 elution yield, which is the reference control. Condition 2 is a pH 3 condition without added salt, which was unable to recover any measurable amount of capsids. The addition of NaCl in conditions 3 and 4 provided a slight improvement in yield, but other additives are required to obtain comparable yields to the pH 2 condition. There are four additives that gave similarly high yield improvement at pH 3–arginine, tetramethylammonium chloride, ethanol, and ethylene glycol.

The next step was to further refine the buffer conditions with the best performing additives. Although ethanol is used in some biopharma processes, it is not a preferred option due to its flammability, so this option was eliminated going forward. The same experiment and analysis were performed with eight more elution buffers containing the remaining three best-performing additives. For this experiment, the neutralized strips were also analyzed by total capsid ELISA to attempt to close the mass balance. The top three conditions are shown in green in Figure 2.

Conditions 3 and 4 show that as little as 250 mM arginine is effective at recovering nearly 100% of the capsids. Conditions 5 and 6 show there is a clear drop-off in the effectiveness of ethylene glycol when the concentration is reduced from 30% to 15%. Considering the most likely next step for AAV downstream processing will be an anion exchange step, ethylene glycol buffer is preferred due to its lower ionic strength, and it will potentially enable direct loading of the affinity elution pool onto the anion exchange column.

As there are reports in the literature suggesting AAV aggregation in low ionic strength buffers, before the process verification run was performed, the stability of the AAV9 capsids in the ethylene glycol buffer

TABLE 2

Results of AVIPure AAV9 wash scouting.

Wash buffer	HCP (ng/mL)	HCP (ppm)	HCP (LRV)	HCDNA (ng/mL)	HCDNA (ppm)	HCDNA (LRV)	Relative yield
20 mM Tris, 200 mM NaCl, pH 7.5 (running buffer wash control)	67	2000	4.5	5.7	180	3.4	100%
0.5 M urea, 50 mM sodium octanoate, 50 mM HEPES, pH 8	52	1600	4.6	4.3	130	3.5	100%
1 M arginine hydrochloride, 50 mM HEPES, pH 7	73	2300	4.5	3.7	120	3.6	97%
0.2 M MgCl2, 10 mM HEPES, pH 7.4	97	3100	4.3	4.3	140	3.5	95%
0.5 M urea, 50 mM HEPES, pH 7	85	2600	4.4	4.8	150	3.5	102%
50 mM sodium octanoate, 25 mM HEPES, pH 8	65	1900	4.5	4.2	120	3.5	103%
1 M tris, pH 7.5	71	2200	4.5	4.1	120	3.5	100%
0.5 M guanidine hydrochloride, 20 mM tris, pH 7.5	73	2100	4.5	5.0	140	3.5	106%

HCP: Host cell proteins; HCDNA: Host cell DNA; LRV: Light reflective value.

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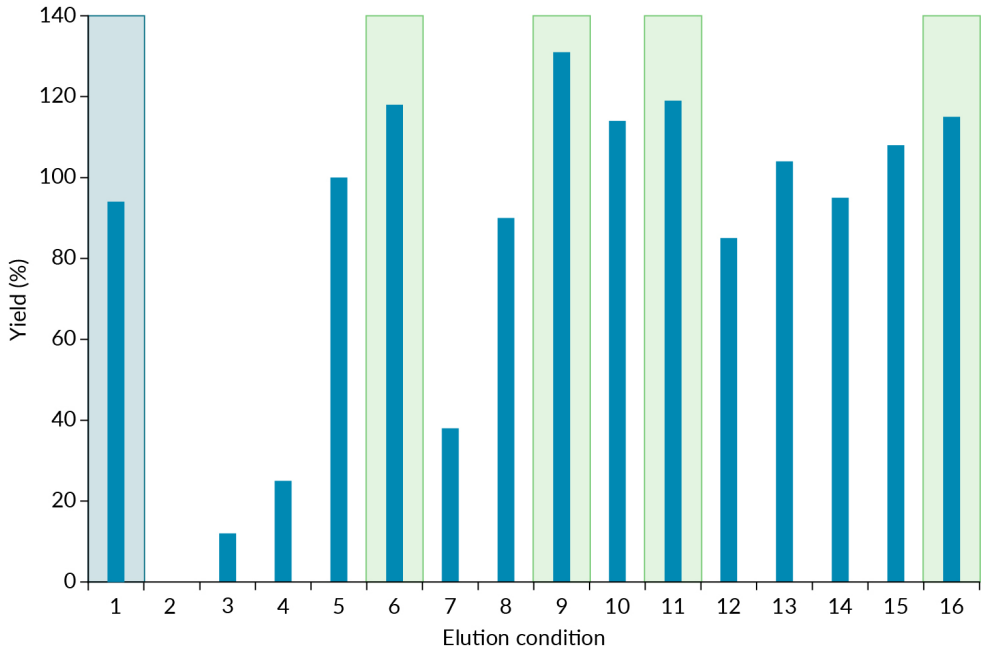
was tested. The purified capsid load and the neutralized elution pool containing 30% ethylene glycol were analyzed using a high-performance size exclusion method. An Acquity UPLC BEH450 SEC column (2.5 µm, 450 Å, 4.6×150 mm) was used, where 3 µL of sample was loaded with a mobile phase of 100 mM sodium phosphate, 150 mM sodium chloride, pH 6.8 + 5% (v/v) isopropanol at 0.3 mL/min and with detection by fluorescence (ex. 280 nm, em. 340 nm). The results shown in Figure 3, do not indicate any evidence of capsid aggregation, so the preferred elution buffer was confirmed.

FIGURE 1

Results of AVIPure AAV9 RoboColumn pH 3 elution scouting.

Number	Elution buffer (all contain 0.1 M glycine)	Yield (%)
1	150 nM sodium chloride, pH 2	94
2	pH 3	0
3	150 nM sodium chloride, pH 3	12
4	1 M sodium chloride, pH 3	25
5	0.5 M sodium chloride, 0.5 M arginine hydrochloride, pH 3	100
6	1 M arginine hydrochloride, pH 3	118
7	1 M magnesium chloride, pH 3	38
8	1 M ammonium chloride, pH 3	90
9	1 M tetramethylammonium chloride, pH 3	131
10	20% ethanol, pH 3	114
11	20% ethanol, 1 M sodium chloride, pH 3	119
12	20% hexanediol, pH 3	85
13	20% hexanediol, 1 M sodium chloride, pH 3	104
14	20% propylene glycol, pH 3	95
15	30% ethylene glycol, pH 3	108
16	30% ethylene glycol, pH 3	115



Before attempting the process verification run, a final round of elution screening was performed to establish if a pH of greater than 3 could be used for elution. In this final round of elution screening, all the buffers contained 30% ethylene glycol, but varied in the buffering agent (glycine or citrate), the amount of NaCl (0 or 150 mM), and the pH (3, 3.5, or 4). As seen in Figure 4, attempting to remove the NaCl or increasing the pH to 4 reduced the recovery to essentially zero. Increasing the pH to 3.5 also showed a precipitous drop-off in recovery. The control condition where glycine was exchanged with citrate, but the pH remained constant at pH 3, indicates that the higher pH of 3.5 or 4 leads to lower yields and is not related to the use of citrate buffer. Based on these results, the original optimal elution buffer was chosen without any further changes.

With the process load, wash, and elution conditions determined, the remaining clarified lysate was used to perform a process verification run using a 1 mL OPUS MiniChrom column. The column was loaded with clarified lysate to a challenge of 2×10^{14} vp/mL resin, representing 80% of the measured DBC. The selected wash and elution conditions and the recommended 1 min load residence time and 4 min elution residence time were used. Each eluate pool was analyzed for capsid titer by ELISA, and HCP and DNA were measured for the neutralized elution pool.

The results of the verification run are shown in Figure 5. Most capsids were found in the elution pool at very high purity, with over 5 log reduction in HCP and close to 4 log reduction of host cell DNA.

In summary, appropriate wash and elution conditions were identified using high-throughput methods and a minimal number of capsids through utilizing the

FIGURE 2

Results of pH 3 elution condition refinement.

Number	Elution buffer (all contain 0.1 M glycerine)	Yield (%)	Strip (%)	Conductivity (mS/cm)
1	150 mM sodium chloride, pH 2 (standard elution control)	94	5	23
2	150 mM sodium chloride, pH 2	0	89	16
3	0.5 M arginine hydrochloride, 150 nM sodium chloride, ph 3	93	6	39
4	0.25 M arginine hydrochloride, 150 nM sodium chloride, ph 3	96	3	32
5	30% ethylene glycol, 150 nM sodium chloride, pH 3	90	7	9.4
6	15% ethylene glycol, 150 nM sodium chloride, pH 3	76	14	12.8
7	1 M tetramethylammonium chloride, 150 nM sodium chloride, pH 3	73	10	81
8	1 M tetramethylammonium chloride, 150 nM sodium chloride, pH 3	67	19	52

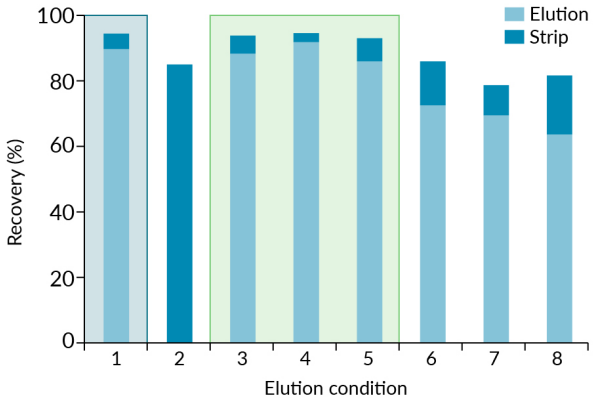


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FIGURE 3

HP-SEC chromatograms for the purified capsid load (top) and the neutralized elution pool for the 30% ethylene glycol, 0.1 M glycine, 150 mM sodium chloride, pH 3 elution condition.

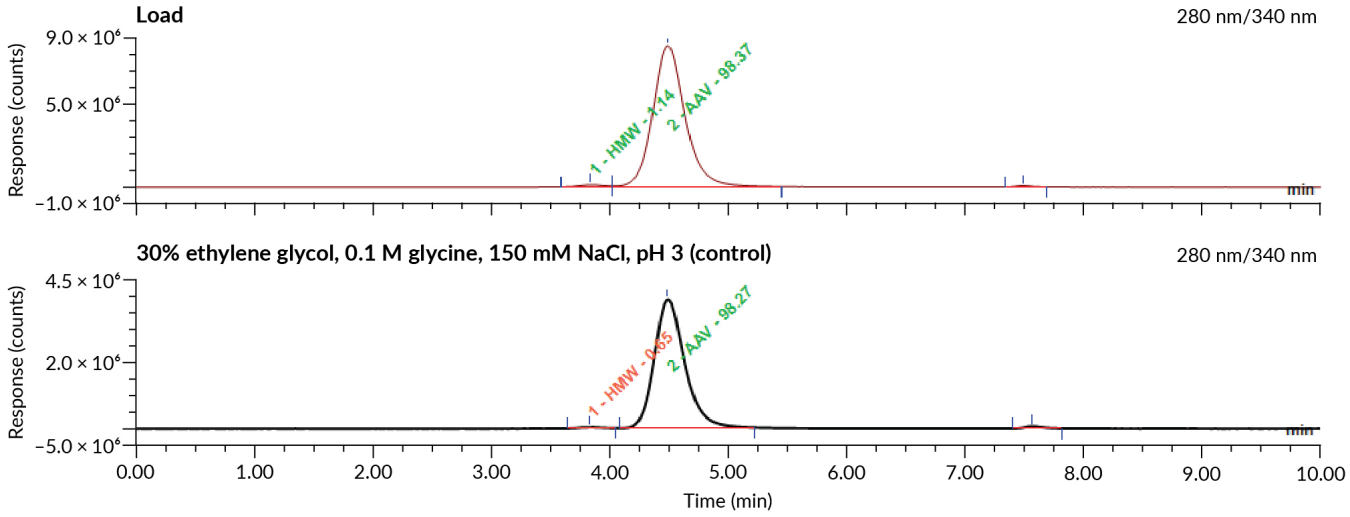


FIGURE 4

Results of elution scouting for pH 3–4.

Number	Elution buffer	Yield (%)	Strip (%)	Conductivity (mS/cm)
1	30% ethylene glycol, 0.1 M glycine, 150 mM sodium chloride, pH 3 (control)	91	6	9.4
2	30% ethylene glycol, 0.1 M glycine, pH 3	0	87	1.5
3	30% ethylene glycol, 0.1 M citrate, pH 3	47	45	10
4	30% ethylene glycol, 0.1 M citrate, 150 nM sodium chloride, pH 3	91	6	3
5	30% ethylene glycol, 0.1 M citrate, pH 3.5	37	51	10.2
6	30% ethylene glycol, 0.1 M citrate, 150 nM sodium chloride, pH 4	41	41	4
7	30% ethylene glycol, 0.1 M citrate, pH 4	0	67	11.6
8	30% ethylene glycol, 0.1 M citrate, 150 nM sodium chloride, pH 4	0	61	5.3

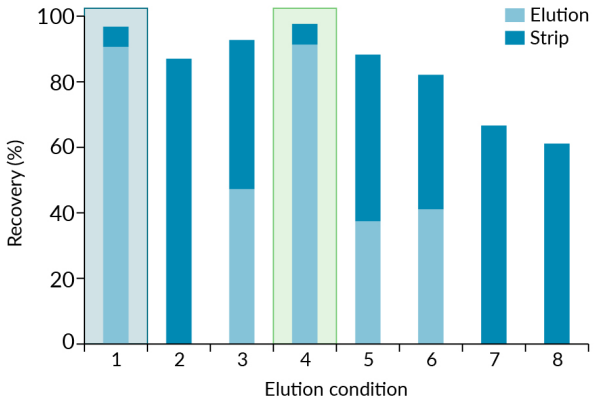


FIGURE 5

Process verification run results using AVIPure AAV9 1 mL MiniChrom column with the optimal wash and elution buffers from the buffer scouting experiments.

Yield analysis

Sample	Titer (vp/mL)	Recovered (%)
Load	2.1×10^{11}	N/A
Flow through pool	1.4×10^{9}	1
Elution	5.2×10^{13}	123
Strip	3.9×10^{13}	8

Purity analysis

Sample	HCP (ppm)	HCP (LRV)	HCDNA (ppm)	HCDNA (LRV)
Load	6.1×10^{7}	N/A	4.3×10^{5}	N/A
Elution	420	5.2	90	3.7

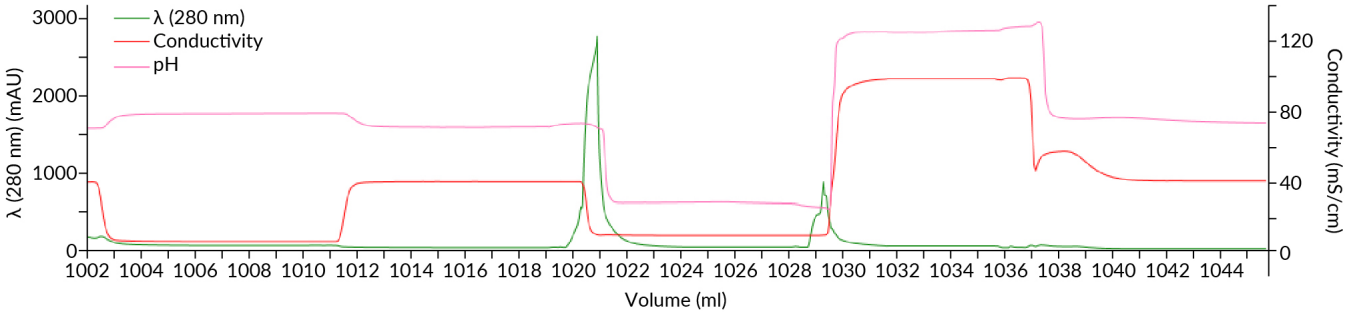
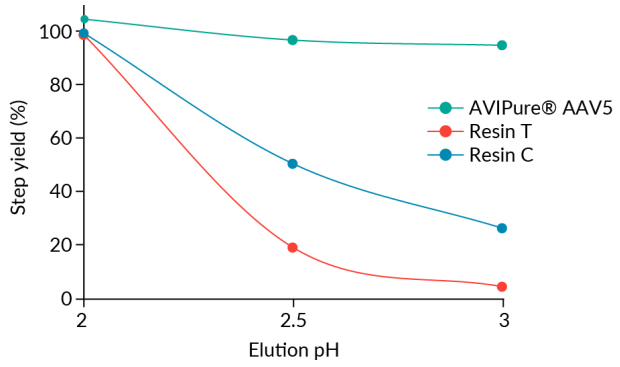


FIGURE 6

Demonstration of yield vs. elution pH for AVIPure AAV5 as compared to other commercial AAV affinity resins.



miniaturized OPUS RoboColumns. The resin capacity was measured as expected to be above 2×10^{14} vp/mL resin, and excellent recovery and purity were demonstrated using the selected process conditions and a 1 mL MiniChrom column.

Case study 2: process validation cycling study with AVIPure AAV5

The second case study is a process cycling study with the AVIPure AAV5 resin using prepacked OPUS columns. AVIPure AAV5 gives nearly complete elution at pH values from 2 to 3. It is the only commercial resin with acceptable yield at pH >2 as demonstrated in Figure 6.

AVIPure AAV5 resin also has the potential to elute at an even higher pH. Figure 7 shows a high-throughput elution screening experiment demonstrating the RoboColumn method combined with a fluorescence detection assay for even faster analysis. Installing a fluorescence microplate reader into the robotic handler allows this experiment to be fully automated.

Several additives offer the ability to elute at pH 4, namely ethanol, ethylene glycol, and propylene glycol. Arginine did not perform as well as it did with the AAV9 resin, highlighting how additives need to be determined empirically with each resin and capsid type.

Column cycling studies are required to validate a resin's lifetime, where a small-scale column is cycled through the entire process step, and the expected performance is demonstrated for each product cycle for as many cycles as are intended to be used in the process. Such cycling studies are also valuable for impurity clearance to show resin reuse does not impact the ability of the resin to remove process

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impurities, such as antifoam or adventitious viruses.

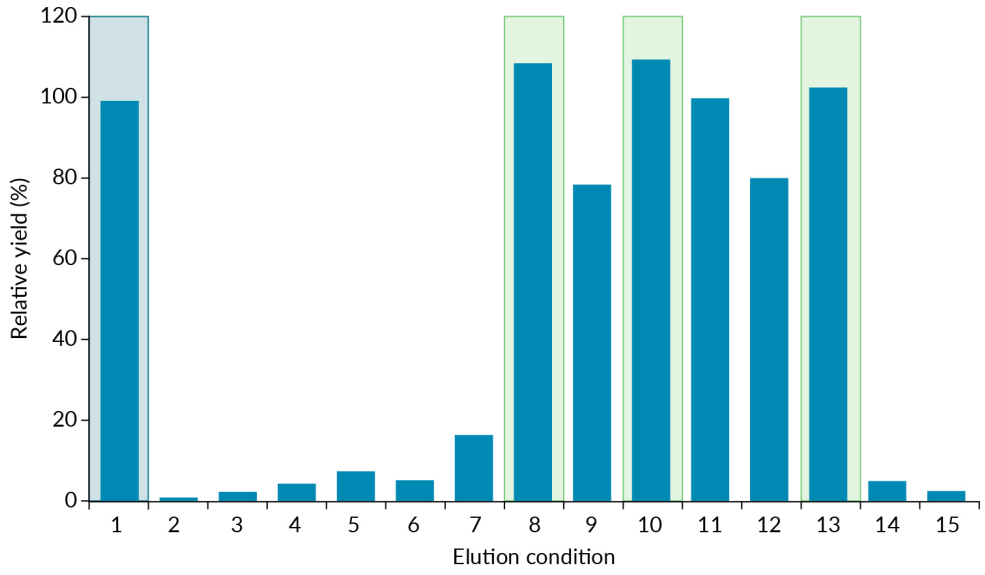
The OPUS ValiChrom columns are ideal for this work, as the columns can be packed to any bed height and easily scale to the larger OPUS manufacturing scale columns that use the same validated packing methods. OPUS MiniChrom columns are packed

FIGURE 7

Elution screening with AVIPure AAV5 RoboColumn.

Number	Elution buffer (all contain 0.1 M glycine)	Yield (%)
1	150 nM sodium chloride, 0.1 M glycine, pH 3	100
2	150 nM sodium chloride, 0.1 M citrate, pH 4	1
3	1 M sodium chloride, 0.1 citrate, pH 4	2
4	0.1 M citrate, pH 4	4
5	1 M ammonium chloride, 0.1 citrate, pH 4	7
6	0.5 M sodium chloride, 0.5 M arginine-HCl, 0.1 citrate, pH 4	5
7	1 M arginine hydrogen chloride, 0.1 citrate, pH 4	16
8	20% ethanol, 0.1 citrate, pH 4	109
9	1 M tetramethylammonium chloride, pH 3	79
10	1 M sodium chloride, 20% ethanol, 0.1 citrate, pH 4	110
11	20% hexanediol, 0.1 citrate, pH 4	101
12	1 M sodium chloride, 20% hexanediol, 0.1 citrate, pH 4	81
13	20% propylene glycol, 0.1 citrate, pH 4	110
14	1 M magnesium chloride, 0.1 citrate, pH 4	5
15	1 M tetramethylammonium chloride, 0.1 citrate, pH 4	2

Plate-based capsid titer method using fluorescence	
Plate reader	Agilent Biotek Synergy 2
Detection	Fluorescence: 284/10 nm excitation, 360/40 nm emission
Gain	Set automatically based on highest concentration sample



using the same packing methods but are only available in discrete column lengths. For the AVIPure AAV5 cycling study, a 1 mL OPUS MiniChrom column was used, and for each product cycle the column was loaded to 80% of the measured capacity with clarified lysate. The wash and elution buffers from resin development were used in the process. The column performance was then evaluated for yield and purity for each product cycle.

A 1 min load residence time and a 4 min elution residence time were used. After each elution, the column was stripped with 0.1 M NaOH for a total contact time of 30 minutes, followed immediately by the next successive cycle for a total of 20 cycles. The results are shown in Figure 8 and demonstrate consistent performance over all 20 product cycles. The chromatograms from all 20 cycles show virtually no change in peak shape, with a consistent elution volume of about 2.5 column volumes.

There are some fluctuations in the yield, but this is overall consistent with an average of 94%. HCP reduction was also consistent with an average of 750 ppm in the elution pool over all 20 cycles. The host cell DNA results are mainly consistent around 200 ppm with a few outliers. Overall, excellent performance, yield, and purity were demonstrated over a full 20 product cycles.

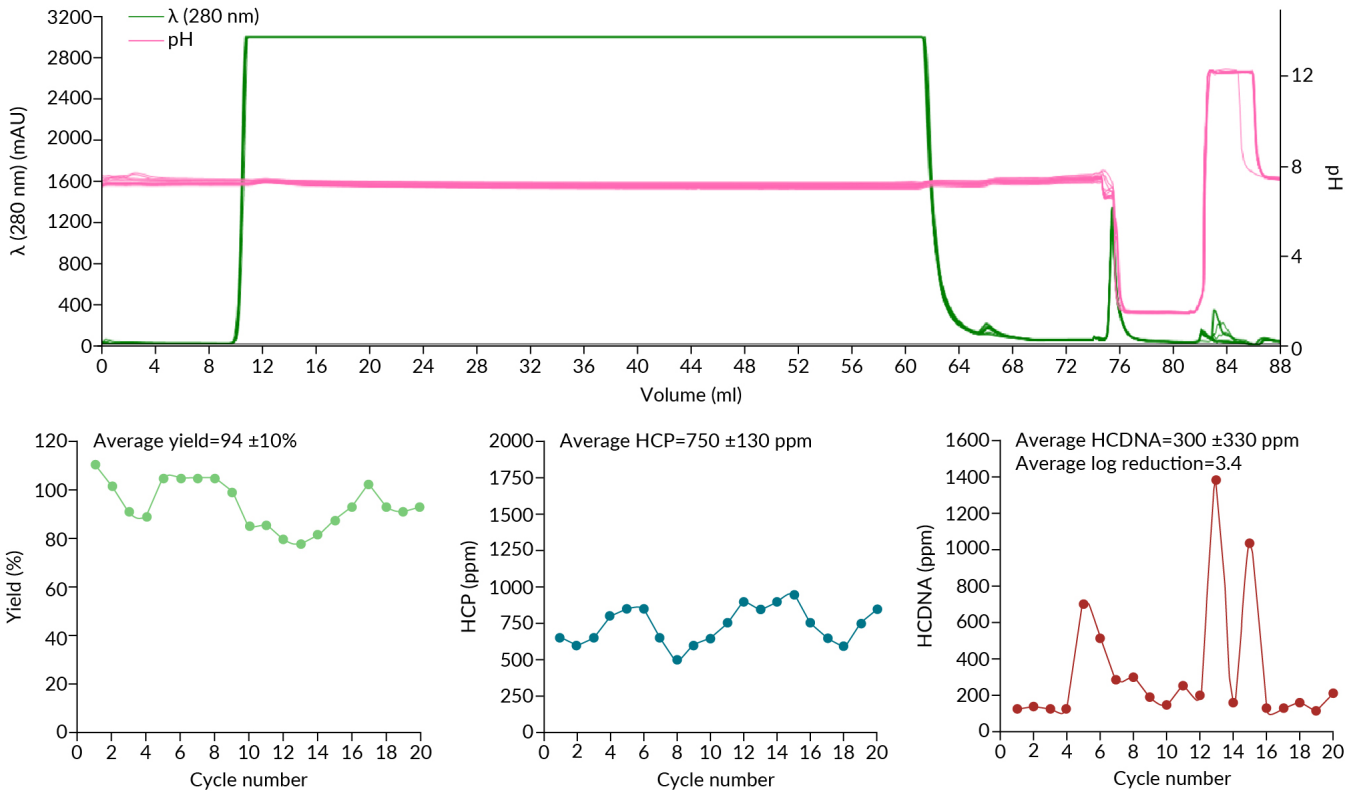
SUMMARY

The methods demonstrated here can be used to effectively accelerate and streamline AAV process development activities. The OPUS product line for reliable pre-packed columns supports an entire product's lifecycle from early development using RoboColumns through clinical and larger-scale productions with OPUS manufacturing-scale columns.

The AVIPure AAV resins are the only caustic stable AAV resins on the market. These

FIGURE 8

AAV5 process validation cycling study results.



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resins offer high capacity, high yield, and high purity while providing more than 100 reuse cycles to reduce downstream processing costs. The ability to cycle AVIPure AAV resins enables commercial-scale production for indications with large dosages and patient populations, in preparation for the future of AAV production.

Q&A

Kelley Kearns

Q Will detergents in the lysate interfere with binding?

KK: The detergents do not interfere with binding. All the data presented here are with clarified lysates in which the cells have been lysed with detergent. Our in-house method is with 0.5% Triton X-100. We have also tested many partner materials containing other detergents, and we have not seen any change in performance with detergents present in the feedstream.

Q How does ethylene glycol improve elution?

KK: We are not entirely sure of the mechanism here. Ethylene glycol is a small, water-soluble molecule with hydrophobic characteristics, and it is known to disrupt hydrogen bonding. So, there are a few different mechanisms by which ethylene glycol may be facilitating dissociation of the capsids from the resin. Unfortunately, we do not know the exact epitope where our ligands bind on the AAV capsids, but we are pursuing that work. We hope to be able to answer this question more fully in the future.

Q If I underload the resin, will it impact the yield? If so, how low can I load before I do see an impact?

KK: We have not seen an impact with underloading. We have loaded as low as 10% of the DBC without seeing any drop in recovery. We recommend slowing down the elution

residence time to a minimum of 4 min to help recover the largest number of capsids. It is important to focus on the elution peak, especially with regard to peak tailing, which can happen when a column is underloaded due to rebinding events. We also recommend running elution in reverse flow to minimize rebinding events.

Q Do the AVIPure AAV resins provide any separation of empty & full capsids?

KK: Unfortunately, no. Our ligands recognize epitopes on the surface of the AAV capsids and we cannot provide any empty-full separation, as is the case for any AAV affinity resin.

Q Do any of your other resins have trouble eluting at pH 3?

KK: We have primarily heard this concern only with the AVIPure AAV9 resin. The tools and data I have shared hopefully provide a starting point to look at different additives to overcome the obstacle for the AAV9 resin.

We have tested all our resins. For the AAV5 resin, the data clearly show that we can elute at pH 3 and even at pH 4 with the addition of some additives. For our AAV2 and AAV8 resins, we see equivalent recoveries at pH 2 and pH 3. I encourage customers to reach out if they see any issues as we can work together to help solve these problems.

Q There is a large difference in resin lifetime in 0.1 M NaOH versus 0.5 M NaOH. Are there any specific reasons for that?

KK: In general, our ligands are highly caustic stable, but as with any protein-based ligand, there is a limit to that exposure. A higher concentration of NaOH will shorten the lifetime of that ligand in the column. We have demonstrated 100 cycles with 0.1 M NaOH and 20 cycles with 0.5 M NaOH. We generally expect a linear relationship where an increase in concentration reduces the number of cycles.

Q What is the procedure for post-elution neutralization of low pH eluate samples?

KK: We recommend 100 mM glycine at pH 2. Ideally, we collect it on a plate that contains the 1 M Tris solution as the neutralization buffer. The volume of the neutralization buffer is typically 10–20% of the elution fraction volume.

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Q What is the typical capsid integrity after elution with pH 2?

KK: This depends on the serotype and any engineering that has been done to the capsid. There are reports in the literature suggesting an impact on capsid integrity below pH 3. Some of our partners do not experience any issues with eluting at pH 2, perhaps due to the engineering of their capsids. In general, we want to be able to provide elution at pH 3 to alleviate any concerns about capsid stability and provide tools to meet any demands in terms of softer elution by adding different elution excipients.

Q Why is the yield percentage at more than 100% in a few elution buffers?

KK: This is related to the error in the analytical methods. The yield number depends on the feed measurement and the elution pool measurement, and the error from both measurements is propagated in the yield calculation. Most of the AAV9 data presented was measured using the total capsid ELISA, where we generally see ±10% error, but there were some figures with ±20% variation. In a single experiment, we see the relative differences correlate very well and we are confident in the data reported relative to the control condition.

Q What bed height do you suggest for AVIPure resins for purification of AAV?

KK: This depends on the flow rate that you are planning to run. With a 1 min residence time, we recommend a 5 cm bed height. OPUS offers those pre-packed columns at 5 cm bed height, and at any scale up to 80 cm inner diameter.

If you are planning on running a longer residence time, such as 4 min, then a 10 cm bed height will be no problem.

Q How specific are the resins if purifying from a mixed AAV population?

KK: This depends on the population. We have primarily been working with a single population of AAV capsid. This needs to be tested empirically.

Q Is there any negativity to running the AAV9 at pH 2 as recommended by Repligen?

KK: The primary concern is that low pH is affecting the stability and ultimately, the infectivity of the viral particle. Infectivity is not something that we generally test for partners

because it involves an assay that is specific to the AAV payload. The impact of exposure to the elution pH on the AAV infectivity needs to be tested empirically, as every partner's capsid is slightly different in terms of engineering and stability.

We can help support customers in terms of improving elution at higher pH or investigating a solution. We have performed the testing with our in-house capsids to understand the infectivity and we do not see any change with short exposure times even at pH 2.

Q Could AVIPure resin be reused in GMP processes? If so, is there a method to check if the strip using 0.1 M NaOH removes everything from the resin?

KK: Yes. Our goal is to support GMP manufacturing with our resins and prepacked OPUS columns. We generally analyze the strip with our total capsid ELISA. For example, for 0.1 M NaOH, we add 1 M Tris at 50% of the volume of that strip fraction to neutralize it and check that with a total capsid ELISA. The goal is to get mass balance and show consistent performance.

Referring to the cycling data, there is little change in elution and strip peaks. We generally achieve close to a 100% mass balance. This would need to be demonstrated for any GMP process validation. Performing a blank column run and analyzing the elution pool can help to determine the effectiveness of the cleaning process and assess if there is any carryover from one cycle to the next.

Q Do you have a suggested residence time and what is the maximum?

KK: We recommend a high flow rate with a 1 min residence time for low titer feeds. For higher titers you can still operate at 1 min residence time without any adverse effects as long as you do not exceed the 2×10^{14} vp/mL capacity, but if you want extra capacity, we recommend increasing the residence time to 4 min.

Residence time can be even longer to increase capacity further, though there is a limit to this. You can expect to achieve a capacity of 1×10^{15} vp/mL resin for high titer feeds with longer residence times.

BIOGRAPHY

KELLEY KEARNS is the Director of Downstream Development at Repligen and has been instrumental in the development and production of bioprocess affinity chromatography resins, essential for the purification of biopharmaceuticals. With over 5 years in this role, he has shown an exceptional ability to solve complex technical challenges, ensuring the production of high-quality, life-saving medicines. Kelley Kearns is a highly accomplished professional with a successful track record in the development and commercialization of late-stage biopharmaceuticals. His expertise lies in bridging the gap between drug development

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and manufacturing, with a focus on process improvement and optimization. In his previous roles, Kelley's contributions have been equally significant. As a Senior Scientist at Dynavax Technologies, he played a key role in supporting routine manufacturing, making critical technical decisions, troubleshooting, and implementing process improvements. At Bristol-Myers Squibb, Kelley managed laboratory studies for downstream process validation and supported commercial process improvements for late-stage biologics. As a Senior Engineer at Merck, Kelley displayed exemplary project management skills, overseeing the design and construction of multimillion-dollar facilities, resulting in enhanced production capabilities and substantial cost savings through process optimization. Kelley holds a Doctor of Philosophy in Chemical Engineering from the University of Delaware, a Master's degree in Chemical Engineering from Rutgers University, and a Bachelor's degree in Chemical Engineering from The Cooper Union for the Advancement of Science and Art.

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Director of Downstream Development,
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AUTHORSHIP & CONFLICT OF INTEREST

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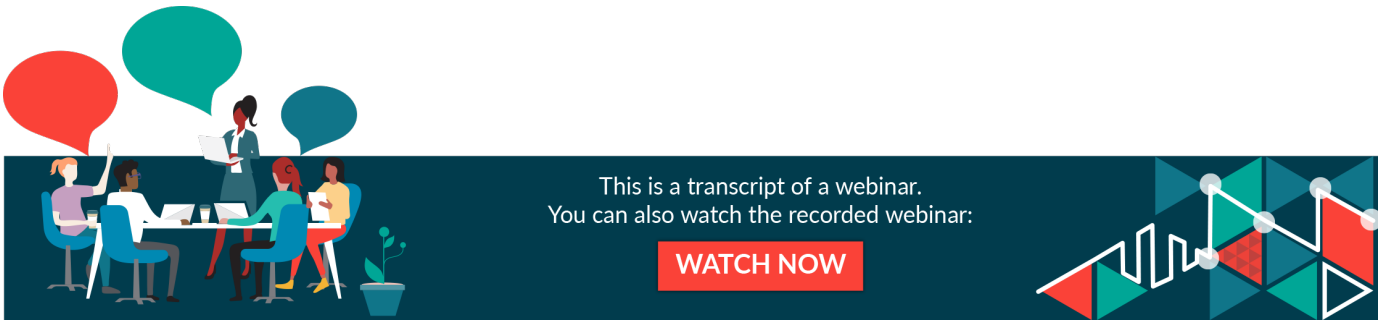

CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Establishing the standards of operational excellence to manufacture commercial cell therapies

Melanie Mansbach & Hui Zheng

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THE CELL & GENE THERAPY INDUSTRY

Between 2015 and 2022, 19 viral vector, autologous, or allogeneic cell therapy products were launched. Lonza supported the commercial launch of three of these products. There are 18 products expected to launch in 2023, showing exponential growth within the industry. This growth is a product of both the success of previous programs and the life-changing and life-sustaining impact of these therapies.

However, along with this interest comes significant challenges. Across the industry, many manufacturers are experiencing production issues and delays, with the cell and gene therapy (CGT) field five times more likely to experience delays due to CMC issues than the monoclonal antibody field. As the need for CGT manufacturing grows, it is even more critical to address these manufacturing challenges. This is especially important in autologous therapy production because every batch is related to a patient.

COMMON CHALLENGES IN CGT DEVELOPMENT

CGT developers often attempt to meet shorter developmental timelines, driven by the availability of expedited approval pathways, such as regenerative method advanced therapy (RMAT) breakthrough and fast-track designations in the US. These designations help to ensure that patients can access innovative therapeutics as quickly as possible, but shorter timelines mean that developers must plan their large-scale manufacturing strategy early in the development process, often before they begin to dose patients. While this results in greater overall timeline reduction, this earlier

timeframe for considering manufacturing represents a significant shift in the paradigm for biologics development and creates challenges for manufacturing efficiency in the long term.

Furthermore, manufacturing is more challenging for biologics than for small molecules, and CGT manufacturing is even more complex. Unlike biologics and small molecules, CGTs are living drugs, and thus are subjected to many positive and negative influences on how they differentiate, grow, and function. In addition, the culture conditions for cell products do not scale up linearly between the lab and the manufacturing plant. For example, cells can be cultured in suspension or on plates, but not all cell types can be cultured in both ways. Cell types also vary in their proliferation rates and the number of times they can undergo expansion while retaining their potency. Other variabilities include differences between cells derived from different donors or different patients. Additionally, many CGTs are developed in the lab using technologies that are not scalable at all.

Moreover, the cost to manufacture a gene therapy is significantly more than conventional biologics. The cost of goods and manufacturing for gene therapy can be US$500,000–1 million, not including R&D, clinical trial, and patient access costs. For the foreseeable future, these therapies will be administered to a very small patient population, often only a few hundred patients worldwide.

Another challenge is the need to appropriately define critical quality attributes (CQAs) and develop appropriate analytical methods. It is important to balance appropriately stringent CQAs to drive product quality, while not setting them outside of the bounds of control for the process. When it comes to navigating the regulatory landscape, guidance is still being defined by regulatory authorities. There is a lack of harmonized regulation among the health authorities which creates an additional challenge.

APPROACHING MANUFACTURING CHALLENGES

At Lonza, a global network is leveraged to contend with these various manufacturing challenges in a few ways. The first is that many documents, policies, and procedures are universal across all sites, and business units and can be shared. The second way is through talent sharing, taking the best practices from other areas and applying them to corresponding areas within the CGT division.

Good documentation practices (GDP) and good manufacturing practices (GMP) are universal, and standard operating procedures (SOPs) and training programs can be utilized to instruct and perform GDP and GMP within the CGT division without reinvention. While the technical content may be different, documents and training material structure and the method of execution can be the same. The approach to training and shift structuring is based on strong error prevention systems that can be universally applied, and a similar approach is taken to run readiness. Virtual reality (VR) is a technical innovation being used within the CGT industry in various ways, such as in sterility training. With VR, training efficiency and consistency can be significantly enhanced.

Deviation management in CGT poses some challenges based on the need to release products on tight timelines. The approach to identifying, rating, investigating, identifying corrective action and preventive action (CAPA), and approving deviations are all the same. These similarities allow the implementation of tried-and-true solutions.

Having established quality systems is important when considering regulations and quality compliance. Authorities, for example, the US FDA, take a systematic approach to inspections. Before approval, the US FDA evaluates establishments through on-site inspections and/or by establishment final review when the firm is named in the CMC section of an NDA, ANDA, or BLA. The

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US FDA checks compliance with Section 501(a)(2b) of the FD&C Act and compliance with 21 CFR.

Across the CGT field, there were 35 facility inspections between 2007 and 2020, resulting in the following findings: 89 on quality, 66 on facilities and equipment, 60 on production, 43 on materials, 34 on lab, and 20 on packaging and labeling. This high number of findings represents a high risk to gaining regulatory approval for commercial production. Lonza's system of leveraging global experience in other areas and setting up a systematic approach for establishing quality systems directly mirrors the regulatory systematic approach to inspections and has led to successful results for multiple Lonza CGT sites.

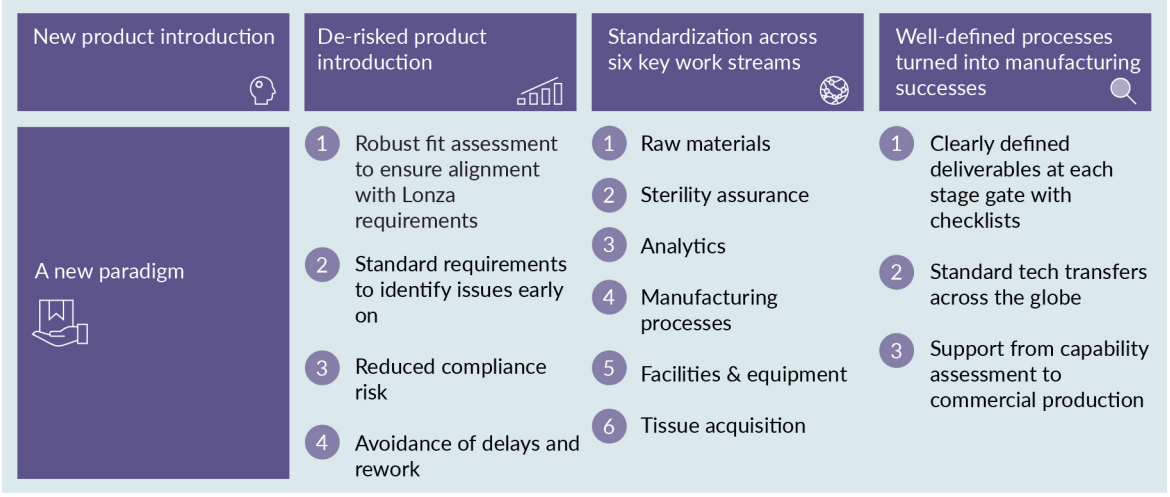
Lonza approaches CGT manufacturing with the intention of establishing customized systems developed and implemented at the divisional level. The global team of CGT subject matter experts (SMEs) has developed processes and procedures to standardize approaches to common requirements, such as the high level of cleanroom classifications, sterile manufacturing, chain of identity (COI) requirements, and managing vector segregation and safety. Leveraging global SMEs and establishing procedures in these areas means that each site and asset can take a common pre-vetted approach instead of working individually.

To address the challenges and provide the best solutions for cell therapy products, Lonza has recently introduced a standard New Product Introduction and tech transfer process, allowing for shortened timelines and faster turnaround to manufacture and deliver quality medicines to patients (Figure 1). This includes a de-risked product introduction, standardization across six key work streams, and well-defined processes.

The centering of production operations around the asset is a further key to Lonza's approach to CGT manufacture. Lonza's asset leadership teams are focused on one type of CGT at one location, with strong team connections and the unified goal of the success of the asset. There is also a high level of communication to the global network, meaning that lessons learned in one location are quickly disseminated to other locations. Because this team is centered on a therapy type and not a particular process, the team can optimally and efficiently distribute tasks across available resources. Since the team has all the departments represented to support operations, they can make decisions and problem-solve at the lowest level possible.

FIGURE 1

Lonza's standard New Product Introduction & Lifecycle Process.



COMMERCIALIZING HIGHLY COMPLEX CELL THERAPY PROCESSES

Case study: Houston

At Lonza Houston, the team has experienced and overcome many challenges that are common across the industry, including particulates prevalent in single-use materials, equipment not designed with CGT commercial manufacturing in mind, manual and open processing steps, and throughput challenges with autologous clinic limitations.

Overcoming these challenges has been eased using the approaches previously described. Successes include completing an FDA PLI audit with no 483 observations, the launch of two commercial autologous cell therapy programs within the last year, and the successful implementation of parallel processing. In 2021, the Houston site achieved its first FDA approval with the Breyanzi program. In 2022, the site gained two more approvals, making Lonza Houston the only CDMO with three licensed CGT products.

Case study: Singapore

Lonza CGT is a global operation with a global approach. The Lonza Singapore team is highly experienced and educated, equipped with a strong continuous improvement mindset to challenge the status quo. The team uses the end-to-end New Product Introduction process to maximize the possibility of getting tech transfer right first time. Continuous improvement also means being open-minded and flexible in response to change and uncertainty.

Challenges overcome by the Lonza Singapore site are representative of current industry challenges as a whole. These include the long lead times found with customer-specific consumables and the existence of manual open processes which bring sterility challenges and risk process efficiency. Recent successes for the Lonza Singapore site include obtaining zero 483 findings in the recent FDA audit in May. The site also achieved a 100% CGT batch success rate in the first half of 2023, which means all batches produced meet expectations. This demonstrates the stability and successful standardization of the manufacturing processes used.

A VISION OF THE FUTURE

To meet the manufacturing demand for CGTs to treat millions of patients, the field needs to move towards all-closed manufacturing processes with a minimum of grade A and B activities. A reduction in manual processes is necessary, which can be enabled by technological developments and automation. A significant cost reduction is required within large-scale manufacturing. Finally, standardized consumables and generalized processes are a requirement.

Q&A

Abigail Pinchbeck, Editor, BioInsights, speaks to **Melanie Mansbach**, Head of Cell Therapy Manufacturing, Lonza Houston, and **Hui Zheng**, Head of CGT Operations, Lonza Singapore

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Q The current CGT processes are largely manual and fixed. How can developers improve them without having to file another BLA?

HZ: Improvement can be split into two parts: product improvement and process efficiency improvement. Normally, product improvements always come with another BLA, but operational access tools can help to make the process more efficient. This means finishing the production and release faster, with fewer people needed to get the job done. For example, one of the OPEX tools we should use is value stream mapping to ensure we have an end-to-end analysis of the process and have identified any bottlenecks. When you zoom into the bottleneck, you can learn more about how to improve the process, documentation, and release testing. This helps establish an efficient, streamlined process and enables delivery of the product to the patients faster.

Q You mentioned that developers may have to plan their manufacturing early in the development process. Can you elaborate on where this causes manufacturing challenges?

MM: When developers are planning for production early in the process, the goals tend to be different. At that early stage, the focus is on finding a process that is successful and repeatable. However, oftentimes this means that efficiencies have not yet been considered, and failure modes have not yet been developed. Those inefficiencies and failure modes are only going to be replicated as you increase your throughput, which creates challenges. It is important to ensure that early on, you have a robust system considering all the possible failure modes and remediating and reducing those risks so that when production is ramped up, the risks have been eliminated.

Q What technologies does Lonza have for process improvement?

HZ: We follow many of the recent technologies. This includes a version of the software for the electronic battery cord. This is a key technology that can reduce human error and make the documentation process smoother. We are also working on a closed system filter to reduce the required manual operations in our processes. VR training is being employed. We want to use the latest technology to ensure that in allogenic production if the production volume increases, visual inspection remains fast. This is related to many automated or semi-automated visual inspection processes.

Q Which stages of the product lifecycle are the most critical to get right from a manufacturing perspective?

MM: Every stage is important, but there are a couple that are slightly more critical than the rest. First, the tech transfer into the manufacturing process is where you can achieve operational

excellence by design. At this stage, you have the opportunity to make the most changes early on before the process is cemented. Being able to get it right the first time and set the process up well is key. The other key stage is the point at which you are getting ready to do regulatory filing for the BLA. Making sure that everything is done correctly is crucial, as any misstep could cause huge delays when working with regulatory agencies.

Q Does Lonza offer smaller packages for testing only?

HZ: This is case-by-case for each site. For the Singapore site, we have a newly built sterility lab, and we would like to work with more customers regarding smaller sterility packages to help them expedite the testing process. We want to ensure all our testing is compliant and ready for BLA submission.

Q What are some of the tools that you use to share lessons learned across areas?

MM: One of the key things is the asset leadership team. Each of our programs and processes has a specific project team dedicated to a single program and they each have a member on the asset leadership team that they directly report to. We ensure that anyone on a specific process shares their lessons learned with that asset leadership team member, and we have regular meetings to discuss learnings across the asset leadership team. We consider each different process that we are running and see if we have applicable or similar areas in other processes. Then, we have those asset leadership team members communicate that back down to the members of the other teams.

Q What do you think is the most limiting factor in CGT success between the manual manufacturing bottleneck and the high variability of the starting material?

HZ: They are both limiting factors in different ways. One way that we can reduce the risk is by doing a lot of thinking at the initial development phase of the product. That might be the best phase for us to close the majority of processes and start to think about utilizing standardized material with a faster lead time or longer shelf life. The more problems we can fix at the beginning, the smoother the transition to commercial scale-up. I believe this is where CDMOs like us can bring the most benefit. We can understand and leverage all the standard utilized commercialization parts to make suggestions from the early development phase.

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Q How does the New Product Introduction process shorten the timelines and create faster turnarounds?

MM: Our New Product Introduction process shortens turnaround times because we are able to have a fixed checklist where we know everything that we need to do before we enter the next stage in processing. This allows us to ensure that we are set up correctly the first time and that we are not repeating steps. We have detailed lists and checklists that allow us to ensure we hit all those key points. It means that we are not doing any rework and we know exactly what needs to happen.

Q Are these lists and checklists digital or are they paper?

MM: We have these checklists in a digitized system, and we run them through a quality tracking system called TrackWise. That way, we ensure that every single task is complete, and we have a quality record to document completion when we process from one module to the next.

Q What role do you think digital tools play in operational excellence for CGT manufacture?

MM: Digital tools and automation are great additions to any operational excellence program. Within CGT manufacturing, it is important to ensure that systems are set up so that digital tools can be used. Often, we are working with very manual processes, and in those cases, digital tools might be more of a hindrance than an asset because of how many changes are being made. Ensuring that CGT processes are set up so that we can make use of as many digital tools and automation as possible helps guarantee successful manufacturing.

Q Can you share how you predict a reliable production schedule?

HZ: For the Singapore site, at this moment, we are working on more allogeneic products. That makes having a reliable production schedule possible.

MM: There are more considerations on the autologous side, as we must consider the clinic and patient scheduling. To have a reliable production schedule, the focus is on reserving manufacturing capacity so that the clinics can set up their patients reliably and know what will be available to them.

Q How early can we predict failure?

HZ: During the whole process, from development to commercialization, there are CQAs that we need to identify from different processes. This is the earliest detection point to see how the process is going. It is important to build in these critical key performance indicators (KPIs) across all departments. A good KPI can tell us a lot about the process and ensure that we can predict any risks early on, from the testing results from the CQAs and the in-process testing.

Q Are you facing any specific challenges in fill-finish operations with autologous cell therapy products?

MM: We learned early on that fill-finish is different for autologous CGT products than other biologics. Many of our programs were initially set up for vials, but for autologous cell therapy, we are filling in bags. There was an early learning curve, particularly with visual inspection; visual inspection of bags and vials is different and requires a different skill set. That was the biggest transition in fill-finish.

Q How does Lonza approach closing the whole manufacturing process?

MM: Closed processes are the optimal state when it comes to cell therapy manufacturing as a whole. We want to have as few open manipulations as possible to significantly reduce the risks associated with open manufacturing process and allow for more consistent results. In a lot of these processes, many stages require open manipulations. We are always actively figuring out where we have opportunities to close the process.

BIOGRAPHIES

MELANIE MANSBACH is a dedicated leader with a career spanning over 15 years, including experience in cell and gene therapy manufacturing and in the US Army. As the Head of Cell Therapy Manufacturing at Lonza Houston, she leads a 250-person team in the production of cell therapy products under cGMP. Her expertise in this specialized field, coupled with her proven track record of team development and process improvement, has consistently delivered outstanding results. Melanie's exceptional leadership and deep knowledge of cell and gene therapy manufacturing continue to drive transformative advancements in the industry.

HUI ZHENG joined Lonza in 2021 and is currently serving as the Head of Operations, CGT at Lonza Singapore. She drives operational excellence and ensures top-quality manufacturing processes. With extensive experience and leadership roles at Novartis, including Head of Project Management Office and Head of Operational Excellence, Hui has a proven track record of successful project execution. Her Master's degree in Biochemical Engineering from Tianjin University added a solid academic foundation to her practical expertise. Known for her dedication and innovation, Hui Zheng continues to make significant contributions to advancing cell and gene therapies.

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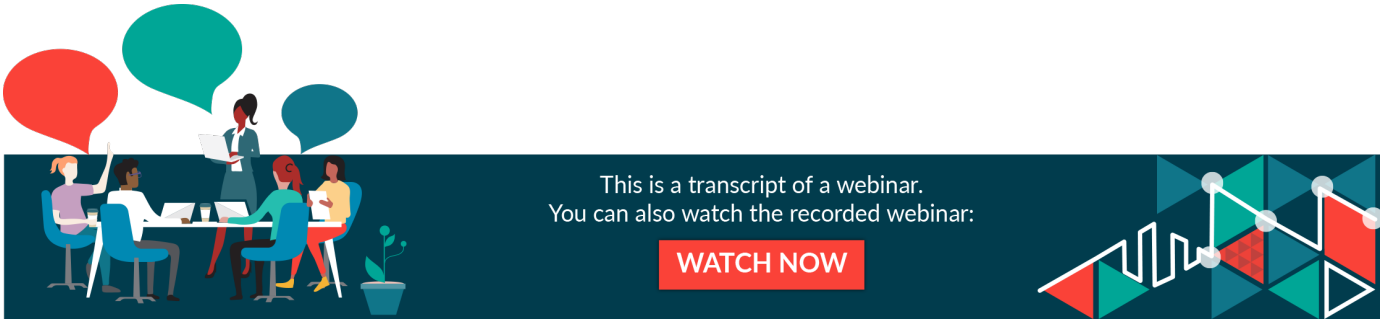

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INTERVIEW

Can AAV continue to deliver the promise of gene therapy?

In this episode, **Charlotte Barker** (Editor, BioInsights) speaks to **Ratish Krishnan** (Senior Strategy Consultant, Merck Life Sciences) and **Elie Hanania**, PhD (Vice President of Process Development Viral Vector Technologies, Avid Bioservices) about the current status and future manufacturing of AAV-based gene therapies, including how to streamline large-scale manufacturing.

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Q Does AAV still reign supreme for advanced therapies?

RK: We have reached a new era of medicine in the realm of advanced and potentially curative therapies. Adeno associated virus (AAV) has undoubtedly established itself as a leading contender for *in vivo* gene therapy due to its safety profile, efficient gene delivery, and ability to provide long-term transgene expression. It is estimated that the viral vector market is growing at roughly 30% compound annual growth rate, and AAV is used in about a third of all gene therapy clinical trials. AAV-based gene therapies have shown remarkable clinical and commercial success over the last decade with groundbreaking treatments approved for rare genetic disorders, ranging from spinal muscular atrophy, inherited retinal diseases, and most recently, hemophilia A.

"Researchers are exploring the strengths of each vector and tailoring them to meet the unique requirements of specific indications. This expanding arsenal of vectors will unlock new possibilities for treating a wide range of diseases, providing hope for patients worldwide."
— Ratish Krishnan

However, I would be remiss if I did not mention the other viral vectors demonstrating considerable promise in clinical applications. Lentiviral vectors dominate the *ex vivo* gene therapy treatment market. They constitute about 50% of the viral vectors used in gene therapy clinical trials. They have gained significant traction because of their unique ability to transduce both dividing and non-dividing cells, along with the ability to deliver a larger payload. Like AAV, lentiviral and retroviral vectors have found considerable commercial success in treating a range of diseases.

Adenoviral vectors have also been extensively studied in clinical trials prior to the emergence of AAV. These vectors are particularly known for their robust transduction efficiency, making them suitable for applications where transient gene expression is desired. However, their immunogenicity has limited their use for long-term therapies.

In addition, herpes simplex virus (HSV) vectors can target and transduce specific nerve cells, making them potential candidates for gene therapies targeting the central nervous system. The US FDA has recently approved Vyjuvek™, which uses HSV for the treatment of wounds in patients 6 months and older with rare and serious skin disorders.

The choice of viral vector depends on several factors, and while AAV remains a front-runner, the continued advancement of other viral vectors underscores the increasing diversity in the field of viral vector-based therapies. Researchers are exploring the strengths of each vector and tailoring them to meet the unique requirements of specific indications. This expanding arsenal of vectors will unlock new possibilities for treating a wide range of diseases, providing hope for patients worldwide.

EH: I believe AAV will still reign supreme, at least for a while. As part of a contract development & manufacturing organization (CDMO), I understand why researchers favor a variety of vectors, but from the manufacturing standpoint, AAV has the advantage of being produced using transfection or infection approaches. It is also robust and can handle harsh conditions during production and purification.

I agree that it will not be the only viral vector in use, as more therapeutic targets and indications are added. Oncolytic viruses will likely be the next reigning advanced therapy, but not for some time, and these may be used in conjunction with other immunotherapy approaches. It may be that one approach may not be as effective as multiple approaches (synergestic impact), and so HSV, oncolytic, and others may increase in popularity over time.

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Q How will AAV evolve in the next 5 years?

RK: Over the past 5-year period, the AAV market landscape has undergone significant changes, mostly driven by process development improvements, commercialization efforts, regulatory changes, and increased investments in this field. The rate of progress in the gene therapy pipeline from preclinical to clinical is on course to match that of established modalities such as monoclonal antibodies. We have moved from the hype of therapeutic potential to the concrete hope of commercialization, and we have now entered a phase of reality where we are starting to see continued success in the commercialization of therapies using AAV.

In the categories of process development, commercialization, and regulation, AAV has become mainstream, with tremendous advancements in all aspects from discovery to commercialization. First, process development improvements have been a key focus, resulting in higher upstream titers and higher downstream recoveries, and more efficient and scalable manufacturing platforms like suspension cell culture are widely utilized now. These advancements have increased the efficiency of viral vector production with the ultimate goal of making these therapies economically viable to the broader population of rare diseases and moving away from the hefty price tag.

Second, commercialization efforts have intensified, with successful clinical trials and regulatory approvals attracting substantial interest from pharmaceutical companies and biotech firms. This surge in interest has led to increased investments in manufacturing infrastructure, expanding production capabilities to meet the growing demand for viral vectors, and large biotech corporations have been continuously exploring merger and acquisition deals with smaller gene therapy biotechs.

Third, regulatory changes have played a critical role in facilitating the transition of AAV research into clinical applications. Regulatory agencies worldwide have recognized the potential of AAV therapies and have worked to establish clearer guidelines for their development and approval. The Center for Biologics Evaluation and Research (CBER) at the US FDA has led the way in this aspect. Looking into the future, we can expect even more exciting developments in the AAV market. There will be a range of therapeutic applications as researchers explore treatments for more prevalent diseases like neurodegenerative disorders.

Advancements in AAV manufacturing and plasmid engineering will enable personalized AAV therapies tailored to individual patient needs, thereby improving treatment efficacy and safety. Ongoing investment in the AAV space will continue to drive its growth. Venture capital funding partnerships and collaborations will fuel further research and development, expanding preclinical and clinical pipelines.

EH: It is important to state that when viral vector technologies emerged, the basic technologies available at that time were designed for monoclonal antibodies. The manufacturing and purification processes were not ideal, and hence I believe that as time passes, there will be improvements in these processes. The ultimate goal is of course to increase titer, yield, and purity of our product.

As Ratish mentioned, we are not just dealing with pediatric hereditary monogenic disorders. Now, researchers are ambitious in trying to tackle more complex disorders, so there is an increased demand for large amounts of AAV with higher titer. Having the infrastructure for scaling up is critical. Getting better plasmids is also important, especially if triple transfection is considered as the primary mode for AAV production. There are now many more transfection reagents on the market that have improved and selective characteristics, resulting in higher titer.

To further tackle the yield issue, innovative approaches, such as producer cell lines, are required. This is a holy grail for researchers, and we have seen great strides forward, but we are not there yet. Finally, the characterization of the AAV is becoming quite important. We need improved assays with superior specificity to enable proper assessment of titer, overall yield, and purity. These are some areas that I foresee becoming more dominant in the AAV field—in production, purification, and characterization.

Q As the use of AAV continues to grow, what challenges stand in its way?

RK: At the macroscopic level, manufacturing scalability remains a critical hurdle to meet the increasing demand for AAV vectors for diverse therapeutic applications. There are also lingering concerns around immunogenicity and host immune responses, which may limit the effectiveness of some AAV therapies, especially in cases of repeat dosing. Lastly, the regulatory landscape, and specifically the harmonization of guidelines across regions, will pose a challenge to the global development and commercialization of these AAV-based therapies.

EH: The biggest hurdle will be scale-up. We already mentioned operational challenges, but as we begin systemic delivery of these large doses, we must be cognizant of the safety profile. AAV is still in its infancy, so we do not have an extensive safety profile. So far it has been tolerable, but with much higher doses, we must be prepared for potential side effects, some of which may be serious. It is something to be aware of.

In addition, when it comes to AAV, we always talk about full capsids versus empty capsids, but of course, now we are aware that this is not black and white. There are also a wide range of partials that have not been extensively researched. We do not know what percentage of full versus empty we truly need to achieve the desired therapeutic effect, or what the impact is of injecting some of the partials will be.

Q What will be the most promising innovations in AAV production to emerge over the next 5 years?

EH: In upstream production, continuous manufacturing and intensification in cell culture will become dominant factors. Using chemically defined media, additives, and boosts, can yield high density of cells, which is critical to produce more virus.

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"As we now have more defined therapies, scientists can consider alternative approaches to produce the required AAV other than triple transfection (such as coinfection using baculovirus or HSV). These may be less expensive for campaign runs..."
— Elie Hanania

In downstream processing, continuous purification with chromatography and tangential flow filtration will emerge.

In addition, the design of plasmids used during transfection will improve in terms of size (smaller plasmids would be a plus) and removal of redundant or non-essential sequences.

As we now have more defined therapies, scientists can consider alternative approaches to produce the required AAV other than triple transfection (such as coinfection using baculovirus or HSV). These may be less expensive for campaign runs, easier to manage, and achieve greater consistency. Scientists are looking at the AAV particles generated by these different approaches to learn how they compare to those produced by triple transfection.

RK: AAV production is on the cusp of transformation, with a lot of innovative trends that are poised to shape the landscape. As Elie mentioned, continuous bioprocessing approaches like perfusion bioreactors will improve productivity.

Improved transfection methods will also be important—the producer cell lines along with plasmid engineering and miniaturization of plasmid promoter elements will further optimize AAV vector production. Lastly, purification techniques like continuous downstream processing and advanced chromatography methods using membranes will significantly improve purification efficiency, thereby ensuring high-quality AAV vectors.

Q What innovative technologies and manufacturing strategies should we consider to streamline AAV manufacturing for more common disease indications?

RK: Streamlining manufacturing is paramount to enhancing patient access and affordability of advanced therapies worldwide. We are seeing AAV-based therapies being approved for high-dose indications and large patient populations. Optimizing productivity, reducing cost per dose, and maintaining high-quality standards will all be important.

This can be achieved by process optimization for improved efficiency and higher yields, scalable manufacturing platforms for adaptability, automation and robotics for reliable production, robust supply chain management, modular facilities for resource optimization, and CDMO partnerships for expertise and cost-effectiveness. These collective efforts will drive the transformation of advanced therapies, making them more accessible to patients in need.

EH: Regulatory agencies will probably have more stringent requirements when it comes to AAV production and purification for relatively prevalent neurological and oncological disorders. Most of these regulations focus on the safety and efficacy of the final product. However, I think some of the requirements need to be vetted by scientists to make sure that they are critical for the process and the product, since these requirements ultimately have an impact on production cost and timeline.

Furthermore, testing and characterization improvements are required to develop precise assays with extended ranges. We also need to have orthogonal methods of testing to be able to confidently affirm that we are generating and delivering what we say we are. Overall, all these approaches need to be robust, scalable, and able to consistently generate the required purity and quality, batch after batch.

BIOGRAPHIES

RATISH KRISHNAN is a Senior Strategy Consultant in the Novel Modalities BioProcessing group for the Americas. A process development scientist by background, he has over 13 years of experience in vaccines, monoclonal antibodies, and viral vector modalities from pre-clinical to late-stage process characterization, validation, and commercialization activities such as BLA authoring. Before joining Merck, Ratish managed process development teams at Novartis and Pfizer. Now, he serves as a global subject matter expert for viral vector manufacturing and provides strategic guidance to internal stakeholders and key customers. He holds a master's degree in Biotechnology from Pennsylvania State University.

ELIE HANANIA has led the process development teams at different cell and gene therapy CDMOs including Fujifilm Diosynth Biotechnologies (College Station, TX), Millipore Sigma (Merk KGaA, Carlsbad, CA), and Progenitor Cell Therapy (Hitachi Company, Mountain View, CA). Elie's expertise spans academic, clinical, and biotech, with diverse experience in viral vectors, molecular and cell biology, virology, and characterization assay development with implementation of empowering technologies in advanced cell and gene therapies.

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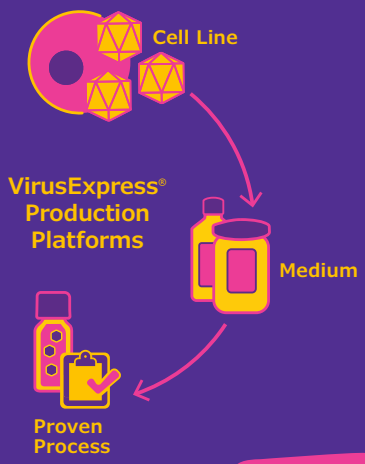

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INTERVIEW

Building with the patient in mind: designing a state-of-the-art facility for viral vector manufacturing

The cell and gene therapy space poses a number of unique challenges when it comes to facility design. In this interview, **Róisín McGuigan**, Editor, BioInsights speaks to Yposkesi's **Louis-Marie De Montgrand**, Chief Operational Officer, and **Morad El Gueddari**, Chief Pharmacist and Head of Quality to discuss the key considerations for designing an advanced therapy manufacturing facility that can meet the needs of future commercial demand.

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Q Could you introduce yourselves and tell me about your current roles?

LM: I am currently the Director of Operations and joined the company to help with the huge transformation involved in switching from a startup/development company to an industrial operations organization. In terms of my background, I am a biologist and have spent

more than 25 years in the biopharma industry, both within big biopharma companies and also in CDMOs, in the cell and gene therapy area.

MG: I am the Head of Quality and the Chief Pharmaceutical Officer. As for my background, I am a pharmacist and a chemical engineer. As Louis-Marie pointed out, our main challenge is bringing the company from the developmental to the commercial side, and this is something we are working together to achieve.

Q When it comes to designing manufacturing facilities, what unique challenges does the cell and gene therapy space pose?

MG: One of the main challenges is that we always have to keep the patient at the forefront of our minds, as our objective is to manufacture a product to create a medicine and cure a disease. The second for our respective job roles is to respect regulatory guidance and ensure we are following GMPs and GMO regulations. The third challenge—which we are addressing with our new facility—is to find a way to enhance our capacity to safely produce at large scale and be commercially ready for tomorrow.

LM: The intention for our new building is to add additional capacity to deliver commercial products. The unique challenge here will be to bring a design that has different flows from the B1 (the existing building). The new building, the B3, will be designed to ramp up high volumes with different client requests and of course, with the up-to-date standards of the US FDA and the French National Agency for the Safety of Medicines and Health Products (ANSM).

Q How did you bring your previous experience to bear when designing a new facility?

LM: In the past, I have been involved in many large capital expenditure projects, and I was able to bring this background to the Yposkesi team. With a mix of our different backgrounds, the entire team brought the quality design and industrial experience needed to create a facility with the best design and up-to-date standards, employing energy efficiency and the latest technology.

Morad designed all the details because he was the sponsor at the beginning of the project. I brought additional background from an industrial operation organization point of view, from

One of the main challenges is that we always have to keep the patient at the forefront of our minds, as our objective is to manufacture a product to create a medicine and cure a disease.

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standardization and harmonization, platform standardization, and equipment and catalog services.

> When designing equipment, we always keep in mind energy efficiency in terms of consumption so that when the building is running at full speed, and we need all that energy for pharmaceutical rooms, we can still be efficient.

MG: As Louis-Marie said, the synergy in expertise between our teammates regarding AAV and lentiviral (LV) vector production is vital. Yposkesi's track record is also important because, since 2013 and the beginning of clinical stage AAV and LV vector production, we have produced many batches in the current building. All of this experience and expertise has been implemented in the new facility. For example, our experience has led us to properly respect GMO and GMP guidance by establishing a unidirectional flow for waste and using a dedicated pass-through airlock decontamination system in order to avoid any potential cross-contamination for the product.

In addition, the building layout has been designed in order to produce at a commercial capacity while still meeting safety regulations and avoiding potential contamination. For example, the bioreactor chiller can be a source of contamination. As it does not need to be in the GMP area, we decided to place it in an unclassified area.

Regarding the quality aspect, the quality management system is exactly the same for the new facility and we decided to link the two buildings with a tunnel. This helps us to avoid problems with technology transfer and also to avoid any potential qualification or validation errors since we already have a functioning quality management system in place. This layout also helps us with something quite specific for the French regulations, the French Bonnes Pratiques de Fabrications. The link between the current building and the new facility will maintain one-fifth of the pharmaceutical officers on-site, allowing us to save time, retain the same quality we already had, and increase our preparedness to commercially produce AAV and LV vectors for cell and gene therapy products.

Q What were the key considerations for ensuring the ability to meet future commercial demands and capacity requirements in such a rapidly evolving space?

MG: As a pharmacist and quality director, I consider this through the lens of the 5M method, which pertains to Machine (equipment), Medium (environment), Method (process), Material (raw materials), and Workforce. We have chosen single-use equipment in order to answer the challenge of producing AAV or LV vectors. Regarding the method, the new facility has been built with production areas identical like mirrors, giving us two independent manufacturing suites that optimize the qualification process. The qualifications are almost copied and pasted, therefore saving time. Regarding the environment, during the design of the building, we took into account our needs in terms of the optimization processes, but also looked at the implementation of innovative, greener, solutions. Regarding the workforce, we have decided to involve people from the current building to optimize training in the new building.

LM: Regarding the consideration of greener solutions, we have implemented renewable energies because it helps us to reduce our costs. It is a capital expenditure, but it is a sustainable solution. When designing equipment, we always keep in mind energy efficiency in terms of consumption so that when the building is running at full speed, and we need all that energy for pharmaceutical rooms, we can still be efficient. When we are in a shutdown or in an inter-campaign situation, we can also reduce our consumption of energy. We have several technologies to reduce energy consumption, like the heating, ventilation, and air conditioning (HVAC) system, that work while still being compliant with all the pharmaceutical requirements.

In terms of the key considerations for ensuring the ability to meet future commercial demands, one of the things that we must consider is that, with such a big building and with our industrial operation, we must reorganize the workforce to work in shifts. Once the building is launched, we will have several products, one after the other, and we cannot stop the process.

In addition, we also must consider and identify the needs for the next 5–10 years on the market. Until now, we have designed commercial manufacturing assets for a 1,000 L size. In case there are some specific requests, we also anticipated a bigger design with a process of up to 2,000 L in upstream, and, of course, with adequate downstream processing.

All of this has been included in this new building design. The new biomanufacturing site will bring a higher capacity and likely be able to meet triple the demand. Our capacity will follow this demand, and the building will be able to deliver at least 40 batches a year.

Q Staff recruitment and retention is another challenging area for cell and gene therapy. How are you ensuring a sufficient workforce to meet your needs?

LM: Yposkesi has been involved with universities and engineering schools for a long time in order to complement our needs. Today, that effort is no longer sufficient. We are going to open the door much more this year. For example, we will have an open house for students on October the 20th, providing the opportunity for students who are really curious and eager to learn about cell and gene therapy to come and see from a practical point of view what an industrial commercial manufacturing building looks like in the new facility, and what a clinical organization looks like in Yposkesi's first building.

...we will have an open house for students on October the 20th, providing the opportunity for students who are really curious and eager to learn about cell and gene therapy to come and see from a practical point of view what an industrial commercial manufacturing building looks like

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We are extending our recruitment strategy because, in the near future, the new building will require almost 80 people at full capacity. We have split our strategy into different skill sets: technicians, engineers, and pharmacists. We are proposing internships and specific programs that give students the opportunity to come to Yposkesi for a few weeks before going back to their university. We are also going to develop some specific businesses with students because we think that they have some new ideas about simplifying processes. Students are really amazing because they have completely new eyes.

The two challenges are about retaining our workforce and involving the current people in the current building in the new facility, and recruiting further workforce and continuing to develop our expertise.

We are planning a ramp-up in recruitment in the next 5 years, and we are putting in all the necessary networking and contract partnerships to bring in all the people we need to be successful. One of the things that will be the most important is to convince people to come and see our capacities because as soon as they come and see, they will understand our way of working, the atmosphere, and the work–life balance. Retention will also be high because they will be allowed to develop themselves and learn new things,. This workforce will ramp up the new facility with us. We are definitely feeling very positive about the future.

MG: Our key focus is recruiting and retaining people for the manufacturing area and specifically for cell and gene therapy. The two challenges are about retaining our workforce and involving the current people in the current building in the new facility, and recruiting further workforce and continuing to develop our expertise. For the first challenge, as Louis-Marie explained, our main strategy is involving our existing expert workforce and all the forces of Yposkesi in the new building. They are involved in every aspect of the project – the qualification, the choice of the equipment like the bioreactors, and even things like the colors used in the new facility. Retaining the current expertise we have is crucial.

As for the second challenge of recruiting more people for the new facility to enhance our capacity to produce commercially, and as Louis-Marie explained we have developed partnerships with local schools and are opening the doors for students to see the different jobs inside a manufacturing facility.

Q If you reflect on your progress so far, what would be your key learnings and advice when it comes to successful facility design for the cell and gene therapy space?

MG: My main advice for the design of a new facility is creating a synergy in expertise and experience. Additionally, always be thinking ahead. In the new facility, we currently have two manufacturing areas in order to produce at large scale, up to 2,000 L of AAV and LV vector product. However, we are also thinking a step ahead, so we have planned for up to four or five additional manufacturing suites. This gives us the capacity to implement a new manufacturing facility if needed in order to be ready to manufacture more product.

LM: We have capitalized on hundreds of batches and our extensive knowledge in order to propose solutions to many current challenges. We are always looking to new technology, trying to implement new analytical equipment, and developing new process equipment in order to keep up with a higher density of cells. We must be agile. On the other hand, we also have to keep cost-effectiveness in mind because to be successful, we have to provide services at affordable prices.

Cost-effectiveness also pertains to standardizing platforms and catalog services to use best practices and accomplish tasks in a shorter timeframe. As Morad said, it is important to always think a step ahead, looking at new technology and new improvements. From a cost-effectiveness point of view, we must also aim to standardize, and keep in mind the fact that we need to achieve industrial-scale processes and by the end, achieve affordable prices for the patient.

Q What's next for this project?

LM: For the commercial manufacturing building, we are in the qualification period. By the end of October, we will finish most of those steps. We are preparing for the environment monitoring and performance qualification (EMPQ) phases, and, of course, the first technical run.

MG: This new facility was authorized by the French authorities in 2022, and we are expecting to be inspected by the French regulators at the end of 2023 or early in 2024 after the first batches. We hope to be ready to produce our new products at large scale by 2024.

BIOGRAPHIES

LOUIS-MARIE DE MONTGRAND has been the Chief Operational Officer at Yposkesi since June 2022. With 20 years of experience in leading operations in Biopharma Industries, Louis-Marie held several positions at major industrial and CDMO actors. He started his career working for Sanofi as an EIT Process Engineer, followed by 10 years at GSK being Head of Production then Head of Technical Services. After that, Louis-Marie became CMO Operations Director at Novasep and VP Operations—Viral Vector Services for Europe at Thermo Fisher Scientific Inc.

MORAD EL GUEDDARI has been the Chief Pharmacist and Head of Quality of Yposkesi since June 2022. Morad joined the company as Head of Operations in 2018. With 15 years of bioproduction and cell and gene therapy experience, Morad held various managerial roles in quality and GMP manufacturing at European pharmaceutical companies, including LFB and CELLforCURE.

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Improving genetic engineering of adoptive cell therapies by a novel culture media supplement

Despina Pleitez, **Terri Jerbi**, **Meredith Safford**, **Jade Scheers**, **Sarah Campion**, **Lori Noffsinger**, **Matthew Hewitt**, and **Alex Sargent**,
Process and Assay Development, Cell and Gene Therapy, Charles River Laboratories

Electroporation (EP) is a popular approach to genetically modifying cells, creating transient pores in the cell membrane that allow the introduction of mRNA, DNA, endonucleases, and/or transposon systems into the cell. However, whilst EP can result in high gene editing and transfection efficiency, it often results in high rates of cell death and a significant drop in cell viability. In this poster, we report that augmenting cell culture media with a defined supplement can improve recovery and viability post-electroporation of T cells and hematopoietic stem cells (HSCs).

METHODS

We tested whether the addition of a pro-survival culture supplement after EP can augment cell recovery and transfection efficiency. **Figure 1** shows an overview of the study design and process flow.

RESULTS

As shown in **Figure 2**, cell recovery increased from 50% to 80–100%, and cell viability increased 30-40% when culture media was supplemented. A two-fold increase in transgene expression was also observed.

In addition, the pro-survival effects of this supplement were not cell type specific, as similar improvements in cell viability, recovery, and transfection efficiency were observed for both T cells and HSCs **(Figure 3)**.

CONCLUSION & FUTURE DIRECTIONS

Collectively, these data demonstrate that supplementation of cell culture media with this novel supplement can improve cell recovery, viability, and transfection efficiency following electroporation. This represents a new and potentially significant mechanism to improve genetic engineering of adoptive cell therapies for the treatment of cancer and other diseases. Future development work will explore whether this supplement can permit cells to undergo more intense electroporation regimes, driving even greater gene editing efficiency whilst maintaining cell health.

Figure 1. Addition of a pro-survival culture supplement after EP.

Day −1: thaw cells
- Cryopreserved CD3+ T cells
- Cryopreserved CD34+ HSCs

Day 0: electroporate (tagged transgene)

Day 1: cell number & viability
- With supplement (+)
- Without supplement (−)
- Non-EP cells=control

Day 4: transfection efficiency
- With supplement (+)
- Without supplement (−)

Figure 2. Cell recovery (left) and cell viability (middle) 24 h after electroporation. Transfection efficiency (right) 4 days after transfection. T cells and HSCs were electroporated using manufacturer's recommended protocol for each cell type. Mock cells were loaded into electroporator but did not receive EP or supplement thereafter. Data shown=mean +/− SD, n=2 donors for each cell type.

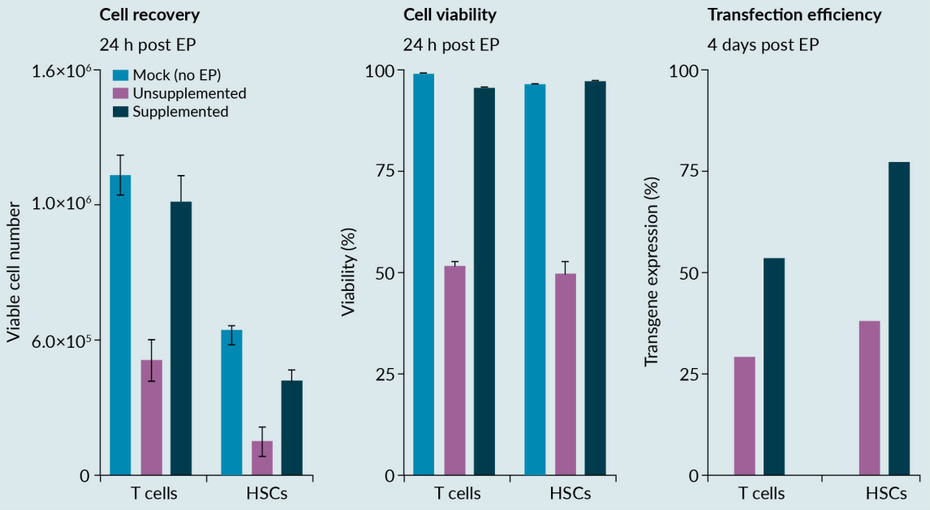
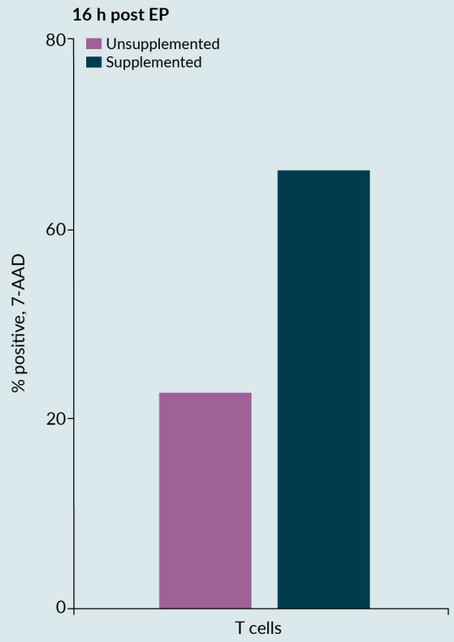


Figure 3. Cell death assessed by flow cytometry using 7-AAD staining. T cells were electroporated using manufacturer's recommended protocol and then cultured for 16 h with or without supplement prior to analysis. Data shown=mean +/− SEM, n=2 donors for each condition.



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INNOVATOR INSIGHT

AAV & lentiviral vector titer determination: past, present, & future



Viral titer determination is essential to the safe and effective dosing of gene therapy products, but methods used to measure viral titer are still improving and evolving. In this article, **Mark White** (Associate Director, Biopharma and Translational Product Marketing, Digital Biology Group, Bio-Rad), **John Bechill** (Principal Scientist, BioProcess Group, Modalis Therapeutics), and **Peng Wang** (Senior Scientist, Viral Vector Analytical Team Lead, Lonza) discuss the need for accurate viral titer determination, traditional approaches to measuring viral titer, and the field's trajectory into the future.

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Q Can you briefly introduce us to your respective organizations? What technologies are you working with and in what applications?

MW: I work in the droplet digital PCR (ddPCR) group at Bio-Rad. For almost a decade, we have been developing specific assays, instruments, and workflows for both AAV and

lentivirus to identify viral titer, vector copy number, and more recently, testing for contaminants such as host-cell DNA and mycoplasma bacteria.

PW: As a leading CDMO, Lonza provides comprehensive solutions to accelerate the development, manufacturing, and commercialization of life-changing therapies. We are constantly investing in and developing our extensive experience across a wide range of modalities including but not limited to AAV, lentivirus, exosomes, and induced pluripotent stem cells (iPSCs).

JB: I am the analytical lead for the bioprocess group at Modalis Therapeutics, a gene therapy company. Our core technology modulates different targets for various types of genetic disorders. We deliver this technology using AAV in a standard platform. We currently deal with early-stage discovery programs and some process development for targets that we are looking to bring to the clinic in future. Specifically, we look at the bioproduction of AAV, the quality attributes of the virus, impurity testing, residual protein and DNA testing, and the potency of the different lots that we produce.

Q Can you review the traditional methods for measuring viral titer for both AAV and LV vectors, and discuss some of the important limitations that have led to a new wave of analytical tools?

PW: Quantitative PCR (qPCR) and ddPCR use a single amplicon to detect a single piece of DNA. The assumption here is that biology will do the work perfectly, but that is not always the case. This is where discussions surrounding full, partial, and empty capsids originated.

We have introduced some new technology to address these challenges. For lentiviral vectors, most people are using cell-based assays, which are lengthy assays with multiple steps. We are looking at each aspect of these assays to work on improving them, including carefully reviewing the associated standard operating procedures (SOPs). It is a difficult challenge, but we are working on it.

JB: In terms of traditional AAV titration methods, 5 years ago, we were still using qPCR-based methods for viral titration of the genome. This was a version of the assay that was done in a normal thermocycler and looked at the cycle threshold (Ct) differences over different types of amplification. The original standard viral genome (vg) titer was wired to a standard of a known vg concentration. Then, dilutions would be performed to create a leaner range, before testing a specific sample of interest and putting it back to the standard that you created. The greatest issue with this was the fact that each time a new standard lot was produced, slight changes were introduced into the assay, so the standard would be slightly different in reality. The use of a standard was a great limitation of qPCR assays.

This changed when ddPCR was introduced. ddPCR allows for absolute quantification without a standard. This has resulted in less variation, which has in turn made it easier to

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tech transfer to different sites. ddPCR also allows and simplifies the use of different primers and probes against different regions. To quantify using different primers and probes previously, you had to ensure that the chosen standard included that specific region. With ddPCR, it is easier to check out several different regions, confirm the viral titers against those regions, determine which ones give you the best values, and select the region that makes greatest sense (as determined by other assays of the capsid titer). It is also easy to look at several things at once.

In terms of utility, ddPCR is easy to scale-up and can test many samples in a high-throughput way. The only limitation of ddPCR is that it requires manual handling for dilution and processing.

I am looking forward to the continued emergence of orthogonal technologies that will allow us to test samples by different methods such as 260/280. However, these technologies are product non-specific, so you still need to utilize a genome titration assay with ddPCR for product specificity. Several of these different techniques for vg titration will hopefully be utilized in the future to give us both high-throughput testing capabilities and a better idea of specific genome titers.

MW: The field started with qPCR because digital PCR was not yet available. As viral titer is critical for understanding the therapeutic dose going into the patient, there has been a focus on getting that right. As the field matures, though, the standards for accurate quantification are increasing. Moving from relying on a standard curve to counting individual DNA molecules specific to a therapy has caused a big shift towards digital PCR, specifically ddPCR.

Historically, most companies were only looking at one component: the gene of interest (GOI). Now, there is more insight and focus on the whole vector, including everything between the promoter and the poly(A) tail. Things are moving forward in terms of our ability to count all the different pieces.

Q How have regulators' opinions and expectations evolved in this particular area over the years? What specific challenges does this present to developers and manufacturers today?

JB: Regulators are looking for well-characterized assays. The transition from qPCR to ddPCR is one of the more rapidly accepted transitions for regulators because of the reproducibility of ddPCR, and its rapid adoption by the field. A lot of information was gathered quickly, so large amounts of data were available to regulators.

Technologies looking at empty and full capsids have made less of an impact in terms of acceptance because there is less information about novel assays and technologies for empty/full quantification versus analytical ultracentrifugation (AUC), which is still the gold standard. It has been a slow transition to accepting any other methods as reliable and qualifiable. Much depends on how well these technologies are characterized and implemented into the process, and reviewed internally, and how observations are supported in the bioprocess.

Q What are some of the key emerging analytical methods for AAV, specifically, and what can they bring to the table compared to traditional approaches?

MW: Over recent years, there has been a push towards molecular-based methods versus more traditional cell culture-based or live virus-based methods, specifically around mycoplasma testing or replication-competent lentivirus testing.

Bio-Rad has released digital PCR-based kits for mycoplasma and is set to release similar kits for replication-competent lentivirus and AAVs. These will run alongside traditional methods. Specifically in lentivirus, especially for autologous therapies, we need rapid release. Being able to turn around a final autologous cell therapy product from live cells collected from the patient within several days is essential, and simply cannot be done using traditional methods.

In our product development, we have been supporting the overall theme of more rapid methods—across the board, speed is a focus for all the measurements we have to make around these therapeutic modalities. As they mature and as the field needs things to be more efficient, it is up to companies like us to start standardizing and speeding up some of these processes.

PW: Due to the nature of AAV, single amplicon-based titer assays are not sufficient. Right now, we are working towards multiplexing and promoting intact titer assays using Bio-Rad's platform to provide meaningful data to help establish a reasonable titer value. We need a titer value that matches the overall impact of the vector, not just the fragment, because we know that AAV is not the perfect packaging solution.

There are many novel technologies coming out to measure full/partial/empty ratio, such as charge detection mass spectrometry (CDMS). These new methods often provide similar or comparable results to AUC. Progress is slow, but by staying open-minded, people do begin to adopt new methods. This is the subject of an ongoing conversation between drug developers, sponsors, and regulatory agencies.

JB: As Peng mentioned, much of the novel technology revolves around full/empty capsid ratios. Several technologies have been released such as photometry, 260/280 dynamic light scattering (DLS), and surface plasmon resonance (SPR)-based methods, which are now increasingly accessible to early-stage companies. As AUC takes a fair amount of time and money to establish, many new technologies have centered around decreasing that burden, allowing people to make more rapid process decisions relating to their full/empty ratios and move towards a higher percentage of full capsids.

However, some of the harder questions for the field today reach beyond full/empty ratio—characterizing partials, for example. Some technologies do not allow the quantification of partials at all. AUC still tends to be the best way to look at partial populations, determining whether you have them and making assertions as to whether they are impacting your product potency in some way.

I want to see better technology being developed in terms of expression. Finding better ways of looking at low-abundant targets is important, especially to open up more therapeutic targets for

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gene therapy to interrogate. ddPCR definitely helps there, though advancements are still needed at both the RNA level and the protein level. Then, we would have two arms, one looking at full/empty, and one looking at the effect of a particular product in a biological system.

Q The same level of process and analytical tool innovation enjoyed by the AAV field hasn't quite materialized for the lentivirus field as yet. Could you comment on what innovations have arrived in the lentivirus space though?

PW: I believe it is time to expand the discussion around full/partial/empty ratios to lentiviral vectors. We presented a poster at the most recent American Society of Gene and Cell Therapy (ASGCT) annual meeting, which investigated the different layers of lentiviral vectors. We found that 50% of the lentiviral vector product was empty. We can work around this, though, and improve processes and molecular design in order to improve full/empty ratio. As we often think of lentiviral vectors as a critical raw material or an intermediate product for *ex vivo* applications, people might think full/empty ratio does not matter. However, if there are potential *in vivo* applications, it is time to look at that ratio.

Q Where could current AAV analytical tools potentially be applied to lentivirus? Equally, where is more bespoke analytical tool innovation required by the lentiviral field?

PW: For lentiviral vectors, we have a whole toolbox to look at various aspects, including p24, particle tracking using nanoparticle tracking analysis (NTA), and infectious titer determination by transduction. Cell-based assays are lengthy and contain multiple steps allowing variability to occur, as we have discussed. We are still working on every single step.

One step we are specifically looking at is the endpoint environment. We are using ddPCR to replace qPCR at endpoint measurements for lentiviral vectors to reduce variability. At the same time, we are also working with AAV on TCID50 (50% tissue culture infectious dose) assays. We recently published a paper in Human Gene Therapy that discussed a comparison of qPCR and ddPCR in the endpoint environment. Our findings showed that we did improve precision on that front. However, there are a lot of opportunities to improve further, and we are prepared to continue working on this to establish more robust and accurate measurements for both AAV and lentiviral vectors.

MW: At Bio-Rad, we have been highly focused on supporting AAV developers—specifically, analytical development and QC groups. For instance, we make a HEK host cell DNA quantification kit for AAV developers. As we were completing our recent Voice of Customer (VoC) program, we added in some lentiviral developers.

The feedback we received that was unique to lentivirus was that host cell DNA is human-derived and most kits on the market generally detect human DNA. But when you have lentivirus in the background of a T cell, those measurement techniques don't work. When we designed

our kit, we made it specific to HEK DNA and not to general human DNA. Therefore, it works for groups working with both lentivirus and AAV. As a kit developer, we want to serve everyone if we can.

PW: As the majority of lentivirus products were produced by using HEK 293T, the kits were developed for HEK 293, and Bio-Rad do a bridging analysis to give a correction factor on that. That is important for us when adopting a kit for application with lentiviral vectors.

Q The application of next-generation sequencing (NGS) is becoming more widespread, but where should and shouldn't it be applied in the viral vector space?

JB: My experience is that NGS is good for identity testing, but it can be difficult to use for making process decisions. Due to the single-stranded nature of AAV, you have to use adapters and linkers to look at AAV using NGS, which creates artifacts. It can be difficult to determine whether you have things like partial populations using this technology. If you see it, then you know it is there, but if you do not see it, you do not necessarily know that it is not there.

I recommend using an orthogonal technique to find good controls, and develop the assays for NGS based on those controls. The field needs more development in terms of being useful for different types of issues with genomes. The longer-read methods will provide the most help. Having one full read means it does not require small alignments of small pieces with some of the other technologies, so you will be less likely to miss any deletions or truncations. We need good controls for finding when things are wrong with NGS in order to make it useful as an analytical tool for bioprocess development.

MW: I agree with John that it is excellent for mapping what might be there. NGS should not be used for counting molecules if you are determining the actual number. With NGS, you can map the potential partials that are there, but you should use an orthogonal technique, like ddPCR, to count how many of those molecules are present and in what ratios. But utilizing both methods together will help you to effectively characterize.

NGS has a great role to play in early development and growing understanding of the process and product. As the process moves towards a higher throughput, lower cost, and faster turnaround time, ddPCR is a great place to land for rapid viral titering across the genome.

PW: I agree with both panelists on that front, but I do want to see NGS adding value somewhere. This requires more development regarding sample preparation and data processing. For general scientists not working in the bioinformatics field, the final reports can be confusing. We often do not know how the data were achieved. As a development team, we want to know all the details. NGS has the potential to be a very good tool as a complementary method.

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Q You have mentioned the fact that we are seeing more novel approaches aimed at accelerating QC testing, particularly to potentially replace some of the lengthier assays, such as mycoplasma and replication competency. How would you weigh up the benefit–risk balance in adopting a novel rapid assay?

MW: We make the kits and rely on customers like John to take those through the regulatory process. It takes a partnership between everybody—the manufacturers of kits, the end users, and representatives from the FDA—to have a conversation about the benefits versus risks to the patient, because risk will trump everything.

Replication-competent tests, in particular, are expensive and take a long time, as they are highly specialized tests that only a small number of people perform. With the newer generations of AAV and lentivirus, I have not heard of anybody finding a positive on those assays. With enough time and enough products making it through, and the field realizing that these things are safe, the risk to the patients will be well-documented enough to move to a molecular-based test as a surrogate. That is the only way we will see that shift.

JB: The hand-in-hand long assays are generally safety assays. People are risk-averse when considering safety assays because these can invalidate a lot quickly if they go wrong. Unfortunately, there are also a lot of culture-based assays, so you have to show the limit of detection (LOD), which is equivalent to outgrowth. However, there have been great strides in moving towards PCR-based assays versus culture-based assays for mycoplasma testing.

We have had internal discussions about whether replication-competent AAV (rcAAV) testing can be a PCR-based assay. The difficulty with this is that there is not much good understanding of what rcAAV is and its genome configuration. People are averse to picking up a PCR-based assay because you could miss something that a culture-based assay might pick up. Having several rounds of infection and growing out rcAAV so that it is detectable above the background of helper sequences is important. Getting a better understanding of when rcAAV occurs, or finding a production system where the risk is low, increases the willingness to adopt a PCR-based assay because the safety margin is higher.

PW: I would also like to discuss adventitious virus testing. Some people suggest that we move to PCR-based adventitious virus testing, but the problem is that while you will be fine for a known virus where you have the sequence in your database, you will not be able to detect a virus that is unknown to your database. I do not think we can get rid of cell culture-based assays because of this issue. We have to prepare our sequence databases adequately first.

Q How can we as a field continue to move the space forward so that lengthy release assays become a thing of the past?

PW: I do not believe we will be able to totally replace these release assays. Adding another tool to speed up the process, however, could be very beneficial.

MW: The only way this could change is by all groups working together. Over the last decade, it has been great to see regulatory bodies, especially the US FDA, being open to learning and hearing about new techniques and technologies. Some therapeutic developers are blazing the trail because the risk to patient safety of a long replication-competent assay is outweighed by certain patients' very poor prognosis. There have been compassionate use cases where accelerated testing is allowed with follow-up. Everybody needs to have a conversation about the risk-benefit together. It is not going to be any one group that will make the change.

Q Thinking about some likely areas for future regulatory scrutiny, vector integrity and identity come to mind. How may regulatory guidance evolve in this area moving forward, and what should we be doing now to prepare to meet these future requirements?

JB: That is a complicated question because it is product-specific. Genome integrity may not influence one product, but it may influence another significantly. In our early-stage programs, we leverage evaluating genome integrity. I like the idea of genome linkage analysis and several groups are looking at this. We do not use this as an absolute—I think of it as a relative assay because positive controls are difficult. At the moment, we are using it as a surveillance technique, looking across our different lots and programs to see if a problem arises, and whether we can correlate it back to using a particular technology.

Other general analytical tools out there look at genome integrity by mass, TapeStation or alkaline gel. These more target-specific technologies will continue to develop, especially as high-throughput methods.

PW: These are some new and handy technologies. Once you fully understand your sample preparation process, you put in two instruments that give readouts, and perform mathematical readings to get the link analysis. At the same time, it is not losing the single amplicon titer—it is actually adding an additional layer to give extra information. We are implementing this in R&D settings, and are working to transfer this to our bioanalytical services groups to add value.

Usually, we give three results to clients: a five prime end titer, a three prime end titer, and the intact titer using linkage analysis. This gives a full picture that can be used to demonstrate how good a process and the molecular design are. It is certainly an exciting new toy that can be added to your toolbox.

MW: The guidance is always outdated, which is the challenge. What is actually published is usually 5 to 10 years old, and everybody is far ahead of that, trying to predict what is going to happen next.

I think a clear theme is that it is the entire genome that is the therapeutic construct, if you will. The gene of interest and its expression into a protein which in most cases is what is doing the work. When we are dosing a patient, we are giving them both the capsid (whether AAV or adenovirus) and the full gene from five prime to three prime. There is a lot more attention

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and scrutiny now towards the genome, especially with multiple papers coming out looking at unusual, truncated genomes and their identity.

With empty/full, a big theme has been looking at partially-filled capsids. These partial capsids may be totally benign, but they may also be impactful. And that may be different for every different therapeutic modality.

We have been trying to help the field answer these questions that have been swirling around with the new QX600 Droplet Digital PCR system with six colors, which allows six different parts of the genome to be observed in one well. As ddPCR is inhibitor tolerant, we can put AAV capsids directly in and lyse them in the droplets, so there is no processing as would be required with NGS, for example. We simply take the raw material that is in the final product and put it in to characterize it.

To explain ddPCR and linkage: linkage is the math that we use to tell whether or not two pieces of DNA or two amplicons were in the exact same droplet. If you get six pieces all linked together, it is highly likely they were a full genome from a single capsid in that droplet. We are pushing our internal teams to create new math and new ways to look at all the components in the same capsid and identify whether they came from a single capsid or not. That is where the trend is heading and we are striving to move with that.

Q How do you approach an uncertain area such as this one, particularly where there is a variety of both established and novel analytical tool options available? For instance, is a matrix approach best? If so, what does that look like?

PW: Matrix approaches are a hot topic at the moment regarding the functional assay. This is a good reason to invest more in your analytical team. If you have a good potential assay that can reflect your mechanism of action (MOA), that is good—however, some products will require an orthogonal approach with other methods surrounding that MOA to provide indirect supporting information. That means a lot of development work. The take-home message is you need to have a good analytical service development team working on a matrix approach. It is not a small project—you should invest time and resources into it.

MW: As a kit developer, we are generally watching and supporting our groups in analytics. Moving to more novel tools is becoming a need. With autologous lentivirus-transduced cell therapies, turning the final drug product around rapidly has driven the field to adopt more novel tools. From what we observe with our customers, they are studying deeply all of the novel tools out there, and looking at the costs and risks involved in order to make their decisions.

Often, we are seeing novel tools being implemented and tracked but not being used for regulatory purposes. Research groups may use these for side-by-side comparisons, so they can get comfortable with the tools for the next round of studies or asset coming through the pipeline.

JB: We use a matrix approach in several ways. The matrix approach allows you to determine whether one assay is behaving correctly. We employ that to view things like partially-full

capsids. We use weight-based techniques to look at the particle size distribution. Then, we use techniques like dual-color or genome integrity assays to determine whether that is a true value.

The matrix approach tells you whether things go up or down together and if the observations you are making are true. We also use it to some degree for looking at purity going into our downstream process. Here, we have a matrix approach where we can look at protein, DNA, and particulates. During our clarification process, we can see if these values go down together, which would suggest that we are achieving good removal of particulates from the system, allowing us to determine the quality of our material going into downstream processing. Matrix assays are an important way to know if you are getting a true answer by seeing several of your assays changing together.

Q The gene therapy field as a whole is notorious for the 'uniqueness' of each product and its corresponding array of QC methods. Where can and can't a platform approach be applied for viral vector-based products? How should we be approaching the evolving regulatory landscape in this regard?

PW: Platform assays are a good starting point. At the early stages of development, you can use platforms as you continue to develop an understanding of your product, but at later stages, you should develop product-specific assays. For example, for any nucleic acid-based modalities, you want control over what is inside your delivery vehicles.

JB: The copy-and-paste approach for platforming is easy but product non-specific, so it can only be used for impurity testing or other general AAV testing. It gets more difficult with things like potency. When it becomes product-specific, each product is going to act differently—for example, a gene-modulatory product will potentially have a different effect. As potency is product-specific and each program has its own approach, there is not much information on how to build those assays. This makes platforming and knowing what is expected by regulators much more difficult.

MW: As a tools and assay provider, it is an interesting challenge to help everybody do as many tests as possible on the same instrument, as that leads to high efficiency. It has been great to see people adopting ddPCR across a range of tests. As a company, we want to create platforms that are designed to run standardized and bespoke assays for any specific therapy. For example, we have a design engine for making assays against any sequence imaginable, alongside a lot of bioinformatics to make sure they work right from the beginning.

Potency assays will be custom in most cases. We are working with some groups who are taking cell culture-based potency assays that are in six-well plates and then, because ddPCR is inhibitor tolerant, they can move to straight cell lysate from a 96-well plate with more reproducibility. It is a platform in that the assay is functionally the same, but the cell type and how much you put in is going to be unique. I do not think we will get away from that. The more we can standardize, the more we can get regulatory bodies on board, allowing the

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analytical development teams to focus on the unique assays. All other assays can simply be bought off-the-shelf.

Q Finally, returning to viral titer, what are some key future directions for innovation in this area, and what does the path look like towards realizing the potential of accurate and precise viral titer determination?

PW: Automation throughput and cost reduction are the major focuses when you have well-developed assays with high accuracy and precision. Innovation is always the key, which is why Lonza invests heavily in R&D teams in CDMO settings. We are working towards the goal of reducing the cost and improving the quality of clients' products.

MW: Standardization is happening. We have been developing methods to look at all the sequences from five prime to three prime at a reasonable cost. ddPCR has become the gold standard for viral titer measurement, specifically at the GOI as a single point, through its application in FDA-approved gene therapy drugs that are now on the market.

Building on that and getting accurate titers of other components will be critical for the future. Then, the field has to solve full/partial/empty capsid measurement. We have to get a handle on how to do that at a larger scale whilst maintaining accuracy and precision. There are many new techniques. AUC is still the gold standard, but I am excited to see full/partial/empty capsid testing evolve over the next 5 years. It will be a key area of innovation that unlocks what we do next.

JB: We need to work towards cost–effectiveness, high-throughput, and less sample manipulation to enable greater sample precision. That allows us to move towards solving the key problem, which I feel has made viral therapeutics less attractive—scalability.

We spend a lot of time focusing on bioprocess conditions, upstream conditions, and downstream conditions. Being able to test many different conditions and watching when our productivity goes up and down is critical. Having techniques that allow us to look at many samples and conditions while not incurring high costs will help our ability to scale processes and produce more virus.

BIOGRAPHIES

MARK WHITE has played a key role in the development of multiple core technology capabilities and assays alongside a multidisciplinary team of biologists and engineers at Bio-Rad and previously at Berkeley Lights Inc. Mark obtained his PhD in Biomedical Sciences at the University of California, San Francisco.

JOHN BECHILL is a Principal Scientist at Modalis Therapeutics within the Bioprocess Group. In the Bioprocess Group, he leads the analytical group which supports upstream and

downstream AAV production. The analytics team focuses on methods of AAV characterization including AAV tittering, residual impurity measurements, and potency assay development. He received his PhD in Molecular Biology from Loyola University, and his career has focused on the development of analytical assays to characterize viral propagation.

PENG WANG earned his doctorate in Chemical and Biomolecular Engineering from UCLA in 2013. After his postdoctoral training at Harvard University in the Department of Chemistry and Chemical Biology, he worked as Senior Research Scientist at MD Anderson Cancer Center. In his current role as Senior Scientist at Lonza Research and Development Team, he is leading an analytics team to develop platform assays for AAV, Lenti, and Exosome products.

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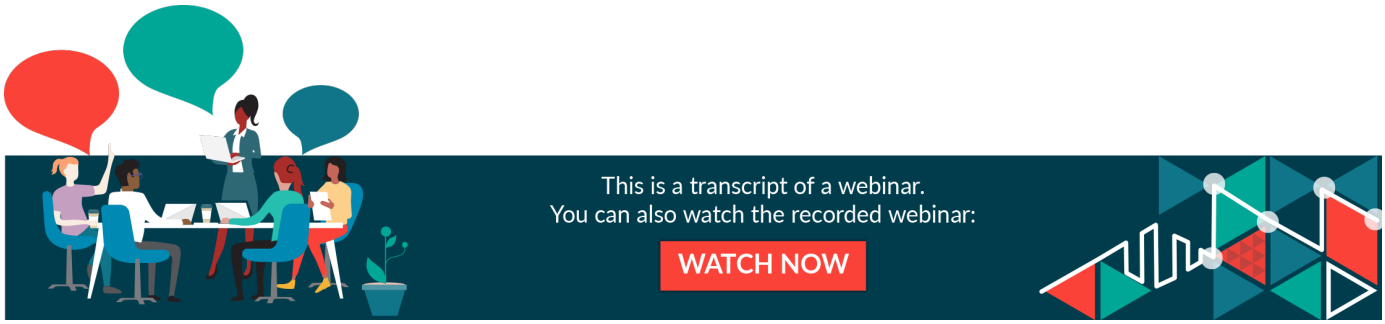

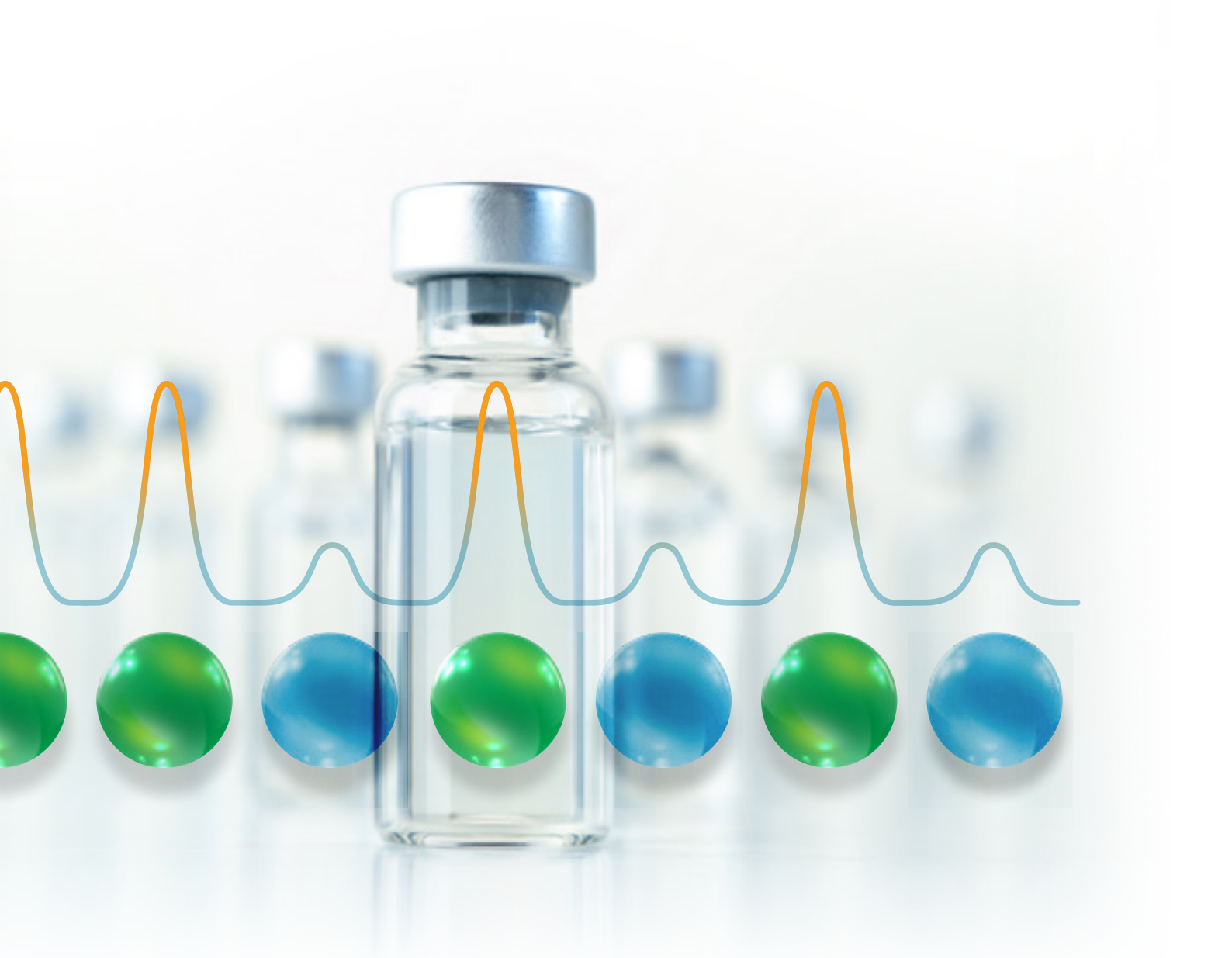

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INNOVATOR INSIGHT

mRNA fragmentation and quality assessment using ion pair reverse-phase analytics

Ana Ferjančič Budihna, Anže Martinčič Celjar, Sergeja Lebar, Andreja Gramc Livk & Aleš Štrancar

Rapid advancement of mRNA technology, as a response to the COVID-19 crisis, prompted an increased need for precise analytical methods to support the fast-paced mRNA process development. Accurate and robust analytics are required to support modifications in the mRNA production process, protocols, raw materials, *in vitro* transcription reaction, purification methods, scale-up, or final formulation processes, to ensure high quality and safety of the final product. This Innovator Insight demonstrates the application of an ion pair reverse phase chromatographic analytical method as a robust analytical tool to determine mRNA fragmentation while also separating *in vitro* transcription components from the main product. The method's efficacy is assessed through a comprehensive stability study of a mRNA standard at different temperatures. The chromatographic analytical results are compared to the ones obtained by the capillary gel electrophoresis, a well-established method for the analysis of fragmented mRNA.

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CHALLENGES OF mRNA FRAGMENTATION MONITORING

mRNA technology is a relatively new alternative to conventional vaccines showing great potential for infectious disease control and gene therapy. The highly effective mRNA-based vaccines enabled curbing of the COVID-19 pandemic and paved the way for the development of a broader range of innovative vaccines and therapies. The rapid pace of development and manufacturing of vaccines is one of the many advantages of the mRNA modality over other platforms, establishing the mRNA technology as a promising tool not only for addressing future pandemics, but

also for combating other infectious diseases like rabies, Zika, and cytomegalovirus infections. Furthermore, numerous mRNA-based therapies are currently progressing through clinical trial pipelines for the treatment of a wide range of diseases, including cystic fibrosis and various cancers [1].

Innovative products, such as mRNA vaccines must exhibit sufficient quality, safety, and efficacy. As modifications are introduced to raw materials, processing steps, and formulation during process development and the scale-up process, the implementation of rigorous analytics becomes essential to ensure the quality and safety of the final product. Inadequately identifying and addressing quality issues can jeopardize the integrity of the product, resulting in unfavorable clinical outcomes, costly delays, and potential challenges in obtaining regulatory approval. Therefore, it is crucial to proactively identify and mitigate any quality concerns to ensure the safety, efficacy, and timely delivery of mRNA-based products.

Ensuring the safety and reliability of a drug substance becomes significantly more manageable within a tightly controlled production and purification environment. To achieve this goal, rigorous QC steps must be executed at every stage of the process, demanding the utilization of robust and accurate analytical methods [2].

To address the need for mRNA characterization methods, regulatory agencies such as the US Pharmacopeia and US FDA are developing a set of analytical methods for mRNA quality. Their goal is to create a shared understanding of mRNA quality attributes with the aim of accelerating product development, guiding successful scale-up of manufacturing, and ensuring best practices and appropriate quality controls for this new modality. US Pharmacopeia guidelines for mRNA vaccine quality suggest ion pair reverse-phase high-performance liquid chromatography (IP-RP-HPLC) as a preferred analytical method for mRNA product-related impurities such as fragmented mRNA [2].

The developed chromatographic method enables detection of *in vitro* transcription (IVT) components while assessing mRNA fragmentation thus accelerating analytics of complex samples.

In this study, the suitability of the CIMac SDVB (styrene-divinylbenzene) chromatographic analytical method for the determination of the extent of mRNA fragmentation using a PATfix analytical chromatographic system was investigated [3]. The results from the SDVB analytics were compared to the data obtained by the widely accepted capillary gel electrophoresis (CGE) method.

VERSATILE mRNA QUALITY CONTROL METHOD

The CIMac SDVB column enables size separation of RNAs alongside the detection of impurities such as DNA template, nucleotides, and capping reagent (Figure 1). The method is also applicable for double-stranded RNA (dsRNA) impurity assessment (Figure 2).

Analysis of the complex IVT sample using the SDVB analytical method is presented in Figure 1. The analytical method enables separation of mRNA from IVT impurities, e.g., DNA template and dsRNA, while NTPs and capping reagents do not bind to the column. They elute in the non-bound peak and cannot be separated. The same analytical method is used for overall mRNA yield determination and estimation of fragmented mRNA.

One of the main impurities in the mRNA production process is dsRNA. The SDVB analytical method allows for the detection of dsRNA species due to its greater hydrophobicity relative to ssRNA, resulting in a longer retention time (Figure 2).

Size separation of RNAs is achieved due to differences in hydrophobicity, where the retention time is correlated with the length of the RNA molecule. Shorter fragments elute before the parent mRNA, making it suitable for mRNA quality assessment (Figure 3).

The separation of RNA molecules by size using SDVB analytics is presented in Figure 3.

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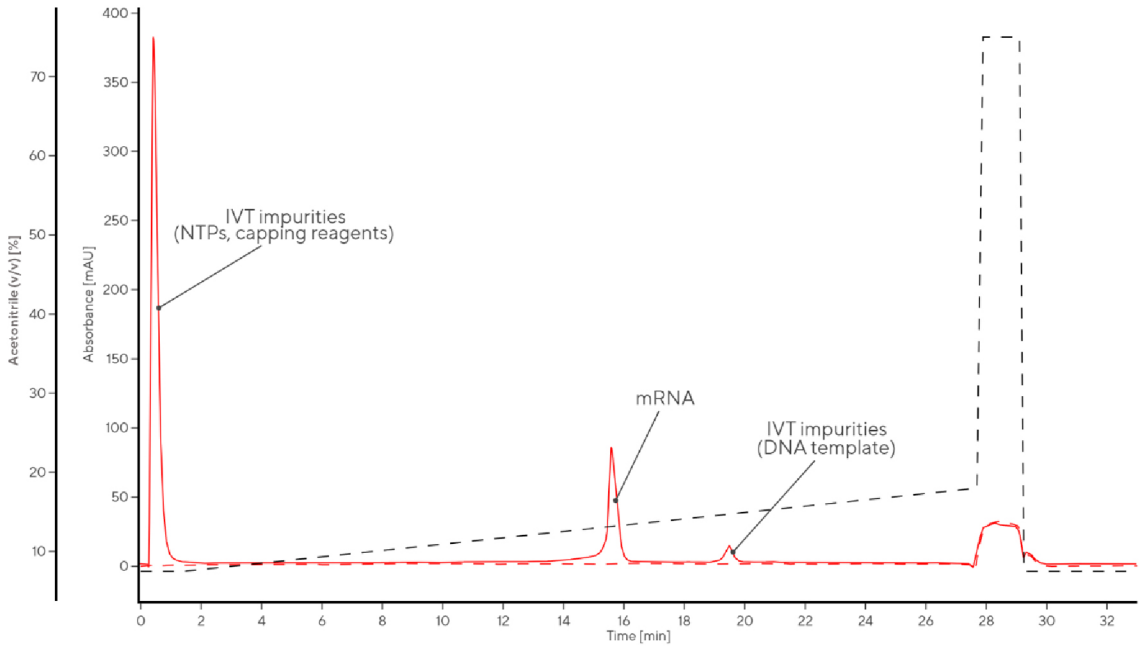
FIGURE 1

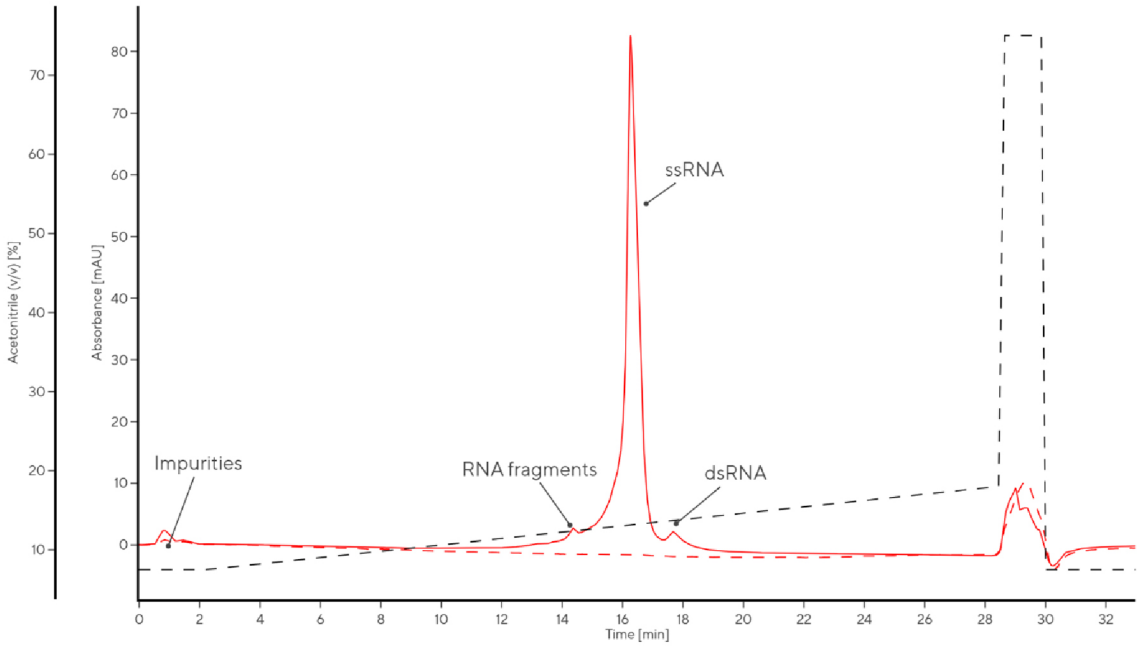
SDVB analysis of IVT sample.



The analytical method enables separation of IVT impurities from mRNA (red trace: UV 260 nm, black trace: acetonitrile gradient).

FIGURE 2

SDVB analysis of an mRNA (4000 nucleotides) with dsRNA impurity detected



Red trace: UV 260 nm; black trace: acetonitrile gradient.

RNA fragments, shorter than 50 nucleotides (nt) elute in the non-bound peak, while baseline separation of RNA fragments in sizes from 50–1000 nt is achieved. The robustness and reproducibility of the method are confirmed using a multiple ladders approach, where the fragments of the same size elute at the same retention time, regardless of the ladder provider.

CGE ANALYSIS OF IVT SAMPLE

RNA fragments are separated under denaturing conditions by size on a bare fused silica capillary filled with separation gel containing urea and polyvinylpyrrolidone. The gel is stained with Sybr® Green II dye, and mRNA fragments are detected using a LIF (laser-induced fluorescence) detector [4,5].

Capillary gel electrophoresis is used for monitoring fragmented RNA; however, impurities such as NTPs and capping reagents cannot be detected when the gel is stained with an intercalating fluorescent dye (Sybr® Green II dye). Another impurity that cannot be detected is dsRNA, due to denaturing conditions. The migration time of linear (lin) pDNA is shorter than the main mRNA peak and overlaps with RNA fragments and therefore cannot be identified. An example of the CGE analysis of an IVT sample, overlaid with lin pDNA, is presented in Figure 4.

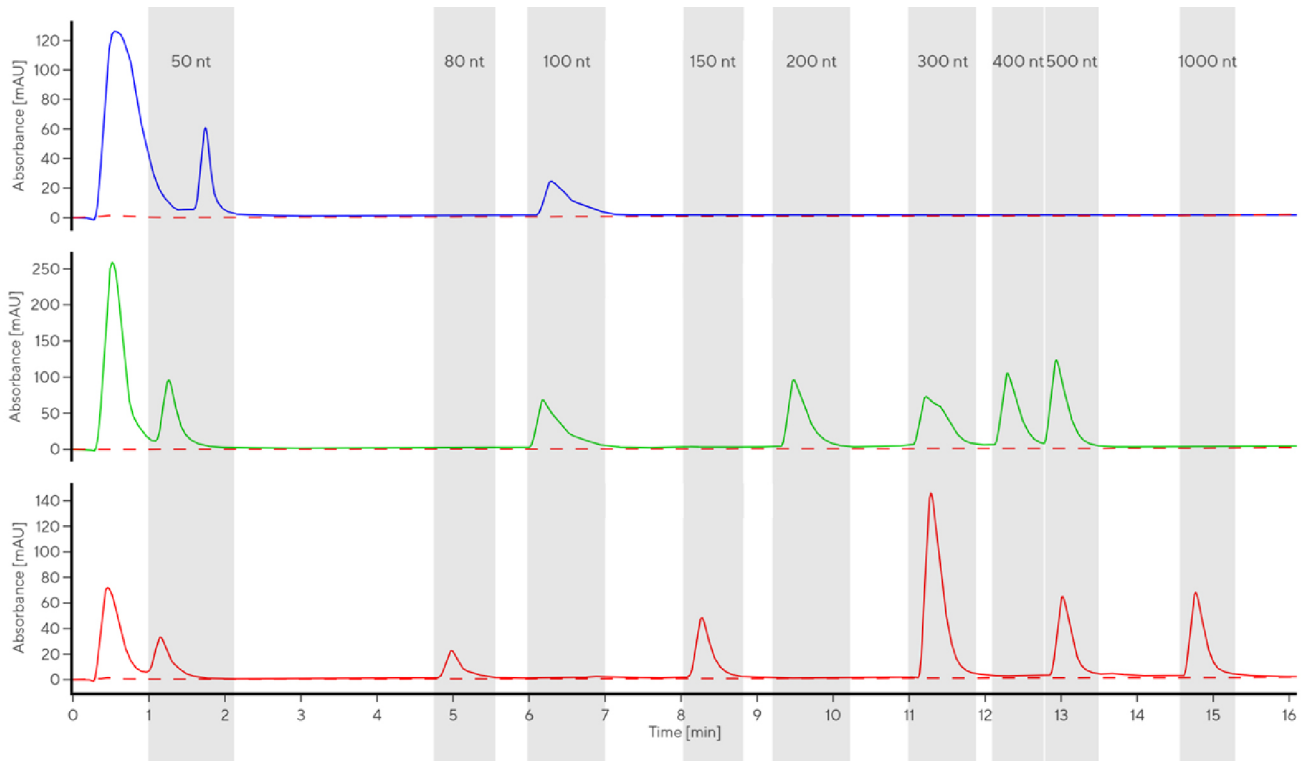
MATERIALS & METHODS

Experiments were performed using mFix4 mRNA analogue standard, a 4000 nt long uncapped mRNA with polyA tail (Sartorius BIA Separations product, Cat. No. BIA-mFix4.1.1).

Chromatographic analysis was performed using PATfix® analytical system (Figure 5) with a quaternary pump, a multiwavelength UV-VIS detector, a column thermostat, and a

FIGURE 3

Overlay of three commercially available small RNA Ladders using SDVB analytics (UV 260 nm).



Blue: Small RNA marker (Abnova); green: RNA marker low (Abnova); red: low range ssRNA ladder (New England Biolabs).

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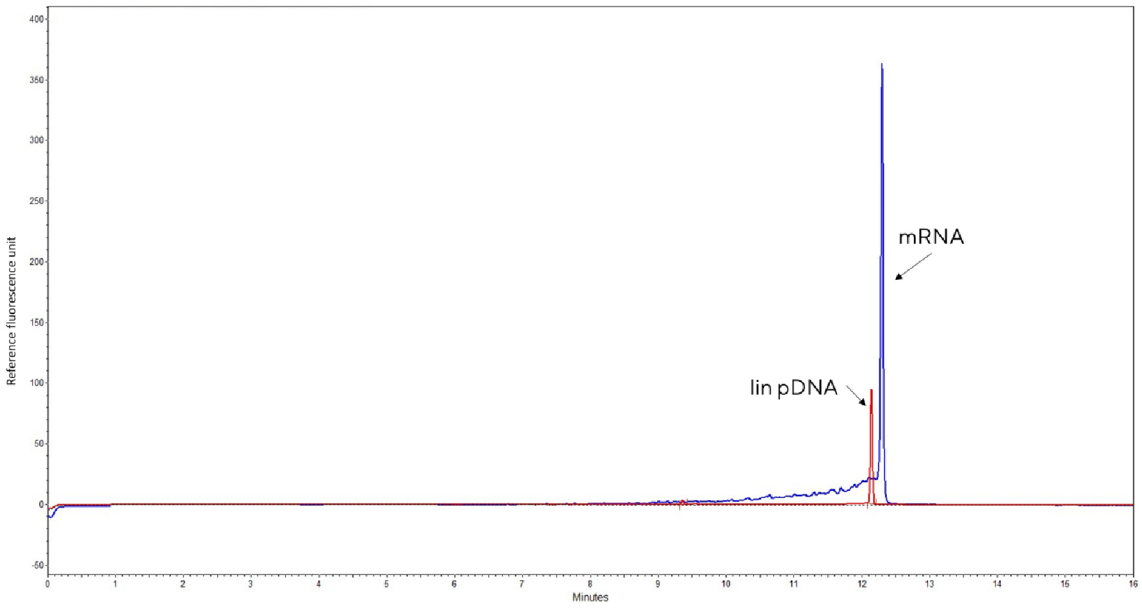
FIGURE 4

CGE analysis of an IVT sample (blue trace) overlaid with the lin pDNA sample (red trace).



mobile phase preheater. PATfix software was used for system control and data analysis.

The mRNA sample incubation was performed using a Thermo-Shaker from BioSan at an elevated temperature. The sample was diluted with mobile phase A prior to injection on the CIMac SDVB reverse phase monolithic chromatographic column from Sartorius BIA Separations (Table 1).

CGE analytics were performed using AB Sciex PA 800 Plus system with LIF detection (Table 2). The sample preparation consisted of a dilution to a target concentration of 1 µg/mL, heating the sample to 65 °C for 1 min followed by rapid cooling on ice and short centrifugation. The sample was injected electrokinetically.

EVALUATION OF FRAGMENTED mRNA

Data analysis to evaluate the extent of mRNA fragmentation, such as peak fronting of the main mRNA peak, was carried out by the PATfix embedded software. The PATfix algorithm determines peak fronting by evaluating the first derivatives (Df) of the absorbance signal (Figure 6), determining the maximum slope of the tangent to the chromatographic response [6,7], which defines the right-hand side border of the peak fronting area. The peak fronting area is proportional to the content of shorter RNA fragments, making it a valuable tool for fragmentation studies. The data analysis was further improved by applying a Savitzky-Golay numerical filter to smoothen the original signal. The PATfix algorithm ensures a robust and reproducible signal integration, independent of the analyst.

FIGURE 5

PATfix® chromatographic system

TABLE 1

SDVB analytical method details and gradient.

Mobile phase A	50 mM TEAA, 7.5% acetonitrile, pH 7.0
Mobile phase B	50 mM TEAA, 18% acetonitrile, pH 7.0
Mobile phase C	50 mM TEAA, 7.5% acetonitrile
Temperature	60 °C
Detection	UV 260 nm
Injection amount	0.5 µg
Column	CIMac SDVB (0.3 mL, 2 µm channels)
System	PATfix® mRNA chromatographic system

SDVB: Styrene-divinylbenzene; TEAA: Triethylamine acetate.

TABLE 2

CGE method details.

Capillary	50 µm bare fused silica, total length 30 cm
Detection	LIF detector (Ex. 488 nm, Em. 520 nm)
Gel	PVP, Urea, Sybr® Green II
Injection	Electrokinetic
Separation	6 kV
System	PA 800 Plus

LIF: Laser induced fluorescence; PVP: Polyvinylpyrrolidone.

Determination of mRNA fragmentation by CGE was performed by peak integration. The red horizontal line represents the baseline while the vertical line represents a dropped line that separates the main peak from fragments as shown in Figure 7.

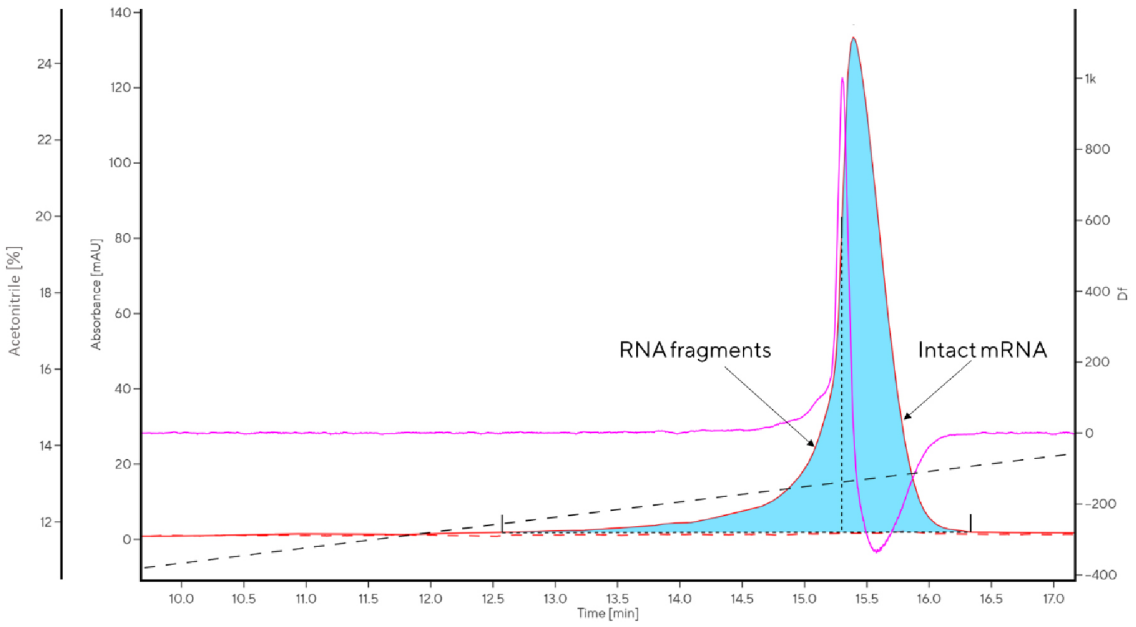
ASSESSMENT OF FRAGMENTED & INTACT mRNA

To demonstrate the method's ability to separate between fragmented and intact RNAs, fractions from an SDVB analytical run of the mRNA sample incubated at 60 °C for 6 h were collected and analyzed by the two analytics. The fractions were collected at the peak split determined by the Df, as shown in Figure 8. The chromatogram of the initial sample and the collected fractions analyzed with the SDVB method is presented in Figure 9.

The chromatogram of the initial sample and the collected fractions analyzed by the CGE method is presented in Figure 10.

FIGURE 6

Determination of fragment mRNA peak with the first derivative of the absorbance signal.



Red trace: UV 260 nm; pink trace: $Df_{UV\ 260\ nm}$; black trace: acetonitrile gradient.

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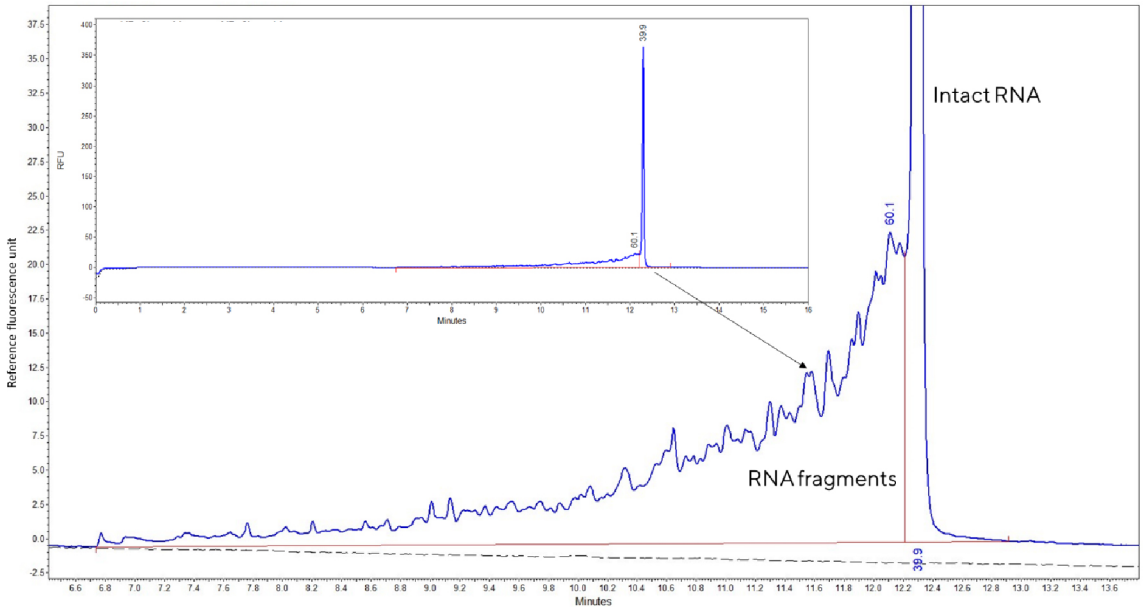
FIGURE 7

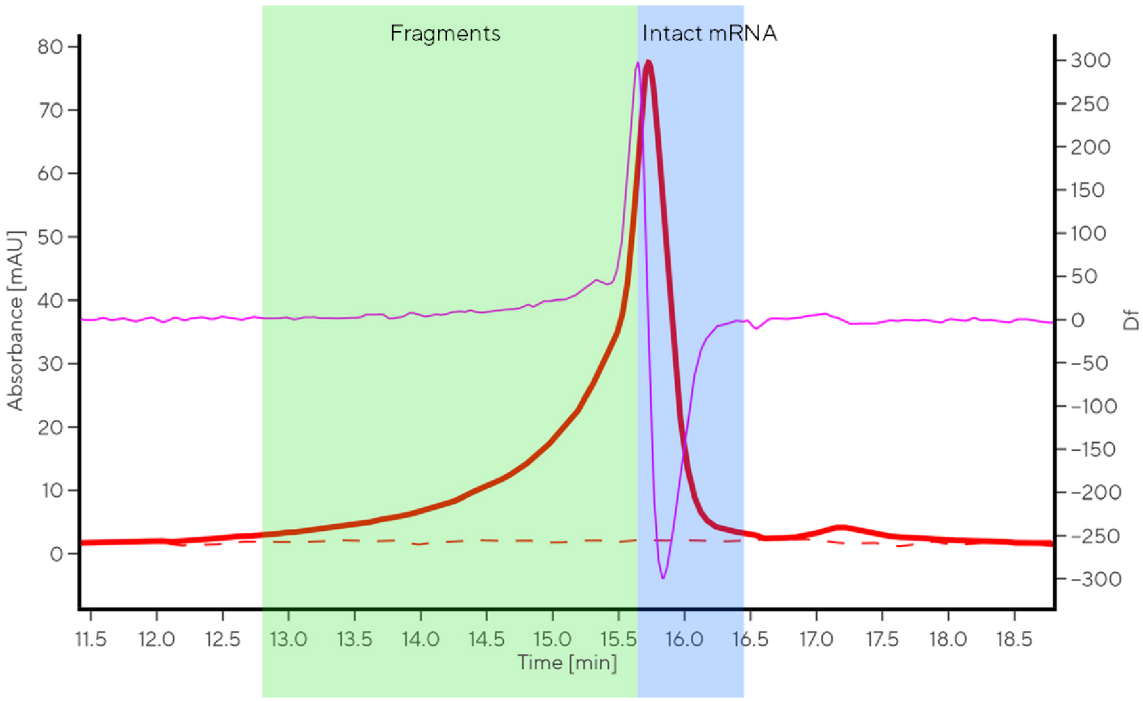
Determination of mRNA fragmentation using CGE analysis.



Blue trace: IVT sample; Black trace: blank sample.

FIGURE 8

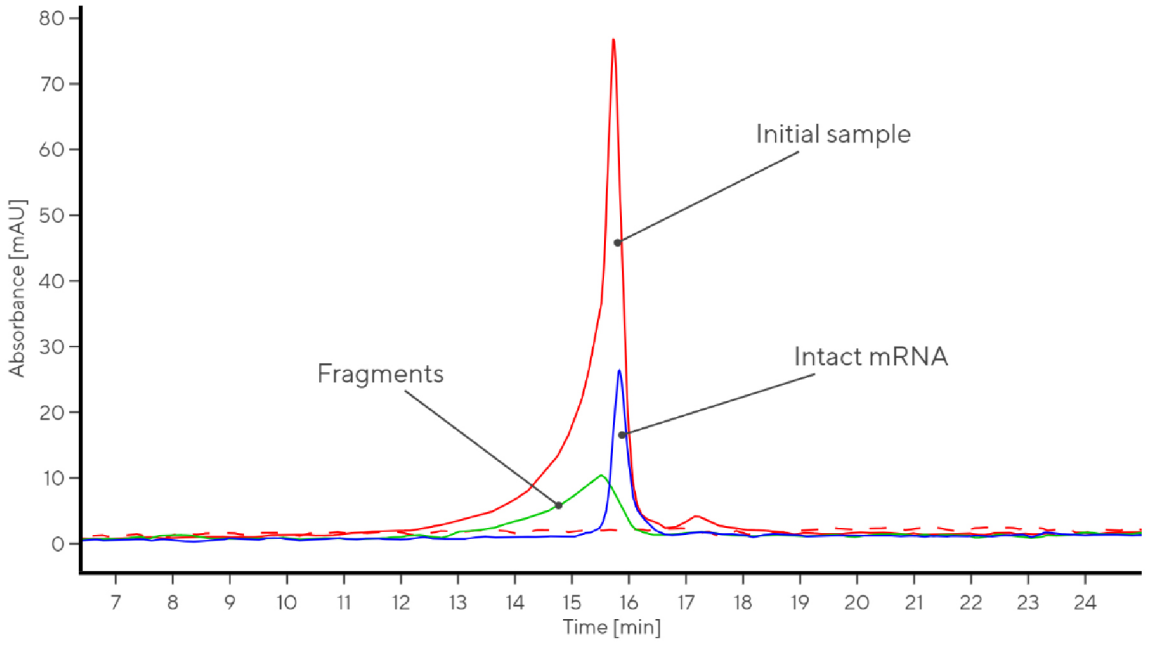
Fraction collection during the SDVB analytical run.



Fragments: green band; mRNA: Blue band; UV 260 nm: red trace; $Df_{UV\ 260\ nm}$: pink trace.

FIGURE 9

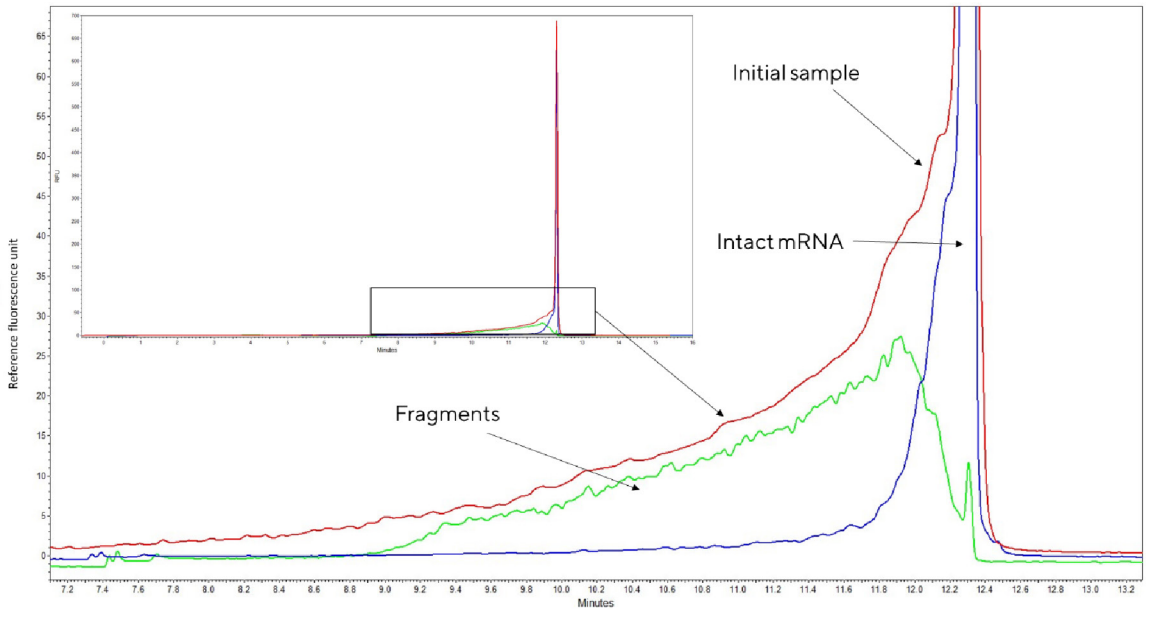
Separation of intact mRNA (blue trace) and fragmented mRNA (green trace) fractions on SDVB column.



Initial sample is presented with red trace (UV 260 nm).

FIGURE 10

Separation of intact mRNA (blue trace) and fragmented mRNA (green trace) fractions by CGE analytics.



Initial sample is presented with red trace.

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The CGE results demonstrate that the collected fronting fraction of the initial sample contains mainly fragments (green trace), and fraction of the main peak is predominantly intact mRNA (blue trace).

mRNA LONG-TERM STABILITY STUDY

The aim of the study is to assess the stability of the mRNA analog standard (500 ± 10 µg/mL in 1 mM sodium citrate pH 6.4) at four different temperatures and determine the storage and shipping conditions for this product. Aliquots of mRNA analog standard were stored for 6 months at four different temperatures: -80 °C, -20 °C, 4–8 °C and 20–25 °C. Stability after 15 freeze-thaw cycles was also tested.

The freeze-thaw sample has gone through 15 cycles of the freeze-thaw sample handling process. The sample was stored at -80 °C between cycles.

Chromatograms in Figures 11 & 12 present the results of the stability study of mRNA analog standard (mFix4) at the initial point and after 6 months, stored at different conditions, analyzed by SDVB analytics. In Figures 13 & 14 electropherograms of the same samples analysed by CGE analytics are presented.

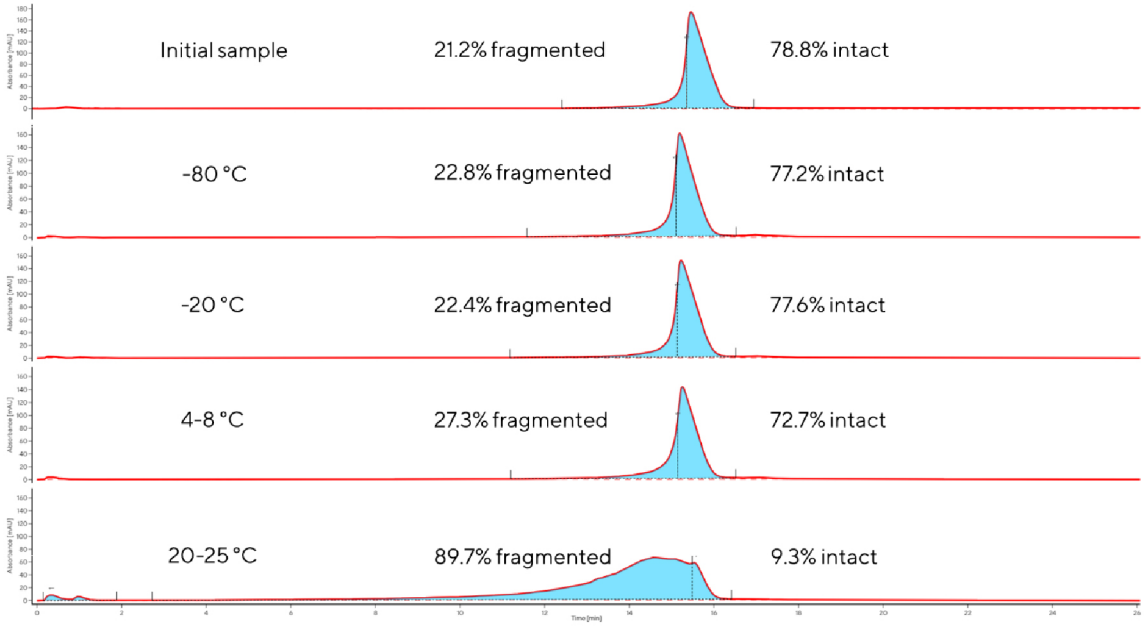
After 6 months of mRNA storage, the peak profile does not significantly change (less than 1 percentage point) from the initial (21.2% fragmentation) when mRNA is stored at -80 °C, -20 °C, and after 15 freeze-thaw cycles. After 6 months at -80 °C, SDVB analytics estimates the fragmentation at 23%, while the orthogonal CGE method estimates the fragmentation at 27%, resulting in a 4 percentage point difference in degradation estimation. The initial sample was not measured on CGE as the method was not fully implemented at the start of the study.

Comparable results were observed after 6 months at 20 °C, where SDVB analytics estimates 23% of fragmentation and orthogonal CGE analytics evaluate fragmentation at 28%. A difference of 5 percentage points comparing both analytics is observed.

With storage at a higher temperature of 4–8 °C slight degradation was observed. Here the results of the two analytics differ noticeably, as the CGE estimates mRNA

FIGURE 11

Stability results of mRNA analog standard at time zero and after 6 months, stored at four different temperatures using SDVB analytics at UV 260 nm.



fragmentation at a higher 40% compared to the 28% estimated by the SDVB analytics. This is yet to be investigated but is, in any case, a surprising result due to the demonstrated strong agreement of both analytics for other samples.

Higher degradation is observed with the sample stored at room temperature for 6 months.

FIGURE 12

Extent of fragmentation of the mRNA analog standard after 15 freeze-thaw cycles (15 x F/T), analyzed by SDVB analytics at UV 260 nm.

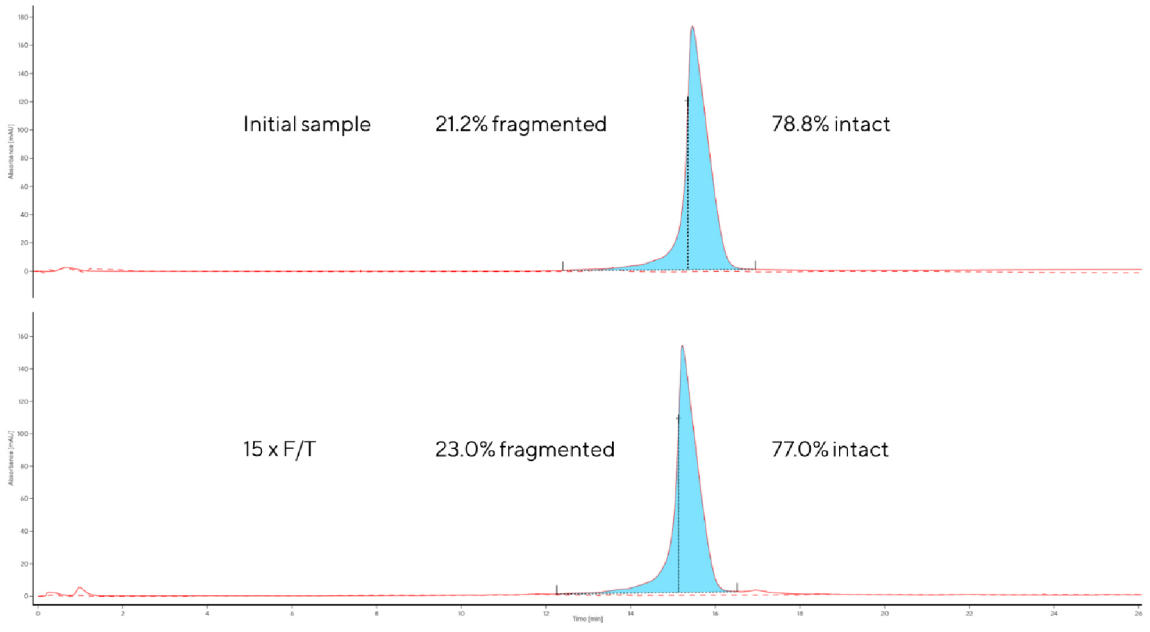
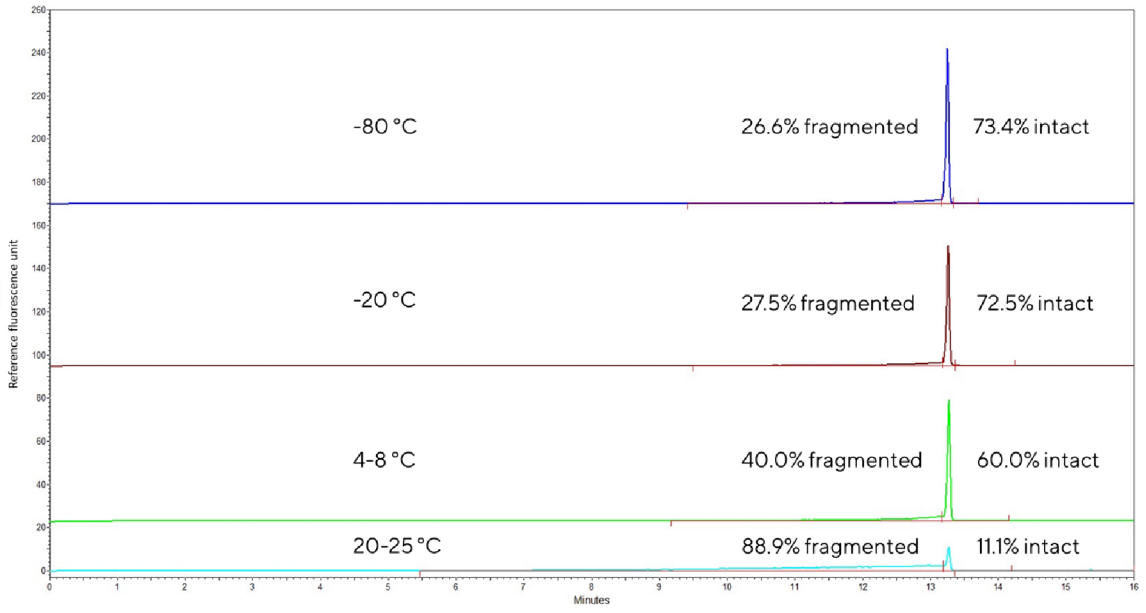


FIGURE 13

Extent of fragmentation of the mRNA analog standard after 6 months, stored at four different temperatures using CGE analytics.



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In this case, most of the sample is degraded, and the result is confirmed by the CGE.

mFix4 standard after 15 cycles of freeze-thaw was tested on SDVB analytics, where 23% of fragmentation was observed. Orthogonal CGE analytics estimated fragmentation at 31.2%, resulting in an 8 percentage points difference between the two analytics.

Orthogonal CGE results support SDVB analytics results with a difference of less than 8 percentage points at all except one temperature checkpoint. The observed increase in fragmentation by CGE analytics can be explained by the fact that these analytics were conducted subsequently to the SDVB analytics and therefore this slight delay could have an impact on the extent of fragmentation. All the data available confirm that SDVB analytics can be employed as an effective analytical approach for sample characterization (Table 3).

HIGH TEMPERATURE STABILITY OF mRNA SAMPLE

IP-RP HPLC chromatography is an established method for RNA stability assessment [8] and is often performed at elevated temperatures [8,9]. The elevated temperature enhances the resolution of oligonucleotides and RNA molecules [10] and therefore the SDVB analytical method was set to an elevated temperature of 60 °C.

The production batch of the mRNA analog standard used in this study differed from the one used in the long-term stability study, resulting in different percentages of fragmentation of the initial sample.

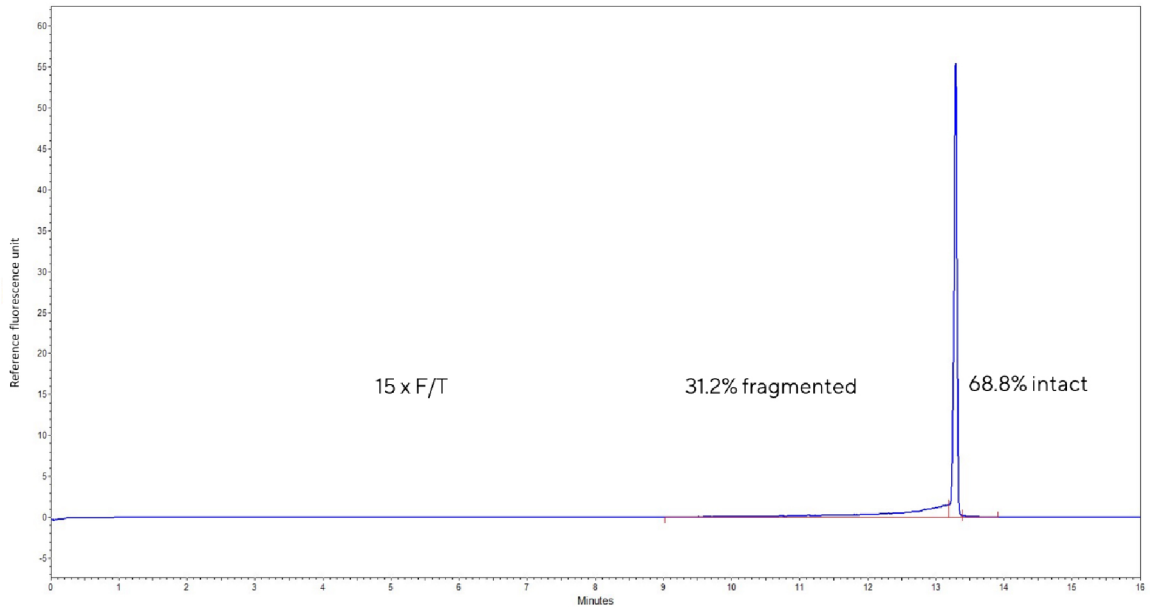
To assess the stability of the mRNA sample during the SDVB analytical run, an incubation at 60 °C was carried out. Temperature study was performed in triplicates using a Thermo-Shaker, with the sample prepared in RNase-free ultra-pure water. To confirm the SDVB results, an orthogonal CGE analysis was performed.

RNA fragmentation of the samples treated for 5, 15, 30, 45, and 60 min at 60 °C was determined by SDVB and CGE analytics (Figure 15).

The primary objective of the study was to determine whether the mRNA sample could maintain its structural integrity for a minimum duration of 30 min (retention time of

FIGURE 14

Extent of fragmentation of the mRNA analog standard after 15 freeze-thaw cycles (15 x F/T), analyzed by CGE analytics.



mRNAs on the SDVB column is 15 min) at the elevated temperature of 60 °C.

Fragmentation of the initial sample determined by SDVB and CGE analytics was 32±2% (Table 4). After 15 min at 60 °C the determined fragmentation by SDVB and CGE analytics increases by 3 and 2 percentage points, respectively. The rate of fragmentation, as assessed by either analytical technique, demonstrates a close match.

As can be seen in Figure 15, after 30 min only slightly increased fragmentation (3 percentage points) was observed with either analytical technique, indicating that during the

TABLE 3

Comparison of mRNA degradation after 6 months at different temperatures using SDVB and CGE analytics.

Temperature/°C	% fragmentation SDVB	% fragmentation CGE
Initial sample	21.2	/*
−80	22.8	26.6
−20	22.4	27.5
4–8	27.3	40.0
20–25	89.7	88.9
15 F/T	23.0	31.2

*Not measured. CGE: Capillary gel electrophoresis; SDVB: Styrene-divinylbenzene.

FIGURE 15

Percentage of mRNA fragmentation at 60 °C monitored by SDVB and CGE analytics.

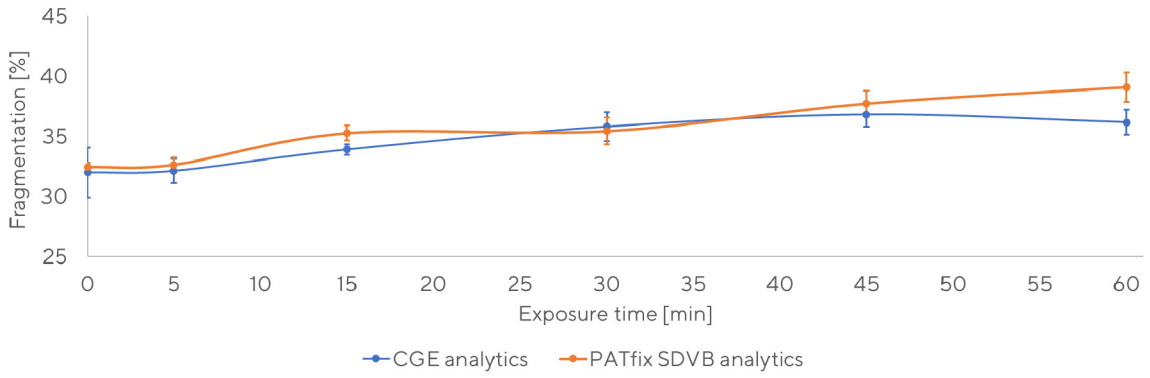


TABLE 4

Comparison of average mRNA fragmentation at 60 °C for 60 min monitored by SDVB and CGE analytics.

Time/min	SDVB analytics		CGE analytics	
	Average/%	RSD	Average/%	RSD
0	32	0.4	32	2.1
5	33	0.6	32	1.0
15	35	0.6	34	0.4
30	35	1.1	36	1.2
45	38	1.1	37	1.0
60	39	1.2	36	1.0

CGE: Capillary gel electrophoresis; RSD: Relative standard deviation; SDVB: Styrene-divinylbenzene.

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SDVB analytical run the mRNA fragmentation degree is very low.

After 60 min mRNA fragmentation exhibited 7 percentage points increase as assessed by SDVB analytics, and CGE analytics calculated 4 percentage points increase, demonstrating a minor variance between the two analytical methods.

ELEVATED TEMPERATURE mRNA STABILITY

In this experiment, the mRNA sample was incubated at 60 °C, and aliquots were sampled after 1, 2, 6, 9, 12, 18 and 24 h. Sample degradation, observed as peak fronting, was estimated at every time point with SDVB (Figure 16) and CGE (Figure 17) analytics.

The initial sample analyzed by SDVB analytics was found to be 28.5% fragmented, while CGE analytics estimated starting fragmentation at 28.7%, resulting in a 0.2 percentage point difference.

After one hour, at 60 °C the fragmentation determined by SDVB is 35.5%, showing a 7 percentage points increase in fragmentation, presenting a slight increase in sample degradation. Findings are consistent with the results from the 60 min degradation study.

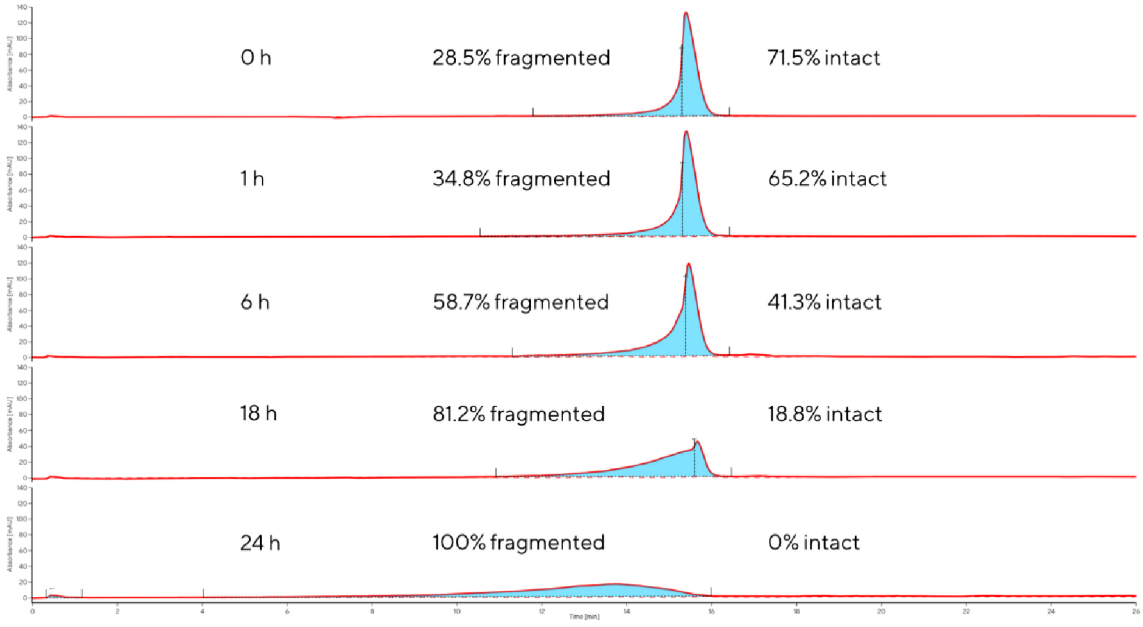
Sample fragmentation further increases at longer contact times, resulting in increased peak fronting. After 24 h, the sample is almost completely degraded. The results from the two analytical methods are comparable (Figure 18).

SDVB ANALYTICAL METHOD OFFERS A SOLUTION FOR mRNA FRAGMENTATION ASSESSMENT

This SDVB analytical method is a comprehensive characterization technique within a single chromatographic run. This approach not only facilitates the effective separation of IVT-based impurities from mRNA but also enables the identification of RNA-based impurities, such as dsRNA and RNA fragments. Given that dsRNA is a prominent impurity with the potential to induce immunogenic responses in patients, its control is crucial [2]. This dual capability of the chromatographic method not only accelerates QC

FIGURE 16

Percentage of fragmentation of mRNA at different times at 60 °C monitored by SDVB analytics.



testing but also offers a comprehensive approach, adding significant value compared to CGE analytics.

The SDVB method selectivity was demonstrated by collecting fronting and main peak of the mRNA sample. Both analytics confirm that fronting of the mRNA, observed on SDVB analytics, contains predominantly fragmented mRNA, and main peak consists of mainly intact mRNA. The SDVB analytical method can be used for estimation of percentage of fragmentation in an unknown sample.

To showcase the applicability of the SDVB method for mRNA fragmentation assessment, a 6-month stability study was conducted. mRNA analogue standard was stored at four temperatures: -80 °C, -20 °C, 4–8 °C, and 20–25 °C. The study also investigated the impact of 15 freeze-thaw cycles.

FIGURE 17

Percentage of fragmentation of mRNA at various times at 60 °C monitored by CGE.

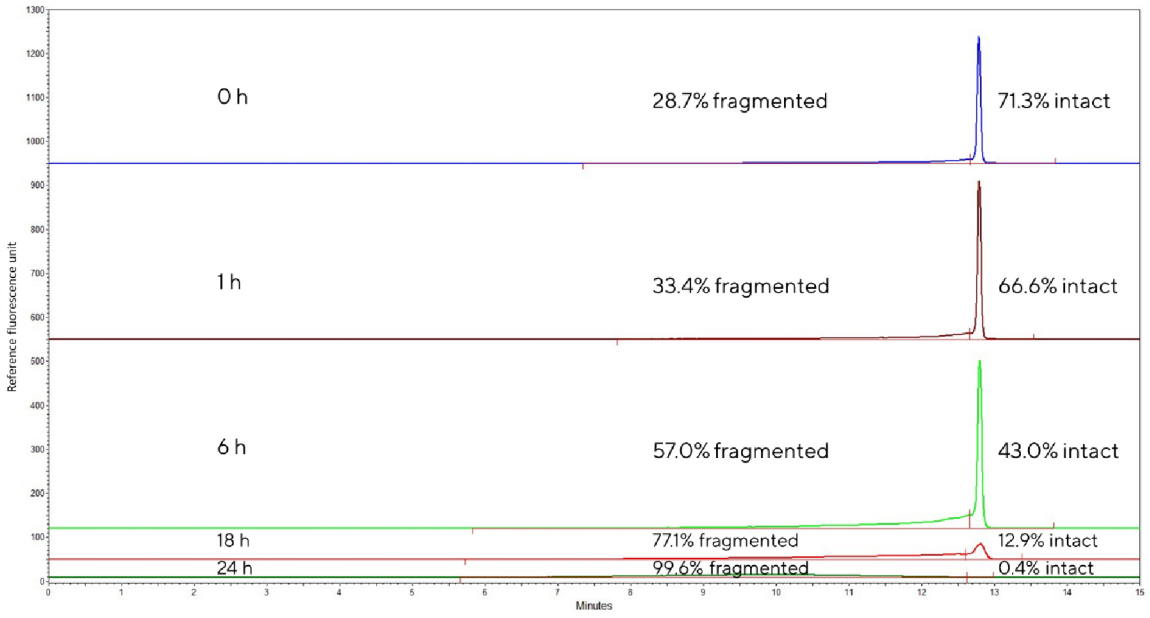
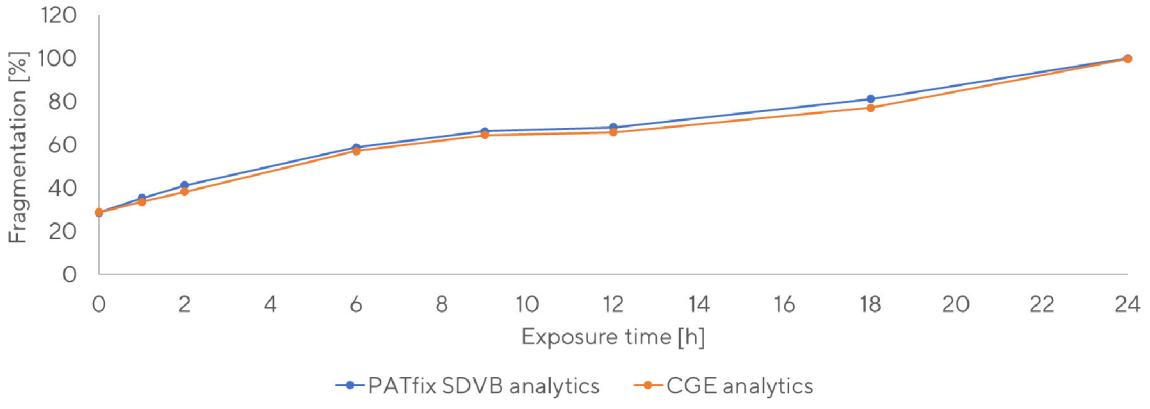


FIGURE 18

Percentage of fragmentation of mRNA at different exposure times at 60 °C monitored by PATfix SDVB and CGE analytics.



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Results obtained by the SDVB analytics show no significant degradation in the samples stored in the freezer at both -80 °C and -20 °C after the 6-month period. Orthogonal CGE analytics supports the findings as a maximum difference between analytics of 5 percentage points is observed. With this experiment, long-term stability of the mFix4 sample at -80 °C and 20 °C for at least 6 months was proved.

After 6 months at a higher temperature of 4–8 °C, both analytics confirmed a rather unexpected result that only a slight fragmentation of the mRNA mFix4 occurred. Although CGE analytics estimate fragmentation at a higher percentage (40%) compared to SDVB analytics (27.3%), the sample presented unusual stability. Furthermore, the mRNA standard stability after 15 cycles of freeze-thaw was evaluated using both analytics, where an 8 percentage point difference between the two methods is observed. These results confirm and extend previous findings, showcasing increased sample stability during the chromatographic purification step in comparison to precipitation, as previously demonstrated [11].

To confirm the SDVB analytical method does not overestimate the RNA fragmentation due to possible degradation during the analytical run, the 60 min degradation study at 60 °C was performed. After 30 min the percentage of fragmentation increased by 3 and 4 percentage points as determined by SDVB and CGE analytics, respectively. Low level of sample fragmentation and the good agreement between the data from both analytics suggests that the data obtained by the SDVB method does not over- or under-estimate the percentage of fragmentation.

Comparison of the high-temperature stability for 24 h study data shows complete agreement between CGE and SDVB analytics. mFix4 sample is unexpectedly resistant to high-temperature incubation after one hour. As expected, at longer contact times and elevated temperature, the peak fronting increased, leading to higher sample fragmentation levels in both SDVB and CGE analytics. After 24 hours of incubation, the sample showed almost complete degradation, as demonstrated by an almost complete absence of the main mRNA peak.

CONCLUSION

The presented method for determining the extent of mRNA fragmentation, using the CIMac SDVB monolithic column in a PATfix chromatographic analytical platform, offers an easy-to-use tool delivering results that are robust, reliable, and in close agreement with those obtained by the CGE.

The SDVB analytical method enables characterization of complex samples independent of the sample matrix in addition to its ability to detect various contaminants such as dsRNA, DNA template, capping reagents, and nucleotides, which is not provided by the CGE analytics. This study demonstrates the importance of robust analytical methods for mRNA product development and quality control, bolstering a safe and effective advancement of mRNA-based therapies and vaccines.

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