NOVEMBER 2022 Volume 8, Issue 10

CELL & GENE THERAPY INSIGHTS

SPOTLIGHT ON:
Cell therapy bioprocessing

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CELL & GENE THERAPY INSIGHTS

Volume 8, Issue 10

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Expanding the analytical toolbox to address complex cell-based therapies

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Today's innovators of cell-based therapies are working with increasingly complex technology platforms to develop treatment strategies that are more precisely controlled and address broader patient populations. Such innovations are built upon an increased understanding of disease pathophysiology that helps to identify the cellular and molecular players that underlie disease progression and drive recovery. Moreover, the emergence of improved *ex vivo* engineering strategies has enabled production of specific cell types with targeted or enhanced functional attributes. While these scientific and technological advances are empowering the next generation of cell-based therapies, they also demand more comprehensive analytical testing strategies to ensure product efficacy and safety.

This article explores the current analytical testing landscape for cell-based therapies, highlighting key analytical objectives, challenges, and emerging strategies. Analytical objectives discussed in this article include characterization of product identity and potency as indicators of efficacy, and key measures of product safety, such as monitoring for cellular impurities and genome integrity. In the context of these analytical objectives, challenges and current mitigation strategies that are specific to autologous versus allogeneic cell-based products are described. Finally, emerging analytical methods that stand poised to disrupt existing industry standards in analytical testing and address remaining challenges are considered.

Cell & Gene Therapy Insights 2022; 8(10), 1355–1369

DOI: 10.18609/cgti.2022.197

STARTING MATERIAL: AUTOLOGOUS VERSUS ALLOGENEIC

A major promise of cell-based therapy is the advent of personalized autologous medicines to regenerate impaired tissues and provide new targeted treatments for diseases lacking suitable medical interventions. A significant breakthrough in this space occurred in 2017, when YESCARTA® and KYMRIAH® became the first two autologous chimeric antigen receptor (CAR) T cell therapies to be approved by the US food and drug administration (FDA) [1]. It can be said that the approval of these therapies marked the dawn of a new era for both immunotherapy and cell-based therapies, and since then, four additional autologous CAR-T products have reached the US commercial market, including Breyanzi® [2], TECARTUS™, ABECMA®, and CARVYKTI™ [3].

Autologous cell-based therapies offer both advantages and disadvantages with respect to manufacturing strategy and corresponding analytical testing. Importantly, use of a patient's own cells as starting material greatly reduces concerns of immunological reactions and does not carry a risk of graft-versus-host disease (GvHD). Autologous therapies therefore have the potential for long-term persistence and therapeutic activity without the use of immunosuppressive drugs, which carry their own health implications [4–5]. Consequently, manufacturing of autologous therapies tends to be less complex than their allogeneic counterparts, which may require additional engineering strategies to address potential immunogenicity, graft rejection, or GvHD.

From the perspective of analytical testing, autologous products tend to require reduced drug product release testing to support patient safety relative to allogeneic products that often involve a more complex manufacturing process and/or carry additional safety concerns. However, the need to manufacture and test autologous products on a per patient basis drives high production costs that limit their widespread application. A second challenge for autologous products is the inherent variability of the patient starting material that then feeds into process, and ultimately drug product, variability. This inherent variability impacts the acceptance criteria for starting material qualification through drug product release, typically requiring broad specification ranges to appropriately capture manufacturing experience. Finally, the required turnaround time for manufacturing and testing can also present challenges for autologous products, especially for patients who are critically ill and require urgent interventions. In these cases, sponsors may try to optimize their manufacturing process to enable streamlined drug product release testing. For example, use of a chemically defined and fully closed process may help reduce the amount of safety tests required for drug product release. The associated cost of having to perform release testing on each patient-specific lot provides additional motivation to reduce the overall testing performed on autologous products, which in turn can impair sponsors' ability to identify critical quality attributes (CQAs) that underlie product efficacy and safety.

While allogeneic products can come with a higher degree of complexity, they also can offer key advantages over autologous products with respect to manufacturing and testing. First, allogeneic products enable use cellular starting material that is optimized for consistency and manufacturing success. This can include starting material from healthy donors qualified for use via stringent eligibility and testing parameters, or even a single cell line used for all drug product manufacturing, as is done with allogeneic pluripotent stem cell (PSC)-derived products. Use of a more controlled starting material affords allogeneic product developers the opportunity to generate therapies that are controlled via tighter in-process and release testing acceptance criteria relative to autologous products. A second key advantage of

DOI: 10.18609/cgti.2022.197

allogeneic therapies is their capacity for scalable manufacturing and subsequent storage as off-the-shelf, on-demand products. Not only does batch production offer substantial opportunity to reduce the cost of manufacturing and testing (i.e., enabling per lot testing to be divided across multiple patient doses), but it also enables allogeneic product developers to consider more comprehensive testing of process intermediates and drug product given the uncoupling of product manufacturing and patient treatment.

The opportunity for increased in-process and drug product testing can be important for allogeneic cell-based products given that they often require increased engineering strategies to achieve their desired safety and efficacy profile. As indicated above, the potential for allogeneic products to be immunogenic can both pose a safety risk and limit long-term engraftment. Rather than relying on co-administration of immunosuppressants, many allogeneic product developers will employ additional unit operations, such as cell selection technology, or genetic engineering to mitigate potential immunogenicity. This in turn drives a need for analytical testing to confirm that the additional manufacturing steps have their desired effect and do not result in unintended changes to product quality. Another consideration that tends to be of greater concern with allogeneic products than autologous products is whether non-target cell types that can impact safety or efficacy persist in the final drug product. Two key examples that are discussed in greater detail below include immunotherapy products that have the potential to contain GvHD-inducing αβT cells and PSC-derived products that may have residual undifferentiated cells or unintended differentiated cell types present in the final drug product. These kinds of safety considerations in addition to any further engineering strategies to enhance product activity tend to drive allogeneic products towards a higher overall analytical testing burden.

The next two sections of this article describe some of the key analytical objectives for cell-based therapies, namely characterization of product identity, potency, purity, and genome integrity. While not meant to be an exhaustive list of all required testing for biologics, these analytical objectives were selected given their tendency to require bespoke analytical testing that appropriately address process- and product-specific attributes. For the first three analytical objectives (identity, potency, and purity testing) two product types are used as illustrative examples – autologous CAR-T products and allogeneic progenitor cell therapies (i.e., products derived from PSCs and/or for which the final drug product population is comprised of progenitor cells that retain proliferative capacity and multipotent differentiation potential). The final topic of genome integrity testing is instead considered with respect to two commonly-used engineering strategies – viral vector-based transgene integration and nuclease-based gene editing.

IDENTITY & POTENCY: DEFINING THE THERAPEUTICALLY ACTIVE CELL POPULATION

Identity

As 'living therapies' cell-based products present notable differences to many classical pharmacological compounds – including greater lot-to-lot variability, reduced stability, and more complex mechanistic and molecular characteristics. These characteristics can be impacted by several factors, including the starting material source (i.e., autologous versus allogeneic), processing methods, critical raw materials, and drug product storage (e.g., cryopreservation) versus fresh administration. Consequently, enhanced analytical methods are required to ensure product consistency and to appropriately characterize the identity and potency of a product's therapeutically active population. This section discusses current industry strategies for identity and potency testing, considering the two aforementioned product type examples

– autologous CAR-T products and allogeneic progenitor cell-based products.

In 2017 the identity assessment used for release of KYMRIAH® defined the therapeutically active population as CAR-positive cells and involved complementary methodology to assay for the presence of the CAR-Transgene by qRT-PCR and surface expression of the CAR protein by flow cytometry [6,7]. At that time, quantification of CAR positivity for product identity testing was viewed as sufficient given that the KYMRIAH® manufacturing process involved enrichment of CD3-positive T cells and removal of the majority of non-T cell types. In March 2022, the FDA issued the draft guidance document, "Considerations for the Development of CAR-T Cell Products" [9] to assist developers of CAR-T and related cell-based immunotherapies. The draft guidance includes recommendations regarding the chemistry, manufacturing, and control (CMC) of autologous and allogeneic products and also provides recommendations regarding identity testing. Specifically, the draft guidance affirms that identity testing of CAR-T products should include testing for the transgene (i.e., CAR) along with 'an assay specific for the cellular composition of the final drug product' [8].While the identity testing applied to KYMRIAH® largely satisfies these FDA recommendations, it has become increasingly clear that not all CD3-positive T cells are functionally equal and that different subpopulations may have varying contributions to patient outcomes.

Active investigation into T cell biology continues to uncover deeper layers of cell subtypes and phenotypes with different functional capacities. For CAR-T products, it is becoming increasing clear that different proportions of CD8 versus CD4 T cells, which can be further subdivided into naïve, memory, and effector phenotypes, impact how quickly and potently administered cells initiate tumor killing and how long they will persist in the body [9–11]. As a result, an increasing number of autologous CAR-T cells and related product types (e.g., allogeneic CAR-T cells, T cell receptor (TCR) modified-T cells, and tumor infiltrating lymphocyte (TIL)-based products) now are routinely assessed by multiparameter flow cytometry for a broad panel of phenotypic markers (Table 1) [13]. Although such phenotypic profiling typically is performed as a part of extended (non-release) characterization rather than as an identity release assay, these data sets are being evaluated in the context of clinical safety and efficacy outcome measures [12,13,14] and may

TABLE 1

Representative phenotypic markers used for T cell-based products.

CD4+ cells	Positive marker	Negative marker	+/- marker
Naïve T cell	CD45RA, CD127	CD25, CD45RO	
Effector T cell	CD25	CD127	CD45RA, CD45RO
Effector memory T cell	CD45RO, CD127	CD25, CD45RA	
Central memory T cell	CD25, CD45RO, CD127	CD45RA	
Resting T reg cell	CD25, CD45RA,	CD45RO, CTLA-4, CD127	
Effector T reg cell	CD45RO, CTLA-4	CD127	CD25
Effector memory T reg cell	CD25, CD45RO, CTLA-4, CD127	CD45RA	
CD8+ cells	**Positive marker**	**Negative marker**	**+/- marker**
Naïve T cell	CD62L, CCR7, CD45RA	CD45RO	
T stem cell memory	CD62L, CCR7, CD45RA, CD45RO		
T central memory cell	CD62L, CCR7, CD45RO	CD45RA	
T effector memory cell	CD45RO	CD62L, CCR7, CD45RA	
T effector cell	CD45RA	CD62L, CCR7, CD45RO	

DOI: 10.18609/cgti.2022.197

ultimately reveal more accurate methods to identify therapeutically active T cell population(s) and/or enable further tuning of *in vivo* function.

Turning now to our second example product type – allogeneic progenitor cell-based products – challenges in identity testing stem from the fact that the target cell population represents one or more stages of a developmental continuum rather than a terminal cell type and because therapeutic activity may rely on subsequent *in vivo* differentiation into one or more cell types distinct from the drug product population. There currently are several clinical-stage programs which involve an allogeneic progenitor cell-based product, such as MPC-06-ID, Mesoblast's phase 3 product candidate for chronic lower back pain [15], spinal cord injury-targeting products comprised of mesenchymal stromal cells (MSCs) [16], PSC-derived neural progenitor cells (NPCs) [17], or PSC-derived oligodendrocyte progenitor cells (OPCs) [18], PSC-derived MSCs for multiple sclerosis [19], PSC-derived NPCs for Parkinson's disease (MSK-DA01 [20], and ISC-hpNSC® [21]), Fate Therapeutic's induced pluripotent stem cell (iPSC)-derived cellular immunotherapy portfolio [23], a range of stem cell-based products targeting diabetes [23], among others.

Typically, a single cell marker is insufficient to define progenitor cells (with CD34 an accepted marker of hematopoietic progenitor cells being a rare exception to this rule [24,25]). Instead, identity testing of progenitor cells often requires detection of multiple markers, as is the case for MSCs – classically defined as CD73, CD105, CD90 triple positive [26,27] – and NPCs – typically defined by a combination of key transcription factors, such as Sox2, Pax6, and MSI1 that are indicators of retained multipotent neural potential [28–30]. However, while such markers are indicative of a given progenitor state, they do not distinguish between cells that will be therapeutically active *in vivo* versus those that will die immediately following administration.

More recently, comprehensive analytical approaches have been applied to NPCs to identify markers that may be predicative of *in vivo* engraftment capability and differentiation fate. For example, preclinical studies have shown that while commonly used markers (e.g., LMX1A, FOXA2) did not accurately predict *in vivo* maturation of dopamine neuron progenitors, transcriptomics were able to identify alternative markers (e.g., SPRY1, CNPY1) which correlated with improved engraftment and higher dopaminergic yield after transplantation into animal models of Parkinson's disease [31]. Independent follow-up work utilizing single-cell profiling yielded similar findings, pointing to additional candidate predictive markers (e.g., CLSTN2 and PTPRO [32]). In the context of programs advancing to the clinic, it was recently announced that Aspen Neuroscience will be utilizing a whole transcriptome RNA sequencing approach termed NeuriTest to identify the optimal cellular phenotype for their dopaminergic neuron progenitor cell product in an upcoming clinical trial for Parkinson's disease [33]. Implementation of identity testing with enhanced *in vivo* predictive power ultimately will enable more accurate quantification of the therapeutically active population and consequently, more consistent product dosing.

Potency

The second key measurement used to evaluate a product's therapeutically active cell population is potency, which is necessary to ensure that a given cell dose will produce the desired magnitude of therapeutic effect. The FDA have accordingly provided nonbinding recommendations to manufacturers of cellular and gene therapy products for developing tests to measure potency in the form of a guidance to industry: "Potency Tests for Cellular and Gene Therapy Products" [34].

Potency assays must evaluate relevant biological activity directly linked to a product's

therapeutic mechanism of action (MoA) and may be conducted as a direct measure of product activity within a living biological system (e.g., *in vitro* culture system or *in vivo* model system) or as an indirect surrogate assay that can be correlated with therapeutic activity (e.g., marker expression or protein secretion). Although potency testing is not required as part of product release during early clinical development in most jurisdictions, including in the United States, one or more validated potency assays are required at the time of product licensure and is viewed as a critical element of product stability and comparability testing [34,35]. For complex cell-based therapies, it is often the case that a matrix of assays is recommended due to the difficulty in selecting a single assay that assesses quality and consistency whilst also predicating clinical efficacy.

Properly designed potency assays should produce quantitative data, be sensitive enough to detect changes in the drug product quality, and ultimately should be practical for routine lot release testing. As such, efforts towards building a suitable potency assay strategy should begin as early as possible during preclinical development, often involving nonclinical mechanistic studies that assess whether a given biological property is necessary and/or sufficient to account for the product's therapeutic activity. In the discovery and nonclinical phases of development, a variety of animal models are often utilized to demonstrate a proof of concept that mirrors the MoA. Results of such *in vivo* mechanistic studies should then guide early adoption and continuous optimization of candidate *in vitro* potency assays that appropriately capture the product's hypothesized MoA.

As clinical development progresses and product knowledge increases, sponsors should develop and implement improved potency measurements that quantitatively assess relevant biological product attribute(s) (as per 21 CFR 312.23(a)(7)). The primary objective of later phase investigational studies (i.e., Phase 3) is to gather meaningful data about product efficacy; intrinsic to this is confirming that administration of a given product dose with defined potency, will perform as expected in patients. Therefore, it is essential that a potency assay or preferably, assay matrix design and acceptance criteria be established prior to start of pivotal clinical studies to avoid potential clinical hold (21 CFR 312.42(b)(2)(ii)). While the path for potency assay development is relatively well-defined, putting this into practice can nonetheless be quite challenging for complex cell-based therapies.

Considering the first example product type discussed above – developers of autologous CAR-T products implement a range of potency strategies to address their products' relatively complex therapeutic MoA. For CAR-T cell products targeting cancer, therapeutic MoA is dependent on recognition of the target tumor-specific antigen (e.g., CAR ligand), activation of T cell effector functions, and direct tumor killing [36]. Since it is quite challenging to design a single potency assay that can robustly and quantitatively measure all of these activities, developers instead will use multiple candidate assays (i.e., a matrix approach) to evaluate product potency during preclinical and clinical development. Individual components of a potency matrix for CAR-T products may include detection of T cell activation markers (e.g., CD69, CD38) [37] or effector cytokine (e.g., IFN-γ, TNF-α) [38] in response to antigen-positive cells, and *in vitro* cytotoxicity against one or more antigen-bearing tumor cell lines [39]. As discussed above, autologous CAR-T products can exhibit broad ranges in drug product phenotypic and functional attributes due to variability inherent to the use of patient-derived starting material. In the context of potency assay development, this can present a challenge for sponsors when trying to determine appropriate acceptance criteria. Clinical development of KYMRIAH® utilized a potency assay that measured IFN-γ secretion following co-culture with CD19-positive target cells, however IFN-γ secretion values varied

 DOI: 10.18609/cgti.2022.197

greatly from lot-to-lot and were not reported as correlating with patient outcome [40]. As developers of CAR-T products consider their own path for potency assay development, it is highly encouraged to seek regulatory feedback on potency assay strategy both prior to, and during clinical development. As is true for many aspects of cell and gene therapy products, regulatory views on acceptable potency assays for CAR-T products are likely to continue to evolve as a wider number of programs accumulate manufacturing and clinical experience.

Turning now to the second example product type – allogeneic progenitor cell-based therapies - the complexity underlying MoA can be even greater given that progenitor cells may exhibit pleiotropic function and/or drive efficacy in stages. For example, MSC-based products are largely viewed as having broad therapeutic functionality, ranging from secretion of trophic factors, stimulation of angiogenesis or neurite outgrowth, extracellular matrix deposition, immunomodulation, and multilineage differentiation potential [41–43]. While such broad therapeutic capability is potentially exciting for addressing unmet medical need, it also can make MoA of MSC-based products difficult to discern. To reduce the risk of clinical setbacks [44], developers of MSC-based products should consider evaluating a broad range of mechanistic endpoints in preclinical studies and implementing a potency matrix that covers some or all of the aforementioned functionalities to maximize their number of shots on goal.

Similar to MSCs, NPCs present the challenge of a capacity for pleiotropism, which can be further confounded by the potential for their *in vivo* activity to occur in stages and ultimately depend on successful differentiation into one more target cell types. As an example of this, BlueRock Therapeutics' PSC-derived NPC product, MSK-DA01, was assessed for *in vivo* engraftment and differentiation capacity in 6-hydroxydopamine lesioned rats to support selection of clinical lots for use in the first-in-human trial for Parkinson's disease [45]. Information provided in the publication indicates that MSK-DA01 testing also includes an *in vitro* dopaminergic neuron differentiation assay [45], which ideally can replace the need for *in vivo* testing and may ultimately serve as a key potency metric. A second example is ReNeuron's fetal-derived, conditionally immortalized NPC product, CTX0E03 that is in clinical development for treatment of chronic deficit resulting from ischemic stroke. Preclinical studies suggest that CTX0E03 therapeutic activity may occur via multiple mechanisms, including immunomodulation, stimulation of angiogenesis, trophic factor secretion, and direct cell replacement [46]. Upon reaching phase II clinical testing, characterization of CTX0E03 potency was limited to evaluation of immunomodulatory capacity using a co-culture assay and *in vitro* neural differentiation potential, suggesting that these may be the primary MoAs for this product [47].

When considering forward compatibility of potency strategy to support commercial release of cell-based products, use of *in vivo* testing or *in vitro* bio-assays present logistical challenges, particularly with respect to assay complexity and reproducibility. Ideally, simpler, surrogate assays that can be correlated with *in vivo* or *in vitro* bioassay results, such as Aspen Neuroscience's NeuriTest, may offer alternative strategies that are more forward-compatible and better support commercial advancement of cell-based products.

PURITY & GENOME INTEGRITY: KEY ELEMENTS OF PRODUCT SAFETY

The second and equally important objective of a product's analytical testing strategy is to address potential safety concerns. A wide array of analytical measures is applied to cell-based therapies to ensure patient safety, some which are broadly applicable across most/all product types, and some that are product-/

process-specific. In almost all cases, cell-based products must be assessed for sterility, endotoxin, mycoplasma and relevant adventitious agents using appropriate analytical methods [47]. Additional safety tests are then applied based on a product's manufacturing process, as defined by the unit operations and raw materials used during production, as well as the product's specific biological attributes. This section focuses on two evolving areas of safety testing for cell-based products that tend to be product-specific:

- Purity, with respect to cellular impurities that may persist in the final drug product population;
- Genome integrity, ranging from assessments for gross chromosomal abnormalities down to single nucleotide changes that carry a potential safety concern.

Purity

A former colleague and visionary of cell-based therapies liked to say, "ask for a 100% pure cell population, and I'll give you one cell." [48]. Although a cell-based drug product may be deemed pure by expression of one – two markers, advancements in multiparameter and single-cell analyses are providing better resolution into cell subtypes and phenotypic profiles present in cell-based products. As discussed above, this has had a big impact on identity testing of T cell based products that are now routinely evaluated for phenotypic markers (Table 1) and progenitor-based products that may utilize higher resolution profiling to more accurately define the therapeutically active population. In the context of purity testing, advancements focus on the use of higher resolution and higher sensitivity test methods, as discussed in this section.

The objective of cellular purity release testing is to monitor non-target cell types that may be present in the final drug product population, which do not comprise the therapeutically active cell population, with special attention paid to any cell types that pose a potential safety concern. During preclinical development of candidate cellular purity assays, quantitative methods should be used to define the cell type composition of the drug product as near to 100% as possible, including any variability in subpopulation frequencies that may occur lot-to-lot. This ensures that all possible non-target cell types are identified, including any cell types that carry any actual or theoretical risk to patient safety [47]. Ultimately, non-target cells that do not impact product quality and do not pose a safety concern may be dropped from drug product release, and instead monitored as part of non-release characterization testing (and eventually may be dropped altogether pending sufficient data). In contrast non-target cells that pose a potential safety concern (theoretical or empirically observed during preclinical testing) must be included as part of drug product release testing.

As discussed above, the strategy employed for purity testing of cell-based products is highly process-/product-specific. For autologous CAR-T products, purity testing typically has been limited to quantification of non-T cells, defined as CD3-negative cells by flow cytometry [49]. In contrast, allogeneic CAR-T products must be monitored for presence of αβ T cells retaining their endogenous TCR given a capacity for these cells to induce GvHD, and previous studies provide guidance on the acceptable limits for this type of cellular impurity [50]. Progenitor cell-based products that involve differentiation of a multipotent or pluripotent starting material must consider whether the starting cell population, or alternative cell lineages that may arise during manufacturing, can persist in the final drug product. Early process development of progenitor-cell-based products should include comprehensive in-process testing to identity persistence or emergence of non-target cells that may then need to be monitored in the final drug product. As a

DOI: 10.18609/cgti.2022.197

specific example, NPC products for the treatment of Parkinson's disease typically need to confirm that serotonergic progenitors/neurons are absent from the final drug product, as these cells have been attributed to the emergence of graft-induced dyskinesias [51]. In each of these cases, monitoring cellular impurities should utilize analytical methods with the appropriate degree of sensitivity and specificity based on intended clinical cell dose and available information regarding a potentially safe limit for the particular cellular impurity.

Purity testing methods for cell-based products currently employ either single cell-based assays (e.g., flow cytometry) or bulk testing methods. Bulk testing methods are well-suited for purity testing because they typically require a relatively small amount of input material, can be faster to perform than single cell-based methods, and offer a sufficient degree of sensitivity. An increasingly common bulk testing method for purity is use of digital droplet PCR (ddPCR), which is used to detect impurity-associated mRNA transcript(s) present in the drug product. For ddPCR-based purity assays, the acceptance criteria typically are set as a one sided limit, at or near the lower limit of quantification or detection. Critically, the proposed purity acceptance criteria should be justified with respect to product safety using relevant *in vitro* or *in vivo* nonclinical models and with consideration for the product's intended clinical dosing strategy.

Special attention must be paid to purity testing of PSC-based products, as residual undifferentiated cells contaminating the drug product have the potential to form tumors or teratomas. Indeed, even a small number of remaining undifferentiated PSCs (e.g., equal to or more 10000 cells) have been shown to generate teratomas *in vivo* [52,53] underscoring the importance of implementing a highly sensitive purity assay for PSC-derived products. To determine the specific level of sensitivity required for PSC-based products, sponsors will perform *in vivo* nonclinical studies in a relevant xenopermissive model system using drug product spiked with increasing amounts of the undifferentiated starting material. PSC-spike groups corresponding to the proposed specification limit, along with a higher spike level that provides some degree of safety margin, should be included to demonstrate lack of tumorigenicity as a justification that the proposed purity assay is supportive of product safety. Finally, the theoretical potential for PSC-derived products to contain impurities from virtually any cellular lineage, underscore the importance of needing to understand all cell types that might arise during differentiation and persist in the final drug product.

Genome safety

Another rapidly evolving area of analytical development is genome safety testing. In the absence of genetic engineering, the use of extended culture alone can induce genome instability. For example, PSCs that were subjected to extended culture have been shown to develop genetic abnormalities in key oncogenes such as BCL2L1 and TP53 [54,55]. Transformed cells with acquired genetic abnormalities, or cells that could later transform post transplantation, are considerably more challenging to detect than residual/remaining undifferentiated cells, as they may be phenotypically silent during manufacturing. The need for improved methods in genome safety testing also is driven by the increasing number of genetically engineered cell therapy products, including products transduced with a permanently integrating viral vector, such as a lentiviral vector (LVV), and gene edited products that utilize a nuclease-based editing system, such as CRISPR/Cas9. This section discusses key genome safety testing strategies currently applied to viral vector-modified and gene-edited cell therapy products, while also indicating areas where further standardization of analytical methods is warranted.

Cell-based products that are engineered with permanently integrating viral vector such as LVV require additional safety measures to

ensure that viral integration does not result in genomic instability. Specifically, release testing of viral vector engineered products must include evaluation of vector copy number (VCN) as an indicator of the potential risk for insertional mutagenesis. Such testing typically is performed by ddPCR [56]. Although VCN <5 has been widely discussed within the industry and by regulators as a benchmark, the FDA currently does not specify an upper VCN limit for vector-engineered products. The draft CAR-T guidance indicates that VCN release criterion should be established based on manufacturing experience and justified by a risk assessment. Use of viral vectors also carries the risk of generating replication competent viral particles [57]. To address this theoretical safety concern, replication competent retrovirus (RCR) testing typically must be included as part of release testing for vector modified products and given the need for high sensitivity, traditionally requires an extended *in vitro* culture assay that can take up to 6 weeks to complete [58].The long duration of RCR testing can pose a challenge for products that are under pressure to reach patients quickly or require fresh administration. More recently, the FDA has indicated that *ex vivo* vector modified cell therapies may justify removal of RCR testing from drug product release given sufficient manufacturing experience demonstrating RCR-negative product [59]. Finally, viral vector insertion site distribution (ISD) analysis often is performed during preclinical development and may be used as supportive data for VCN specification setting [8]. Interestingly, ISD analysis recently was shown to correlate with patient outcome for a CD19 CAR-T product in clinical testing for chronic lymphocytic leukemia [60], suggesting a potential future role for ISD analysis in product optimization.

Cell-based products that are gene edited, such as those modified using CRISPR/Cas9 or related nuclease-based editing platforms offer a more targeted approach to genetic engineering but still require comprehensive genome safety testing. The primary safety concern for gene-edited cell products is that the editing process itself has the potential to result in off-target editing or translocations events that could compromise genome integrity and product safety. For nuclease-based editing platforms, editing specificity is predominantly determined by use of one or more guide RNAs (gRNAs) to target a given genomic site. Most gRNAs will exhibit some level of off-target binding to genomic sites whose sequence is a close match to the target sequence. This can facilitate Cas-mediated double strand break (DSB) at non-target genomic regions, which can then be modified with insertion/deletions or give rise to a translocation event between two separate DSB sites [61]. More recently, therapeutic developers are exploring use of base editors, prime editors, and epigenome editors that further reduce the risk of inducing off-target editing or translocation events [62,63].

To help guide developers of gene-edited products, the FDA recently issued a draft guidance document detailing the type of information that should be included in an Investigational New Drug application to demonstrate product quality and safety [64]. As described in the draft guidance, preclinical development of gene-edited products should include a series of analytical studies to characterize the potential for any off-target editing or chromosomal translocation events resulting from the gene editing process. Analytical studies should include multiple orthogonal methods to identify candidate off-target editing and translocation events, using a combination of *in silico*, biochemical and cell-based assays, and at least one unbiased genome-wide approach. Based on the collective output of the orthogonal screening methods, a candidate list of off-target editing, and translocation sites should be assessed in a verification study using a targeted method with sufficient sensitivity to detect low frequency events (e.g., amplicon-based sequencing). Bona fide off-target editing or translocation sites that are found to be recurrent (i.e., across multiple manufacturing runs, and if relevant, across multiple

DOI: 10.18609/cgti.2022.197

donors) in the verification study should then be included in drug product release testing using an appropriately sensitive and controlled test method, such as ddPCR. An additional test method often performed on gene-edited products is some form of *in vitro* autonomous cell growth assay, which typically involves extended culturing of the gene-edited drug product in the absence of growth factors. While the draft guidance has helped normalize the types of genome safety testing applied to gene-edited products, there remains a need for further standardization of how the analytical procedures are performed both with respect to their experimental execution and subsequent data analysis. Continued engagement between therapeutic developers and regulatory authorities will help bridge this gap, as will increased information sharing within the scientific community regarding preferred analytical strategies, pitfalls, and successes.

FUTURE DIRECTIONS

The field of cell-based therapeutics continues to evolve at a fast pace with future challenges and prospects apparent on the horizon. Key remaining challenges for autologous cell-based products include the high cost of manufacturing and testing, as well as the inherent variability of patient-derived starting material and corresponding difficulty in setting release testing specifications that ensure product quality without compromising patient inclusion. Allogeneic products have the potential to overcome these limitations, but with a higher demand for complex engineering strategies that can only be addressed by more complex analytics. The arrival of hypoimmunogenic cells [65] is an exciting example of how allogeneic products have the potential to generate universally applicable therapies. However, it remains to be seen whether such immune stealthing strategies will require some form of kill switch to be incorporated into the product design to ensure an *in vivo* clearance mechanism is in place should it be needed.

As further complexity is incorporated into cell-based products, the need for enhanced analytics to monitor product safety and activity becomes even more important. Increasing use of high content analytical modalities, including single cell-based techniques is expected to provide significant advances in our understanding of human disease and to help guide design of optimal intervention strategies. Currently, there exist more than 30 single-cell sequencing technologies allowing for the simultaneous interrogation of different genetic and epigenetic information [66]. The promise of high content single cell profiling was recently demonstrated for CAR-T based therapies based on research conducted at the Broad Institute of MIT and Harvard and independently at Stanford University. By applying single cell RNA sequencing to a wide range of clinical CAR-T products with available patient outcome data, researchers at these two institutions found that the presence of regulatory T cells in CAR-T drug product negatively impacted clinical efficacy, thus identifying a previously unchecked cellular impurity with the potential to impact CAR-T product quality [67,68]. By a similar token the emergence of single-cell multi-omics platforms, such as the Tapestri Platform (Mission Bio) [69] and current/in development platforms by isoplexis [70] are poised to substantially disrupt the current analytical landscape given their capacity for integration and correlation of *in vivo* proteomic activity with transcriptomic profiling; high content, high resolution platforms such as these are expected to reveal more predictive biomarkers and guide design of optimized cellular therapeutics. Finally, multiple advances are still expected in the single-cell multi-omics field. Missing modalities such as ChIP-seq and metabolomics will be valuable additions to complete the portfolio of analytical readouts. Likewise, yet to be explored is the potential of combining single-cell omics with fluorescent cell imaging, such high-content image-based morphological profiling i.e., Cell Painting (Molecular Devices) [71].

As the field of cell-based therapeutics continues to evolve, the future of analytical testing strategies in turn must expand to include methods that probe deeper into products' underlying biology, push the limits of assay sensitivity, and all the while maintain forward-compatibility as products march onward to market.

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Contributions: All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.
Acknowledgements: None.
Disclosure and potential conflicts of interest: The authors have no conflicts of interest.
Funding declaration: The authors received no financial support for the research, authorship and/or publication of this article.

ARTICLE & COPYRIGHT INFORMATION

Article source: Invited; externally peer reviewed.
Submitted for peer review: Aug 18 2022; **Revised manuscript received:** Oct 25 2022; **Publication date:** Nov 15 2022

CELL & GENE THERAPY INSIGHTS

CELL THERAPY BIOPROCESSING

SPOTLIGHT

COMMENTARY

Cell therapy 101 (part 1) Challenges of early clinical development: a 'thumbnail sketch'

Michael D Leek

Cell therapy has long been regarded as a possible therapeutic panacea, having potential to restore structure and function of damaged and diseased tissue and whole organ systems. Historically, therapeutic targets have included treatment of diabetes, liver failure, kidney disease, CNS disorders, connective tissue disorders, and more recently, cancer. Casual observers will see cell therapies as a 'bright new shiny toy', a potential cure for cancer, based on some of the CAR-T data recently generated. This, however, is not the case: those of us working in the cell therapy space back in the late 80s remember the days when such products were classified as medical devices, and the first autologous and allogeneic products were starting to emerge from companies such as Advanced Tissue Sciences and Organogenesis. Many of the challenges we faced in those early days still exist. We are still grappling with logistics of shipping cell-based products from clean room to clinic, regulatory uncertainty, patient-to-patient variability with autologous approaches, donor-to-donor variation of allogeneic products, and high cost of goods. Moreover, uptake of cell therapies by established pharmaceutical companies has been slow, as cell-based products fail to achieve the requirements around scalability, reproducibility, and cost that many major corporations see as mandatory. Numerous academic and industry-based groups around the world are working on a wide variety of cell-based projects; however, not all of these groups understand the complexities of commercializing the technology from bench to bedside. In addition to gaining appropriate funding to enable progression from the laboratory to clinic, all of these companies face the challenge of navigating a complex regulatory landscape, plus transitioning to GMP manufacture. Particular issues which need to be addressed include:

1. Transfer from research to a clinical-grade product
2. Manufacture product to GMP

3. Quality testing – what needs to be done and how?
4. What are the regulations, and how do we adhere to them?
5. What are the challenges for commercialization?

This article aims to address the above in a broad-brush manner, subsequent 'Cell therapy 101' will review specific areas in more detail.

Cell & Gene Therapy Insights 2022; 8(10), 1413–1427

DOI: 10.18609/cgti.2022.207

As a commercial reality, therapeutic use of cell-based products is unlikely to become commonplace for many patients in the near future. However, based on the number of academic initiatives, startups, and biotech companies active in the field, it's clear that a significantly expanding number of clinical studies are being initiated and an increasing number of products are progressing to authorization.

One of the problems with commercialization of cell therapies is product-to-product variation complexity of manufacture. Unlike conventional drugs, cell-based products cannot be terminally sterilized or easily produced in large batches. These complications lead to further hurdles, as broad regulatory guidance cannot be easily applied to products with such biological diversity. Furthermore, convoluted manufacturing processes often result in unscalable or over-costly products with little sustainable commercial merit.

Companies or academic groups considering clinical studies with cell therapies therefore face a number of difficult technical, regulatory, and commercial challenges in progressing from the lab to patient. These challenges fall into five broad categories:

- Technical transfer – provisional assessment of commercial feasibility;
- Production to GMP standards (including shipping to clinic and GDP);
- Quality systems (including in-process and final product release);
- Regulatory affairs;
- Establishing and implementing a clinical program

Each of the above categories are highly specialized areas of expertise. As a consequence, it is unlikely that an academic group or company new to the sector will be able to justify the expense and resources to cater for each area. This presents a potential situation where promising cell-based technology could hit a 'brick wall' caused by escalating costs and complexity. Numerous academic institutions have attempted to circumvent some obstacles by building in-house clean rooms. Unfortunately, possession of such a facility without the appropriate staff, quality, regulatory, and clinical infrastructure may prove counterproductive, and further delay progress towards the marketplace.

TECHNOLOGY TRANSFER: PROVISIONAL ASSESSMENT OF COMMERCIAL FEASIBILITY

Technology transfer is perhaps one of the most overlooked facets of product development, and the main area that leads to increasing complications as products progress from Phase 1/2 towards pivotal studies.

Products developed via an academic or preclinical startup route formulated using research-grade materials to a quality/scale that is not clinically/commercially feasible

– the philosophy that 'quality starts at the beginning of a development program' cannot always be adhered to' – as a consequence, several transitional steps need to be taken before a process is suitable for manufacture in a clean room. These steps include:

Replacement of research-grade materials with suitable clinical-grade products

This a particularly important issue when considering manufacture of cell therapeutic products, as many growth factors and cytokines used on the research bench are not commercially available to clinical-grade. The consequence of this is that the resultant GMP-compliant product may be significantly different to the research product. This was an issue for one US biotech company when they switched from research grade laminin to a clinically-acceptable version – as their embryonic stem cell phenotype changed under revised culture conditions, the company eventually established the root cause of this phenotype change was linked to impurities in research-grade materials and modified the media accordingly.

Modification of the process to facilitate suitable scale-up

In the race to get products into the clinic, companies occasionally lose sight of the final goal – to develop a product that will generate significant sales. Cell therapy does not lend itself to scale-up as products cannot generally be campaign manufactured and stored with ease for several years (like conventional drugs). As a consequence, the manufacturing process needs to be scalable to meet increasing demand from Phase 1 to Phase 3 and in-market.

Analysis of all raw materials to ensure minimal animal components or antibiotics (if present) are carried through the process

Many research-grade cell culture media contain animal components such as bovine serum, and regulators are becoming increasingly concerned that trace elements may be carried over into the final product, increasing the chance of potential zoonoses. As a consequence, wherever possible, it will be preferable to remove animal components by using alternative reagents. However, in many cases this is not possible. In such instances, regulators may require direct evidence that no animal elements have been carried over into the final product.

Design review to identify critical steps in the process: establish what latitude (if any) is needed during each critical step

Although many products are initially developed empirically, by the time the product enters the clinic, regulators will expect to see some evidence of design control. This can be performed on many levels – however, the most simplistic and effective method involves:

- Breaking the manufacturing process into a number of steps;
- Asking the question of what would happen if this step was missed out or modified;
- Asking if the final product would be altered significantly. Such design review will aid the Qualified Person (QP) in making batch release decisions in cases where BMR-deviations have been flagged during manufacture.

Introduction of appropriate sterility tests

This is a quality issue which will be discussed in more depth later. However, during the research phase little attention may have been taken with regard to product sterility. Introducing appropriate sterility tests throughout

manufacture will establish that the process is under control and provide support data for subsequent real-time release.

Establishment of product shelf-life

Understanding cell viability is key to developing an efficacious and commercially viable product – for instance, a two-day shelf life will not be commercially acceptable if product manufactured in the UK needs to be shipped to the USA.

Written product specification, along with appropriate release criteria

This is essential to define the product, in order to ensure the same formulation can be released in a reproducible manner over time.

Develop and test assay systems for product release

In order to product that leaves the clean room meets the release criteria, test methods need to be validated to ensure they are robust and not themselves subject to unacceptable variation. Regulators will expect to see an increasing amount of data supporting the validity of test methods as products progress from Phase 2 to Phase 3 studies (as the release tests will define what is being evaluated, and what is being sold).

Initiation of 'engineering runs'

Initiation of 'engineering runs' in simulated 'clean room-type' environment. Often the final stage of technology transfer, engineering runs would typically be performed by operators who will make the clinical product. The purpose of such engineering runs is to identify potential 'glitches' in the process and 'iron' them out before clinical manufacture begins in earnest.

Once all of the above are completed, the tech transfer should be finished and prototype product ready for manufacture in a GMP clean room. However, in-parallel a commercial 'sanity check' could be performed looking at:

- Market need – the potential indication, competitive landscape;
- Extrapolation of cost of goods sold (always a worthwhile exercise during the product development cycle) – in the early days of cell therapy, one company produced a tissue engineered skin replacement that cost US $ 1000 to manufacture, but was only reimbursed by Medicare at $645. More recently, some of the approved CAR-T products cost hundreds of thousands of dollars - these costs cause some payers to regard them as a treatment of last resort;
- Ease of product usage. Early cell therapy products were either provided frozen, with complex thawing protocols which left the pharmacy, nurses, and clinicians bewildered, or they were provided with a shelf-life of less than 2 days. Both extremes are obviously unacceptable with regard to user compliance.

Prior to commencing GMP manufacture, there should be a final design review meeting when all of the above are audited to ensure both process and product are ready for scale-up into the clinic.

GMP MANUFACTURE

The application of GMP to manufacture of investigational medicinal products (IMPs) is intended to ensure that trial subjects are not placed at risk, and that the results of clinical trials are unaffected by inadequate safety, quality or efficacy arising from unsatisfactory manufacture. Furthermore, the application of GMP is intended to ensure that there is consistency between batches of investigational medicinal product used in clinical trials, and that changes during development of an investigational medicinal product are adequately documented and justified.

DOI: 10.18609/cgti.2022.207

The production of investigational medical products involves added complexity when compared to marketed products. This is largely due to absence of fixed manufacturing routines and variety of clinical trial designs, with consequent packaging designs for randomization and blinding bringing an increased risk of product cross-contamination. Furthermore, there may be incomplete knowledge of the potency and toxicity of the product and a lack of full process validation, or, marketed products may be used which have been repackaged/modified in some way (this is often the case when two or more companies collaborate using each other's proprietary materials).

These challenges of IMP manufacture require recruitment of personnel with a thorough understanding of, and training in, the application of GMP to investigational medicinal products. Co-operation is required with trial sponsors (for the purpose of this review – generally cell therapy companies) who undertake the ultimate responsibility for all aspects of the clinical trial including quality of the IMP. The increased complexity in manufacturing operations requires a highly effective quality system.

The principles of GMP state that specifications (for starting materials, primary packaging materials, intermediate, bulk products, and finished products), manufacturing formulae, and processing and packaging instructions should be as comprehensive as possible given the current state of knowledge. They should be periodically reassessed during development and updated as necessary. Each new version should take into account the latest data, current technology used, regulatory and pharmacopeial requirements, and should allow traceability to the previous document. Any changes should be carried out according to a written procedure, which should address implications for product quality, safety, and efficacy, along with stability and bioequivalence.

Based on the above, the GMP function should be responsible for maintaining a Product Specification File (PSF). This should be continually updated as development of the product proceeds, ensuring appropriate traceability to the previous versions. It should include, or refer to, product specifications – starting materials, packaging, intermediate, bulk, finished product; manufacturing methods (as documented in batch manufacture records); in-process testing and methods; relevant clinical trial protocols; stability data; storage and shipment conditions.

The above information should form the basis for assessment of suitability for certification and release of a particular batch by the QP.

For every manufacturing operation or supply there should be clear and adequate written instructions and written records – the batch manufacturing record (BMR). Information in the PSF should additionally be used to produce detailed written instructions on packaging, quality control testing, storage, and shipping.

Batch records should be kept in sufficient detail for the sequence of operations to be accurately determined. These records should contain any relevant remarks which justify the procedures used and any changes made, enhance knowledge of the product, and develop the manufacturing operations.

An additional requirement of GMP, correct labelling of clinical trial prototypes is often overlooked by startup companies producing Phase 1 products, and should comply with generic requirements including those shown below (a good starting point is EU GMP Annex 13 (section 26) – some labeling requirements are shown in **Box 1**.

All the above indicate that GMP manufacture is not simply a case of 'gowning-up', going into the cleanroom, and making the product according to a BMR.

GMP manufacture is a specialized area requiring recruitment of highly-trained individuals with the necessary discipline and diligence to ensure that product is manufactured to the highest standards consistently with minimal batch-to-batch variation.

BOX 1

Example IMP labelling requirements.

- Name, address, telephone number of the sponsor or investigator (main contact for information on the product, trial and unblinding), trial reference code.
- Pharmaceutical dosage form, route of administration, quantity of dosage units, for open trials, the name/identifier and strength/potency.
- Batch and/or code number to identify the contents and packaging.
- Trial subject identification number/treatment number, visit number.
- Directions for use, storage conditions.
- 'For clinical trial use only' (or similar wording).
- Period of use (use-by date, expiry date or re-test date as applicable).

Shipping cell-based therapies from cleanroom to clinic presents numerous challenges. For instance, fresh products inevitably have limited shelf life and need to be temperature-controlled (typically between 2 and 8 °C). As a consequence, getting viable product to the patient in a timely manner requires detailed logistic planning and continuous monitoring of time/temperature. Principles of Good Distribution Practice are often focused on wholesale distribution of approved medicinal products, however these principles can equally be applied to investigational medicinal products may provide guidance on how to supply clinical trial material (the section on supplementary requirements to Annex 13 – dealing with storage and transportation of IMPs provides some help). General GDP principles include monitoring of defined conditions during storage and transportation, detailed protocols for receipt and storage at the investigational site, robust data collection based on detailed SOP's, and clear understanding of product acceptance criteria by the pharmacy/clinic.

QUALITY SYSTEMS

The Quality team generally will be involved in three main activities:

1. Quality control
2. Quality assurance
3. Validation

Quality Control is concerned with sampling, specifications and testing as well as the organization, documentation, and release procedures which ensure that the necessary and relevant tests are carried out. The quality team also ensure that materials are not released for use, nor products released for sale or supply, until their quality has been judged to be satisfactory. Quality Control is just not confined to laboratory operations, being involved in all decisions which may concern the quality of the product. The independence of Quality Control from Production is considered fundamental.

With regard to conducting clinical trials, specifications and quality control checks should include measures to guard against unintentional unblinding due to changes in appearance between different batches of packaging materials.

A comprehensive set of in-process tests and controls may provide assurance of sterility and product specification when final product testing is problematic (for instance, with short shelf-life cell therapies). Consequently, real-time release may be authorized for specific parameters as an alternative to routine testing of final product. Authorization for real-time release should be given, refused or withdrawn jointly by those responsible for assessing products together with input from GMP inspectors.

Production processes for investigational medicinal products are not expected to be validated to the extent necessary for routine production but premises and equipment are expected to be validated. It is, however, a regulatory requirement that evidence of virus inactivation and removal of other impurities of biological origin should be demonstrated, to assure the safety of biotech-derived products.

Validation of aseptic processes presents special problems when the batch size is small. Process consistency can usually be established

DOI: 10.18609/cgti.2022.207

by ongoing simulation of the sterile process, thereby minimizing product loss.

Validation studies should reinforce GMP and be conducted in accordance with defined procedures. It is a requirement of GMP that manufacturers identify what validation work is needed to prove control of the critical aspects of their particular operations. Significant changes to the facilities, the equipment, and the processes, which may affect the quality of the product, should be validated. A risk assessment approach should be used to determine the scope and extent of validation.

When any new manufacturing technique or method of preparation is adopted, steps should be taken to demonstrate its suitability for routine processing. The defined process, using the materials and equipment specified, should be shown to yield a product consistently of the required quality.

Significant amendments to the manufacturing process, including any change in equipment or materials, which may affect product quality and/or the reproducibility of the process should be validated.

In the EU, release of IMP from the facility should not occur until the QP has certified that requirements of EU 536/2014 are met – including some of the elements listed in Box 2.

Within the EU, QP's are central to success of the clinical development program, as he/she has final responsibility for release of product. As a consequence, a QP should maintain knowledge and experience and be up to date with technical and scientific progress and changes in quality management relevant to the products requiring certification.

Quality is incorrectly regarded by some as a disciple void of innovation. However, evaluating new assays to streamline product release, reduce manufacturing excursions and implement constructive change is the backbone of most Quality Management Systems (QMS). This is demonstrated by adoption of BacT systems for sterility testing which can produce reliable data in hours (compendial sterility testing typically previously took 14 days to complete). Such innovation has significantly reduced 'vein-to-vein' time for autologous therapies.

REGULATORY AFFAIRS

Since the enactment of the first drug law in 1848, legislation surrounding drug development has evolved into a maze of regulations which has become at times difficult to navigate. Not only are existing regulations constantly reviewed and updated, the increasingly rapid rate of development in the pharmaceuticals and biologicals field creates new issues that need to be addressed by new legislation. Furthermore, for companies manufacturing cell therapies there is an additional complication – most of the rules are designed to legislate for drugs which generally can be (i) terminally sterilized, (ii) campaign

BOX 2

Example of QP release requirements for IMP.

- Batch records, including control reports, in-process test reports, release reports, demonstrating compliance with product specification, protocol, and randomization code.
- Deviations or planned changes, consequent additional checks/tests, completed and endorsed by staff authorized to do so according to the quality system.
- Validation status of facilities, processes, and methods; examination of finished packs; where relevant, any analyses or tests performed after import of product components.
- Product stability reports.
- Source and verification of conditions of storage and shipment.
- Audit reports concerning the quality system of the manufacturer.
- Documents certifying that the manufacturer is authorized to formulate investigational medicinal products for export.

manufactured, (iii) produced in extremely large batches, and (iv) stored at room temperature for several years.

Whenever a new medicine is developed, before it can be sold and supplied to patients, it has to have a licence (in the EU this is a Marketing Authorization – the number printed on the pack). In order to gain a licence, all the scientific information that has been generated during development has to be gathered, reviewed, summarized, and presented in an application to a Regulatory Agency.

Gaining regulatory approvals is not a cheap or clear-cut process. Key documents such as the CTA (clinical trial authorization), IMPD (investigational medicinal product dossier) and IB (investigator brochure) are complex to prepare, and need to adhere strictly to accepted formats imposed by regulatory bodies.

Furthermore, specific regulations from the UK, EU, and USA need to be taken into account when developing novel cell therapies, as each regional requirement will differ substantially! Understanding of regulatory requirements can save or cost a company substantial amounts of money, depending upon the quality of the advice presented.

In addition to some of the more generic factors above, autologous cell-based approaches present bespoke regulatory considerations related to factors such as use of precious (final) product for retained sampling and release-to-clinic; and shifting release criteria across a patient population with highly-variable starting material. Allogeneic products face different considerations related for instance to provenance/suitability of allogeneic donors (see the section on 'hot topics' below) and complexity of switching allogeneic donors mid-trial or between clinical phases. None of these issues are insurmountable, and early regulatory dialogue with appropriate agencies usually helps...

CLINICAL SUPPORT

The majority of clinical trials are concerned with the evaluation of a therapeutic drug or device, some can also be concerned with other forms of treatment, such as surgical procedures, radiotherapy, and quality of life studies.

Clinical trials generally fall into four phases:

- Phase 1: to determine safety of the drug
- Phase 2: to provide evidence of efficacy of treatment and establish optimal dose
- Phase 3: efficacy/side effects compared to other drugs/treatments/placebo
- Phase 4: large-scale epidemiological study (mainly industry)

In conducting a clinical trial (on a cell therapy) a number of key criteria are taken into consideration some of these are outlined in Box 3.

One fundamental question is, 'how many patients do we need'? Statistical methods can be used to determine the number of patients required to meet the trial's principal objectives. However, more practical matters such as availability of patients and resources must also be taken into account. The estimated time period for patient recruitment to any trial will of course depend on prevalence of a given disease.

The important role of appropriately trained clinical research organizations (CRO's) in performing clinical trials cannot be underestimated for cell therapeutics. The need for accurate recording and processing

BOX 3

Clinical planning: key criteria.

- Purpose of the trial, what is the indication?
- Design the trial (including writing the protocol IMPD and IB).
- Which patients are eligible?
- Required size of study.
- End points for evaluating safety and treatment efficacy.
- Specific regulatory considerations, such as long-term follow-up of allogeneic treatments.

DOI: 10.18609/cgti.2022.207

of patient data is fundamental to any clinical trial. If data stored on the master data file are incorrect, conclusions of the study will also be incorrect. While collecting and handling data, it is necessary to ensure that submissible data is accurate, and complete. As a consequence, the CRO's role in ensuring efficient data management and ongoing monitoring of data arising is a priority.

Cell therapy clinical trials present unique challenges on several fronts. In many cases, lack of appropriate *in vivo* clinical models make it difficult to generate reliable/appropriate predicate pharmacokinetic and pharmacodynamic data. In such instances, some regulatory agencies may consider a '3+3+3' dose-escalation approach in last-line of treatment patients. Other complexities include variation in standard of care from country to country - for instance, use of total body irradiation and/or lymphodepletion in cancer patients is currently proving an 'interesting' discussion topic.

SUMMARY: CURRENT 'HOT TOPICS' IN CELL THERAPY

Having provided a brief synopsis of some considerations when developing cell therapy products, this section highlights some of the current considerations and challenges when developing cell therapy products.

Patient-to patient variation across autologous therapies

Many treatments currently in-market are autologous CAR-based T cell products (such as Kymriah® and Yescarta®). These products are often used in fourth or fifth line cancer patients who have (i) advanced underlying systemic pathologies resulting in severe loss of immune function, and have (ii) received numerous previous therapeutic products. Consequently, some patients will not yield sufficient cells to meet starting material qualification; others may have anergic cells which may not expand readily in vitro or are dysfunctional (exhibiting loss of potency). This problem could be addressed by treating patients earlier (first or second line, when their immune system may be stronger) and developing theranostic assays to identify and screen-out potential non-responders.

Sourcing allogeneic cell therapies (starting materials, adventitious agents), donor screening/selection, donor-to-donor variation

With allogeneic therapies becoming more commonplace, the search for suitable donors is becoming increasingly competitive. Such donors need to be screened for adventitious agents (such as HIV, hepatitis B/C, syphilis. In some tissue-specific material additional testing may be needed – for example in leukocyte-rich tissue, HTLV is required) and need to meet all of the quality and potency requirements to manufacture reproducible cell-based products. Setting appropriate release criteria which allow use of subsequent donor cell banks is a subject for careful consideration as criteria need to be sufficiently narrow to ensure potency and therapeutic potential, and sufficiently wide to make allowance for donor-to-donor variation. Failure to consider the above could result in regulators perceiving new donor banks as a material change to product specification, this could lead to expensive bridging studies or requirement for additional clinical trials.

Shipping & logistics: fresh to frozen, thawing protocols

One of the unresolved complexities of developing cell therapy products centers around logistics of shipping from clean room to clinic. Many companies elect to ship product between 2 °C and 8 °C, and whilst product shelf-life is an important consideration, it is possible that following prolonged shipping, cells undergo phenotype stress in the same way human counterparts often suffer following long-haul flights. At present there is no

regulatory requirement to demonstrate that every batch of product leaving the clean room has remained within specification throughout its journey to the clinic. it is however incumbent upon those of us developing cell therapies to ensure the shipping process does not adversely affect key release criteria attributed to the product. Shipping product frozen may overcome some of the vagaries encountered at 2–8 °C – however, ensuring reproducible thaw at the pharmacy or bedside is a difficult process to police. Moreover, as cell therapies become mainstream, it is untenable for a pharmacy to facilitate several different thawing protocols in a single day – lack of controlled freeze/thaw processes is a roadblock to uptake of cell therapies, and an area where standardization and automation would be extremely beneficial.

Process optimization, closed systems; fill-finish; scale

Cell therapy companies should be constantly looking to optimize key processes. Reducing the number of open manipulations will significantly reduce potential for product contamination, automated fill-finish will become a key criterion as companies manufacture large batches of allogeneic frozen product, and in a drive to reduce cost of goods, companies will transition from small 1- and 5-liter bioreactors (such as the G-Rex) to systems with infinitely higher GMP capacity.

Regulatory landscape

Whilst there is a certain degree of harmonization between countries and continents, keeping abreast of local regulatory requirements is an essential commercial consideration. Understanding regional variance can save considerable time and money. For instance, those companies intent on treating patients with allogeneic products in the EU and USA should consider that the US FDA does not favor donors who lived in some EU countries between 1980 and 1996. As a consequence, it makes sense to consider US-sourced donor banks to facilitate multinational trials without the need for region-specific, bespoke cell banks.

Commercial considerations, manufacture, in-house versus contract out

Historically, investors in biotech have not supported infrastructure such as cleanroom builds and the extensive investment in quality, GMP, and support staff needed to utilize and maintain such facilities. It is however apparent that given (i) complexities of manufacture (compared to conventional drugs), and (ii) high costs of contract manufacture, that in-house manufacture may represent a cost-effective and time-saving alternative to use of third-party contractors. This is certainly the case when complicated manufacturing processes (such as allogeneic CAR-Ts) are concerned.

Many of the points raised in this article have remained pertinent since the first cell therapies were developed back in the mid-80s. Whilst cell-based products are gaining exceptional clinical and commercial momentum, we are still several steps away from fully 'pharmaceuticalized' products which are cost-effective to manufacture, fully reproducible from batch-to-batch, and simple to ship from cleanroom to clinic. Less than ten years ago, however, no one had heard of CAR-T – today, 'last chance saloon' cancer patients are being given new hope.

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DOI: 10.18609/cgti.2022.207

AUTHORSHIP & CONFLICT OF INTEREST

Contributions: *The named author takes responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*
Acknowledgements: *None.*
Disclosure and potential conflicts of interest: *The author has no conflicts of interest.*
Funding declaration: *The author received no financial support for the research, authorship and/or publication of this article.*

ARTICLE & COPYRIGHT INFORMATION

Article source: Invited; externally peer reviewed.
Submitted for peer review: Oct 18 2022; **Revised manuscript received:** Nov 16 2022;
Publication date: Nov 29 2022.

FASTFACTS

A flexible & fully automated process for CAR T cell manufacturing

Sophia Lollies, Miltenyi Biotec

Chimeric antigen receptor (CAR) T cell therapy is leading a revolution in cancer cell therapy, with its recent success in hematologic malignancies. One of the largest challenges in the field of CAR T cell manufacturing is the complexity and labor intensity of the process steps. The CliniMACS Prodigy platform is a flexible and fully automated cell manufacturing system designed to cover the entire clinical workflow from starting material to cell product, providing an all-in-one solution to CAR T cell manufacturing challenges.

THE CLINIMACS PRODIGY PLATFORM

The CliniMACS Prodigy platform is designed to cover an entire clinical workflow for CAR T cell manufacturing and consists of four main parts **(Figure 1)**.

The CliniMACS Prodigy instrument is a fully automated platform offering integrated solutions to streamline cell processing workflows. An integral capability of the device is the fully automated washing, fractionation, and cultivation of cells to increase reproducibility and standardization. This instrument can be further modulated with the CliniMACS Electroporator, the CliniMACS Formulation unit, and the CliniMACS workbench, giving centralized control for standardized procedures. MACS GMP reagents are optimized for a full end-to-end approach with proven performance and are validated in numerous manufacturing processes to ensure a high quality final cell product.

In addition, the closed tubing set and consumables provide a closed and safe environment with multiple compartments for every process step within a GMP-compliant cell manufacture workflow. Cell washing, sample preparation and separation, and genetic modification and formulation can be performed inside different compartments of the tubing set. The CliniMACS Prodigy software guides the user throughout the whole process with a flexible configuration of either customized or standardized applications.

Figure 1. The four-part CliniMACS Prodigy platform.

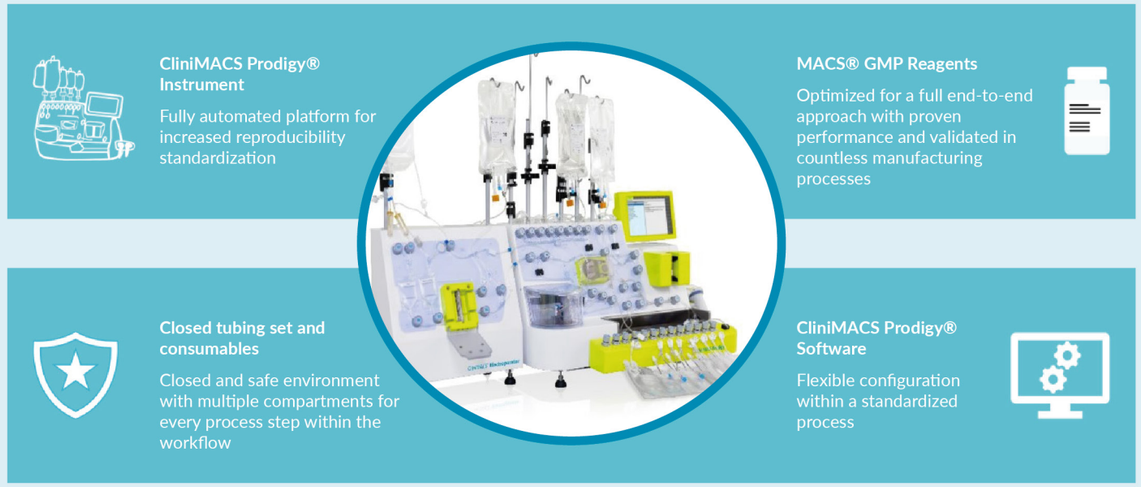
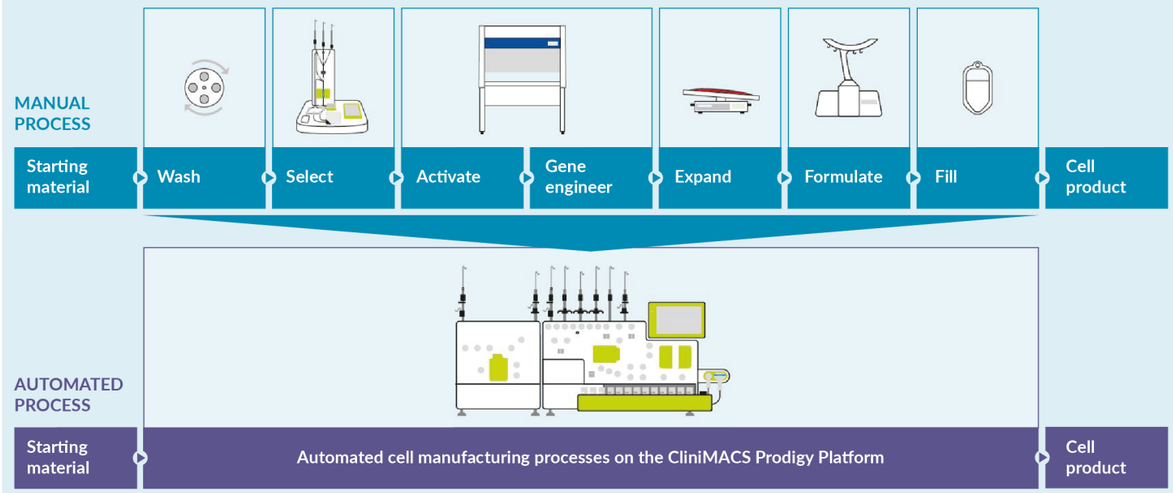


Figure 2. An outline of the automation of previously manual processes using the CliniMACS Prodigy platform.



FROM MANUAL OPERATION TO AUTOMATION

Manual operations can lead to product risk and failure, as well as increased operational and capital expenditure. Open steps require high cleanroom requirements and are often labor intensive with the need for highly skilled staff, varying device protocols, and differing service contracts. Many of these risks can be mitigated by utilizing a hands-off, end-to-end process platform from R&D to commercial manufacturing.

The CliniMACS Prodigy platform offers end-to-end automation in a closed system **(Figure 2)**. The benefits of automated cell manufacturing include reproducible and consistent results, as well as reduced operator hands-on time. No extensive training of personnel is required, and production capacities are easily scalable. This in turn reduces costs and lowers the need for multiple devices.

CELL & GENE THERAPY INSIGHTS

Cell & Gene Therapy Insights 2022; 8(10), 1267; DOI: 10.18609/cgti.2022.185

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SPOTLIGHT

INTERVIEW

Advancing cell therapy manufacturing towards industry 4.0

David McCall, Commissioning Editor, *BioInsights,* speaks to **Thomas Bollenbach,** Chief Technology Officer, Advanced Regenerative Manufacturing Institute (ARMI)

THOMAS BOLLENBACH, PhD is Chief Technology Officer at the Advanced Regenerative Manufacturing Institute (ARMI) BioFabUSA. Tom is responsible for providing leadership to the institute's technical programs, which include the development of enabling platform technologies for human cell and tissue manufacturing, Quality by Design-based process development methodologies for scalability and GMP-readiness, and development and demonstration of modular, automated and closed manufacturing systems. Prior to joining ARMI, Tom served as Vice President of Research and Development at Harvard Apparatus Regenerative Technology, where he led the development of cell- and biomaterial-based tracheal, bronchial and esophageal implants. Tom joined HART from Organogenesis, where he implemented IND-enabling preclinical programs for bioengineered living skin, and maintained strong cross-functional interactions with Clinical Operations, Manufacturing and Business Units to provide scientific support to corporate strategies. Tom received his BSc in Biochemistry from the University of Waterloo, a PhD in Biochemistry from the University of Notre Dame.

Cell & Gene Therapy Insights 2022; 8(10), 1399–1406

DOI: 10.18609/cgti.2022.205

Q What are you working on right now?

TB: ARMI is a not-for-profit manufacturing innovation institution in the Manufacturing USA network, with a focus on enabling scalable, consistent, and cost-effective manufacturing of cells, tissues, and organs. Right now, we have three major focus areas: to adapt manual manufacturing processes to scalable, modular, automated, and closed manufacturing systems; to develop robust empirical methods for developing final product and in-process critical quality attributes (CQAs); and to develop new enabling technologies for real-time sensing and monitoring of critical attributes that are relevant to living cells (not just the typical glucose, lactate, pH, but actually measuring specific proteins, lipids, and metabolites relevant to the production of those cells). We are then adapting those programs to existing manufacturing processes, which are contributed to by external partners in a new 30000ft^2 early-phase clinical development manufacturing facility in Manchester, New Hampshire. Over the next two years, we will be adding to that footprint with a 100000ft^2 facility for full good manufacturing practice (GMP)-complaint manufacturing. We are working on building the enabling infrastructure and then immediately applying that to real world, clinical and commercial processes.

Q What stands out for you in terms of recent advances towards automation and fully closed systems for cell therapy manufacturing, and why?

TB: The current compact systems that are available are fully automated, closed, and compatible with proprietary single-use systems. This design potentially limits manufacturers of anything other than autologous products at small-scale. However, I have seen an increase in the number and diversity of closed, automated platforms being offered by original equipment manufacturers (OEMs) that we consider to be modules of larger scalable closed manufacturing systems. We also design and implement fully scalable 1–100+ L systems that are fully closed and automated and will incorporate smaller subsystems.

We need to move towards modular systems. For example, if you are going to treat diabetics with allogeneic pancreatic islets derived from stem cells, you will need to produce many thousands of doses per week, which is not feasible in the current fully integrated closed systems.

Q What does the latest cost of goods (CoGs) analysis for closed/automated cell therapy bioprocessing tools tell us?

DOI: 10.18609/cgti.2022.205

TB: As expected, one of the findings was that a reduction in labour costs as a huge factor in driving down CoGs for these products. However, one of the striking things about the last round of analysis was that partial automation, not the fully automated solution, was more cost-effective.

"As expected one of the findings was that a reduction in labour costs as a huge factor in driving down CoGs for these products....one of the striking things about the last round of analysis was that partial automation, not the fully automated solution, was more cost-effective."

If you look at cost of business, there are so many factors involved. I am not sure if any of the analyses have taken into consideration the effect of closed systems on facilities costs, for example. If we do not need to manufacture in ISO 5 or ISO 7 spaces but can do so in a controlled non-classified space (CNC), which would represent a huge advance. The cost to build and operate in a CNC space is significantly lower by several orders of magnitude than that of ISO 5 or ISO 7 cleanroom space. We have taken steps to move towards that and started to build our manufacturing systems in ISO 8 (or roughly class 10k) suites. However, trying to demonstrate manufacturing in a controlled non-classified space may dissuade anybody who wanted to work with us and potentially, the regulators, too. People don't seem to be ready for this large of a disruptive step change in the manufacturing conventions, even if we can admit that a fully closed system should not need a clean room.

There are other technologies that these systems should start incorporating that will further drive down cost. Analysis, not only restricted to closed and automated bioprocessing tools but also in terms of quality control (QC) and sensorization levels, needs to increase significantly to reduce costs. One of the things we have been tackling over the last few years is developing real-time sensing capabilities that can automatically measure CQAs both in-process and for the final product. This means we will not need to perform a laboratory experiment to release the product, moving the industry towards real-time release, and will have the ability to make changes in process or discard manufacturing batches earlier that won't meet their final product QC specifications. This is also true of sterility testing: if you are cutting down your manufacturing time by two weeks because USP<71> is not required, this will obviously decrease the cost markedly because of the cost of testing but also because of the commercial implications if your product's shelf life is short, you ship at risk, and the sterility test comes back positive, triggering a recall.

Q What for you are the next steps in continuing progress towards 'Bioprocessing 4.0' and the digitization of cell therapy manufacturing?

TB: We are integrating several complementary approaches. The automated closed manufacturing lines that we build are truly modular and mobile. As we are running simultaneous processes with different cells or tissues, we are looking at how to move equipment around based on the need for that piece of modular equipment at a certain time. You may have a centrifuge that is used for an hour in the entire month-long process. Where can you move that piece of equipment so that it is being used continuously? By sharing equipment, you can drive down the overall capital cost for the facility.

We are also connecting all of our hardware systems, including those modules on the automated lines and the analytical instrumentation in our core facility. All data generated is connected through software housed in the cloud. The next layer for us will include automated data collection, storage and management, the analytics engine, and our manufacturing execution system to automate the linkages between the manufacturing equipment. We will use existing sensors for the simple things we measure inline, including raw materials, chain of custody, and chain of identity for individual patient cells for a particular manufacturing batch. Ultimately, that connected system will be used to demonstrate distributed, automated manufacturing over some distance.

As an institute, we work on standards development with standards development organizations (SDOs). We contribute as an ecosystem with nearly 200 members to the development of standards for communications between individual modules of the manufacturing lines. One of the hardest things that we had to do when we built our first integrated line was figure out how to ensure all these pieces of equipment communicate through a supervisory process controls system. Standardizing communications protocols between equipment OEMs is going to be important, as will standardizing and streamlining data collection and data processing.

Q What learnings can the field take from recent regulatory setbacks, particularly relating to cell therapy potency assay development?

TB: Potency is the thing that scares everyone. In my opinion, the challenge is that we cannot rely on hypothesis-driven approaches in tackling the development of potency assays. I heard as recently as yesterday that a company was strongly considering a particular marker for a potency panel because it was strongly secreted by the cell type that they were developing, and based on the phenotype of the cell, it made sense to measure it. Furthermore, that marker was accepted by the US FDA. However, at best, all this does is confirm on a physiological basis that a certain cell type has been produced, not that the cell will have any efficacy in the patient.

Much of this thinking is based on the guidance for potency, which suggests that potency should be linked to the mechanism of action. Unfortunately, other than in a few obvious cases, we do not understand the complexity of cell biology well enough to know what the best potency marker might be to predict how cells might behave once in a patient.

DOI: 10.18609/cgti.2022.205

Our approach is to characterize the products as extensively and deeply as possible *in vitro*, which involves multi-omic characterization, flow cytometry, and anything that can measure what the cell contains and what it secretes. Then, we apply the modern and emerging tools of data analytics to draw correlations between preclinical or clinical outcomes and the *in vitro* data to determine the subset of that data that correlates with the clinical outcome. This helps to narrow down the set of candidate markers for potency to be validated later as clinical trials progress. The challenge here is that this requires an expensive measurement and data infrastructure. However, we and others have that infrastructure in place in the form of core facilities that are available to the ecosystem as a service.

In other words, cell potency, like any CQA, needs to be derived empirically rather than linking it solely to a preconceived mechanism of action based on imperfect models of biological activity. An example of that is our new project funded by the Juvenile Diabetes Research Foundation (JDRF) to look at a data-driven approach to understand donor-to-donor variability in the manufacturing of stem cell-derived islet clusters for type 1 diabetes. We are going to run the process and characterize the cells and the conditioned culture medium at each step to begin to understand the factors that correlate with final product quality. We want to know how the CQAs that we measure *in vitro* correlate with *in vivo* data in rodent models and eventually, in patients, in order to find the important markers. People may take for granted that islet clusters will produce insulin if you expose them to elevated levels of glucose, but some lines do not secrete insulin until they have been implanted. We don't want to discard those batches based on poorly-developed potency assays. We need better markers for QC to be able to predict whether they are going to be efficacious in the clinic.

Q What progress have you seen lately in improving cell differentiation approaches for cell therapy?

TB: The best progress that has been made is in high density design of experiments, which are robotics-based approaches to derive culture media formulations empirically. These are promising as they do not rely explicitly on the somewhat ill-defined rules of developmental biology, especially for therapeutic cell types. The historical approach has been to start with a pluripotent stem cell and differentiate it based on some combination of growth factors and cytokines. Hopefully, this gives as pure a population as possible of a target cell type. This approach would be great if we had a deeper insight into developmental biology, but these new empirical methods allow us to take a reasonable look at what growth factors might be important in achieving a particular cell type, and then use robotic approaches to mix and match those in various combinations to determine the optimal mixture. That speaks to the difference between the cell as a true representation of Mother Nature versus the cell as a therapeutic medicine. When treating a particular disease – for example, myocardial infarction with cardiomyocytes – you do not need it to look exactly like the cardiomyocyte designed by Mother Nature. You simply need a cell type that engrafts reasonably well in the heart and

delivers some mixture of growth factors to cause healing of the tissue. It does not matter if we replicate Mother Nature, as long as we have something that is safe and efficacious.

Q How do you see the regulatory path for bio-fabricated tissues and organs evolving? What will this mean for the commercial viability of tissue-engineered medicinal products (TEMPs)?

TB: This is a hot topic here in the US. I don't believe that they will be overseen by HRSA as donated allogeneic organs, or as '361 tissues' as most donated allogeneic tissues are. Due to the complexity of the manufacturing processes involved, I believe that they will be regulated as medical products by FDA. I believe that most, if not all future products will be regulated within the Center for Biologics Evaluation and Research (CBER) if their manufacture and/or clinical activity is dependent on living cells. One might argue that certain organs should be regulated as devices based on their primary mode of action – for example, hearts and lungs because they function as pumps or ventilators. Other organs that function as chemical factories, like the liver, should be regulated as biologics. CBER most likely will regulate these under BLA as a biologic or a biologic-device combination. FDA has regulations (21 CFR Part 4) regarding how to conduct GMP manufacturing for combination products. I think that the way that the regulatory path has evolved supports the activities that we are doing in terms of moving toward 'Bioprocessing 4.0'.

In terms of commercial viability, I do not think regulation will necessarily sink any manufacturer – however, failure by a firm to conduct sufficient product characterization and/or to move toward QbD in manufacturing of tissues and organs will put that firm at a distinct competitive disadvantage in profitable commercialization versus firms that do invest up front.

Q How does ARMI address the challenge of finding a qualified workforce?

TB: We have ongoing workforce development efforts as part of our DOD-funded BioFabUSA program. We focus on the intersection between the workforce and new automated technologies. We have the luxury of being able to build on an industry that is nascent and has relied on relatively highly skilled or highly trained labor, with PhD-level scientists performing process development and carrying out manufacturing. In my experience, the strongest workforce in manufacturing these products has not been the over-educated scientists doing the basic research, it is the credentialed, certified, apprentice students in dedicated programs. We want to simplify manufacturing to the point where it involves the equivalent of a machinist and a machine operator, with staff who can run a CNC machine but do not need to know all

DOI: 10.18609/cgti.2022.205

the details of the engineering or the machine itself. We are building credentialing and certification programs that train those people. We have put our first cohort of students through a program that we developed, and we ended up hiring them all ourselves!

Q What are your key goals and priorities in your role and for ARMI for the foreseeable future?

"We look at the future as automated, scalable, integrated, done in controlled non-classified spaces, and with empirically-derived but highly accurate measurements that scale with the process."

TB: We are a manufacturing innovation institution, so we get to push the boundaries of what is thought of as possible. Our goal is to drive towards the expectations of Industry 4.0 by developing technologies capable of digital data collection. This means moving manufacturing towards real-time release and then mitigating the need for large quality groups to drive down the cost of goods. The vision of the future is one where QC is done by monitoring sensor outputs and not doing laboratory work. We have a public/private partnership agreement with CBER, where we can begin to socialize these technologies. We are working with them to be able to deliver some of these technologies on a periodic basis for evaluation. That benefits the product developers as they do not have to undertake the burden of presenting both their technology and a new bioprocess technology to the FDA themselves. We look at the future as automated, scalable, integrated, done in controlled non-classified spaces, and with empirically-derived but highly accurate measurements that scale with the process.

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AUTHORSHIP & CONFLICT OF INTEREST

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

Acknowledgements: *None.*

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

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

ARTICLE & COPYRIGHT INFORMATION

Article source: Invited.

Interview conducted: Oct 27 2022; **Publication date:** Nov 22 2022

DOI: 10.18609/cgti.2022.205

FASTFACTS

Standardizing flow cytometric assays during cell manufacturing

Annika Graband, Product Manager, Cell Analysis Reagents, Miltenyi Biotec

Flow cytometry is a popular tool in the analysis of cell and gene therapies due to its versatility and ability to analyze a range of markers in the same sample. The method can be used at almost every step of the drug development process, including research, in-process and quality control for clinical manufacturing, and even patient monitoring. Due to its prevalence in cell and gene therapy manufacturing processes, minimizing any possible variation is key in order to establish reliable assays

VARIATIONS IN FLOW CYTOMETRY

Standardization is key in flow cytometry, due to the current challenge of high levels of irreproducibility in pre-clinical research. Irreproducibility of results causes tremendous economic damage and leads to longer drug development times and in turn, to higher costs for approved drugs and treatments.

The main sources of variation to avoid within flow cytometry are summarized in **Figure 1**. They include variability in donor samples, due to both the patient and the collection procedure used. The antibodies used can show unspecific binding with variable performance from lot to lot. The instrument itself can be a source of variation, such as if it is not set up correctly, or if daily quality control is a complex and manual process. Even after collection of the raw data, there is still the possibility to introduce variation in the data analysis and reporting, for example, different operators will gate a sample differently. Finally, a main source of variation is if protocols are unoptimized and include several manual handling steps.

Figure 1. Sources of variation in flow cytometry

Donor variability
- Patient driven
- Pretreatment driven
- Collection driven

Reagents
- Lot-to-lot differences
- Specificity (e.g., Fcγr binding)

Instrumentation
- Pipetting variability
- Manufacturing tolerances
- Operational tolerances

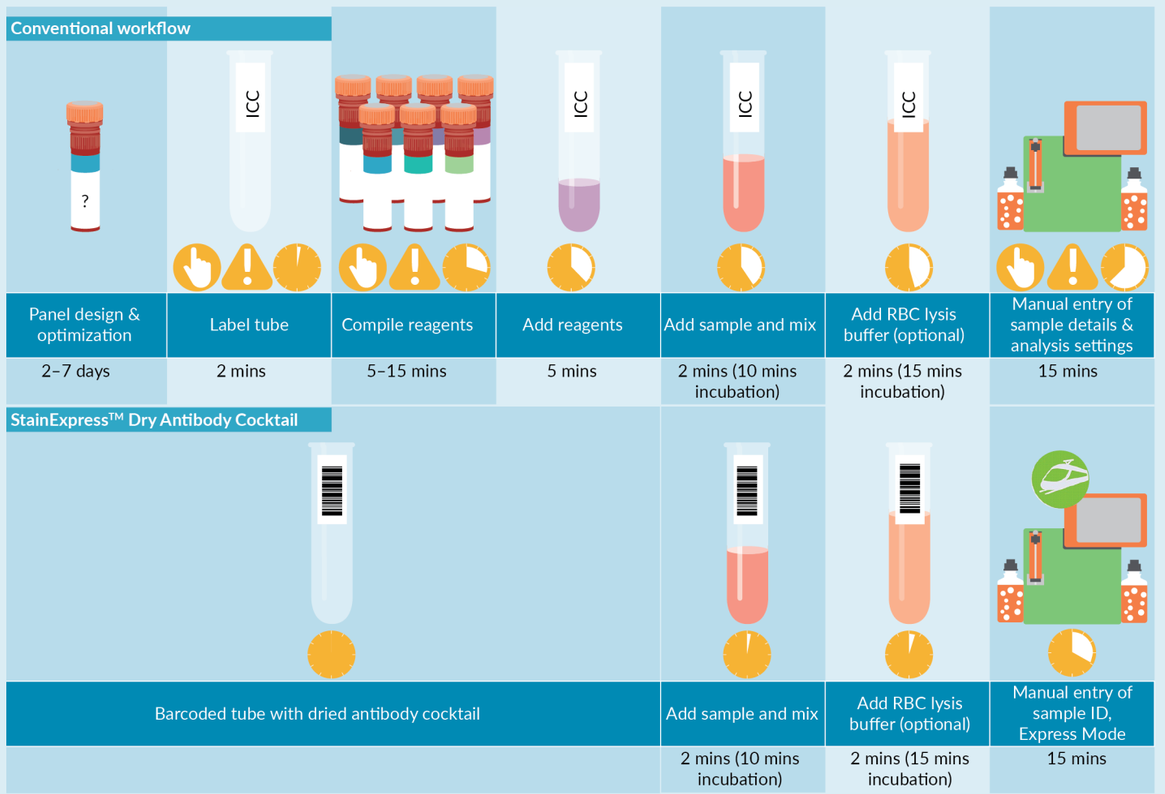
Analysis & reporting
- Human factor

Protocols
- Non-optimized procedures
- Manual handling

REDUCING VARIATIONS IN FLOW CYTOMETRY

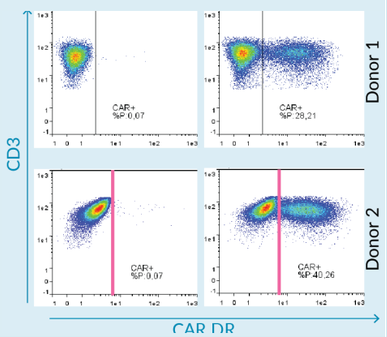
StainExpress™ Dry Antibody Cocktails are pre-formulated panels of dry antibodies for routine applications, designed with reducing variability in mind. These antibody cocktails are based on REAfinity™ technology, using REAfinity recombinant antibodies. This dry and temperature-stable formula ensures high lot-to-lot consistency and stability and minimizes non-specific background signals. They are designed to streamline the flow cytometry workflow, eliminating multiple handling steps to reduce operator variability and save time during assay set-up **(Figure 2)**. StainExpress tube barcoding allows automated sample data acquisition and entry, with integrated analysis software on MACSQuant Analyzer flow cytometers to save further hands-on time. The overall prevention of variability offered by the StainExpress kits can enable users to achieve multi-center and inter-trial comparability of results.

Figure 2. Comparison of the conventional workflow with the StainExpress Dry Antibody Cocktail.



Strategies for reducing variations in flow cytometry can also include the analysis and reporting stages. Software modules with pre-defined settings and algorithm-based analysis allow for faster and more reproducible sample analyses. Gating adjustment based on algorithms can be used to avoid variations caused by human factors during data analysis **(Figure 3)**.

Figure 3. MACSQuantify software with Express Modes enables the transfer of an instrument setting from one device to another



CELL & GENE THERAPY INSIGHTS

Cell & Gene Therapy Insights 2022; 8(10), 1213; DOI: 10.18609/cgti.2022.179

In partnership with:

Miltenyi Biotec

Feeder free expansion of a clinically relevant number of human NK cells

Erica Heipertz, Staff Scientist, Thermo Fisher Scientific

Over the last decade, CAR-T cell therapy has emerged as a revolutionary treatment for blood cancers. However, as we begin to look forward to other immunotherapy options, one that is gaining increased attention is NK cell therapy. NK cells have several advantages over T cells, including their ability to function in an antigen-independent manner, which makes them a viable option for an allogeneic, 'off the shelf' therapy. NK cell therapy also has relatively few side effects, potentially, and recent studies suggest it may be a viable therapeutic option for solid tumors.

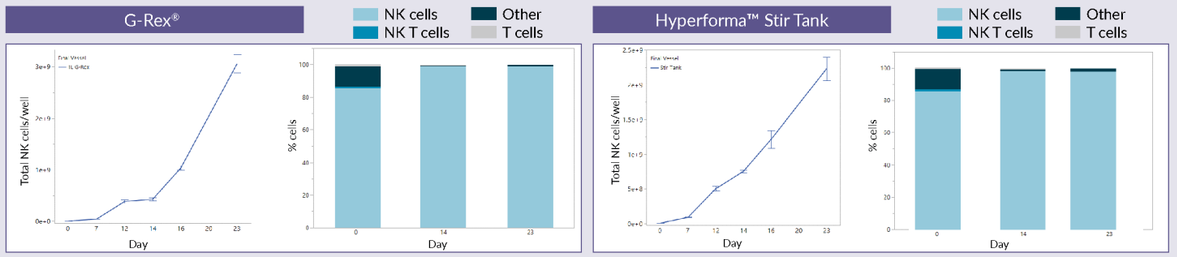
Cell & Gene Therapy Insights 2022; 8(10), 1285. DOI: 10.18609/cgti.2022.189

Thermo Fisher Scientific's CTS NK-Xpander Medium is able to expand human NK cells without the need for feeder cells. The expanded NK cells maintain cell surface maker expression, including CD56 and CD16, and maintain robust cytotoxic activity. CTS NK-Xpander Medium is manufactured animal origin-free at the primary level. However, supplementation with human AB (hAB) Serum is recommended for best results. CTS NK-Xpander Medium is designed to meet regulatory compliance requirements for ancillary materials used in cell and gene therapy manufacturing.

NK cells can be enriched from multiple sources, including PBMCS and cord blood, and can be differentiated from CD34⁺ cells and iPSCs. These NK cells can be expanded using CTS NK-Xpander Medium supplemented with hAB serum and IL-2. Expanded NK cells may be genetically modified and/or cryopreserved prior to infusion into the patient.

NK cell therapy requires a larger number of NK cells per therapy dose, and multiple doses may be required. Therefore, CTS NK-Xpander Medium must be scalable in many different large-scale bioreactors. We expanded enriched NK cells in both a static culture environment and a stir tank bioreactor. In both, NK cells expanded in CTS NK-Xpander Medium reached over 400-fold expansion and yielded over 2.5 billion NK cells in a three-week period.

Figure 1.



NK cells cultured in CTS NK-Xpander Medium and G-Rex ® (1L Scale) expanded an average of 500-fold after 23 days in culture and are CD56+ CD16+ CD3−. Total NK cell yield: 3 Billion NK cells

Day	Vessel	Seeding density/volume increase	Total volume	hAB (%)
0	6 well G-Rex	7e6 cells/mL	15 mL	5 %
5		Increase volume to 40 mL	40 mL	
7	100 mL G-Rex	Remove 80% of media and increase volume to 100 mL; transfer to 100 mL G-Rex	100 mL	
9		Increase volume to 375 mL	375 mL	
11	1L G-Rex	Increase volume to 750 mL	750 mL	
14		Increase volume to 1 L	1L	
15+		Mimic perfusion: 80% daily media exchange		

NK cells cultured in CTS NK-Xpander Medium and Hyperforma™ Stir tank (2L Scale) expanded an average of 400-fold after 23 days in culture and are CD56+ CD16+ CD3−. Total NK cell yield: ~2.5 Billion NK cells

Day	Vessel	Seeding density/volume increase	Total volume	hAB (%)
0	6 well G-Rex	7e6 cells/mL	15 mL	5 %
5		Increase volume to 40 mL	40 mL	
7	100 mL G-Rex	Remove 80% of media and increase volume to 100 mL; transfer to 100 mL G-Rex	100 mL	
9		Increase volume to 375 mL	750 mL	
11	2 L stir tank	Increase volume to 750 mL	1125 mL	
14		Increase volume to 1690 mL	1690 mL	
15+		Increase volume to 2 L	2 L	

Figure 2. NK cell purity and phenotype

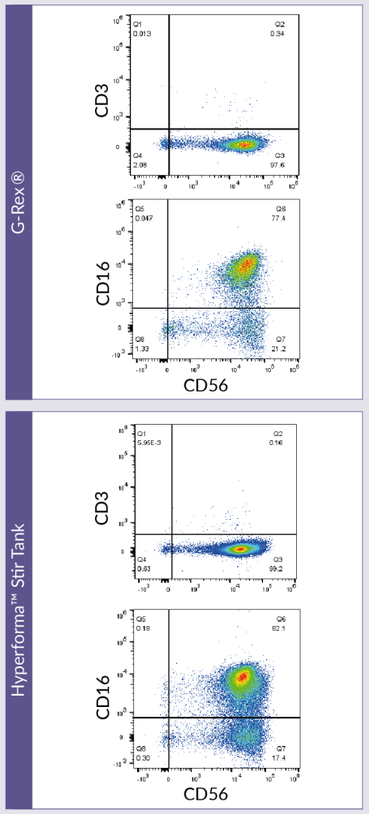
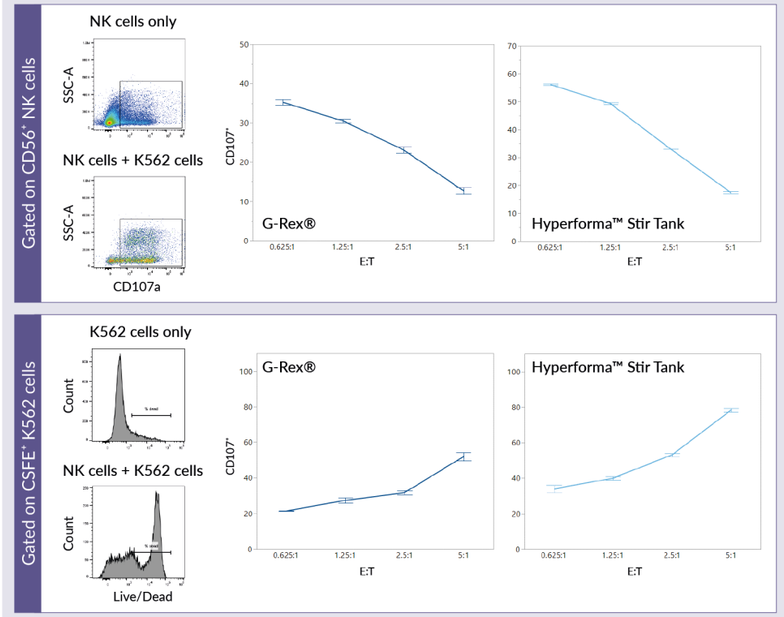


Figure 3. NK cell function.



NK cells expanded in CTS NK-Xpander Medium are CD56+ CD3-, with minimal NK-T and T cell contamination. (Figure 2). In this study, post-expansion, the NK cells were over 95% CD56⁺ CD3-.

For NK Cells to be successful as an allogeneic therapy, they must retain their functionality post expansion. The functional ability of the NK cells expanded with CTS NK-Xpander Medium was tested after expansion in both the G-Rex® and HyperformaTM Stir Tank by co-incubating them with CFSE labeled K562 target cells (Figure 3). After two hours, NK cell cytotoxicity was measured by flow cytometry using the Attune NXT flow cytometer. NK cells maintain their functionality and are able to degranulate as shown by their CD107a expression, and kill the K562 target cells in a dose dependent manner.

CELL & GENE THERAPY INSIGHTS

CELL THERAPY BIOPROCESSING

SPOTLIGHT

Producing cell-based therapeutic products with lot-to-lot consistency from highly variable starting cell products

William E Janssen, WEJ Cell and Gene Therapy Consulting Services, **& Scott R Burger,** Advanced Cell and Gene Therapy, LLC

VIEWPOINT

"Lot-to-lot consistency of therapeutic products is inextricably linked to product efficacy, safety, and generalizability of application."

Cell & Gene Therapy Insights 2022; 8(10), 1277–1281

DOI: 10.18609/cgti.2022.185

Among the many challenges involved in development of chemistry, manufacturing and control (CMC) for cellular therapies and cell-based gene therapies (CGT), has been the sourcing and acquisition of raw materials. Although there has been tremendous growth in the areas of specialized GMP growth media, cryostorage solutions, containers and processing equipment, the need to adapt materials intended for research use in early-phase clinical studies of novel cell and gene therapy products remains. Even when GMP materials are available, the pathway to identifying which are optimum for specific cell types is seldom clear. However, these concerns generally pale compared to the challenges posed by the most critical material used in the manufacture of CGT products, namely donated, viable, functional cells.

Owing to the heterogeneous nature of the human donor pool, as well as to differences in collection methods between collection facilities, cellular starting material is intrinsically variable and heterogeneous [1–3]. Some of the variable parameters of the starting material can affect the downstream manufacturing process. By extension, then, the final manufactured product, too, is to some extent inherently variable. For reasons of patient safety and benefit, scientific integrity, and regulatory compliance, CGT final products must be as consistent as possible. The manufacturing process, therefore, should be designed to include procedures that reduce variability. Currently, however, this is not measured in a process-focused way.

Variability of a product may be a function of multiple parameters, some of which may be critical to the product's intended function, while others may be immaterial. Which variability-associated parameters are critical and which are not must be determined based on intended function. Variability-associated parameters include final cell dose, purity of final cell type, functional state of final cell type, and the composition of cellular impurities.

CGT manufacturing processes are made up of serially-linked discrete steps. For example, the process for manufacturing a simple natural killer (NK) cell product might be summarized as:

1. A leukapheresis cell collection;
2. Gradient separation of light-density cells;
3. Incubation with CD56 monoclonal antibody conjugated to ferromagnetic particles, followed by selection on a magnetized column;
4. Suspension of the selected cells in a restrictive growth medium and multiple days of incubation for cell expansion;
5. Collection and washing of post-expansion cells; and
6. Final aliquoting of the cells into vessels for storage and eventual distribution.

At the inception of each step, the population of cells present will have measurable parameters, such as, in the foregoing example, the fraction of the cells that are $CD3^-$ and $CD56^+$. At the conclusion of each step, the same parameters are again measurable. If, then, over the course of multiple iterations of the same production step this parameter is measured before and after, a mean, μ, and a variance, σ (defined as the sum of the squared differences between each measurement and the mean value for that measurement, divided by the number of iterations minus 1). Focusing on the variance, σ, for each discrete processing step, the difference in variance following the process compared to at the inception of the process can be computed to show the change in variance associated with each processing step. We can refer to this computed

measure as Δσ, the change in variance associated with the particular step.

Some processing steps will have a positive Δσ, that is, an increase in variance for the specific parameter being measured. Conversely, many processing steps will be associated with a negative Δσ, conveying a reduction in variance. An example of the latter is a positive selection operation intended to select for a specific target cell population while discarding cells not in the targeted population. The CD56 positive selection in the NK cell production sequence described above should yield a highly enriched population of $CD56^{+}$ cells. The selection operation, therefore, should have reduced variability, or to use the nomenclature in the preceding paragraph, $\Delta\sigma<0$.

Each process step (unit operation) in a CGT production scheme has multiple measurable parameters, each of which is associated with a mean (μpre) and variance (σpre) for the parameter at inception of the step, a mean (mpost) and variance (σpost) for the same parameter at completion of the step, and a change in variance, Δσ (σpost-σpre) (Figure 1).

The variability associated with the final manufactured product is simply the sum of the variability in the starting cellular product plus all of the Δσ measures. That is:

$$\sigma\_final=\sigma\_pre+\sum_{(j=1,N)} \Delta\sigma j$$

Thus, if the preponderance of the individual processes in a manufacturing scheme have negative Δσ then the variability associated with the starting cellular material will be reduced, and the final manufactured cell product will have a greater lot-to-lot consistency.

We submit that the above-described abstract structure represents a useful framework for development of manufacturing schema that incorporates inter-lot consistency as a critical endpoint. In practice, the application of this model follows a limited number of specific, concrete steps:

1. From critical quality attributes and critical process parameters, identify the critical quantifiable parameters for the final cell product. Obvious parameters are identity, purity, inclusion of non-active elements, dosage and potency. There may, however, be others that are specific to a particular CGT product. For example, a critical parameter for a dendritic cell-based vaccine might be the density of major histocompatibility complex molecules on each cell's surface;

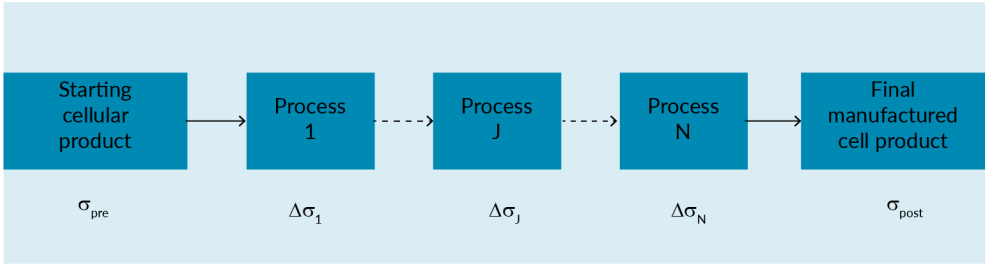
2. Rank the critical parameters in order of likely impact each may have on the desired function of the final CGT product;

3. Identify the Δσ for each process step in development, and for each critical parameter. Identify where multiple measured parameters may have negative Δσ for the same process step;

4. Optimize, insofar as possible, inclusion of process steps to achieve the lowest variability for all critical parameters in the final product. That is, achieving the most consistent final product.

FIGURE 1

Generic CGT product manufacturing schema, progressing from heterogeneous, donated cellular starting material and progressing through N processing steps to a final CGT product.



Lot-to-lot consistency of therapeutic products is inextricably linked to product efficacy, safety, and generalizability of application. Unlike therapeutic products that have tightly controllable raw materials, products based on viable, living cells are derived from a heterogeneous donor pool. To a greater degree than small-molecule drugs or traditional biologics, end-product consistency for CGTs depends on control of individual manufacturing process steps. We propose a quantitative approach to manufacturing process analysis and improvement based on determining the change in variability of measurable critical parameters associated with each manufacturing unit operation. This, in turn, fosters understanding of the contribution of individual process steps to process and product variability, facilitating development of manufacturing processes that maximize lot-to-lot consistency.

BIOGRAPHIES

WILLIAM E JANSSEN, PhD has worked for more than 30 years at translating lab bench models into cell and gene therapy products for administration to patients. In the course of these efforts he has developed and refined methodologies for all aspects of cell based therapy from collection, through manufacturing and administration, including regulatory aspects. Dr Janssen has also been responsible for facility design, drug master file creation, development of staff training programs, process engineering, technology transfer, SOP development, process validation, comparison studies and integration of processes, equipment and raw materials. He has also been both a proponent and implementer of informatics solutions for management of cell and gene therapy development and manufacturing data.

SCOTT R BURGER, MD, is the Founder and Principal of Advanced Cell and Gene Therapy, a consulting firm specializing in cell and gene therapy product development, providing expertise on manufacturing, regulatory, and strategic aspects of these products. Dr Burger has over 30 years of experience developing cell and gene therapy products and has consulted for over 160 companies in North America, Europe, Australia, and Asia, advising on a wide range of cell therapy and gene therapy products for immunotherapy and regenerative medicine applications. Dr Burger has consulted on over 100 regulatory submissions for cell therapy or gene therapy products at all stages of development. He has served as an expert witness in cases involving cell and gene therapy intellectual property, commercialization, FDA regulatory affairs, and GMP compliance, and as a subject matter expert for NIH-NHLBI, CIRM, PACT, and DMRDP review panels. He has been an invited speaker at internal FDA workshops and is frequently asked to speak on cell and gene therapy manufacturing and regulatory topics at international conferences. A graduate of the University of Pennsylvania School of Medicine, Dr Burger completed training in clinical pathology and transfusion medicine at Washington University in St. Louis and is author of over 200 scientific publications and presentations, and recipient of numerous honors and awards.

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DOI: 10.18609/cgti.2022.185

AUTHORSHIP & CONFLICT OF INTEREST

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

Acknowledgements: *None.*

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

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

ARTICLE & COPYRIGHT INFORMATION

Article source: *Invited; externally peer reviewed.*

Submitted for peer review: *Oct 10 2022;* ***Revised manuscript received:*** *Oct 17 2022;* ***Publication date:*** *Nov 3 2022*

CELL THERAPY BIOPROCESSING

SPOTLIGHT

Key considerations for early investment in the process development of cell-based therapeutics

Kelly Kemp, Senior director of CMC and Head of the Process Development, Analytical Development and Quality Control teams, ViaCyte, a Vertex Company, **& Sebastian Rieck,** Director of the New Product Sciences team, ViaCyte, a Vertex Company

VIEWPOINT

"During early process development, every relevant aspect of a product's lifecycle and cost-of-goods should be taken into consideration."

Cell & Gene Therapy Insights 2022; 8(10), 1407–1412

DOI: 10.18609/cgti.2022.206

On October 3, 2022, David McCall, Commissioning Editor of *Cell & Gene Therapy Insights*, spoke to Kelly Kemp and Sebastian Rieck about current challenges in cell therapy process development and manufacture. This article has been written based on that interview.

CURRENT CHALLENGES IN CELL THERAPY MANUFACTURING

The logistics for getting a cell-based product released and to the clinic in time for treating patients can be challenging, as can questions of yields and scalability to meet clinical forecasts. It is critical for developing a viable product life cycle to understand and carefully evaluate the potential bottlenecks for supplying a cell therapy to the market early in its development. The cell therapy field is working with different cell types that can be more fragile and not as amenable to off-the-shelf solutions that many current biologics are using for their production processes. How do we scale to provide for 1000, 10000, or more subjects and ensure we still have a comparable product? One must develop a well-defined process and well-characterized product and avoid any variability in potency. Furthermore, when the cell product has been differentiated from pluripotent stem cells, for example, to a new cell type, the identity and purity of the cells must be maintained through the scale-up process.

Materials are often a challenge in bioproduction, too. In today's world, having a single-sourced or custom material adds risk to the process and business. With an increase in supply chain issues stemming from the COVID-19 pandemic, there is a need to establish quality agreements, as well as diversify and secure secondary suppliers. It is increasingly important to partner with material suppliers, especially as one proceeds through the different phases of clinical development. Moreover, it is key to ensure vendors have the ability to work with your forecasts and timelines. The quality of materials used for cell therapy products can also be a challenge when looking to find GMP-grade materials or alternatives to human- and/or animal-derived materials. Material risk assessments are essential when introducing components into the production process and developing plans to upgrade critical materials is advised. Finally, the price of media and growth factors contribute significantly to the cost of goods, and working with vendors or contract manufacturing organizations to reduce these costs is key in helping to enable better affordability and availability of these therapies to patients.

Other challenges in cell therapy manufacturing include the fact that with some novel products it can be hard to find contract development and manufacturing organizations (CDMOs) with the right expertise, either on the processing side or on the analytics side. In addition, many of these treatments require a large number of cells. If cell potency could be improved – through gene editing and directed differentiation, for example – that in turn would reduce the number of cells required per dose, alleviate the scale-up requirements, and potentially decrease the cost per dose. Moreover, post-production procedures can impact a product's accessibility to patients. Key decisions in several areas such as product storage, product shelf-life, distribution, handling logistics, and compliance can contribute positively to a product's lifecycle.

LIMITATIONS IN AUTOMATION

At ViaCyte, the production of pancreatic endoderm cells is a ~30 day process, in

which the time and complexity of culturing leads to significant aseptic risk. In cell therapy manufacturing, managing aseptic risk, through process validation, operator training, and environmental monitoring, for example, and moving toward a closed and perhaps automated process is an optimal direction for the future.

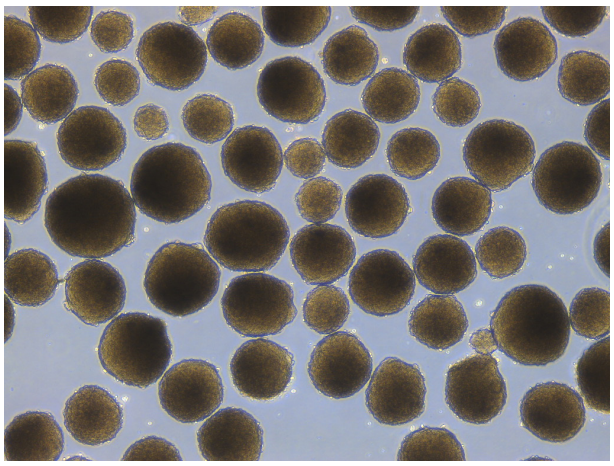
In terms of automation, one of the process development challenges in the cell therapy field is leveraging off-the-shelf technologies, as opposed to modifying equipment for scaled-up processes. Often, cell therapy production methods are novel and customized; how do we get an off-the-shelf and/or automated process for these cell therapies? In most cases, the industry is still aligning on how to work with technology providers to develop the robotics needed for automation, thus this question remains to be answered. As the field grows, the hope is that in early development, standardized equipment can be leveraged, and there will be a move away from customization to one-size-fits-all platforms. For now, to navigate the need for tailored solutions, process development engineers work closely with collaborators in the cell therapy field – and luckily there are some great collaborators out there: Invetech, PBS Biotech, Cytiva, Pall, Millipore Sigma, Thermo Fisher, among others. The bioprocessing industry is increasingly realizing the importance of partnerships as the cell therapy field grows. Yet, even with collaboration, it is essential to realize that development of new or customized equipment and processes takes time – it is therefore critical to start down the road to scale-up and automation as early in development as possible.

LIMITATIONS IN IMPROVING YIELD

One of the challenges for ViaCyte (which produces cell aggregates), as well as other for companies in the field, is working with cell clumps or spheres, or even with organoids, instead of single-cell suspensions (Figure 1).

FIGURE 1

ViaCyte's PEC aggregates.



Due to the physical properties of these multicellular bodies, cryopreservation is often not very efficient and requires optimization to obtain viable high post-thaw yields.

Cryopreservation can be a huge hurdle for these multicellular products, though there are opportunities available to leverage controlled rate freezers and optimize the freezing profile to obtain better yields. New cryopreservation media are being developed and small molecules identified to improve this critical process step. Alternatively, some groups are investigating the dissociation of the multicellular spheres prior to cryopreservation to improve post-thaw yields. This approach has in certain cases even demonstrated potential to act as a positive selection step for a specific cell population. The toxicity of dimethyl sulfoxide (DMSO), a typical cyropreservative, is another challenge in this area. With scale-up often comes longer processing times, which could lead to longer exposure to DMSO toxicity and decrease yields. Therefore, it is important to characterize these critical process parameters and ensure production can be performed within the time limits of the process capabilities.

Beyond cryopreservation, optimizing the culture parameters in the bioreactor can also contribute to improved yields. Media formulation development to identify the

optimal concentrations of nutrients such as pyruvate, glucose, or amino acids, as well as understanding the best media exchange process (e.g., batch fed or perfusion) for a specific cell type can improve cell yields.

DECREASING COST OF GOODS

A high proportion of the cost of goods for cell-based products often is attributed to the culture media, supplements, and single-use materials. When introducing closed systems and custom media, costs per dose can be high when producing at a small-scale. However, in the long-term, working toward efficiencies of scale-up while maximizing facility and personnel productivity with back-to-back production will ultimately lead to a decrease in cost per dose. For many cell therapy companies, it is at phase 3 or at the beginning of commercial production that those efficiencies start to be seen.

A process can be made more robust through automation whilst achieving lower cost of goods. Single-use materials are often quite expensive, so if the facility and capabilities are available, using stainless steel and clean-in-place could be validated to offset costs. Utilizing CROs can offer advantages in reducing overhead costs, especially when few lots are produced per year - for example, outsourcing standardized analytics. Finally, some media components are expensive - if the IP and/or expertise permits, producing these growth factors or other media supplements in-house or in collaboration with the vendor or a contract manufacturing organization (CMO) could offer cost savings.

COMPETENCE FOR ASSAY DEVELOPMENT IS A KEY SCALE-UP ASSET

Investment in a robust assay development proficiency is the cornerstone of a well-structured Quality by Design (QbD) strategy for maintenance of product comparability, including potency, during scale-up. The maintenance of a cell product's critical quality attributes (CQAs), or the physical, chemical, biological, or microbiological characteristics that the production process should control to be within appropriate limits, ensures the desired product quality required for therapeutic benefit. Control of the various critical process parameters (CPPs) that impact the cells' CQAs should facilitate robust and consistent large-scale production of a therapeutic stem cell-derived product. The establishment of consistent, qualified, and reliable assays that can be used to establish and routinely measure these CCPs/CQAs ensures a clear understanding of, and ability to maintain, the input/output parameters that influence product performance.

ADVICE FOR SHORTENING PROCESS & PRODUCT DEVELOPMENT TIMELINES

The key to quick development is maintaining quality and managing risk.

Today, the US Food and Drug Administration has more pathways offering rapid review or accelerated approval – initiatives like the Breakthrough Therapy designation. However, even if one is not following a fast-track path, working closely with the regulatory agencies is still critical. In the US, having a pre-investigational new drug meeting to get the conversation started early is useful. Later, having Type C chemistry, manufacturing and control meetings will ensure alignment on your strategies and potentially reduce cost and time to market. Hearing that the production or control strategy is not adequate after submission of a biological license application can be avoided with these early regulatory conversations.

Access to key personnel, including highly qualified scientists and engineers, is also important. People with the right mindset are key, especially in cell therapy, where you want people to creatively push the boundaries. The cell therapy field is committed to patients, which in turn creates a culture of

DOI: 10.18609/cgti.2022.206

amazing energy and drive to identify solutions that will improve health and quality of life for patients. Having a variety of personnel with broad expertise and backgrounds is also valuable, providing the benefits of diversified thinking. ViaCyte is successful in its diversity, equity, and inclusion (DEI), ensuring that groups have diversity not only in terms of professional experience, but also in general background. This helps the team ultimately generate stronger approaches to development of cell therapies. In addition, being goal-oriented and having cross-functional collaboration between departments (rather than forming silos) is crucial to support efficiencies and productivity.

Again, working closely with vendors is key, as is strong engagement with key opinion leaders. Technology landscaping is central to select optimal platforms for development and scale-up. Find out whether there is an off-the-shelf solution or a way to minimize the pieces needed to establish a cell production process. In terms of mindset, remember that strategic investment early will pay out later via the extended lifecycle of the product.

ACTION POINTS

Novel cell therapies require innovation. This is essential, whether it is for process automation or for improving yield, throughout therapeutic product development. Collaboration with vendors – whether for materials, equipment, custom-made equipment, or automation – is key. Focus on or invest in development processes earlier to complete steps in time to satisfy critical clinical and regulatory timelines.

Initiate collaborations with CMOs/CDMOs in which both parties work together to produce creative and innovative solutions for high quality cell products. Identify scale-up platforms with the greatest runway for the product lifecycle. Simplify where possible; consider whether the scale-up process can leverage one production platform. During early process development, every relevant aspect of a product's lifecycle and cost-of-goods should be taken into consideration to improve efficiencies and ultimately maximize the ability to provide safe and effective cell therapies for patients.

BIOGRAPHIES

DR KELLY KEMP is the Senior Director of Chemistry, Manufacturing and Controls at ViaCyte (a Vertex company), working to characterize and optimize the manufacturing process as well as develop new analytics for an innovative stem cell-derived product aiming to treat diabetes. With a passion for biology and technology, as well as a proficient understanding of cGMP and regulatory requirements, she has had a strong career focus on scaling up cell production processes and implementing process improvements. She earned her PhD in Developmental Biology from the Free University of Brussels, Belgium, and continued her training as a postdoctoral fellow at the Salk Institute for Biological Studies in La Jolla, CA, focusing on the transcriptional regulation of human pluripotent stem cells. She has led process development teams for cell-based products at Shire, Stemedica Cell Technologies, and Vital Therapies.

DR SEBASTIAN RIECK is a Director of New Product Sciences at ViaCyte (a Vertex company), creating new cell initiatives to improve potency of an innovative stem cell-derived product aiming to treat diabetes. With a passion for product development, he has had a strong career focus on translating regenerative medicine products by discovery and process development. He earned his PhD in Pharmacology from the University of Pennsylvania, Perelman School of Medicine at the Institute for Diabetes, Obesity and Metabolism in Philadelphia, PA. He continued his training as a postdoctoral fellow at the Vanderbilt University in Nashville, TN, focusing on the epigenetic regulation of pancreatic progenitors. He has been a part of and led R&D and process development teams for cell-based products at Johnson & Johnson (BetaLogics), Novo Nordisk, and ViaCyte.

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Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*
Acknowledgements: *None.*
Disclosure and potential conflicts of interest: *The authors have no conflicts of interest.*
Funding declaration: *The authors received no financial support for the research, authorship and/or publication of this article.*

ARTICLE & COPYRIGHT INFORMATION

Article source: Invited; externally peer reviewed.
Submitted for peer review: Oct 4 2022; **Revised manuscript received:** Nov 16 2022; **Publication date:** Nov 24 2022

DOI: 10.18609/cgti.2022.206

CELL & GENE THERAPY INSIGHTS

CELL THERAPY BIOPROCESSING

SPOTLIGHT

INTERVIEW

Enabling scalable manufacture of exosomes for future clinical & commercial requirements

David McCall, Commissioning Editor, *Cell & Gene Therapy Insights*, spoke to **Peter Jones,** Vice President of CMC, Evox Therapeutics

PETER JONES has over 30 years of experience in the biopharmaceutical industry and is currently Vice President of CMC at Evox Therapeutics. Prior to this, he was Executive Director, Process Development at Autolus Therapeutics, and held several positions of responsibility at Oxford Biomedica, including Senior Director, Head of Operational Strategy, Head of Technical Operations, and Head of Manufacturing Development. Peter also served in multiple senior roles at GlaxoSmithkline and Genzyme in bioprocess development and manufacturing. Peter has a BSc (Hons) in Applied Biology from the University of Hertfordshire and a MSc (Eng) in Biochemical Engineering from University College, London. He is a Chartered Chemical Engineer, Fellow of the Institution of Chemical Engineers and Royal Academy of Engineering. Visiting Professor in Manufacturing of Advanced Therapies at Aston University.

Cell & Gene Therapy Insights 2022; 8(10), 1269–1276

DOI: 10.18609/cgti.2022.186

Q What are you working on right now?

PJ: Evox Therapeutics is a privately held, Oxford-based biotechnology company, founded in 2016, that focuses on harnessing and engineering the natural delivery capabilities of extracellular vesicles, sometimes known as exosomes, to develop an entirely new class of therapeutics for treating diseases with significant unmet needs.

The company is based on exosome technologies developed at the University of Oxford and Karolinska Institute in Sweden. It has developed a proprietary DeliverEX™ technology platform with the most comprehensive intellectual property estate in the exosome therapeutics space with full freedom to operate. This consists of an analytical toolbox, tailored targeting technology and a scalable manufacturing process.

Through its precision engineering approach, we have been able to engineer the exosome machinery to precisely load a wide variety of therapeutic drug cargoes (antisense oligonucleotides, proteins, small interfering RNA (siRNA), messenger RNA (mRNA), viral vectors, and gene editing) into the lumen of exosomes, as well as modifying the surface of exosomes to enable tissue-targeted delivery. *In vivo* functional delivery with all these drug cargoes has been generated. Studies in rodents and non-human primates have also demonstrated the ability of exosomes to greatly expand the efficiency of cell delivery and highlight their exceptional safety profile, even upon repeated exosome administration.

Broadly, we work on the delivery of short RNA therapeutics for gene silencing, and the delivery of gene editing technologies including CRISPR-Cas9, mega nucleases and Cre recombinases. We are also working on a novel approach to gene therapy using adeno-associated virus (AAV) vectors with our exo-AAV – namely, the encapsulation of the vector into exosomes. AAV alone has shown incomplete cellular transduction in multiple tissues, including the liver. Exo-AAV dramatically improves efficacy and broadens cellular uptake, thereby lowering dose and alleviating current safety concerns with high-dose AAV gene therapy. By encapsulating the AAV within an exosome, it offers the potential to overcome the elicitation of a humoral immune response, by protecting the AAV from neutralizing antibodies (NAbs) which would otherwise limit gene transfer. Protection from NAbs opens the potential for re-dosing of all patients, irrespective of any pre-existing NAbs, and potentially allowing repeat dosing of exo-AAV. Given the immunogenicity, cytotoxicity, and empty-capsid challenges that afflict AAV development, exo-AAV represents a compelling clinical alternative approach for efficiently delivering genes to a variety of cells and tissues such as the liver, central nervous system (CNS), immune cells, retina, and lung treatment in the future.

Q What are exosomes?

PJ: Exosomes are nano-sized, lipid membrane-enclosed vesicles that are produced by most mammalian cell types. They are 'nature's delivery vehicle', carrying a

DOI: 10.18609/cgti.2022.186

variety of therapeutic drug cargoes such as RNA, DNA, lipids, carbohydrates, peptides, and proteins (including oncoproteins, tumour suppressors, transcriptional regulators, and splicing factors) and transferring their therapeutic drug cargo into target recipient cells or tissues.

> "Exosomes are emerging as a promising new drug delivery carrier and there is also excitement around their potential as a new modality of therapeutic agent."

One of the challenges with exosomes is that they are very heterogeneous in nature, made up of different sizes and types of extracellular vesicles. Exosomes are a sub-class of extracellular vesicles (EVs), a term coined by the International Society for Extracellular Vesicles (ISEV), which categorizes vesicles based on their biogenesis or release pathway. There are three main sub-classes of EVs based on their biogenesis:

1. Microparticles/micro vesicles that shed directly from the cell membrane and have a size range of 50-1000 nm;
2. Apoptotic blebs derived from dying cells of typically 50–4000 nm size;
3. Exosomes which are smaller (typically 30–150 nm in diameter) and are released from multivesicular bodies (MVBs) rather than the cellular membrane.

Exosomes have been shown to be important mediators of cell-to-cell communication by which cells efficiently and safely exchange materials. They are formed through the invagination of the endosomal membrane to form an endosomal MVB which fuses with the plasma membrane, releasing the intraluminal vesicles as exosomes. Native exosomes are the exosomes naturally released by cells, whilst 'engineered' exosomes are those loaded with a therapeutic drug cargo using the exosome machinery to precisely control the exosome contents.

Q Why are exosomes getting so much attention?

PJ: Exosomes are emerging as a promising new drug delivery carrier and there is also excitement around their potential as a new modality of therapeutic agent. With an increasing understanding of exosome biology and function, the pharma/biotech industry is getting closer to harnessing their therapeutic properties to deliver a range of therapeutic drug cargoes that can be beneficial to patients. They are showing potential in a wide variety of research, diagnostic, and therapeutic applications. There are also strong positive safety indicators that exosomes are non-toxic and non-immunogenic as shown in numerous exosome trials over the past 20+ years and evidenced by blood transfusions that contain large amounts of allogeneic exosomes. Recently, US-based Codiak BioSciences Inc. also demonstrated a favourable safety and tolerability profile for its exosome platform from clinical data generated during its Phase 1 trials.

Exosomes' ability to convey information and stimulate cellular activity has given rise to the concept that they can serve as a delivery system for therapeutic drugs to target specific diseases, injuries, or viruses, especially because exosomes are able to pass through the blood-brain barrier. There is potential to use exosomes as an alternative vector for the delivery of proteins or nucleic acid drug cargoes in therapeutic applications that fight diseases, modulate immune responses and repair tissues. If this can be achieved, the commercial potential for exosomes will be extensive.

Exosomes exhibit many desirable features of an ideal drug delivery system. The surface membrane of an exosome provides a protected and stable internal microenvironment, allowing drug cargoes within exosomes to travel long distances within tissues without degradation. The external surface of an exosome can be engineered with specific surface markers to target a particular cell or tissue type, thus potentially lowering unwarranted side effects.

Q Tell us about the key current challenges in exosome manufacture as you see them

PJ: Engineered exosome therapeutics is a large clinical and commercial opportunity intrinsically linked to providing evidence that exosomes can have a therapeutic benefit or be a more effective drug delivery system compared to other modalities e.g., lipid nanoparticles (LNPs). Another key challenge is scaling up the bench scale processes currently used to provide enough safe, high quality, and efficacious engineered exosomes to meet clinical and commercial needs.

The challenges to exosome manufacture are not necessarily in the upstream production, as all mammalian cells produce exosomes (typically, several thousand exosomes per cell per day). The upstream process in most cases is like conventional biologics, based on adherent 2D cell culture using planar technologies or serum-free 3D suspension culture using batch, fed-batch, or perfusion processes.

The key to large-scale exosome manufacturing is the downstream process used to purify and resolve exosomes away from the secreted proteins and nanoparticles. The different subclasses of EVs overlap in size, which can lead to further complexity, increased heterogeneity, and reduced potency of the exosome-based drug product. There are a growing number of orthogonal analytical techniques to allow precise characterization of the product and optimization of the purification scheme for exosomes. Methods used to identify, isolate, quantify, or characterize their physical properties are essential for successful drug development. In-depth knowledge of an exosome-based product's structure and biological activities facilitates easier process design to ensure the drug attains critical product safety, purity, and potency quality attributes, and provides a better understanding of product stability.

To develop commercially viable and regulatory-compliant manufacturing platform processes, it is important to develop efficient separation, purification, and loading steps. Evox is developing a suite of proprietary enabling technologies to overcome these potential bottlenecks and allow us to consistently generate commercially viable yields and reduce the cost per dose of our products.

DOI: 10.18609/cgti.2022.186

Q What are the issues for this emerging field to navigate on the CMC side of things?

PJ: Currently, there is not a full understanding of the relationship between exosome characteristics and function, which impacts on the industry's ability to develop scalable solutions to separate specific exosomes from others. Isolation and purification of exosomes from cell debris is complex, and it is even more difficult to isolate a particular subpopulation of exosomes. As exosomes are products of cells, their manufacture depends on the ability to produce large quantities of cells without altering certain cell behaviours and characteristics. The potential for changes in cellular phenotype during technical transfer (e.g., scale-up and equipment change) is a key consideration. Product characterization relies on a raft of analytical tools to enable the successful development of the upstream and downstream processes, quality control, and final product release testing.

Initial work performed by academic laboratories relied mostly on ultracentrifugation, which, while yielding highly pure samples, is not scalable commercially. We and others have been employing size and charge-based column chromatography separation methods to purify exosomes. Chromatographic purification steps can separate complexes based on their size (size exclusion chromatography), charge (ion exchange chromatography), or hydrophobicity (hydrophobic interaction chromatography). We are also exploring the use of Capto™ Core multi-modal resins for intermediate purification, polishing, and immunoaffinity-based purification of exosomes that relies on the conservation of surface proteins to enrich exosomes more specifically.

Q Can you tell us more about Evox's strategy and approach to manufacturing?

PJ: Evox's strategy and approach to manufacturing is to invest significant resources in the continued development of its DeliverEX™ technology platform to enable exosome therapeutics to be manufactured at scale. Since it was formed, Evox has developed a variety of proprietary exosome-adapted cell lines based on CAP® (CEVEC's Amniocyte Production) cell line and human embryonic kidney (HEK293) suspension cell sources, enabling consistent and stable exosome engineering. To date, Evox has developed upstream processing platforms supporting both adherent and suspension cell culture at scale. Also, its scalable approach to downstream purification is tailored to exosome therapeutics manufacturing. It has established in-house capabilities supporting 200 L production of high-quality material to support its research and development activities and has experience of working with contract development manufacturing organisations (CDMOs) to upscale its manufacturing platform up to 2000 L under GMP conditions.

Evox has also invested heavily in its exosome-based platform focused on developing efficient methodologies to engineer exosomes to contain high numbers of copies of drug cargoes. This can be done either through exogenous loading (purifying the exosomes upfront and afterwards

loading the therapeutic drug cargo into the exosome), or by endogenous loading (the cell makes the exosome and therapeutic drug cargo, which is loaded into the exosome through genetic association to an abundantly expressed protein or fragment). At Evox, we have employed all these approaches, routinely loading hundreds to thousands of copies of drug per exosome without limit on drug size or structure. Dependent on the engineering approach, therapeutic drug cargoes can be either loaded on the exosome surface or incorporated into the exosome membrane or lumen. Luminally loaded can either be tethered onto the inner surface or be freely soluble within the exosome.

Q What are the chief bottlenecks or obstacles to industrializing exosome manufacture for future commercial applications – and what will be key to addressing them from both the technological and the strategic standpoints?

PJ: From the technological perspective, as the clinical experience is still limited, we are at an early stage in addressing some of the chief obstacles to industrializing exosome manufacture to meet what could be a huge commercial opportunity. However, there are important lessons we can learn from other modalities such as monoclonal antibodies (mAbs), recombinant proteins, and viral vectors.

For example, the molecular mechanisms that regulate exosome biogenesis and secretion are still poorly understood and the exact mechanisms of action of many regulatory elements are not entirely clear. Such insights could lead to increased upstream volumetric productivity and improved product quality, as well as the rational design of more appropriate cell lines and optimised culture media formulations capable of supporting higher density cell cultures. Most of the media formulations currently used in exosome production were originally developed for other biomanufacturing applications.

There are also potential improvements to be made in upstream processing and how best to leverage recent advancements in other modalities using intensified continuous and connected bioprocesses (based on alternating tangential flow filtration (ATF) perfusion, continuous capture, and continuous polishing). This would be aimed at increasing productivity and reducing the cost per dose, whilst limiting the need for increased scale and higher capital costs.

The heterogeneity of extracellular vesicle preparations can be challenging, and the downstream processes must be optimized for a given exosome application and the proper analytical tools developed. The development of novel approaches for the isolation of exosomes is necessary to improve overall recoveries and scalability, and to reduce cost of goods. Currently, to standardize the purification process, much of the field's focus is on developing improved immunoaffinity resins with high capacity and specificity for exosomes, especially to resolve engineered exosomes from empty vesicles and other residual impurities.

The heterogeneity of particles within exosome products also requires a huge amount of orthogonal testing to differentiate the particles of interest from the product and process-related impurities. Additionally, the number of product attributes to be measured is far greater than in more traditional products. This applies across all stages of CMC development, from cell

DOI: 10.18609/cgti.2022.186

line development to final material release and characterization. High-throughput methods for accurate quantitation and sizing of particles remains a key challenge in this area, as this is critical to determining product yields. Current gold standards such as nanoparticle tracking analysis (NTA), and tunable resistive pulse sensing (TRPS) are highly informative but cannot be adapted to rapid and automated analysis of high sample numbers. Evox are pioneering alternative approaches to such quantitation to overcome this bottleneck.

"One of our main areas of focus is on exosome-delivered AAV for the treatment of phenylketonuria and other rare diseases."

Whilst many companies in the exosome space are still largely at the preclinical stage, there are an increasing number moving into the clinic. To meet the increased demand, there needs to be credible alternative GMP manufacturing options. It is therefore essential that more CDMOs see the commercial opportunity and invest in developing future exosome-related manufacturing services. From the strategic perspective, more CDMOs are recognizing the potential of exosome therapeutics, as evidenced by Lonza's acquisition of Codiak's exosome manufacturing facility in Lexington, MA (US) and its high-throughput exosome manufacturing technology. This is further evidenced by the recent announcement of the partnership between RoosterBio and AGC Biologics to accelerate cell and exosome manufacturing capabilities.

There are commercial opportunities also for suppliers to be more forthcoming in addressing these obstacles especially in terms of developing more appropriate analytical tools, improving throughput of existing assays and addressing the many bioprocessing and scale up challenges facing companies developing exosome-based therapies. Besides suppliers, we should also encourage more academic labs into the space, to help generate process understanding and insights surrounding the fundamentals of exosome biogenesis and purification.

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

PJ: Over the next several months, Evox's focus will be to execute our pipeline-driven platform strategy and to rapidly progress our various exosome therapeutic programmes to the clinic and beyond. The DeliverEX™ platform has unlocked a wide range of opportunities for us to exploit engineered exosomes as a delivery vehicle. Multiple different drug types can be engineered into or onto exosomes covering almost the entire breadth of drug modalities.

The CMC team will focus on supporting the advancement of our products to the clinic and conduct further platform development. One of our main areas of focus is on exosome-delivered AAV for the treatment of phenylketonuria (PKU) and other rare diseases. We will also continue to work on enhancing exosome-mediated delivery of other drug cargoes including antisense oligos, siRNA, mRNA, and gene editing.

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

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *Jones P discloses he recieves payment or honoraria for lectures, presentations, speakers bureaus from Royal Academy of Engineering Visiting Professor of Manufacturing of Advanced Therapies at Aston University.*

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

ARTICLE & COPYRIGHT INFORMATION

Article source: *Invited.*

Interview conducted: *Sep 8 2022;* ***Publication date:*** *Oct 24 2022*

DOI: 10.18609/cgti.2022.186

CELL & GENE THERAPY INSIGHTS

Supply Chain Channel

RAW & STARTING MATERIALS QUALITY, SOURCING & MANAGEMENT

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SUPPLY CHAIN CHANNEL: RAW & STARTING MATERIALS QUALITY, SOURCING & MANAGEMENT

November 2022
Volume 8, Issue 10

CELL & GENE THERAPY INSIGHTS

STARTING MATERIALS SOURCING & MANAGEMENT

CHANNEL CONTENT

INNOVATOR INSIGHT

Cell & gene therapy orchestration: supply chain management in real-time

Andreas Göbel & Dr Camila Mendes

With more Cell & Gene Therapies (CGT) entering the market, many pharma companies are currently looking into their supply chains and adapting them for the complexities and requirements that come with these novel therapies. Many startup biotech companies are doing the same thing, but with the advantage of being able to start with a greenfield project. The complex, highly collaborative supply chains of CGT require control and visibility to fulfill the very high requirements for safety, resilience, and speed that come with the underlying drug products. A very high degree of process complexity and the involvement of many different contributors, each with numerous intermediates and handovers, combine to ensure that it is almost impossible to manage more than 50 patients per year with simple tools such as Excel spreadsheets and web portals. There is a 'magic tool' that can provide the necessary support here – one that has been talked about for several years now: the Cell & Gene Therapy Orchestration Platform (Orchestration Platform). Biotech companies may require Orchestration Platform features that support regulatory requirements, such as chain of identity and custody, logistics management, and general collaboration. But as they move with their assets through preclinical, clinical, and eventually, commercial, it becomes clear that each of these different phases come with added requirements for the Orchestration Platform. Step by step, they will require features on different levels (workflow and integration, manufacturing, operations, tactical and strategic), depending on the product lifecycle and organizational state. In this article, we will detail all levels of a complete end-to-end supply chain management approach for personalized therapies. We will also highlight critical features of and key considerations for an Orchestration Platform and describe the phase in the product lifecycle in which each single level begins to generate its full value.

Cell & Gene Therapy Insights 2022; 8(10), 1303–1314

DOI: 10.18609/cgti.2022.191

The more biotech and pharma companies in the emerging high-growth area of CGT want to scale their production and global access to their products, the more they come to realize, they need to put more effort into their supply chain management.

This is to be expected right from the beginning, as the first patients are onboarded into first clinical trials. Whereas traditional pharma products involve supply chain and operational complexity only at the commercial phase, CGT setup and operational challenges continue throughout the clinical stages and into commercialization.

Supply chains in CGT are complex for a variety of reasons, starting with but not limited to complex manufacturing processes, high levels of coordination during the product and patient journey, and new regulatory compliance requirements. The good news is that the answer to the problems stemming from these complex supply chains is a single, consolidated Orchestration Platform.

An Orchestration Platform is usually a computerized application that coordinates all multi-enterprise critical activities across the supply chain and supports improved supply chain management in make-to-order environments by implementing full supply chain protocols (clinical, laboratories, manufacturing, and logistics). The Orchestration Platform executes these protocols through a workflow engine, providing a therapy control tower and data capture with reporting that realizes the chains of identity and custody.

To understand the need for such a platform and related methods, it is essential to look at the history of conventional production planning. "Today most mid-sized and large manufacturing enterprises throughout the world use a planning method and tool called Material Requirements Planning (MRP). This method and tool were conceived in the 1950s with the increasing availability, promise, and power of computers." [1].

DEFINITION OF MRP

"A set of techniques that uses bill of material data, inventory data, and the master production schedule to calculate requirements for materials. It makes recommendations to release replenishment orders for material. Further, because it is time-phased, it makes recommendations to reschedule open orders when due dates and need dates are not in phase. Time-phased MRP begins with the items listed on the MPS and determines (1) the quantity of all components and materials required to fabricate those times and (2) the date that the components and material are required. Time-phase MRP is accomplished by exploding the bill of material, adjusting for inventory quantities on hand or on order, and offsetting the net requirements by the appropriate lead times" [2].

From 2010, around 80% of manufacturing companies implemented an ERP system, simultaneously implementing a compatible MRP module.

Today, the methods and principles of MRP have been improved and adapted to tackle the ever growing "volatility and uncertainty of demand, and the complexity and ambiguity of product portfolios and supply chain networks in which companies now operate (VUCA)" [3].

From that point of view, the supply chains of CGTs are nothing other than a 'very VUCA' environment, and it seems to be logical to further extend existing supply chain management methods to overcome the specific challenges that arise here on top of traditional manufacturing models.

It is very likely that Cell and Gene Therapy Orchestration Platforms and related methods, as a special way of supply chain management, will establish themselves in the same way that MRP did in the past for traditional manufacturing models. We will see personalized therapy vendors implement Orchestration Platforms alongside their ERP, similar to what happened with MRP.

However, Cell and Gene Therapy Orchestration today is vastly non-standardized, in contrast to traditional supply chain management methods.

DOI: 10.18609/cgti.2022.191

The following describes a general scheme explaining the different domains of a Cell & Gene Therapy Orchestration Platform, based on established pharma supply chain management methods but enriched with the specific needs of CGT. From this, a peer-reviewed discussion shall commence to establish standardized terms, principles, and methods related to Cell and Gene Therapy Orchestration.

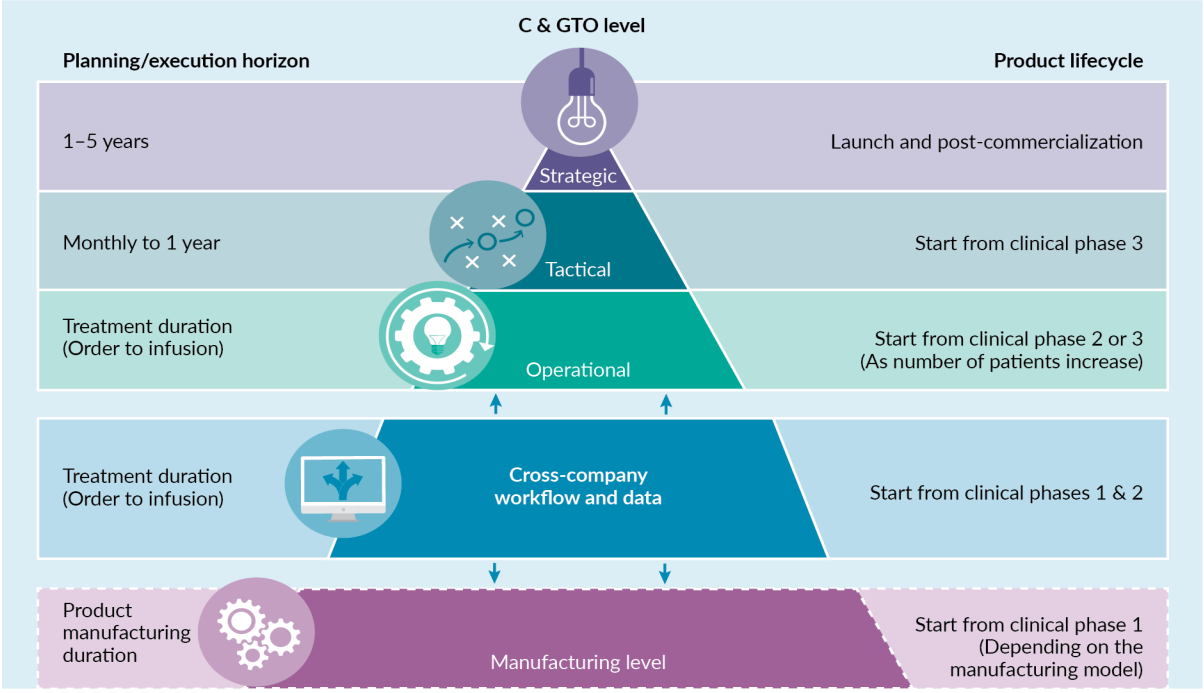
DEFINITION OF CELL & GENE THERAPY ORCHESTRATION

A set of techniques that uses order data, inventory data, supply chain network data, master data and a set of different but dependent service schedules to calculate the optimal supply chain sequence to execute a predefined supply chain protocol for the purpose of a cell and gene therapy in a make-to-order environment. It makes recommendations to release replenishment orders for consumable supply materials. Further, because the supply situation of patients living cells is highly variable and volatile, it makes recommendations to reschedule the therapy partially or completely when supply dates move, manufacturing is delayed, or logistics services cannot be provided. Cell and Gene Therapy Orchestration (C&GTO) begins with the clinical order involving an initial scheduling process determining an infusion date proposal, the possible starting material extraction appointments the number of drug product batches to be produced and materials required to fabricate those, and the dates when the materials are required. C&GTO, by default, fulfills regulatory requirements like chain of identity (COI) and chain of custody (COC) and optionally, additional features like label and document management."

Based on established supply chain management methods such as LEAN supply chain management, the different features of a platform implementing the C&GTO paradigm can be grouped by different levels (Figure 1).

FIGURE 1

Supply chain management planning addresses different types of problems according to the decision horizon.



All those levels from long-term (strategic), medium-term (tactical) and short-term (operational) are part of the holistic C&GTO concept. The C&GTO concept has the cross-company workflow & data level at its core but is also connected to higher supply chain planning levels and to day-to-day activities in the manufacturing level at the bottom. In this picture, we present an overview of the C&GTO levels according to the planning or execution horizon and additionally linked to the product lifecycle phase when each level becomes relevant.

IMPLEMENTING SUPPLY CHAIN PROTOCOLS ON THE CROSS-COMPANY WORKFLOW & DATA LEVEL

No matter the phase of development, companies need to set up supply chain workflows and upstream and downstream integrations with supply chain partners, building a 'supply chain protocol'. At this stage, the entire supply chain protocol from order to infusion – including logistics and manufacturing is defined and configured in a single system for real-time transparency, automation, and visibility of each step in the therapy's journey. It also contains the full data model to be filled during protocol execution from connected data flows or by manual input.

The possibility to freely define different flows and dynamic data models, as well as an engine executing the defined therapy supply chain protocol, are the key elements of this level and form the backbone of every C&GTO.

The established protocol definition, execution, and data consolidation in one system ensures that all actions and notifications are tracked by collecting information from multiple stakeholders and connected systems, and connecting them into one central source of truth, which allows users to act quickly on the actions that are required to drive the therapy for each patient. This is also equally important for reporting and data analysis over the entire process.

BOX 1

Features for CGT orchestration platforms at cross-company workflow level.

At the cross-company workflow level, CGT supply chain orchestration platforms need to offer features like:

- **Therapy Control tower**: The therapy control tower is more than a usual dashboard. It has not only a transparent overview of all scheduled, ongoing, and past therapies with a condensed view on status but also presents a concrete proposal for action. It usually comes with the ability to drill down on every single process step and provides the full capability of acting as a supply chain responsible person.
- **COI COC and COCn**: The COI ensures that a patient gets the treatment specifically produced for them, including tracking for each patient throughout the 'vein to vein' process. This ensures treatment of the patient with the correct cells. COC ensures chronological documentation that records the sequence of custody, control, transfer, and analysis – not just in manufacturing but throughout the logistics and treatment processes. Additional to both – COI & COC – there is also the chain of condition (COCn) that tracks the temperature and other key variables critical to quality and viability of the treatment from the starting material throughout the final drug product.
- **Real-time tracking**: For full transparency and especially as an input for other levels of the orchestration platform, real-time tracking gives just-in-time insights into geographical movements and temperature conditions of transported goods, and into process details like handovers and step state status changes.
- **Configurable workflows**: Fully flexible and configurable workflows are one of two core elements of a non-bespoke C&GTO platform, because nobody knows what tomorrow's therapy supply chain and manufacturing protocols will look like.
- **Dynamic data model**: This is the second core element for building the foundation of a fully flexible orchestration platform. It is particularly crucial because data models of different therapies may differ substantially.
- **Generic and dedicated data integration**: Data integration describes a way to consume data from external interfaces and having data consumed from owned interfaces. It may seem to be a contradiction, but one needs both: on the one hand a generic integration infrastructure to support future systems and devices, which are not yet known. On the other hand, one needs the capabilities to integrate into today's commodity systems, like MES and ERP systems, with easy-to-configure dedicated data integration modules. Also, the orchestration platform itself must be consumable, by both users and machines. For a machine-to-machine communication it needs to expose application programming interfaces (APIs) based on well-established standards, like HTTP REST or more specifically in a clinical environment, FHIR.
- **Data protection**: Compliance with global data protection regulations like GDPR, HIPAA, and French HDS is a must, since every personalized therapy touches patient health records and person-related information.
- **Labelling**: Several steps of supply chain protocol imply the management, printing, and/or tracking of labels, such as ISBT128-compliant blood bag labels, logistics service provider labels, and sample labels.

DOI: 10.18609/cgti.2022.191

KEY CONSIDERATIONS FOR IMPLEMENTING A CROSS- COMPANY WORKFLOW & DATA LEVEL

Flexibility & scalability

A supporting IT system for a company's first CGT can be complex, costly, slow, over-specific, not scalable, incomplete, and unsuitable for having several different therapies in their portfolio or pipeline without careful thought and planning. By adhering to C&GTO principles, the cross-company workflow and data level must be a flexible solution suitable for a range of therapies, and not tightly specific to one therapy. A flexible system architecture supports the implementation of various therapies' protocols. Additionally, systems must be easily scalable for adjusting into new market requirements, and scalable for global clinical and global commercial set-up.

Standards

Common platform cloud solutions and industry standards are essential to enable diverse value chain partners to connect to the integrated vein-to-vein orchestration system. Adoption of common user-interface standards across life science companies, suppliers, and medical centers will simplify operations, reducing the administrative burden and risk of error from using different systems for each cell therapy manufacturer.

To manage COI and COC, orchestration platforms need further integration to systems in the manufacturing & operational levels. Implementing a fully integrated, digital vein to vein platform and supply chain, dramatically increases throughput and unlocks the potential for full automation with the goal of a "self-driving therapy supply chain". This will increase both the speed and accuracy of the manufacturing process while reducing the likelihood of COI and COC errors.

INTEGRATIONS & REMOTE CONTROL ON THE MANUFACTURING LEVEL

In the traditional biomanufacturing business, there is typically an ecosystem of many different manufacturing plants for three processes: drug substance, drug product, and packaging. In CGT, these processes vary enormously in terms of capacity and complexity. In the personalized CGT manufacturing processes, where each batch is unique to an individual patient, the production of such living cell-based products is inherently variable, and the manufacturing process must be able to accommodate this variability. Many of these challenges can be traced back to process challenges like paper-based quality assurance/quality control (QA/QC) and the failure to establish the CMC process for CGT early on. To make sure, this complexity does not affect quality, efficacy, compliance, and accountability in the entire vein-to-vein process, an orchestration platform should support an integration into existing Manufacturing Execution System (MES).

Further, the required flexibility should also support new or even future manufacturing models. For example, in point-of-care manufacturing models, the manufacturing process must be fully covered by the orchestration platform. Here, the manufacturing protocol becomes part of the larger supply chain protocol, being converted into standard operation procedures (SOPs), which effectively 'remote control' on-site operators during their daily work.

KEY CONSIDERATIONS WHEN IMPLEMENTING FEATURES AT THE MANUFACTURING LEVEL

Real-time & remote release

Traditionally, biopharmaceutical manufacturers release involves review of in-process control data, batch records, test records, and off-line release testing of drug substance and

drug product. The current QC sampling and testing process is burdensome, though – it is labor intensive with many potential opportunities for errors in sampling, labeling, transporting, storing, and testing. This can take several weeks after production, causing significant delays in product release.

CGT challenge all QA/QC steps and require a fully digitized and faster product release while maintaining product safety. It is required that in the QC processes, deviations are detected through real-time feedback by sensors and systems, and a more proactive QA rather than reactive investigations to improve process control, leading to quicker product release.

During the real-time manufacturing process, the captured data must meet the needs of QA and QC, whilst allowing sign-off by remote QC specialists. Furthermore, eliminating or minimizing these opportunities for errors will make the overall manufacturing and 'testing' processes easier and more predictable, which translates to a more reliable supply chain.

CROSS-COMPANY SCHEDULING ON THE OPERATIONAL LEVEL

When the number of drug product batches increases in late-stage clinical phases or after commercialization, there are new challenges on a more operational level, namely with the scheduling of those tasks in the supply chain protocol that require an appointment or a certain amount of a limited production capacity. Once a therapy is ordered, its' delivery must be optimized towards the 'time to patient' KPI, within agreed lead times. However, due to patients' poor health condition, the cell collection schedules can change at very short notice, in turn influencing the subsequent schedule and thus requiring a re-scheduling of all subsequent steps.

The flexibility needed for scheduling and re-scheduling can be achieved in a C&GTO platform by tightly integrating the schedules of as many parties as possible with bi-directional data flow. The scheduling needs to cover all upstream (doctor, patient, logistic) and

BOX 2

Features for CGT orchestration platforms at manufacturing level.

At the manufacturing level, C&GTO platforms need to offer features such as:

- **MES integration**: Manufacturing execution systems, MES for short, are used to track and control data points between patient, donor, raw material, and material management. With a MES, biomanufacturers achieve higher maturity levels in their manufacturing, as they enable manufacturing automation (less manual processing steps in processing a batch) and integrate vertically and horizontally into the manufacturing technology ecosystem.
- **Standard operating procedures (SOP) management and execution**: SOP management and execution turns the supply chain and manufacturing protocols of the cross-company workflow level into high-quality SOPs. They can be executed by manufacturing operators and healthcare professionals.
- **Manufacturing devices integration**: New manufacturing models, like point-of-care approaches, require the tight integration of various manufacturing devices into the orchestration platform for automation and documentation purposes. In more traditional manufacturing, this is covered by MES systems.
- **Electronic batch record**: With Electronic Batch Recording (EBR) systems, the patient's material can be correctly identified at any time during the process. EBR systems also record manufacturing locations or used equipment. This caters to CGT regulations which specify that the cells need to be identifiable at any stage of the manufacturing process, to secure CoI. Next to the severe effects of administering the wrong drug (i.e. based on cells from another patient), this also prevents the cost generated by failed batches.
- **Labeling**: Shop-floor systems should be integrated to enforce the traceability and manage the patient data and information to be printed on labels. Digital controls in the manufacturing facility will leverage and extend new industry labeling and tracking standards to ensure chain of custody, chain of identity, and chain of condition (tracking of transport conditions).
- **Document management**: This ensures a secure, documented lifecycle management for SOPs, work instructions and manufacturing documentation.
- **Deviation management:** Deviation management is the process of detecting, evaluating, and correcting deviations from approved instructions or SOPs. C&GTO platforms streamline the entire deviation management process with the traceability required and provides processes as well as documentation to corrective and preventive activities. This allows organizations to deal efficiently with deviations by automating the data collection and complying with regulatory requirements.

DOI: 10.18609/cgti.2022.191

downstream (manufacturing, logistic, infusion) processes. The biggest advantage of the scheduling automation is the amount of time saved by avoiding manual communications through ineffective channels such as phone or email. Additionally, booking errors stemming from manual scheduling or rescheduling become more unlikely with this approach. Ultimately, the scheduling automation can help to scale the number of batches that can be produced due to an optimal usage of resources, notwithstanding dynamic timing variabilities.

KEY CONSIDERATIONS FOR IMPLEMENTING FEATURES AT THE OPERATIONAL LEVEL

Fully automated & advanced scheduling

Scheduling enables therapy-driven production planning and detailed scheduling for make-to-order environments. It should support real-time, cross-company scenario planning, optimization, and order sequencing. An automated decision support can be reached via integrated cognitive planning engines. Decision-makers can obtain insights into the impacts of detailed scheduling on the supply chain in real-time. Automated scheduling should link advanced scheduling capabilities seamlessly into a cross-company workflow level.

SUPPLY CHAIN SIMULATIONS ON THE TACTICAL LEVEL

Tactical supply chain simulations based on the current network master data allow for predictive planning. This predictive planning can involve an integrated view of logistics, supply, and clinical, revealing insights into the best possible supply chain parameterization to produce as many batches as possible depending on the rather unpredictable level of demand. The entire network of stakeholders involved, through upstream and downstream linkages, in the different processes and activities are checked with regards to inventory management, planning processes, logistics availability, etc.

CGT challenges the tactical level design and simulations, as vendors have to deal with scenarios without inventory buffer for the main starting material (human blood or samples from the patient, in the case of autologous or allogeneic CGT) and further supply and demand uncertainties.

The CGT Sales and Operations Plan (S&OP) that describes the intended procedures for production and distribution of the cell therapy product is also a challenge, because it is impossible to have long-term forecasts for the consumption of materials, which would help to establish appropriate levels of inventory at the cell processing facility as well as throughout the organization's complete supply chain.

BOX 3

Features for C&GTO platforms at operational level.

To achieve this, the operational level needs the following features:

- **Cross-company schedule management**: This means accessing every partner's relevant planning system to ensure access to and integration of the available calendars, slot schedules, and service booking systems of the different parties contributing to a therapy's supply chain.
- **Cross-company scheduling**: Operational real-time scheduling upon a therapy order is utilized with the aforementioned calendars, slot schedules, and service booking systems. Depending on the level of maturity of the integration scenario, this cross-company scheduling and rescheduling can be optimized and semi- or fully automated
- **Inventory management**: To avoid frequent rescheduling reactions of the cross-company scheduler due to supply issues with raw materials and production equipment, decentralized inventory management is key to avoid supply shortages, thus optimizing schedules holistically throughout the therapy supply chain.
- **Logistics scheduling**: Logistics scheduling supports transport service ordering processes. This can even be automatically integrated into the end-to-end scheduling of a therapy, depending on the maturity of the logistics service provider's digital infrastructure for service ordering and consumption.

Key elements of tactical level simulations

- High master data quality;
- Proper parameterization of the supply chain protocol with data points from the master data;
- Choice when it comes to service providers and sites.

SUPPLY CHAIN DESIGN OPTIMIZATION ON THE STRATEGIC LEVEL

Supply chain management addresses different types of problems according to the concerned decision horizon. At the strategic level, long-range decisions are concerned with supply chain configuration: onboarded clinics, number and location of suppliers, laboratories, own production facilities, contract manufacturing organizations, logistics service providers and warehouses, etc.

In CGT, the complexity and scalability of the end-to-end supply chain must be considered as the major factor for strategic decisions determining the commercial therapy performance. This leads to selections around a geographical supply chain network configuration with fallbacks and alternative routes.

Key elements of an end-to-end CGT supply chain network

- Clinical network → clinics and apheresis centers;
- Supply network → supplier selection;
- Production network → manufacturing site location and CMO selection;
- Distribution network → distribution structure and logistics service provider selection.

A full supply chain network design should anyhow be part of an orchestration platform master data configuration because details of this configuration are required during the supply chain protocol execution. Optimizing the existing designs based on insights from the tactical level is the foundational essence of commercially successful CGT products.

FLEXIBILITY & MODULARITY

Independent from the aforementioned levels, an orchestration platform should provide the right degree of flexibility, as nobody can predict how tomorrow's asset will look at the various different levels described.

With increased demand for end-to-end supply chain management, it is important to understand the variety in concrete challenges that sets one company's situation apart from the next. Even though all companies want to form an overall supply management ecosystem, the company size and background make all the difference:

New players in CGT – e.g., new-entrant biotechs with a strong scientific background – usually conquer the complexity of supply chain management step by step. This also means that they do not need all levels from the start. They typically master the levels as their business matures (Table 1).

- In their preclinical stage, they need to establish a supply chain protocol design based on the patient and product journey.
- Entering Phase one clinical trials, they meet challenges on the manufacturing level, e.g., selecting between centralized or decentralized models, requiring a manufacturing protocol design.
- Starting with Phase three and commercialization, they face the number of patients increasing and the next set of challenges on the operational level – for example, how to collaborate more efficiently upstream and downstream, how

DOI: 10.18609/cgti.2022.191

TABLE 1

When to implement which level.

	Workflow & integration level	Manufacturing level	Operational level	Tactical level	Strategic level
Asset state	Pre-clinical	Phase 1	Phase 3 – commercialization	Post-commercialization	Post-commercialization
Asset evolvement					
Implementation process	Supply chain protocol design	Manufacturing protocol design	Scheduling optimization	Data mining and evaluation	Supply chain improvements
Orchestration platform implantation activity					

to adhere to compliance rules, etc. These are questions addressed by scheduling automation and optimization.

- Post-commercialization, they want to understand all the operational planning on the tactical level and identify and predict bottlenecks. By using data mining and simulations they can reassess the supply chain distribution network.
- From this stage, they can deduce and implement supply chain improvements at the company's strategic level.

This calls for a modular system of the C&GTO platform to solve supply chain challenges, giving emerging players the freedom to choose only those modules that they need at any given time in their growth process.

There is another, more technical reason for a modular concept: an orchestration platform needs to be flexible in answering to future requirements that are as yet unknown. These might be with new logistics partners that need to be added, new compliance regulations (e.g., for new markets), or even completely different manufacturing models (e.g., point-of-care manufacturing). Here, it is helpful if only one module needs to be changed, tested, and rolled out. Thus, modular solutions are more responsive to future changes in the environment for CGT.

That said, one should not look for a non-bespoke solution (this describes a solution where every feature is statically built-in code, or hardcoded). Instead, the platform should offer a generic core that can be configured accordingly, depending on the supply chain protocol at hand. This has the huge advantage that the cost for maintaining the solution in a fast-paced environment as CGT is lower. The sweet spot is a modular solution with out-of-the-box features that is still highly configurable, as no CGT vendor's processes resemble the next. Optimally, out-of-the-box features are standardized components, that come along pre-qualified, significantly reducing the amount of work to be performed on the obligatory 'validation of computerized systems' project.

CONCLUSION

Know & control your processes

The end-to-end supply chain process for a personalized therapy can be understood as a 'supply chain protocol'. An orchestration platform can guide through the design phase of such a 'supply chain protocol' and even act as the driving engine on top of the defined process after implementation.

Manufacturing execution in CGT is different

The term execution in MES is gaining a different meaning in CGT, where it relates to 'remote-controlling' human operators or, at the most, involves several different devices that still need the operator as a connector. An orchestration platform can act as such an MES, becoming an integral part of a supply chain protocol execution.

CGT scheduling is production planning optimization in real-time

The traditional production planning optimization systems never had a direct connection into the effective execution. CGT require exactly this connection for scheduling automation and according rescheduling with follow-up events as part of the supply chain protocol. An orchestration platform can establish this connection optimally by bringing a scheduling engine as an integral component.

The commercial performance of a CGT product is governed by supply chain optimization

'The manufacturing process of personalized medicines starts with the raw material extraction and ends with the administration of the resulting product.' [4].

In a commercial context, this means that the greatest optimization potential for the performance of a therapy product is buried inside the supply chain network design itself. An orchestration platform, with simulation and supply chain network modeling capabilities can support fulfillment of this potential.

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DOI: 10.18609/cgti.2022.191

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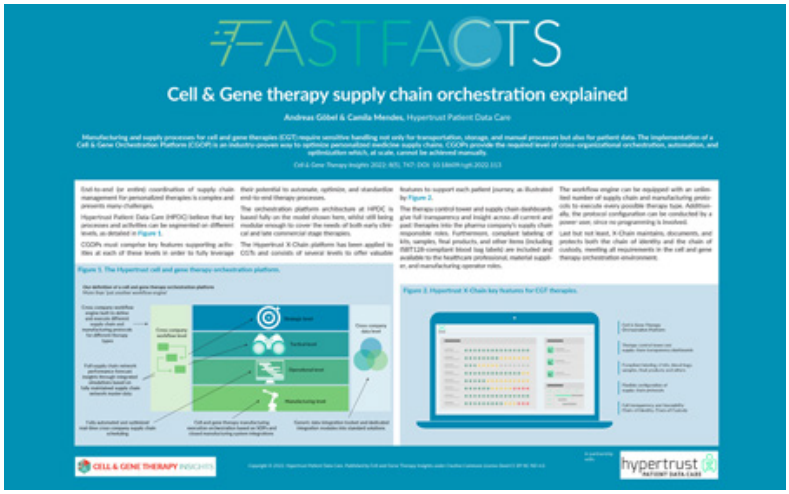

AUTHORSHIP & CONFLICT OF INTEREST

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

Acknowledgements: *None.*

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

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

ARTICLE & COPYRIGHT INFORMATION

Article source: *Invited; externally peer reviewed.*

Submitted for peer review: *Sep 16 2022;* ***Revised manuscript received:*** *Oct 26 2022;* ***Publication date:*** *Nov 4 2022.*

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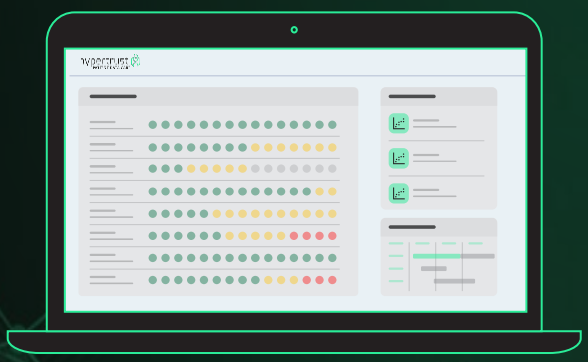

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STARTING MATERIALS SOURCING & MANAGEMENT

CHANNEL CONTENT

The living cell supply chain: a product-specific, rational approach to management of cellular starting material donors & donations

William E Janssen, WEJ Cell and Gene Therapy Consulting Services, **& Scott R Burger,** Advanced Cell and Gene Therapy, LLC

VIEWPOINT

"In cell therapy and cell-based gene therapy products, donated, intact living cells are arguably the most critical of materials for CGT product manufacturing"

Cell & Gene Therapy Insights 2022; 8(10), 1375–1379

DOI: 10.18609/cgti.2022.201

Among the many challenges involved in development of chemistry, manufacturing and control (CMC) for cellular therapies and cell-based gene therapies (CGT), has been the sourcing and acquisition of raw materials. Although there has been tremendous growth in the areas of specialized GMP growth media, cryostorage solutions, containers and processing equipment, the need to adapt materials intended for research use in early-phase clinical studies of novel cell and gene therapy products remains. Even when GMP materials are available, the pathway to identifying which are optimum for specific cell types is seldom clear. However, these concerns generally pale compared to the challenges posed by the most critical material used in the manufacture of CGT products – the viable, functional cells that comprise the starting material.

Cellular starting material is intrinsically heterogeneous, in that any population of donors will possess all of the genetic diversity that is inherent in the human species. Moreover, there are a plethora of inter- and intra-cellular functions that vary with environmental factors, including individual stress levels, nutrition, circadian cycles and symptomatic and asymptomatic disease. As such, not only will cells collected from two different individuals have demonstrable differences, but cells collected from the same individual at different times may differ as well. Finally, a cell collection rarely if ever nets a homogeneous population of cells and within these heterogeneous cell populations, such as in an apheresis collection, the distribution of cell types in any given collection will vary, both between individual donors and also between collections from the same donor.

In addition to the inherent variability of the living cell raw material used in CGT products, the potential for a presence of infectious agents must be considered, particularly if the infectious disease is transmitted in the form of viruses that incorporate their genome into the host cell genome.

The variability in the cellular starting material for CGT products and the potential for presence of infectious agents stands in contrast to the tight controls that may be placed on non-cellular raw materials that are used in CGT production and in the manufacturing of traditional pharmaceuticals.

Procurement of cellular starting material for GMP manufacturing of CGT products often poses problems for developers of CGT products. This is due in part to the complexity, variability, and heterogeneity of cellular starting material, but also to uncertainty about regulatory expectations and requirements for use in GMP manufacturing.

One common misapprehension is the concept of 'GMP cell collection'. As consultants, we are often asked what constitutes a GMP cell collection, but in fact, GMPs are not applicable to cell collection. The sections of the Code of Federal Regulations (CFR) that describe GMPs [1,2] do not address donated biological material of any sort. Cell and tissue collection is the province of Good Tissue Practices (GTPs) [3]. To be suitable for use in GMP manufacturing, cellular starting material must be collected in compliance with GTPs.

Other common questions related to procurement of cellular starting material for GMP manufacturing of a CGT product include:

- What environment is necessary at the point of collection? ;
- What testing of the donor is necessary? The FDA has promulgated through regulation [4] and guidance [5,6] specific minimum infectious disease screening and testing. Beyond this, is there a one-size-fits-all list of tests? How should the testing regimen be tailored to fit the intended final CGT product? ;

- What other selection criteria should be employed in recruitment and collection of the 'right' donor? Specific HLA types, minimum counts of the cell type that is required for the CGT end product, minimum and maximum overall cell counts, and absence of specific genetic markers may apply.

To expect regulatory entities to provide fixed, one size fits all answers to the above questions is unfair to the regulators, and potentially problematic for the manufacturers of CGT products. Regulatory guidance documents are of necessity written in general terms, to be broadly applicable to a wide variety of CGT products. Moreover, these documents must, of necessity, be limited to the minimum standards that should be in place for the variety of CGT products. It falls to the CGT product developer to define product-specific aspects of procurement and collection of cellular starting material. This requires a methodical, science-based approach, and assessment of risks the cellular starting material could pose to the CGT product.

Two principles should guide how to address these questions. First, practices should be based on actual science, not hypotheticals. Second, what is the final product intended to be, and what risks might be posed to that product by the starting material?

For example, an early-stage CGT company recently asked us whether apheresis collection of cells should be performed in a surgical suite, with donors attired in surgical patient gowns. Is there a scientific basis for this, or does it simply reflect a belief that a more controlled environment surely must improve the quality of apheresis collection? The track record for leukapheresis performed in standard blood bank donor room settings spans decades, encompassing millions of successful collections. Such a large dataset provides well-established figures for expected percentage of contaminated apheresis products, and the most common sources of contamination. One could, if necessary, further determine whether reported contaminants were detectable shortly after cell collection. All of this information would lead to the reasonable conclusion that performing apheresis collections in a standard donor room setting is satisfactory, and that proper skin prep, not donor gowning, is critical to prevent contamination of apheresis products.

Application of the second principle could include assessing the significance of a donor having an undetected genetic abnormality, and whether that would have a negative impact on the manufacture of the final product, or on the efficacy of that product as a therapeutic entity.

Using these two guiding principles, then, a rational approach for management of the cellular supply chain emerges:

1. Determine what the optimal cellular starting material might be (e.g., leukapheresis, whole blood donation, skin scraping, surgically resected tissue), and what sources of such material are available (e.g. blood donor centers, paid volunteer cell collection companies).

2. Determine what screening and testing of donors and their donated products should be performed, based on regulatory and accreditation requirements and identified risk factors. At minimum, of course, should be the legally required testing for adventitious viruses and other infectious agents [4,5]. Considerations should include risk factors such as donor characteristics and health issues that could be problematic for a recipient of the CGT product, and viruses that may preferentially infect cells that make up the final CGT product. What donor qualification screens are for protection of donor safety, and what are for protection of patient/recipient safety? Note that a qualified donor may still donate a nonqualified product.

3. Based on pre-clinical and early clinical development data, identify minimum

number(s) of critical cell type(s), maximum numbers of contaminating cell types, and how these may be assessed in donor testing prior to collection of cellular starting material. What makes a product unacceptable? Are there product disqualifiers that are (potentially) curable?

4. With the above information in hand, assess the collection centers that are available to serve as sources for these materials. Collection centers should be audited similarly to vendors of non-cellular raw materials.

5. Document all donor and product testing and screening to be performed, with clear delineation of responsibility for performance, and what documentation will be made of both results consistent with acceptance of cellular starting material and results that require rejection.

6. It should be noted that all of the above steps assume that the cellular material that is to be obtained is collected from a living donor. Collection of cellular materials from cadaveric donors differs in several respects from living donor collection, but many of the steps described above still apply.

Detailed inclusion of the above steps and resultant data in regulatory submissions can reasonably be expected to facilitate the approval process. Much of this information should also be included in the Quality Target Product Profile (QTPP) [7,8] for the CGT product in development.

In cell therapy and cell-based gene therapy products, donated, intact living cells are arguably the most critical of materials for CGT product manufacturing. A risk-based, science- and data-driven approach, as outlined above is an effective strategy for qualification of these complex starting materials.

BIOGRAPHIES

WILLIAM E JANSSEN, PhD has worked for more than 30 years at translating lab bench models into cell and gene therapy products for administration to patients. In the course of these efforts he has developed and refined methodologies for all aspects of cell based therapy from collection, through manufacturing and administration, including regulatory aspects. Dr Janssen has also been responsible for facility design, drug master file creation, development of staff training programs, process engineering, technology transfer, SOP development, process validation, comparison studies and integration of processes, equipment and raw materials. He has also been both a proponent and implementer of informatics solutions for management of cell and gene therapy development and manufacturing data.

SCOTT R BURGER, MD, is the Founder and Principal of Advanced Cell and Gene Therapy, a consulting firm specializing in cell and gene therapy product development, providing expertise on manufacturing, regulatory, and strategic aspects of these products. Dr Burger has over 30 years of experience developing cell and gene therapy products and has consulted for over 160 companies in North America, Europe, Australia, and Asia, advising on a wide range of cell therapy and gene therapy products for immunotherapy and regenerative medicine applications. Dr Burger has consulted on over 100 regulatory submissions for cell therapy or gene therapy products at all stages of development. He has served as an expert witness in cases involving cell and gene therapy intellectual property, commercialization, FDA regulatory affairs, and GMP compliance, and as a subject matter expert for NIH-NHLBI, CIRM, PACT, and DMRDP review panels. He has been an invited speaker at internal FDA workshops and is frequently asked to speak on cell and gene therapy manufacturing and regulatory topics at international conferences. A graduate of the University of Pennsylvania School of Medicine, Dr Burger completed training in clinical pathology and transfusion medicine at Washington University in St. Louis and is author of over 200 scientific publications and presentations, and recipient of numerous honors and awards

DOI: 10.18609/cgti.2022.201

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

Acknowledgements: *None.*

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

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

ARTICLE & COPYRIGHT INFORMATION

Article source: Invited.

Revised manuscript received: Nov 2 2022; **Publication date:** Nov 14 2022

CELL & GENE THERAPY INSIGHTS

SUPPLY CHAIN: RAW & STARTING MATERIALS QUALITY, SOURCING & MANAGEMENT

CHANNEL CONTENT

INTERVIEW

Innovating in iPSC differentiation & engineering

David McCall, Commissioning Editor, BioInsights, speaks to **Emily Titus,** Senior Vice President, Technical Operations, Notch Therapeutics

EMILY TITUS obtained her PhD from the Institute of Biomaterials and Biomedical Engineering at the University of Toronto, where she used a combination of laboratory and bioinformatics approaches to define and interpret gene regulatory networks controlling embryonic stem cell fate decisions. At Notch, she oversees the Technical Operations function, which includes Process and Analytical Development, Manufacturing Sciences, Engineering and Project Management. Previously, she held the position of Vice President, Technology Advancement at CCRM, where she built cell reprogramming, genome engineering and pluripotent stem cell differentiation programs and led the company incubation program that culminated in the launch of Notch Therapeutics.

Cell & Gene Therapy Insights 2022; 8(10), 1437–1443

DOI: 10.18609/cgti.2022.209

Q What are you working on right now?

ET: Notch Therapeutics is developing induced pluripotent stem cell (iPSC)-derived immune cell therapies. At Notch, I oversee the Technical Operations function. This

includes process and analytical development, engineering, technology transfer and manufacturing sciences. I work on cell line development for the iPSC lines that will be used to create our products, as well as on scaling up our differentiation process. Our core technology is a bead-based differentiation platform, and we work in-house on the development and manufacturing of that custom bead reagent.

Q Can you tell us more about the reasons why Notch has pursued its own particular approach to developing allogeneic cellular immunotherapies, and in particular, why iPSCs were chosen as a source of starting material?

ET: While the allogeneic field has made fantastic progress in improving the number of doses per manufacturing run, we recognize that a healthy donor cell source will have a natural limitation to the eventual batch size that can be produced. With iPSCs, however, we envision an unlimited supply of consistent starting material. We can gene edit the iPSCs and create a clonal master cell bank in which every single cell has our selected edits. Then we can think about running a batch size that could support an entire clinical trial, for example. I think of iPSCs as the third generation of engineered T cell therapy starting material – we want to push the limits of how standardized and off-the-shelf we can make this kind of product.

Q You've worked in pluripotent cell programming for more than a decade at CCRM and then Notch – can you take us on the journey as you have experienced it through the rapid development of the field over this period – both in terms of the emergence of iPSCs and the application of increasingly sophisticated engineering tools?

ET: It all began during my graduate school experience, when the stem cell field first learned that we could reprogram mouse cells to a pluripotent state. Soon after we learned that we could do the same with human cells. My grad school lab quickly pivoted from working with mouse embryonic stem cells to working primarily with human iPSCs. In those early years working with embryonic cells, we mostly thought about how to use them to study developmental biology and develop drug screening platforms, but we did not think seriously about using them for human medicine and building cell therapies. This quickly changed once we were able to harness human iPSC reprogramming technology.

In the beginning, we faced significant technical challenges. It was much more difficult to perform reprogramming without the commercial products available to us now. It was hard to reproduce differentiation protocols from literature because the reagents and techniques were

DOI: 10.18609/cgti.2022.209

"...iPSC and genome editing fields have been advancing, engineered T cell therapy has progressed tremendously and taught us what applications we could pursue in the oncology setting."

not consistent from lab to lab. Over the ten years that I worked on establishing these techniques at CCRM, I saw rapid progress in standardization of research tools and techniques such as feeder-free media, defined culture matrices, improved passaging techniques, and even suspension-based culture systems. It has become much easier to think about producing iPSCs and their differentiated progeny at scale and with the consistency and quality required for clinical application.

Once joining Notch, I was fortunate to continue working closely with my former colleagues at CCRM to translate our research reprogramming protocols into a manufacturing process that was used to establish our clinical iPSC lines within their good manufacturing practice facility. Most recently, we've been able to use genome engineering tools to accomplish precision editing, including knockouts of certain genes and site-specific insertions of transgenes. With these tools in place, we can now turn our focus to thinking about the design of clonal products that we could create.

At the same time as the iPSC and genome editing fields have been advancing, engineered T cell therapy has progressed tremendously and taught us what applications we could pursue in the oncology setting. There has been a convergence of these ground-breaking technologies, and as they come together, we believe that we have a path towards treating human cancers with edited iPSC-derived immune cells. To me, the progress has been very rapid – we went from Nobel Prize-winning scientific discoveries to being on the verge of clinical application in an impressively short amount of time.

Q Coming to the present day, what do you regard as the state of the art in gene editing applied in the creation of master iPSC banks, currently? What is possible today?

ET: Companies like ours plan to create cells with multiple edits. To enable an allogeneic product, we must introduce edits to overcome both graft versus host disease (GvHD) and rejection of that product once it is infused into the patient. At Notch, we are working on ways to improve the efficiency of gene editing. If we wanted to have a triple knockout cell line with multiple transgene insertions, how could we make that as quickly as possible? If you introduce the edits sequentially, it can take a long time to build a product. We have been

working on maximizing the efficiency of any given single edit and thinking about different strategies for multiplexed engineering. This must all be done using processes and materials that will permit the use of the cell line in the clinic. In addition, in anticipation of scrutiny on the clonality of the master cell bank, we have employed high-throughput cell printing and imaging instrumentation that can document that our edited cell lines are derived from a single cell. We are focused on creating an integrated process by which we generate edited iPSCs reproducibly with high efficiency and ensure that in each campaign, given the number of clones that can be screened, we can find a clone that meets all the criteria of a clinical candidate.

Q Can you expand on the some of the successes and the remaining obstacles in successfully differentiating iPSCs for specific immune cell therapy modalities?

ET: I have always considered T cells to be one of the most challenging cell types to differentiate. Early on in the iPSC field, we saw evidence that we could differentiate into many useful cell types, including cardiomyocytes, neurons, retinal cells, and pancreatic cells to name a few. But it has been a long time coming to see T cells being differentiated, to be able to specify which type of T cell is produced and to ensure it has therapeutically useful functional properties. Adding to this challenge, the field has also struggled to produce hematopoietic precursor cells with lymphoid potential. Without sufficient and consistent sources of the precursor cell, downstream exploration of the right conditions for T cell maturation was naturally hampered.

To solve this, Notch first focused on developing high efficiency and scalable hematopoietic precursor manufacturing. These precursor cells are cryopreserved to establish a consistent input material for the subsequent stage of differentiation. Next, the Notch bead technology is used to drive rapid expansion and differentiation to progenitor T cells in scalable suspension bioreactors. Entering into the final stage of differentiation, we have ample material to compare various media and processing steps to optimize the final maturation to CD8 single positive αβ T cells. The final step is to iterate and compare the iPSC-derived T cells to primary T cells to achieve the functionality and potency desired in a clinical product.

Q How is the analytical side of things keeping up with innovation in this field? Are there any shortfalls that require attention?

ET: We are heavily editing iPSCs and we need to develop assays that can monitor for any mistakes introduced during that process. Innovation, standardization, feedback from regulators will all be helpful in developing approaches to survey for off-target edits.

DOI: 10.18609/cgti.2022.209

Secondly, iPSCs can acquire culture-associated abnormalities and as such, iPSCs are always going to be scrutinized for their genomic stability throughout reprogramming, *in vitro* expansion, and differentiation. At Notch we think about how to best apply a genomic integrity tracking and monitoring program across our research and development work to determine whether any of our processing steps have introduced a genomic instability. No single genomic integrity assay tells the whole story. Whether deploying karyotyping, array comparative genomic hybridization (aCGH), or analysis of specific regions that are likely impacted by abnormalities, the resolution or coverage of any one particular assay will have limitations.

Q What are the remaining steps to successful commercialization in the area of iPSC-derived cellular immunotherapies - what will they involve and require?

ET: Many future challenges remain to be solved for iPSC therapies to progress from clinical to commercial success. Many of those challenges will be addressed in a stage-appropriate manner. That said, when we began this work at Notch, we recognized that iPSC therapies have a unique catch. The iPSC line that you use for clinical development will ideally also be used long-term in the commercial product. You do not want to have to go back to the beginning and start with a new cell line because you did not keep commercial use in mind when sourcing and testing the original donor material or you did not have adequate documentation and traceability of materials during the initial derivation and editing of the line. Furthermore, your development team would ideally love to be working on the clinical cell line as early as possible while they test the function of edits and optimize the differentiation processes. There is enough information existing now to support companies to generate their iPSC starting material so that it can be used in the long-term.

Another key aspect of getting ready for commercialization is scaling up your manufacturing process. In my team, our approach is to work on scalable systems that can be translated to larger volumes without completely changing the culture format. In particular, the transition from adherent to suspension-based culture is a challenging step. We do not want to have to re-discover our differentiation process down the road. Although not widely used for iPSC expansion and differentiation protocols, I am a big proponent of working in stirred-tank bioreactors, and we have now demonstrated each stage of our process in a platform that spans volumes from 200 mL to 50 L. Now I have confidence that I can stop scaling at 1, 3 or 10 L, as dictated by our stage of

"Many future challenges remain to be solved for iPSC therapies to progress from clinical to commercial success. Many of those challenges will be addressed in a stage-appropriate manner."

development, but I know that the equipment is available to scale-up further when required. Investment in early translation to a scalable platform will pay dividends in the future.

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

ET: At Notch, we have made good progress on our cell differentiation process and its scalability. A key priority now is to define a product profile that we want to invest in moving into the clinic. We want to be able to iterate on a product design. One thing that is notable about building iPSC products is that the design is locked in once you introduce the edits into the cell line. If you do not have the throughput to generate and test multiple designs, you are not going to be able to quickly solve challenges you might see in preclinical development that would prevent you from moving a product forward. From an operational perspective, my priority is to think about how to design our workflows at Notch to enable that rapid iteration of product design, to ensure we make efficient progress towards the clinic.

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DOI: 10.18609/cgti.2022.209

AUTHORSHIP & CONFLICT OF INTEREST

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

Acknowledgements: *None.*

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

Funding declaration: *The author received financial support for the research, authorship and/or publication of this article from Notch Therapeutics.*

Article source: Invited; externally peer reviewed.

Submitted for peer review: Oct 27 2022; **Revised manuscript received:** Nov 22 2022; **Publication date:** Nov 28 2022

STARTING MATERIALS SOURCING & MANAGEMENT

CHANNEL CONTENT

Building resilience into post-pandemic cell & gene therapy supply chains

Tiffany Clement, Cell Therapies Core Inventory Management & Support Services Manager, Moffitt Cancer Center **& Cheryl Cox,** Director of Operations of the Cell Therapies Facility, Moffitt Cancer Center

VIEWPOINT

"There is a need in the industry for customers to know who they are ordering from, and to establish a purchasing partnership with manufacturers to ensure that raw materials of sufficient quality are reliably delivered."

On October 19th 2022, David McCall, Commissioning Editor of *Cell & Gene Therapy Insights*, spoke to Moffitt Cancer Center's Tiffany Clement and Cheryl Cox about trends and issues in cell and gene therapy supply chain management. This article has been written based on that interview.

Cell & Gene Therapy Insights 2022; 8(10), 1381–1384

DOI: 10.18609/cgti.2022.202

COVID-19'S IMPACT ON THE CELL & GENE THERAPY SUPPLY CHAIN

COVID-19 has impacted supply chain for numerous sectors. However, cell and gene therapy is unique due to the complexity of manufacturing. Furthermore, we rely on partnerships with both industry collaborators and academic Principal Investigators doing their own studies, each of whom have different needs regarding budgets and funding.

Not only did COVID-19 shift the supply chain to an environment where materials became scarce, but it also raised prices of those items. On the academic side, many people were priced out of completing their research due to these price increases. Funding and budget constraints have been an unexpected side-effect of the pandemic. Additionally, many Centers have faced issues with limited staff, as well as challenges surrounding recruitment of high-risk patients with compromised immune systems. COVID-19 has certainly highlighted the weak spots of this sector.

Many protocols for investigational new drugs (INDs) indicate specific manufacturers that must be used for supplies. This makes substitutions impossible, even down to certain pipette tips or plastic cryobags. Alterations to suppliers require completion of an IND amendment. It can be difficult to find replacements and perform the change controls to be able to purchase the supplies and equipment to move forward.

POSITIVE CHANGES STEMMING FROM THE PANDEMIC

One positive that came from the pandemic is the flexibility in sourcing that was established when COVID-19 hit. Previously, IND amendments were required to get alternative sources approved, but this process became more relaxed in the COVID-19 era. Materials and processes that were once standard had to change and alternatives had to be found. Today, new vendors and manufacturers are entering our space, bringing with them greater competition and consequently, better pricing for similar products. This allows more options and greater freedom to source other materials while keeping to a budget.

As the move to alternative materials is made, the flexibility of processing can also increase. Process improvements were made possible that previously would not have been attempted if the same material had been used every time.

FUTURE STEPS TO MAKE SUPPLY CHAINS MORE RESILIENT

It is important to consider purchasing power in forming partnerships with suppliers. There is a need in the industry for customers to know who they are ordering from, and to establish a purchasing partnership with manufacturers to ensure that raw materials of sufficient quality are reliably delivered. On the front end, two or three potential suppliers should be identified early in development. Looking at multiple suppliers at this stage

reduces risk later in processing. It is key to ensure that these partnerships are formed with a clear understanding of the production schedule that the manufacturers are working to. This will enable you to see where the constraints are.

Adjusting inventory models has been a particular focus of ours. Many of the inventory models in the cell and gene therapy space are based on the minimum and maximum amount of a product that we want to keep, which accounts for turnaround time from the vendor to the dock. However, it has become apparent that some of these inventory models are not holding up well in this space, and in the coming years this will need to be revisited. For now, there are some adjustments to be made to account for extended back orders. For us as a department, it has been important to be transparent with new potential partners regarding the materials being used and the issues that have been seen since 2020.

Early in process development, it is important to establish more than one vendor for critical materials, such as medias and bags. During validation, consider your options and incorporate them into your validation processes. This will mean that at the point of writing your IND, you can establish a preferred vendor, in addition to other validated vendor options. This will allow for quick vendor changes to occur and reduces risk to the patient population.

FURTHER DISRUPTION TO SUPPLY CHAINS IS TO BE EXPECTED MOVING FORWARD

As we continue to move past the issue of scarcity of materials due to overordering, extended back-order timeframes will become apparent due to lags in manufacturing. It is likely there will be an increase in issues on the side of the manufacturer of raw materials and consumables. Many of these manufacturers are attempting to adjust naturally to an influx of orders, but they lack the infrastructure to support the increase in demand. Alongside this, there is also likely going to be an increase of quality issues in products. As manufacturers continue to ramp up production to meet demand, quality issues may arise due to substitution of raw materials that can no longer be produced.

BIOGRAPHIES

TIFFANY CLEMENT is the Cell Therapies Core Inventory Management & Support Services Manager at the Moffitt Cancer Center in Tampa, Florida. She maintains over 10 years of experience in supply management, working directly with a variety of industries to improve all aspects of supply management, including process redesign, automated warehouse design, operations strategy planning, organizational change, and strategic sourcing. She is a graduate of Saint Leo University in Florida and holds a Master of Business degree in Healthcare Management.

CHERYL COX is the Director of Operations of the Cell Therapies Facility at Moffitt Cancer in Tampa Florida. Cheryl has provided leadership for cellular therapy facilities for eight years. At Moffitt worked on the production of various cellular therapies clinical trial products, overseen BMT Standard of Care, and the implementation of commercial FDA approved products. Cheryl is an approved FACT auditor, Women in Science, a Lab committee member of ISCT and an active member of SITC.

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AUTHORSHIP & CONFLICT OF INTEREST

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

Acknowledgements: *None.*

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

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

ARTICLE & COPYRIGHT INFORMATION

Article source: Invited; externally peer reviewed.

Submitted for peer review: Oct 19 2022; **Revised manuscript received:** Nov 2 2022; **Publication date:** Nov 11 2022.

DOI: 10.18609/cgti.2022.202

CELL & GENE THERAPY INSIGHTS

RAW & STARTING MATERIALS QUALITY, SOURCING & MANAGEMENT

INTERVIEW

The drive for standardization in cellular starting material collection for cell therapy

David McCall, Commissioning Editor, *BioInsights,* speaks to **Anh Nguyen Jones,** Senior Director, Cell Therapy Patient Operations, GentiBio

ANH NGUYEN JONES has over 13 years of Cell and Gene Therapy experience advancing multiple cell therapy programs through clinical and commercial development working closely with Clinical, Commercial, Quality and Technical Operations. Anh joined GentiBio in 2022 as the Senior Director of Patient Operations, leading the teams responsible for the management of cell collection centers, patient scheduling, cell logistics and chain of identity. Prior to joining GentiBio, Anh was Director of Global Apheresis Operations at Bristol Myers Squibb (previously Celgene, Juno Therapeutics) where she led a global team that built and maintained the global network for cell collection centers in support of all cell therapy clinical trials and the commercialization of Breyanzi and Abecma across the globe. Prior to BMS, Anh was in varying roles within Quality Assurance and Apheresis Operations at Dendreon, commercializing the first FDA approved cancer vaccine, Provenge. She holds a BSc in Biochemistry at the University of Washington.

Cell & Gene Therapy Insights 2022; 8(10), 1393–1397

DOI: 10.18609/cgti.2022.204

Q What are you working right now on?

ANJ: GentiBio is developing engineered regulatory T cells (Tregs) that establish immune tolerance and provide tissue-specific immune suppression to treat patients living with autoimmune and autoinflammatory diseases. Our lead candidate is GNTI-122 for new-onset type one diabetes.

My recent work has been on oncology products. Therefore, learning about the different disease states and cell collection starting materials has been exciting. It has been quite a journey to seeing the different treatment modalities that are available to patients today, and I have been very fortunate to work at the forefront of amazing science and technology over the last few years.

Outside of GentiBio, I am participating in the Cell Collection Standards for Cell and Gene Therapies project. This is led by the Parenteral Drug Association (PDA) and the Standards Coordinating Body for Gene, Cell, and Regenerative Medicines and Cell-Based Drug Discovery (SCB). It is aimed at developing a standardized cell collection manual that can be utilized across industry. There has been an increase in the number of gene therapy companies which has led to a high demand for the same resources at treatment sites, cell collection centers, and cell processing labs. Furthermore, each company has its own nuanced way of utilizing the cell collection starting material, whether it be fresh or cryopreserved, and so the PDA is setting the standards to ensure that the collection manual is always in the same format. This will enable those who receive the manual to know exactly where to look for the information they need regarding the collection requirements, parameters, definition for collection, standardization for the collection start and end time, product sealing requirements, tubing needs, and methods of testing. This initiative involves collaboration with academic institutions such as the Association for the Advancement of Blood & Biotherapies (AABB) and Foundation for the Accreditation of Cellular Therapy (FACT) as well as industry partners.

On top of that, I am part of the Industry Working Group for Site Certification and Qualification of Cell Collaboration Centers and Treatment Sites, which has very similar objectives to the Cell Collection Standards project. As previously mentioned, one of the things we recognized early on was that treatment sites, collection centers, and cell processing labs become overburdened with companies requesting audits for these sites. Some of these centers are already FACT accredited, AABB certified, and inspected by the FDA – how do we reduce that audit burden on those centers so that they can focus on the patients instead of the administrative paperwork? The site certification is building a risk-based approach so that new companies know how to qualify and where to start. Our goal is to reduce the time and effort required for manufacturers and sponsors to qualify and audit these centers, and reduce the amount of time and available staff the centers themselves require to support this process.

Q What are the challenges or considerations for autologous and allogeneic Tregs as a starting material for cell therapy?

DOI: 10.18609/cgti.2022.204

ANJ: Specifically for autologous therapies, there is still a one-to-one model with the patient as the source or a company to generate a batch of cell therapy product. The model itself is a challenge, of course, as you must ensure that the patients are eligible for the trial or the product and are healthy enough to undergo a cell collection procedure. Once the patient is deemed eligible, the cell collection appointment must be aligned with the manufacturing schedule. Additionally, there is a chain of identity and chain of custody challenge with ensuring that the right product is returned to the right patient every time. That level of complexity requires a high-touch, personalized cell therapy supply chain approach. It requires a team of individuals to track and monitor the cell therapy patient journey from cell collection to manufacturing to the delivery of the product to the administration location. Overall, the challenge is with scalability: operating with a one-to-one process is obviously limiting in this regard. Other challenges include low volumes of cell collection materials to start these processes. Autologous cellular starting materials are often high cost. Finally, we need to ensure consistency in the starting cell collection material, and ensure that these collection centers are able to collect based on a company's specific requirements.

For allogeneic therapies, there is no dependence on the patient, and a single healthy donor's cell may be utilized to generate several batches of cell therapy products to be administered to multiple patients. In general, this will lower the cost of goods, but the biggest challenge here is defining your donor pre-screening requirements, eligibility criteria, and the tests that are required by regulatory agencies. Additionally, most companies require tests to identify 'ideal healthy donors'. I have been fortunate to work with companies that understand the process and product characterization data. This informs decisions on starting materials and target drug product quality profiles, helping us to understand and determine what incoming cell collection parameter best corelates with our downstream manufacturing of the drug product.

Q What are the improvements and innovations needed in this space?

ANJ: During my time working in product commercialization, I have realized the importance of flexibility. There are some operating models where you need fresh cell collection starting material, while others need cell collection material that is cryopreserved. Both have their pros and cons. By allowing centers to cryopreserve their cell collection starting material, you create logistical and schedule flexibility not only for those centers but also for your manufacturing site. If you were to manage fresh products, you limit where your manufacturing facilities can be located, and how much time you have available to get your cell collection material to the manufacturing site. If you can start with a cryopreserved material, you have more flexibility in terms of shipping the cell collection starting material. Furthermore, you are not limited by time constraints and shelf-life. However, one of the key considerations with cryopreserved materials is that they involve standardization in terms of cell collection manuals and site

certification. In the past, some manufacturers have provided either too vague or overly specific instructions for how to cryopreserve material, leading to variability. By providing clear instructions on the critical processing steps, we can help cell collection centers and adjacent cell processing labs reduce cell loss during the freeze/thaw process. Moving forward, standardization of the cryopreservation process must be considered to reduce variability, especially as the industry is constantly expanding.

"Standardization is critical in many aspects, not only for cryopreservation of the cell collection starting material or the procurement of fresh cell collection starting material through leukapheresis, but also of the manuals and qualification process."

Standardization is critical in many aspects, not only for cryopreservation of the cell collection starting material or the procurement of fresh cell collection starting material through leukapheresis, but also of the manuals and qualification process. In terms of adoption and optimization of orchestration, I see many companies offering cell orchestration platforms that are targeted towards commercialization. These processes are more modular, so people can purchase only those components they need rather than the whole system. This also relates back to standardization, as depending on whether the desired product is autologous or allogeneic, different pieces of the platform may be needed depending on what is scale- and phase-appropriate.

Q Can you summarize the key goals and priorities for yourself and for GentiBio over few years?

ANJ: As we approach the clinic with GNTI-122 as our lead candidate, we are looking at establishing phase- and risk-appropriate processes and programs to scale with the growth of the organization and its goals. Right now, we are spending time building the infrastructure that we need to start our clinical trial. For patient operations, we are building the procedures needed to qualify a cell collection center, and figuring out how our collection manual is going to be built based on what we need our manufacturing process to be. Moreover, we are developing communication plans and our scheduling process, whether it is paper-based or an electronic system. It will also be exciting for me to see what the output of the working groups in which I am involved will be, and to share that with the rest of the industry and the other organizations that are just starting up in the space.

DOI: 10.18609/cgti.2022.204

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AUTHORSHIP & CONFLICT OF INTEREST

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

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *The authors has no conflicts of interest.*

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

Article source: Invited.

Interview conducted: Oct 10 2022; **Publication date:** Nov 22 2022

NOVEMBER 2022 Volume 8, Issue 10

CELL & GENE THERAPY INSIGHTS

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INNOVATION INSIGHT

Diagnostic & therapeutic uses for extracellular vesicles: insights into a rapidly evolving field

Stefan Wild (Moderator), Joana Correia, Antonin de Fougerolles & Joshua Welsh (pictured from left to right)




Extracellular vesicles (EVs) are an important means of intracellular communication secreted by cells and can be utilized for diagnostic as well as therapeutic purposes. In this roundtable discussion, four industry experts discuss challenges in the field of EVs, and their potential for use.

Cell & Gene Therapy Insights 2022; 8(10), 1247–1255

DOI: 10.18609/cgti.2022.183

SW: What is driving the growing interest in EVs in therapeutic education and research? Where are we now, and what is next for this area?

JC: Exogenus was founded seven years ago, and back then there were only a handful of companies worldwide exploring EVs as a therapeutic tool. Now, the field has totally changed. We see many companies working in the field, some of which are reaching the clinic. We are at the turning point of this new field of therapeutics. The use of EVs for diagnostics is moving fast. Although this is a challenging scientific area, with some remaining bottlenecks, it is an interesting time to be working in the field.

AdF: A revolution has occurred in the last ten to fifteen years in genetic medicines. Gene editing and gene therapies have now developed to a point where making the drug is no longer the main issue. These technologies suffer from a difficulty in the safe and effective delivery of drug payloads.

Most of those are only effective in one cell type. I helped develop the lipid nanoparticle and conjugate and the eye technology while at Alnylam Pharmaceuticals, which works well but only for hepatocytes. Likewise, at Moderna, we developed mRNA, but that is only largely being used in a vaccine context. Many great innovations are being held back by an inability to deliver safely and effectively. Lipid nanoparticles are one option where a great deal of work is ongoing.

One thing to consider is the means that cells in nature use to communicate and deliver payloads. These payloads could be proteins and RNA. As our knowledge of extracellular vesicles (EV) biology expands, we are now able to use that knowledge to load varying drug classes into the exosomes and thereby deliver them to new tissues in a safe, non-immunogenic way.

SW: What is going on right now in the research field of EVs?

JW: Interest in EVs generally is growing at a huge rate, irrespective of application. There is a huge body of literature showing functional studies of EVs and their ability to modulate cells downstream, and potentially deliver cargo. One of the things that has helped facilitate this is the growing use of technology that allows people to detect EVs.

The idea of EVs as therapeutics is going to be important, though we need to be careful that what we are quantifying are actually EVs. There are many manuscripts in the literature that identify EVs as the active component of a functional study, when this is in fact extracellular RNA or proteins. We must take care to demonstrate that EVs are the active component and not co-isolates with EVs. This is important particularly in therapeutic applications, to

DOI: 10.18609/cgti.2022.183

allow us to properly isolate the active component of a therapeutic without the risk of downstream adverse effects. It is a tricky topic because we are working in the 30-150 nm region with the vast majority of these EVs that are being used as therapeutics.

SW: Size is a critical point; many technologies are limited due to the small size of the EVs.

"Size is a critical point; many technologies are limited due to the small size of the EVs."

- Stefan Wild

Q What are the main differences or advantages of EVs compared to cell therapies?

AdF: Analytics are key. The sorts of tools that we have at hand, from an industrial perspective, allow us precise analysis of the EV population and their location.

In terms of EVs versus cell therapy, both can work well. EVs have the advantage in that you can quantitate their number and the amount of active ingredients you have. Whereas, in cell therapy, much of that will happen once it has been injected into a patient. Both are useful.

Many people working in cell therapy are trying to substitute EVs for cell therapy. My concern there is getting a good understanding of the active ingredient in the cells or in the EVs. We at Evox have always been direct and deliberate in terms of how we approach engineering our EVs to contain a specific drug of choice so that we know what the active ingredient is, and how to quantify that.

Q **SW:** Where do you see the main difference between cellular therapies and EVs?

JC: Cell therapies have developed to show a lot of potential, although there are some bottlenecks that we have encountered over the last few decades, such as their stability. Some of them show immunogenic reactions. Cell therapies have been developed to substitute or reconstitute tissues and EVs can potentially do that through their uptake into the host tissues, which is a different mechanism of action.

EVs are typically more non-immunogenic. They are also more able to cross biological barriers due to their size and their biochemical and their physical characteristics. They also have the possibility to target different tissues that would be more difficult with cell therapies, as we can modify and engineer EVs to be able to target specific diseases.

JW: The stability of EVs is going to be very useful, as is the ability to produce them. Thousands of EVs can come from a single cell, and this abundance can be utilized.

AdF: There are certain advantages to EVs, such as the ability to engineer ligands on the surface of the EV to preferentially direct them to certain tissues. This kind of direction is more difficult to do with cell therapy. We have also had a lot of success in loading and delivering a range of drug cargos into the same EV. We can put Cas proteins and guide RNA into the same vesicle for gene editing. This is not something that would be possible from a cell therapy perspective, particularly as those payloads need to be inside the recipient cell.

> "Cell therapies have been developed to substitute or reconstitute tissues and EVs can potentially do that through their uptake into the host tissues, which is a different mechanism of action."
>
> **- Joana Correia**

Q **SW:** Where is the biggest hurdle we are currently facing in EV research for clinical applications?

JC: From a technical perspective, purification is a sensitive topic. There are many groups working in the field, and we now know much more than we did ten years ago, though challenges do remain for the industry. Standardization of technologies to analyze and evaluate the concentration of the active ingredient in final clinical products is still under discussion by regulators. We need to find ways to standardize to have good, measurable products.

Biodistribution and pharmacokinetics are challenging for the clinical development of products. There are technical hurdles to face because ensuring EVs are delivered to the correct tissues can be difficult.

AdF: From a Chemistry, Manufacturing, and Controls (CMC) perspective, the key challenges are downstream purification and analytics, which go hand in hand. As the analytical capability has improved, so has the ability to identify and characterize the EV population of interest. From a drug development perspective, the key is making sure that what you are delivering is reproducible and quantitative so that the preclinical studies can be supportive of your CMC process. This is where the field has evolved over the last two or three years dramatically. We now have scalable processes that are ready for the clinic.

In general, there is a gap between how many academic researchers approach quantification, and how we are developing it from an industrial perspective. There is also a challenge with

DOI: 10.18609/cgti.2022.183

some publications on EVs where we do not have a complete sense of what was actually looked at. Standardizing that knowledge base is a more broad challenge for the field.

JW: From an isolation point of view, many people use differential ultracentrifugation, and there is a platform for reporting EV isolation methods called EV track. There are over a thousand different combinations of how people perform differential ultracentrifugation to get their EV preps. The state of the field is in complete heterogeneity when it comes to isolating EVs. Many people are even starting to use a combination of methods. There is no perfect method from the research side, though some methods are better than others, and some can introduce artifacts. It has been shown that you can isolate EVs in two different ways, and they can have two different effects downstream. There can be subsets of EVs depending on the source of EVs.

It is undesirable to have a therapeutic with a component that could potentially be inhibiting or competing with the desired effect. There are no tools yet that can measure the entire diameter distribution of EVs in a high throughput fashion, although there is a real effort in the field to push these forward. There are also some standardization studies in Europe with metrology agencies so that our characterization methods can be standardized. Once we have better analytics, we can then go back and assess our upstream processes.

Q **SW:** The engineering of EVs becomes more and more popular. What would be on your wishlist for additional features to engineer onto EVs?

AdF: Our approach to EV therapeutics is taking advantage of what nature has given us. They are efficient at delivering payloads, and they tend to be non-immunogenic. A range of those molecules are important in EV biogenesis and uptake. We put in our drug, or in some cases our drug plus a targeting agent. The rest of the EV remains largely unchanged when we do that. This creates a robust platform to be repeated for various drugs without changing the underlying platform nature of the EV. In our five years focusing on the engineered exosome space, we have added ligands to cross the blood-brain barrier, target particular cells *in vivo*, load different types of drug cargo or RNA cargo protein, and cleavable linkers designed to free the drug once delivered. We have a whole toolbox designed and ready to apply.

> "From a Chemistry, Manufacturing, and Controls (CMC) perspective, the key challenges are downstream purification and analytics, which go hand in hand."
>
> **- Antonin de Fougerolles**

JC: Our experience is a bit different – we have been relying more on the potential of cells to do their job. Our aim

is to identify cells that match the needs of a disease, and then fine-tune those cells with microenvironment or genetic changes so they can produce more potentially therapeutic EVs.

There is still much to explore in the field not only from human-derived EVs, but also from other organisms. We have been seeing some interesting publications on the potential of EVs derived from other organisms, such as bacteria. There is much room to grow in the field, even without modification or engineering of EVs. I personally believe more in their potential as therapeutic vehicles.

"I would like to see improvements, from a titration point of view, for example, in knowing the concentration of EVs that are put into a dose."
- Joshua Welsh

JW: From a quality control (QC) point of view, the ability to engineer EVs is going to be incredibly important and useful. Having engineered proteins on the surface as positive controls to use as reference materials is going to be of great utility for the field. We currently lack standardization of EVs. It would be helpful to get us all talking in the same units. Engineering traverses the therapeutics, and it is going to be critical for the field to move forward in a standard way.

Q **SW:** As we near the end of the discussion, I would like to look into the future. What are your predictions about future clinical applications and how far do you think we are from these?

JW: Many industrial processes and how far they are from approval remain a mystery. I would like to see improvements, from a titration point of view, for example, in knowing the concentration of EVs that are put into a dose. At the moment, there is not a single particle technology that can detect the full distribution of EVs from the majority of cell lines with a modal point of ~50nm. Nanoparticle tracking analysis (NTA) is not standardized, and it cannot detect most things <100 nm in a heterogenous population. Resistive pulse sensing has a cutoff of 70 nm, while the most sensitive flow cytometers also have a limit of detection in the 60–70 nm region as well

Current dosages are somewhat of a guess in their orders of magnitude, and I would like to see some standardization in quantitative units before that goes forward at a clinical level. It is not reliable to change doses by orders of magnitude.

AdF: This is an evolving thing. Counting both academic and industrial partners, there are over a dozen different trials that are ongoing with exosomes. Some of them are engineered

DOI: 10.18609/cgti.2022.183

with specific drugs, and some are not. Ultimately, we are only going to learn by advancing things through the manufacturing process and gathering data from trials.

In terms of quantitating the EVs, we look at it a different way; we quantitate the active pharmaceutical ingredient (API) because that is ultimately driving our drug. This is irrespective of the EV dose. We still want to know EV dose, however, which is why we are moving towards an engineered approach. From a field perspective, there are some promising trials, but this is going to be an evolving process, as is true with any technology.

JC: We cannot forget that EVs are complex biological entities, and they do have their own protein and RNA content. Focusing on the API is the most essential part, but it is important to consider the need to characterize what is around that.

I am happy with the development of the field. Seven years ago, it was interesting to be part of the first steps of technology development, but there were very few industries, so we were alone. I am happy to see a large increase in industry interest in the field. Now, pharmaceutical industries are looking to EVs as a solution to their needs. This can bring them into clinical applications. We in the scientific community need to promote that, by generating good data, comparisons, and pointing out the fragilities of the technologies.

Hopefully, in some years from now we will see patients being treated with this tool. EVs are taken much more seriously these days as compared to ten years ago.

BIOGRAPHIES

STEFAN WILD received his degree in chemistry at the Ludwig-Maximilians-University Munich, Germany in 1998. He completed his PhD in biochemistry in 2001. From 2002 to 2005 he was working as research scientist for Memorec Stoffel GmbH. Since 2005 he is senior scientist R&D at Miltenyi Biotec. As manager, he is responsible for the development of new tools to investigate extracellular vesicles.

JOANA CORREIA co-founded Exogenus Therapeutics in 2015, after successful participation in COHiTEC program for technology commercialization. Having been awarded several honors, including three entrepreneurship prizes (Everis Foundation Award, Young Entrepreneur ANJE, and Prémio Empreendedor XXI BPI/CaixaBank), Joana has raised €3.5M in funds for R&D, and is currently a leader in the field of EV-based therapeutics recognized internationally. With a PhD in Human Biology, she dedicated 20 years of her career to R&D in the area of human diseases and healthcare, is inventor of two patents, has several publications in high-impact journals, and is an invited researcher of the Center for Neuroscience and Cell Biology (CNC, Coimbra, Portugal). Joana is an enthusiastic entrepreneur and activist, with a creative mindset and a strong drive to solve healthcare challenges.

ANTONIN DE FOUGEROLLES has been CEO of Evox Therapeutics since late 2017. Evox is developing a new class of therapeutic by using nature's delivery system, small vesicles called exosomes, to deliver in a non-immunogenic and repeatable way a wide variety of genetic medicines

(gene therapy, gene editing, RNAi, mRNA, etc) to tissues and cells that are currently inaccessible with other approaches. Tony has 25 years of biotech R&D experience and has played a key role in developing and advancing three new drug modalities towards the market (mRNA, RNAi, Nanobodies) and helped build three multi-billion dollar businesses from start-up stage (Moderna, Alnylam, Ablynx). Over his career, Tony has raised over US $150m in equity financing, been the author and Principal Investigator on over $80m in grants, has over 60 scientific peer-reviewed publications, and is an inventor on over 100 issued US patents.

JOSHUA WELSH received his PhD in 2017 at the University of Southampton, where his thesis focused on small particle flow cytometry development and standardization in collaboration with Thermo Fisher Scientific. He then joined Jennifer Jones' lab in the Translational Nanobiology Section at the US National Institutes of Health where he is focused on developing EV assays and software for translational medicine using cross-platform integration, with a particular focus on cancer. Dr Welsh is currently an ISAC Marylou Ingram Scholar, a member the ISEV-ISAC-ISTH EV Flow Cytometry working group, a member-at-large of the ISEV Executive Board, member of Current Protocols in Cytometry Editorial Board, Chair of the ISEV Rigor and Standardization: EV Reference Material Task Force, member of ISAC Data Standards Committee, and currently co-organizing the MISEV22 guidelines. More information at: www.joshuawelsh.co.uk

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

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *JW discloses he has royalties on US NIH intellectual property. He is a member-at-Large, ISEV Executive Board, member of ISEV-ISAC-ISTH EV flow cytometry working group, Associate Editor at Current Protocols, member of ISAC Data committee and Chair at ISEV Rigor and Standardization, EV Reference Materials Task Force. JSC discloses she is a shareholder and employee of Exogenus Therapeutics The other authors have no conflicts of interest.*

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

ARTICLE & COPYRIGHT INFORMATION

Article source: This article is based on a expert roundtable discussion which took place on Sep 16 2022, and can be found here.

Expert Roundtable discussion conducted on: Sep 16 2022; ***Revised manuscript received:*** Oct 18 2022; **Publication date:** Nov 30 2022

DOI: 10.18609/cgti.2022.183

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CELL & GENE THERAPY INSIGHTS

INTERVIEW

CAR-T cells: from cancer to autoimmunity

Charlotte Barker, Editor, BioInsights, speaks with
Dimitrios Mougiakakos, Professor & MD, Department of Hematology & Oncology, University Hospital Magdeburg

PROFESSOR DIMITRIOS MOUGIAKAKOS, MD has been the director of the Hematology and Oncology department at the Otto-von-Guericke University in Magdeburg, Germany since November 2021. He studied medicine in Hannover and performed his residency at the Universities of Freiburg, Regensburg, and Erlangen, Germany. During his postdoctoral studies at the Karolinska Institute in Stockholm, Sweden he focused on immunotherapeutic concepts against malignancies and against hyper-inflammatory conditions. Moreover, he has a special interest in understanding the metabolic perspective of heterocellular crosstalk and to use these insights for improving immunotherapies. Following his return to Erlangen he was strongly involved in developing and leading the local cell therapy program, where patients with refractory SLE were treated with anti-CD19 CAR-T cells for the first time.

Cell & Gene Therapy Insights 2022; 8(10), 1143–1150

DOI: 10.18609/cgti.2022.171

Dimitrios Mougiakakos joins us to discuss the exciting potential for CAR-T cell therapy for autoimmune disease and how his team approached GMP manufacturing in the clinical setting.

Q How did you first become interested in cell and gene therapy?

DM: As a student, I was very interested in the immune system. I studied medicine at the Medical School of Hanover and my doctoral thesis was on dendritic cells as a key antigen-presenting cell population of the immune system in the context of leukemia.

My interest in immunology continued and as a young hematology resident, I decided to pursue post-doctoral training in Rolf Kiessling's group at Karolinska Institute in Stockholm. Professor Kiessling, the discoverer of natural killer cells, was very much interested in using dendritic cells as a vaccine for cancer patients, so that brought me into contact with cell therapies.

While in Sweden, I also developed a very strong collaboration with Katerina le Blanc, a pioneer in using cell therapy to treat hyper-inflammatory conditions, such as after bone marrow transplantation. Between these two groups, I saw cell therapy both driving and controlling immune responses.

When I returned to Germany and continued my residency at University of Erlangen, I was naturally very interested in continuing to work with cell therapy and went on to launch the clinical chimeric antigen receptor T cell (CAR-T) program at the university's Department of Hematology and Oncology.

Q What led you to your recent work treating system lupus erythematosus ('lupus')?

DM: As a hematologist, I don't typically treat lupus patients. The initial focus of the clinical CAR-T cell program was on patients with lymphoma or other malignant entities – either commercial products or in the experimental setting.

However, my group at Erlangen had a strong connection to the department of rheumatology and immunology. I was a member of several consortia together with colleagues from rheumatology and we often met at the university café to drink coffee, eat snacks, and – most importantly – exchange ideas!

During those discussions, the idea of using CAR-T cells for autoimmune conditions came up. We and many others observed an intriguing off-target effect when treating patients with CAR-T cells targeted at B cell-derived malignancies. As well as killing CD19-expressing tumor cells, the CAR-T cells also kill the non-malignant B cells, which are also CD19-positive. We speculated that this would represent a beneficial effect in autoimmune disorders caused by B cell dysfunction, such as lupus.

Q What is the benefit of using CAR-T cell therapy over monoclonal antibody (mAb) therapies to target abnormal B cells in lupus?

DM: Our colleagues in rheumatology have used mAbs against the CD20 receptor, also found on B cells, in lupus patients, but the results were not as good as they expected. One explanation is that antibodies do not work alone but require other co-factors

DOI: 10.18609/cgti.2022.171

in order to be efficient. When a mAb binds to a target cell, in this case a B cell, it can lead to a direct cytotoxic effect; however, it's more effective to have the complement system co-acting during this time. The abnormal B cells involved in lupus produce autoantibodies that cause constant inflammation. This inflammation is mediated by complement activation, leading to a depletion of the complement factors.

Another problem is the myeloid compartment. When an antibody binds to its target cell it can activate monocyte macrophages that ingest the target cell, B cells in this case. In lupus patients, this phagocytosis can be impaired, and this may also contribute to a lack of efficacious antibodies.

Current evidence suggests that you cannot achieve a full B cell depletion in lupus patients by using mAbs, whereas CAR-T cells can bypass these limitations.

Another problem with previous attempts to control lupus using mAbs might have been the target, with most studies to date utilizing anti-CD20 antibodies. During B cell differentiation from raw B cells to long-lived plasma cells, there are changes in the phenotype. CD20 is already lost at the plasma blast stage. We know that plasma blasts accumulate in lupus patients, and this might indicate that we have not targeted the B cell population that is responsible for producing the harmful autoantibodies. This is speculative but it led us to use CAR-T cells that are not directed against CD20, but rather CD19.

A 2019 study provided support for this hypothesis, showing that CD19-directed CAR-T cells could effectively treat lupus in a mouse model [1].

We hoped that these findings would translate to human patients, with CAR-T cells leading to a deep and long-lasting depletion of B cells and driving an 'immunological reset'.

Q What have been the results of the human trials so far?

DM: In an early stage of our discussions, our colleagues in rheumatology mentioned a female patient in her early 20s, whose lupus was having a profound impact on her life. Like many people, I didn't know a great deal about lupus. I learned that in Europe there is an incidence of 100 cases per 100000 people, so we expect 300000 patients per year. Lupus can affect all organs in the body and can dramatically affect quality of life, requiring ongoing treatment. There is no standard of care for patients with refractory disease so there is a high unmet clinical need in patients that do not respond to the state of the art.

The young woman being treated in the rheumatology department had several organs affected, including her kidneys, heart, and lungs. Our colleagues had exhausted all treatment options, but her disease remained refractory, leaving her unable to attend school or go about her regular life as she had before the onset of the disease. We discussed the possibility of CAR-T therapy with this patient and her close relatives, and she told us she would like to go ahead.

Uncertain whether it would work, we set about producing the CAR-T cells. The first challenge was efficiently collecting the T cells from the patient. Patients with lupus normally have low T-cell numbers and have received several agents that can negatively affect T-cell function so we were unsure whether the cells would function correctly. But we collected enough T-cells,

transduced them *in vitro*, expanded those cells, and were able to transfuse one million fresh CAR-T cells per kilogram of body weight. To our delight, it worked!

Toxicity levels were very low, with no severe signs of the most common CAR-T cell-associated toxicities – cytokine-release syndrome or neurotoxicity. We saw a strong expansion of the CAR-T cells within the patient's own body. By day nine she reached peak expansion, with almost a third of all of her T cells being CAR-T cells.

Within three weeks, all autoantibodies were at zero, kidney function was improved and complement concentrations were back to normal. One year after CAR-T cell treatment, the patient is off medication, has no symptoms, and is effectively back to normal. CAR-T cells are still detectable in peripheral blood and for this patient, it appears that we have achieved the immunological reset we hoped for.

We have now treated six patients. The data are now being evaluated for publication and are very encouraging. The good efficacy of CAR-T cell therapy in patients with lupus is also encouraging in terms of other autoimmune disorders.

For any physician, it is our greatest achievement to see our patients doing better. Just last Thursday we had a get-together with the first few patients and treating physicians and it was wonderful to see how the patients benefited from this treatment and how happy they are with the results.

Q What were the operational challenges of setting up cell therapy in your clinical setting?

DM: The use of approved commercial cell therapy products is already very demanding, involving various certification processes to make sure that the medical team (administration, nurses, physicians) is fit to carry out such treatment and that the structure of your hospital can keep up with the requirements for cell therapy – the whole hospital structure is under evaluation.

When you want to produce CAR-T cells within your setting, there is another level of challenge. In terms of regulatory requirements, it is a huge thing to carry out because you are producing three things in one: an immunotherapy, a cell therapy, and a gene therapy.

And there are different requirements set up by the regulatory organizations for all those three therapeutic forms, and CAR-T cell producers must fulfill them all. This requires a lot of time and investment, a highly specialized team with experts in good manufacturing practice and good clinical practice, and the appropriate facilities. We were very lucky to

"One year after CAR-T cell treatment, the patient is off medication, has no symptoms, and is effectively back to normal. CAR-T cells are still detectable in peripheral blood and for this patient, it appears that we have achieved the immunological reset we hoped for."

 DOI: 10.18609/cgti.2022.171

have the support of the university and the university hospital in Erlangen to set up our own CAR-T cell unit.

Q How did advances in technologies help you overcome those challenges?

DM: Technology is developing rapidly in this area. We used the CliniMACS Prodigy® (Miltenyi Biotec) instrument, which allowed us to carry out a semi-automatic manufacturing process, with the device taking care of culturing and expanding the cells. This has several advantages. First, the process is very standardized. Second, it removes people from the bench who would otherwise be required to carry out the culturing, so we can reallocate our staff towards quality management and facility management of clean rooms. That was beneficial for the whole program because it is not only more efficient as an organizational structure, but also reduces the user-dependence of the manufacturing process.

We also benefited greatly from switching from generating the cell product on the bench to generating it within a closed system.

Q Do we need decentralized CAR-T cell production in the academic setting?

DM: It is a considerable investment; however, I believe it's important. Of course, I don't think that a decentralized CAR-T cell production will produce high-throughput cell products, like a company facility.

However, industry and academia have different perspectives, and having the two sides work together is a big advantage. Academia can do things that no company would have done. When you treat patients every day, and you have your own facility next to you, many ideas come up via this close interaction.

Q What challenges remain? Where do you see technology could make an impact?

DM: Challenges remain in three key aspects of cell therapies, particularly with CAR-T cell therapy – efficacy, timing, and safety.

Safety does not appear to be of major concern, at least in the fields where we have utilized CAR-T cells to date. I believe we can treat our patients in a very good and standardized fashion. People throughout the world have done a great job interacting and exchanging experiences to establish protocols that are beneficial for our patients. In my own experience using CAR-T cell therapies, we can manage most of the side effects. However, it's still possible to make it even safer; for example, there are developments like constructs with off-switches that allow us to deactivate CAR-T cells.

Timing is an important factor, especially when treating patients with malignant disease that is refractory or resistant to conventional therapy. Collecting the cells and generating the CAR-T

cell product can take two to three weeks. During this time, the tumor continues growing and causing problems, and we sometimes lose patients before they can receive the treatment.

There are different strategies in order to address timing. Studies have been carried out using allogeneic CAR-T cells, which can be matched to the patient, or genetically modified. Another possibility is to speed up the production time. A recent paper described generating expanded CAR-T cells in 48 h, which is amazing. A combination of those approaches will help us to treat more patients in a timely fashion.

Then we have the issue of efficacy. CAR-T cell therapy works in many malignant diseases, but it doesn't work 100% and we need to understand why. We need to understand how the disease escapes the CAR-T cell therapy, for example in solid tumors.

CAR-T cells do not work as well in solid tumors as they do in lymphoma and leukemia. There are various strategies to address this problem; one very interesting way is to reactivate CAR-T cells within a patient. This is based on evidence that in trying to enter the dense tissue of a solid tumor, a CAR-T cell 'forgets' its target, becoming dormant. You can reactivate it by vaccinating the patient with the target antigen, helping the CAR-T cell remember the target and continue its journey through this hostile microenvironment. These strategies are currently being evaluated within clinical trials and I believe we are on the right track to increasing efficacy.

Diseases outside of cancer bring other challenges. The immune system is very pleiotropic and has many functions, being involved in all homeostatic processes. Therefore, I think cellular therapies could be of use in many diseases, including autoimmune conditions and fibrosis. There have been several murine models evaluated that support the notion that CAR-T cell therapies will not be limited to the field of cancer treatment.

Q In which areas do you see the brightest future for cell and gene therapy?

DM: The field has changed the way we treat cancer patients and there is still huge potential to increase efficacy and apply CAR-T cells in a plethora of new indications.

Based on my personal experience and our observations using CAR-T cells in lupus, I think that autoimmune disorders could be the 'next big thing' in CAR-T cell treatment. We have seen amazing outcomes in our patients, exceeding the response to existing therapies.

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DOI: 10.18609/cgti.2022.171

AUTHORSHIP & CONFLICT OF INTEREST

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

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *Mougiakakos D discloses he has a grant/contract from Gilead. He recieves consulting fees from Janssen, Roche, Novartis and Gilead. He also recieves Payment or honoraria for lectures, presentations, speakersbureaus, manuscript writing or educational events from Miltenyi Biotec, BMS, Celgene. He also recieves support for attending meetings and/or travel from Janssen, Gilead, Takeda, Roche, Abbvie.*

Funding declaration: *The author received financial support for the research, authorship and/or publication of this article from Miltenyi Biotec, BMS, Celgene.*

ARTICLE & COPYRIGHT INFORMATION

Article source: *This article is based on a Podcast which took place on Aug 02 2022.*

Podcast held on: *Aug 02 2022; Revised manuscript received: Sep 23 2022;* ***Publication date:*** *Oct 11 2022.*

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CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Process development excellence to de-risk & accelerate commercialization of cell & gene therapies

Behnam Ahmadian Baghbaderani

Cell and gene therapy applications are rapidly growing, though a wide range of challenges remain in the field, especially from a manufacturing perspective. Highly debated topics include scale-up or scale-out, product quality, manufacturing timelines, robustness of analytical methods, and the characterization of cell and gene therapies products and processes. Process development is the foundation for the successful commercialization of cell and gene therapies. Lonza development services start working with our clients at the earliest stages to understand the clients' needs, focusing on the application and respective process and analytical development requirements. This fuels the development activities focused on establishing a robust and reliable process that meets the GMP design considerations for that specific application, which can then be transferred into manufacturing.

Cell & Gene Therapy Insights 2022; 8(10), 1329–1338

DOI: 10.18609/cgti.2022.193

THREE-STEP DEVELOPMENT STRATEGY

To help customers develop a robust manufacturing process in a phase-appropriate and streamlined manner, Lonza utilizes a three-step development strategy; the first step is diagnosis to de-risk, the second step is to develop and industrialize, and the third step is to confirm the manufacturing process and deliver to good manufacturing practice (GMP).

Diagnose to de-risk

The first stage of process development (PD) is focused on transferring the existing processes

that clients may have established, or developing a baseline process based on the concept and agreeing on the target criteria for that specific application. This step entails performing feasibility runs using the agreed upon baseline process, understanding the target criteria, and then performing a comprehensive manufacturability assessment. This step will help to identify any existing gaps in meeting target criteria. Subsequently, a risk assessment is conducted to ensure clients are aligned with the gaps that have been identified. In the next step of development, this manufacturability assessment will be used to carry out appropriate process and analytical development studies to ensure the manufacturing process would meet the GMP requirements and build appropriate mitigation strategies to de-risk the manufacturing.

Develop process & industrialize

Once a good understanding of the process and analytical methods performance and gaps are established, the true PD begins. Focus shifts to study design in order to address the previously identified gaps to meeting the target criteria. In this step, the goal is to perform process development and incorporate appropriate analytical methods in the process that can allow us to meet the GMP design considerations. To develop a GMP-compliant process, one of the recommended best practices is to incorporate process control and process monitoring or process analytics to help better characterize the process. This will help build understanding of how modifications to the process and development studies impact the process performance moving from one unit operation to another. Importantly, as we scale and optimize the process, this will guide how we develop the final product to ensure it meets the quality requirements that we aim for, including consideration of assays and analytical methods that allow us to properly release the final product. Performing stability testing is one critical aspect of development to ensure the final product consistently meets all quality requirements over time.

Depending on the phase of application (i.e., early- versus late-stage clinical application), the type and focus of the studies can differ. Once the timeline requirements are understood, the focus of the development studies is adjusted for early-phase and late-phase application to meet the expected target criteria and pre-defined milestones.

Confirm & deliver to GMP

The third step of development is focused on running the process end-to-end and ensuring that the process can meet the target criteria. This step involves working with the Manufacturing Science and Technologies (MSAT) team to establish any required draft documentation and confirm that the process is robust and reproducible.

'OFF-THE-SHELF' BIOASSAY LIBRARY

There is a wide range of analytical methods and assays required for cell and gene therapies, focusing on process monitoring, decision-making, and release for safety, identity, strength, and purity purposes. However, the timelines for the development of both the manufacturing process and the analytical methods for different applications can be tight. For some applications, there could be a need for specific assays that should be custom developed or modified to be incorporated into the process.

Lonza's off-the-shelf bioassay library is made up of assays that are pre-developed, optimized, and / or qualified, and can be applied to similar applications with proper evaluation. A given analytical method or assay from the library can be custom modified over a 5–7-month period, depending on the status of the assay and process requirements. However, there are existing developed and / or qualified assays that could be incorporated

DOI: 10.18609/cgti.2022.193

into similar applications with minimal evaluation and modifications. This would significantly condense the development timeframes and reduce the risk of failure of the manufacturing campaign potentially resulted from issues related to the analytical methods. These assays may require product-specific qualification, and implementation into QC labs.

DEVELOPMENT OF COMMERCIALLY VIABLE GENE THERAPIES: A CASE STUDY

Scale-up of the upstream process remains one of the main challenges in addressing demand for viral vector, and final viral vector product requirements. Figure 1 shows Lonza's AAV HEK293 process, which is based on transient transfection using suspension culture.

When scaling-up from a 3 to 250 L 3D suspension bioreactor, reliance on the traditional approach of maintaining the power volume (P/V) ratio may result in suboptimal titer at harvest at large-scale (Figure 2A). For this reason, Lonza focuses on scale-up studies based on the evaluation of a variety of different factors, including hydrodynamics of the bioreactor, CO_2 accumulation as well as timing of the stripping, and mixing time for transfection complex. Following optimization of appropriate process parameters, the relative productivity can consistently improve across different scales (Figure 2B).

A wide range of PD parameters are considered to ensure a process is both optimized and scalable, as well as meeting the target criteria. As highlighted earlier, PD relies on incorporating appropriate process control and monitoring assays for characterization purposes to navigate through any possible challenges that are encountered, thus avoiding suboptimal unit operations. For instance, absorbance ratio is a simple and straightforward analytic incorporated during the chromatography step for purification of AAVs to evaluate and maximize full-versus-empty ratio. In a recent study performed by Lonza, AAV materials from a 10 L upstream production were processed through a CIM monolithic chromatography column. It was found that the fractions with absorbance ratios ranging between 1.2 and 1.5 were high on impurities, whilst those fractions with an absorbance ratio of less than 1.2 contained more empty capsids and were potentially more highly aggregated. Mock pools were subsequently created and the samples were re-evaluated with alternative methods, such as analytical ultracentrifugation (AUC) to validate understanding of the process performance.

To ensure successful delivery into GMP manufacturing for both clinical and commercial products, process robustness must be confirmed. The sampling strategy should be finalized via close collaboration with MSAT teams taking place in the development phase. Technical support for tech transfer and manufacturing is offered, including process investigation and root cause analysis, if needed.

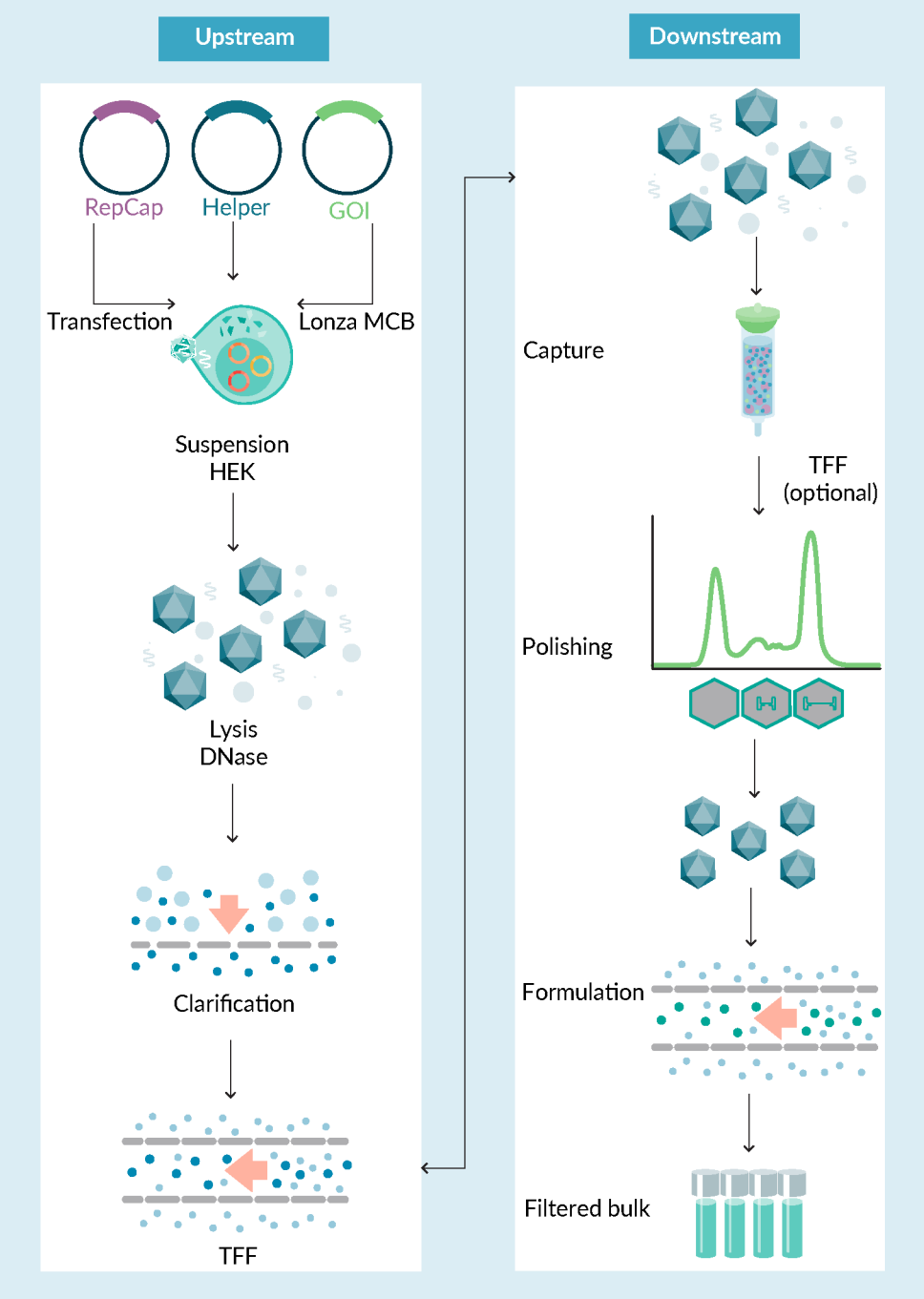
ADDRESSING REMAINING CHALLENGES TO SUPPORT COMMERCIALIZATION

It is certainly an exciting time for cell and gene therapies as more applications are moving from early clinical to late-stage clinical applications. Lonza is focused on supporting the path to BLA filing and commercialization. The key to successful commercialization is appropriate risk assessment using Failure Mode and Effects Analysis (FMEA). A collaborative approach to analyzing FMEA findings alongside the client allows us to focus on process robustness, understanding the scope of process limit evaluation, and process characterization and assay validation.

One current major challenge relates to processing of the number of samples that can be generated. For example, studies conducted in support of late-stage activities can generate thousands of samples. For most analytical technologies, there is a trade-off between high throughput and the precision and accuracy of the method. Therefore, there is a need to design and improve current

FIGURE 1

A transient transfection-based MFG process for AAV using HEK293, including upstream production of viral vector in a 3D suspension bioreactor, purification in downstream through a capture column and polishing step, and formulation of the bulk product.



methods to accommodate high number of samples produced for process characterization activities.

Management of samples (keeping track of sample locations, chain of custody) is also a challenge. Lonza utilizes a global LIMS system to track sample storage. LIMS and other sample management systems can be customized to accommodate development needs. Operational excellence strategies and practices are also essential to reduce the risk of losing samples and to gain efficiencies. Processing the samples using more automated assays, which require less hands-on time, can allow

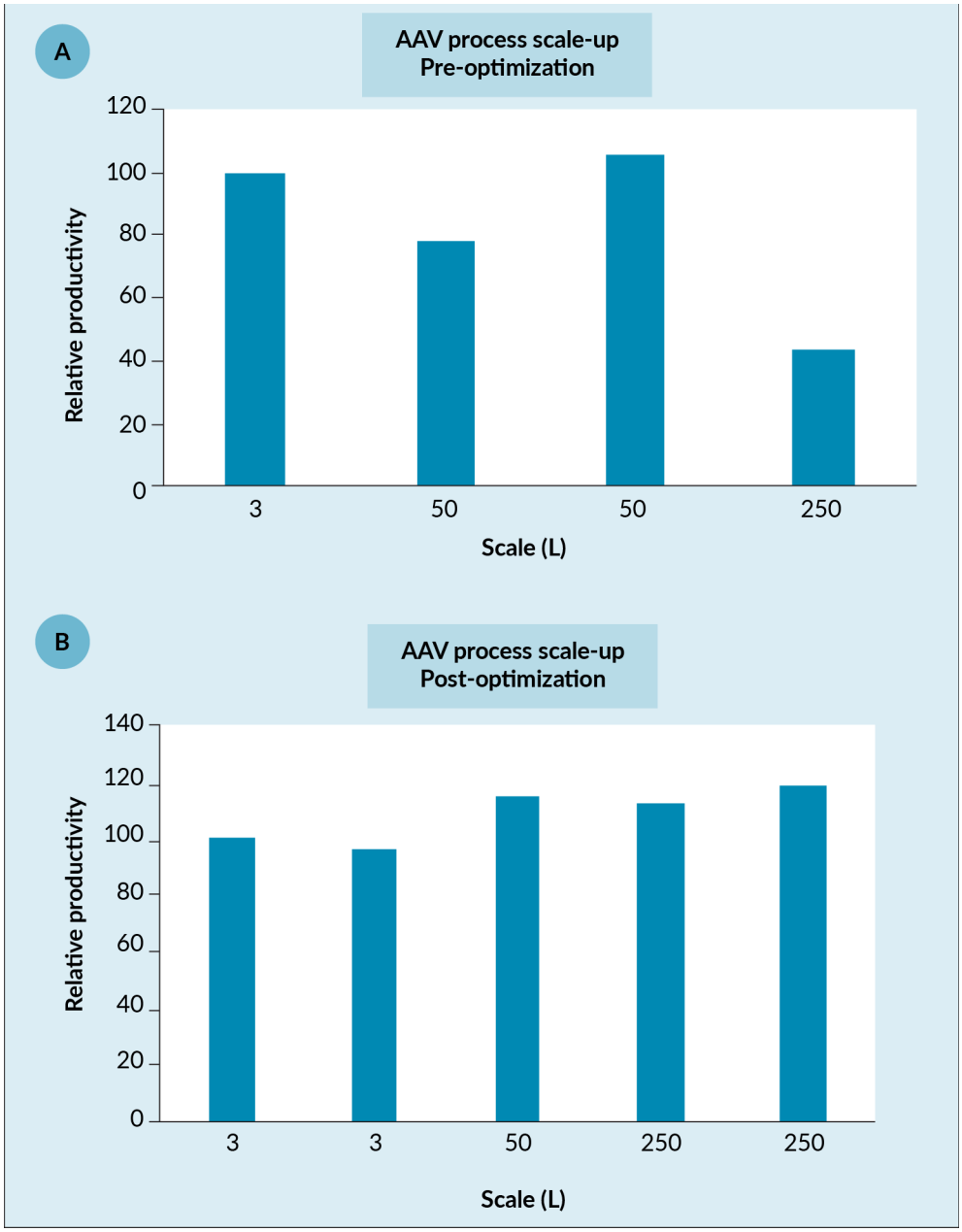
DOI: 10.18609/cgti.2022.193

us to perform the process characterization studies in a more efficient manner.

Finally, regarding the challenges and potential solutions relevant to characterization of cell and gene therapy products and final product release testing, there needs to be a specific focus on using appropriate types of assays and analytical methods, as well as assay robustness. AAV-based gene therapies require specific assays, such as testing empty versus full capsid. The most promising methods potentially replacing the gold standard of AUC are high-performance liquid chromatography (HPLC) and ultra-performance liquid chromatography (UPLC) methods. Off-the-shelf capillary isoelectric focusing is another method that could hold promise for evaluation of empty versus full capsid. As new technologies emerge and develop, it is important to incorporate them into the process during the PD studies for evaluation.

FIGURE 2

The productivity scale-up challenge, as shown by AAV Based transient transfection.



Q&A

David McCall, Editor, BioInsights
speaks to (pictured) **Behnam Ahmadian Baghbaderani, Global Head of Process Development for Cell and Gene Technologies, Lonza**

Q What analytical methods do you have available for exosome characterization?

BAB: We have invested significantly in exosomes characterization, specifically. We have established development services, both PD and bioassays services in the Europe and United States as well as a manufacturing site offering exosome manufacturing platform in large scale 3D bioreactor. We are using a wide range of technologies and a comprehensive list of analytical methods covering bulk analysis, single particle analysis, and cargo analysis. For example, we use nanoparticle flow cytometry -based technology as a highly reliable method for single particle analysis. There are also assays that could be applicable depending on the particular use of the exosome – for example, potency assays. Once again, we have established an array of assays for characterization, process testing, and release that can be offered to customers, in addition to customizing new assays.

Q Does Lonza develop flow cytometry assays for in-process and quality control (IPC/QC) cell manufacturing on different flow devices?

BAB: Flow cytometry-based assays are one of the most widely used technologies for the evaluation of identity, purity, and even for potency in combination with other methods. We have used flow cytometry for ongoing process testing, process characterization, and as a release assay. We have focused on using different generations of assays. Our focus is to make sure that these technologies comply with GMP and The International Council for Harmonization (ICH) of Technical Requirements for Pharmaceuticals for Human Use guidelines. Depending on the indication and analytical requirements, where we use the flow cytometry differs. We also believe in developing the methods that are efficient and reduce the technician time and / or operator to operator variabilities.

DOI: 10.18609/cgti.2022.193

Q What is the most typical rate limiting step or bottleneck during commercialization readiness, and what steps are Lonza taking to mitigate that kind of risk?

BAB: When it comes to commercialization, there are a wide range of initiatives and focuses that need to be harmonized and orchestrated during the commercialization readiness campaign. Firstly, it is important to work based on existing data from clinical runs and tech transfer runs to perform the FMEA assessment. This data availability is an area of challenge, in particular, there is a major gap on the assay and analytical side. This is considering the fact that assays and analytical methods sometimes have not been properly validated, or are not robust, reliable, and fit for purpose.

When it comes to commercialization, we need to ensure the process characterization studies including process limit evaluation are properly designed and executed before going into the validation strategy. Those are some of the bottlenecks and challenges that we will see during the FMEA risk assessment, and we want to work with our customers to put proper mitigation strategies and evaluation in place before going into the next level of process performance qualification (PPQ) and validation studies.

Q How do you work with customers that might want full commercialization support, minimal support, or a hybrid of the two?

BAB: For a CDMO, it is important to collaborate and build partnerships with the customers. We have established a step-by-step, harmonized, phase-appropriate development and manufacturing service. We work with the customer to ensure any existing process and methods can be transferred from customer to Lonza, and properly evaluate and use a risk assessment-based approach to define the focus of PD, whether this is related to the early phase clinical or to commercialization readiness. We want to build these relationships and partnerships by being open and transparent from the beginning. If there is no process in place, we can still work with the customer on the development using the existing proof of concept and agreed upon target criteria to develop such processes and methods.

Q In your experience, what are the most overlooked parts of PD by developers?

BAB: One of the challenges that I see often is technology. The technology development is not keeping pace with the recent development of commercially viable cell and gene therapy applications. Many of the technologies that we have seen on the process or analytical sides were developed for biologics, and may not be fit for purpose. Some of the technologies being established may require major modification before they can be used for cell and gene therapy.

On the other hand, many new technologies specifically developed for cell and gene technologies might be missing the full package of commercialization readiness or some aspects of GMP compliance and qualification.

In addition, we often deal with materials that do not have all the qualifications needed for the GMP applications. Sterility assurance is another area that could be improved when it comes to the equipment and materials used in cell and gene therapies.

Q Is Lonza open to using any cell manufacturing technology or device from different providers for PD?

BAB: When it comes to the development of client processes and protocols, we are open to new technologies. We believe that innovation is important, and new, innovative technologies can be incorporated into the manufacturing process to meet robustness and applicability requirements for CGT applications. At the same time, we need to carefully evaluate these technologies to ensure they can address manufacturing and analytical challenges, and fit the expected GMP compliance requirements. Also, we need to work with our customers to implement these technologies, considering the timeline and phase appropriateness.

Q Can you talk about a success story that you're most proud of for your PD team?

BAB: Over the last ten years of focusing on the development of cell and gene therapies, I am proud of the customers who have worked with us from early development, and continued towards late phase commercialization readiness. We have a few customers that have made it past this phase, and I am proud of empowering industrializing cell and gene therapies. I am also very proud of building a highly talented and experienced development experts across Lonza cell and gene therapies network, allowing us to extend and expand our services to a large number of customers globally.

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DOI: 10.18609/cgti.2022.193

AUTHORSHIP & CONFLICT OF INTEREST

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

Acknowledgements: *None.*

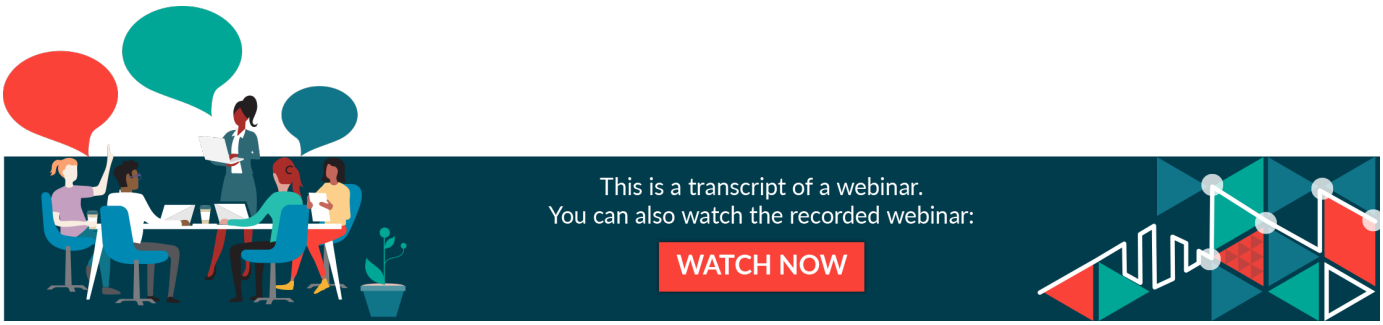
Disclosure and potential conflicts of interest: *The author is a Lonza employee and stockholder.*

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

ARTICLE & COPYRIGHT INFORMATION

Article source: *This article is based on a Webinar with Behnam Ahmadian Baghbaderani carried out on May 5 2022.*

Webinar held: *May 5 2022;* ***Revised manuscript received:*** *Nov 2 2022;* ***Publication date:*** *Nov 16 2022.*



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Roadmap to success in AAV purification. In-process control, high throughput & novel column modalities as necessary means for control over scalable AAV process

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Manufacture and purification of recombinant adeno-associated viruses (rAAV) require development and optimization of processes to ensure the best possible quality of the final rAAV product. To do so, different strategies in upstream can be used to achieve the highest possible viral titer and lowest amount of impurities, both of which further influence downstream. Second challenge involves removal of cell debris where different pre-treatments can be utilized. In the next step, optimized capture of rAAV on a cation-exchange chromatography should be developed to remove impurities and achieve a high recovery of rAAV. In the end, several chromatographic options are available to remove empty and defected capsids, so only functional viruses can be isolated. Here, the process of manufacturing and purification of rAAV has been designed using monolithic columns to achieve this important goal of preparing rAAV for the use in gene therapy.

Cell & Gene Therapy Insights 2022; 8(10), 1315–1328

DOI: 10.18609/cgti.2022.192

Purification of rAAV using chromatographic columns is one of the key purification process solutions in gene therapy industry. Common rAAV industrial manufacturing platform is based on two chromatographic steps: capture and polishing. During capture step, rAAV is

bound, concentrated, and partially purified using cation exchange or affinity chromatography. In the next step, rAAV is further purified and enriched using anion exchange column or ultracentrifugation. The overall bioprocess yield and purity of the final product depend not only on downstream steps, but on upstream process as well, since certain harvest attributes can largely affect column capacity, and consequentially downstream process efficiency. This is partially addressed utilizing different pre-treatment strategies designed to simplify and increase productivity of early downstream steps, but there is no universal solution for highly variable impurity profile during rAAV production in cells. Manufacturing process ends with buffer exchange to formulation buffer, followed by fill and finish (Figure 1) [1–4].

Therapeutic applications of rAAV-based gene therapy vectors require removal of process and product related impurities, as they represent serious safety threats. Their removal burdens the economics of the manufacturing. The most critical subset of process-related impurities are host cell impurities (hcDNA, hc proteins and chromatin complexes) as well as plasmids and transfection reagents used for rAAV production. On the other hand, the most critical subsets of product-related impurities include non-potent rAAV capsid (empty rAAV, partially filled rAAV, rAAV capsid containing hcDNA insert or portion of plasmid DNA), capsid-capsid complexes and capsid-DNA complexes [2,5,6]. Fully scalable monolith based rAAV downstream platforms can eliminate both process-specific and product-specific impurities. An optimized rAAV production process and sample pre-treatment further raise the quality of input material for monolith chromatography.

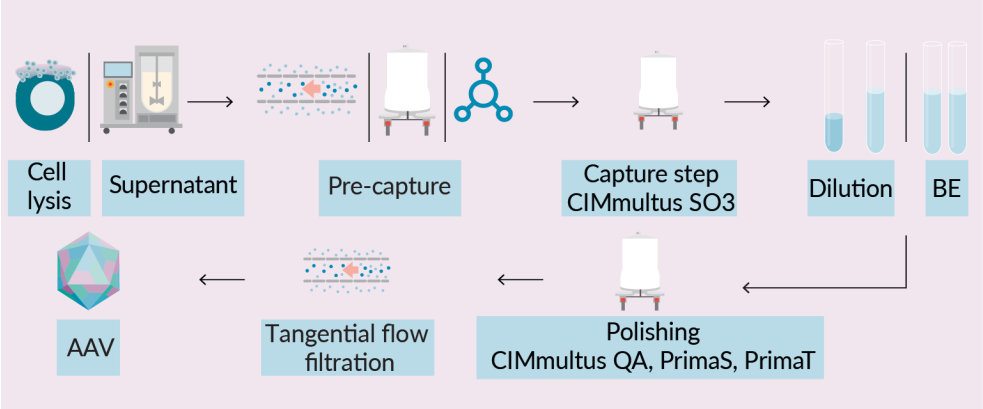
The following article shares experimental data showing how the processes of rAAV production (upstream) and purification (downstream) can be designed and optimized to achieve high viral titer, low impurities together with a sufficient percentage of full capsids. To enable process development and in-process control fast, reliable and cost-efficient, as well orthogonal to PCR and ELISA, analytics using high pressure liquid chromatography (HPLC) is needed [7,8].

INCREASE THE PRODUCTIVITY OF DOWNSTREAM WITH CONTROLLED AAV PRODUCTION

One of the key goals of rAAV upstream process development is achieving high viral titer together with a sufficient percentage of full capsids. Physical titer can nowadays be efficiently measured with digital or qPCR starting from relatively crude sample. However,

FIGURE 1

Schematic representation of downstream processing of complex rAAV samples using monolithic columns.



BE: Buffer exchange; TFF: Tangential flow filtration; AAV: Adeno-associated virus.

DOI: 10.18609/cgti.2022.192

methods that can reliably address empty/full rAAV ratio, such as AUC, SEC-MALS and TEM [8], would all require sample purification and concentration, in some cases for multiple logs, in order for sample to fall within their detection range. For that reason, the most used method for empty/full rAAV ratio assessment in crude upstream samples is a combination of ddPCR and ELISA data. These two methods differ in sample preparation requirements and basic principles for detecting the target, thus limiting the reliability of the obtained information. ddPCR/ELISA is also time consuming, and therefore less useful for rAAV in-process control in real time.

To overcome these limitations, PATfix™ Valve Switch (PATfix VS) analytical method was developed based on ion-exchange high performance liquid chromatography (IEX-HPLC) (Figure 2A). This is an automatized two-column analytics, in which acidified and clarified samples are first captured on CIMac™ SO3 column, and then redirected to CIMac AAV column, on which empty and full rAAV capsids are separated along the salt gradient. This system is equipped with multiple detectors, including UV and Multi-Angle Light Scattering (MALS), that provide chromatograms for each sample. Selective quantification of empty and full capsid populations is done through PATfix software that calculates surface area of respective peaks. With this approach, both empty and full rAAVs can be recorded using the same detector in a single measurement of the sample.

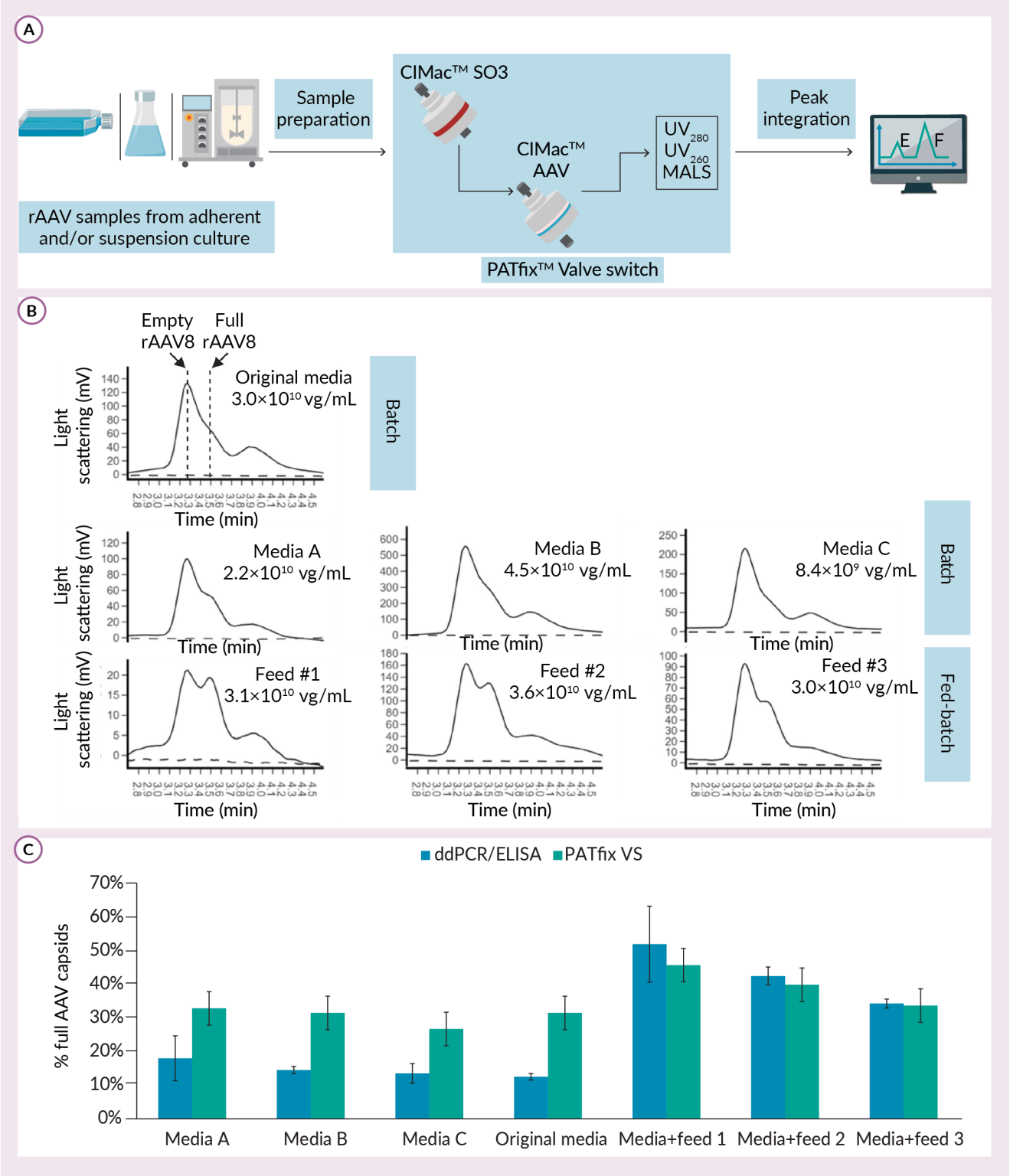
Due to its low limit of detection and sample volume required (less than 1×10^{10} of total capsids, up to 10 mL of sample), PATfix VS is suitable for early upstream process development. This is achieved with the use of improved MALS detector that functions well with relatively impure samples and has better sensitivity than UV. We used the advantage of this property for screening of different chemically defined media and feeds in rAAV8 upstream process optimization. For that experiment, cells were previously adjusted to each media, and rAAVs were produced through triple transfection in batch and fed-batch mode (Figure 2B). Compared to batch process in original media, only media B batch and original media + feed number two fed-batch resulted in titer increase. With regards to empty and full rAAV8 ratio, batch runs showed from none to modest improvement, while two fed-batch conditions incorporating feed number two and feed number one resulted in a visible increase of percentage full capsids, from 31 to 40 and 45%, respectively. Results obtained with ddPCR/ELISA method were well aligned with PATfix versus with respect to fed-batch, but not with batch samples (Figure 2C). Taken together, these results point towards partial alignment of two approaches for detecting empty/full capsid ratio. Discrepancies in PCR/ELISA were described before where poor accuracy and excessive variability were observed [8,9]. Regardless of the method used, improvement of titer and % of full capsids were not improved with a single approach tested in this experiment (Figure 2B & 2C). Nevertheless, upstream process optimization should be directed towards maximization of both parameters [10].

There are other factors known to affect the empty/full rAAV ratio, such as cell line type, viable cell density, conditions of transfection, plasmid DNA, transfection reagent and others, that can be optimized using PATfix VS analytics as a guide [10]. Moreover, the same method is applicable as at-line in-process analytics during production runs. Based on analysis of samples taken at different time points after transfection, operator can adjust the duration of the process by determining the point of time when production slows down, or the empty/full ratio starts becoming unfavorable.

Due to its robustness the method was successfully applied for analysis of empty/full ratios in complex upstream samples as well in early downstream steps, such as for example pre-capture TFF (data not shown). This allows for link-up between upstream and downstream to create more holistic approach to AAV bioprocess [10].

FIGURE 2

Analysis of empty and full rAAV8 capsid production in different USP media (in different dilutions).



A) Scheme of PATfix Valve Switch analytical method that is used as analytical tool for batch and fed-batch screening. B) Chromatograms show zoom-in view of elution gradient on CIMac AAV column, where empty and full capsids (marked with dashed line and arrow) are visualised using MALS. Percentage of full capsids calculated from ddPCR/ELISA approach and PATfix VS are compared for listed samples. C) Standard deviation of ddPCR/ELISA is calculated as per two independent functions with Gaussian distribution. For PATfix VS standard deviation is a value of 5% which was measured experimentally for repeated analysis of AAV8 standard.

DOI: 10.18609/cgti.2022.192

OPTIMIZING PROCESS CONDITIONS FOR SEROTYPE INDEPENDENT CAPTURE STEP

Downstream starts with collection of supernatant or lysis of cell culture in bioreactor. The next main goal is to reduce host cell impurities in the process of pre-capture. TFF is usually used for the pre-capture step, but to achieve the best process yield and product purity for selected upstream and serotype, one should test a wide toolbox of pre-capture methods or, if sample is very complex, even a combination of them, including – OH chromatography, flocculation, TFF, solid phase extraction, and/or nuclease treatment [2,11]. When performing capture step on monolithic columns, acidic precipitation of impurities is a part of AAV process.

Next step in the downstream process when using monoliths is the capture step using a cation exchanger in which rAAV is bound while most of the contaminants are removed [2,11,12]. On a CIMmultus™ SO3 column, majority of proteins are removed in CIP peak while small portion also in flowthrough during the sample application step. This results in better performance of the polishing step. rAAV binding is performed at pH 3.5 and elution is achieved with ascending salt concentration (Figure 3). Due to the structural nature of the monolith, rAAV does not succumb to shear stress which allows the usage of high flowrates. As a result of a high binding capacity of the column, rAAV is concentrated to target levels (1×10^{13} to 5×10^{13} vg/ml). Last but not least, the cation exchange monolithic columns allow for capturing of any serotype or AAV chimera, making these columns a perfect tool for AAV library screening [2,11–14].

The process of finding the best possible condition for SO3 chromatography can be time consuming since different parameters must be optimized to ensure the best possible binding of rAAV to the matrix. To speed up the process of screening many samples or conditions, a standard 96-well design offers a great advantage. CIM® SO3 0.05 mL Monolithic 96-well plates have been developed for this purpose.

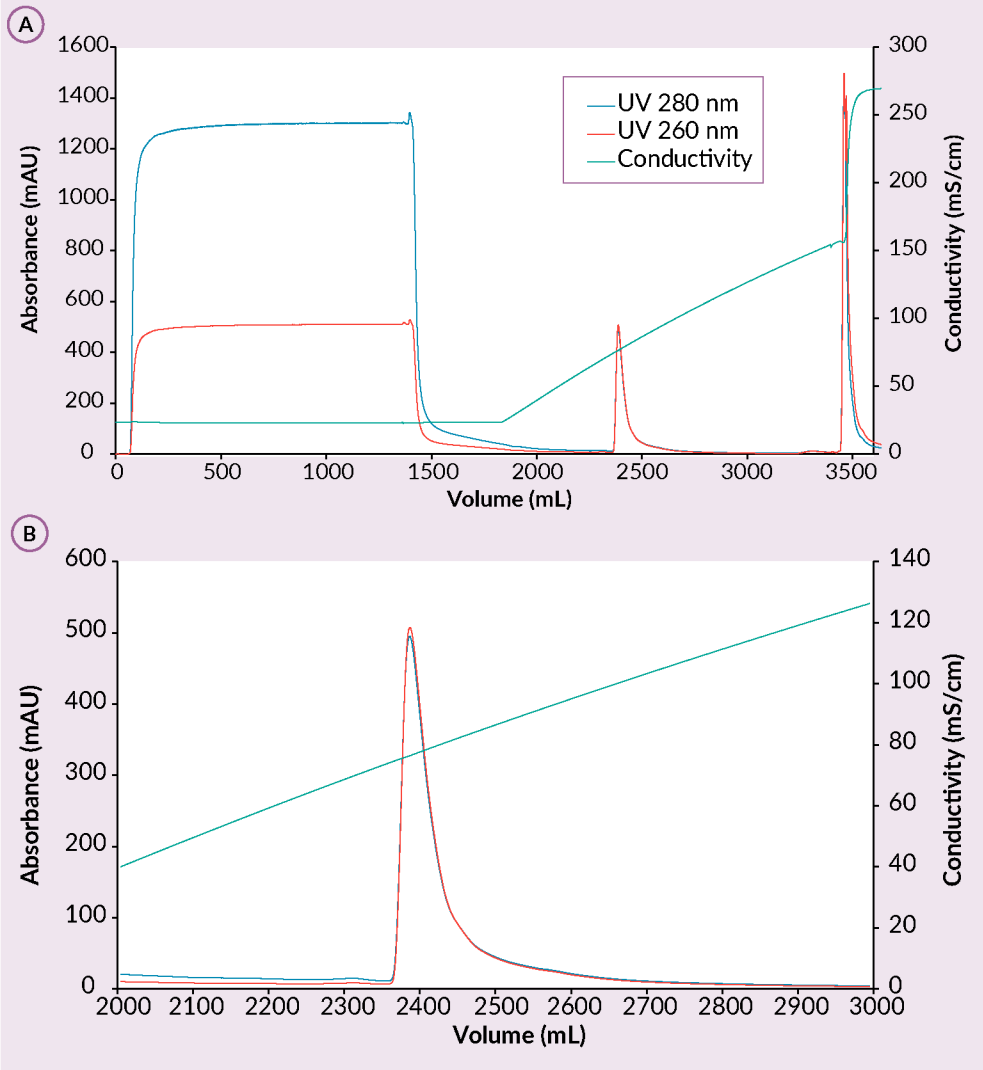
An experiment was performed to test different pre-treatment methods and screen buffers of different pH, sodium chloride concentrations and use of Poloxamer 188 to determine the optimal combination of parameters for the SO3 capture. Three different pre-treatment options were explored – acidification, TFF and TFF coupled with salt tolerant DNase. For the used sample the TFF coupled with salt tolerant DNase was identified as the best pre-purification step and was used to screen buffers using CIM SO3 0.05 mL Monolithic 96-well plates. DNase-treated TFF retentate rAAV2/9 was acidified, filtered, and loaded to the CIM SO3 0.05 mL monolithic 96-well plate. Plate was washed with different mobile phases A (MPA; different pH, sodium chloride and poloxamer conc.). Elution was performed using 4 CV of mobile phase B (MPB; buffers with corresponding pH and poloxamer conc. with 2 M NaCl). To determine optimal combination of parameters for the SO3 capture step wash and elution fractions were analysed using fluorescence microplate reader, ELISA and SDS-PAGE [14].

Fluorescence readings (FLD) of elution and wash samples from the buffer screen experiment on the SO3 plates showed stronger signal in wells where the rAAV was detected. For wash fractions, wells with higher pH and/or sodium chloride concentration in mobile phase A showed inefficient binding observed as high fluorescence signal. On the other hand, higher FLD values in elution fractions correlate with successful rAAV binding. Efficiency of rAAV capture was verified with capsid specific ELISA analytics (Figure 4A) and the impurity profile with SDS-PAGE [14].

The most optimal combination of parameters for mobile phase A from the CIM SO3 0.05 mL Monolithic 96-well plates was chosen – pH 3.5 and 500 mM NaCl in presence of Poloxamer 188. This mobile phase A was further used for experiments on CIMmultus (Figure 4B). It was shown that sample obtained by TFF with salt tolerant DNase coupled with the most optimal mobile phase A gave the highest vector recovery, highest protein/

FIGURE 3

(A) Example of a CIMmultus SO3 chromatogram and (B) a zoom in on the elution.



Conditions: sample AAV2/8 mobile phase A: 50 mM formic acid, 0.2 M NaCl, 1% sucrose, 0.1% poloxamer 188, pH 3.5. mobile phase B: 50 mM formic acid, 2 M NaCl, 1% sucrose, 0.1% poloxamer 188, pH 3.5, column CIMmultus SO3-80mL, method A to 100%B in 20CV, 3CV CIP (1M NaOH, 2M NaCl).

DNA reduction and highest dynamic binding capacity. SO3 step recovery was 87.70%, while protein and DNA content were reduced by 99,98% and 99,25%, respectively. Dynamic binding capacity was approximately 1.44×10^{14} capsids per milliliter of SO3 column. This confirmed successful optimization of the capture step [14].

CIM SO3 0.05 mL Monolithic 96-well plates are efficient and fast tool for rAAV capture step screening and fine tuning of mobile phases, including automating of procedure and analytics steps. Obtained results can be applied to CIMmultus preparative line, which is scalable to large industrial volumes.

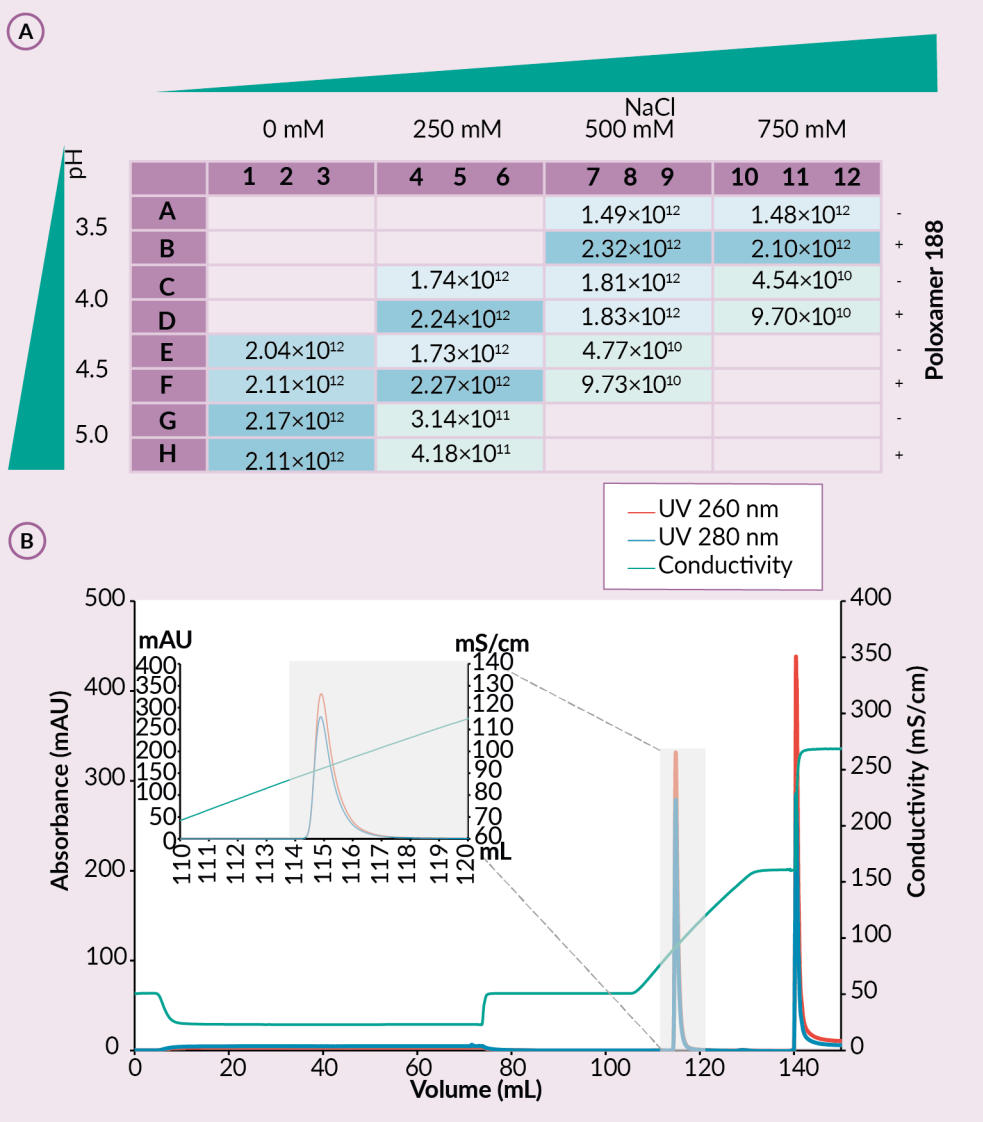
DIFFERENT CHROMATOGRAPHIC POSSIBILITIES FOR FULL ENRICHMENT. SEPARATING EMPTY AND PARTIAL CAPSIDS FROM FULL AAV

In each AAV downstream process, one of the key steps is enrichment of full capsids. Usually, only a part of rAAV capsids are functional capsids. Besides ultracentrifugation

DOI: 10.18609/cgti.2022.192

FIGURE 4

(A) ELISA analytics of elution fractions for rAAV titer and (B) elution from CIMmultus using optimized conditions.



Conditions: sample AAV2/9. mobile phase A: 20 mM formic acid, 0.5 M NaCl with the presence of poloxamer 188, pH 3.5. mobile phase B: 20 mM formic acid, 2 M NaCl, with the presence of poloxamer 188, pH 3.5, column CIMmultus SO3-1mL, method A to 100%B in 25CV, then step to 100% B in 10CV, 3CV CIP (1M NaOH, 2M NaCl).

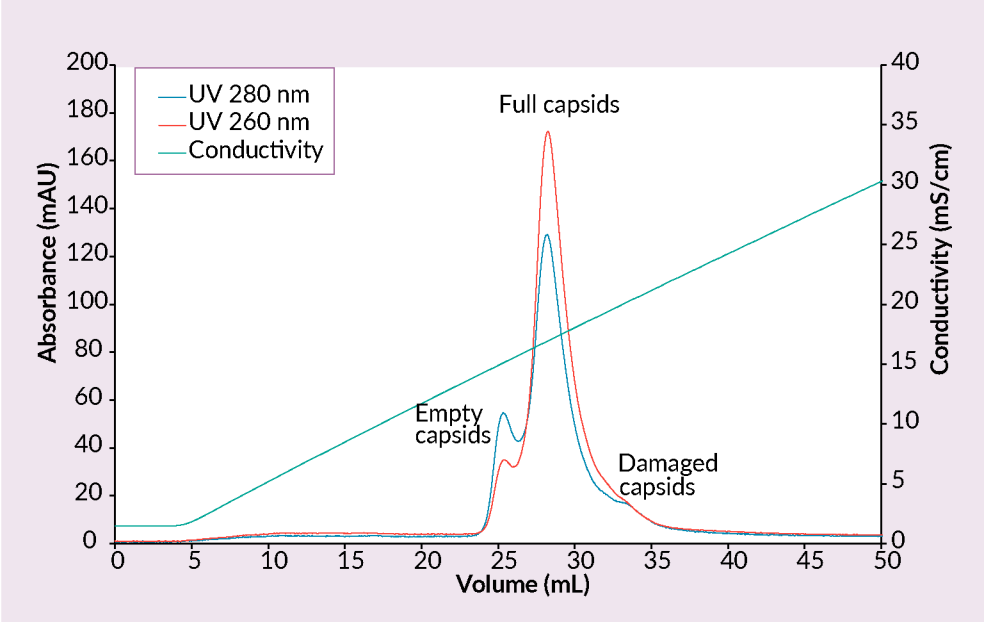
the most common approach is liquid chromatography using ion exchange chemistries, which is based on particles' charge differences. It enables separation of the full (F) capsids and product related impurities including non-functioning AAV capsids (empty, partially filled, misfolded and wrongly packaged genome or other DNA containing subspecies) [15–17].

Quaternary amine (QA) is well known and several times patented method [18,19] for the separation of empty and full capsids. CIMmultus™ QA monolithic columns exploit anion exchange mechanism; sample is loaded onto the column at low conductivity (2–5 mS/cm) and elution is usually achieved with ascending salt concentration. pH ranging from 8.5 – 9.5 should be used (Figure 5). Presence of magnesium has effect on elution and removal of empty capsids.

New ligands were explored to provide researchers with alternative options if the

FIGURE 5

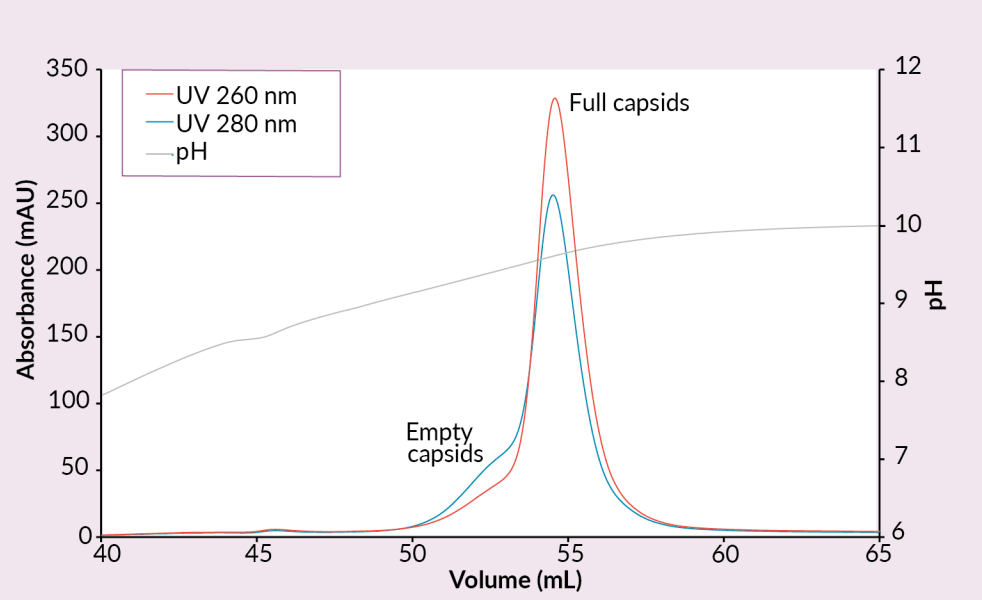
Separation of empty and full AAV capsids by anion exchange chromatography with a salt gradient.



Conditions: sample AAV2/8 (SO3 eluate), mobile phase A: 25 mM BTP, 1% sucrose, 0.1% poloxamer 188, pH 9.0, mobile phase B: 25 mM BTP, 0.5 M KCl, 1% sucrose, 0.1% poloxamer 188, pH 9.0, column CIMmultus QA-1mL, method A to 50% B in 50CV, 5CV CIP (1M NaOH, 2M NaCl).

FIGURE 6

Example of a CIMmultus PrimaS chromatogram.

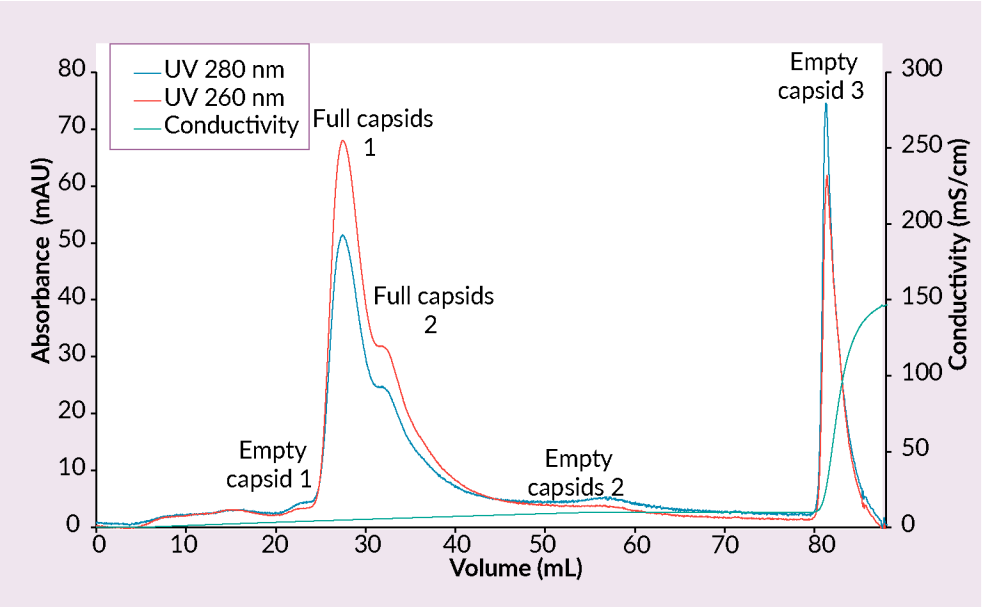


Conditions: sample AAV2/8 (SO3 eluate), mobile phase A: 10 mM Tris, 10 mM BTP, 2 mM MgCl2, 1% saccharose, 0.1% poloxamer pH 7.00, mobile phase B: 10 mM Tris, 10 mM BTP, 2 mM MgCl2, 1% saccharose, 0.1% poloxamer pH 10, column CIMmultus PrimaS-1mL, method A to 100%B in 30CV, 5CV CIP (0.1M NaOH).

DOI: 10.18609/cgti.2022.192

FIGURE 7

Example of a CIMmultus PrimaT chromatogram.



Conditions: sample AAV2/8 (SO3 eluate), mobile phase A1: 25 mM HEPES, 1% sucrose, 0.1% poloxamer pH 7.0, mobile phase A2: 50 mM Tris, 13.6 mM borate, 1% sucrose, 0.1% poloxamer pH 9.0, mobile phase B1: 50 mM Tris, 9.6 mM borate, 50 mM MgCl2, 1% sucrose, 0.1% poloxamer pH 9.0, mobile phase B2: 50 mM Tris, 12 mM borate, 2 M NaCl, 1% sucrose, 0.1% poloxamer pH 9.0, column CIMmultus PrimaT 1mL, method A2 to 100% B1 in 50CV, then step to 100% B2 for 10CVs, 5CV CIP (1M NaOH, 2M NaCl).

QA does not provide satisfactory full rAAV enrichment. Therefore, to create new opportunities for eliminating empty and other non-functioning AAV capsids from rAAV preparations CIMmultus PrimaS™ and CIMmultus™ PrimaT columns were developed.

CIMmultus PrimaS is a multimodal ligand which exploits a unique combination of weak anion exchange and hydrogen bonding that achieves different selectivity compared to the QA ligand [11,20]. Binding is performed at low conductivity (2.5 mS/cm) with the presence of magnesium (2mM), while the best separation is achieved when eluted with ascending pH gradient (pH from 7 – 10) at low conductivity.

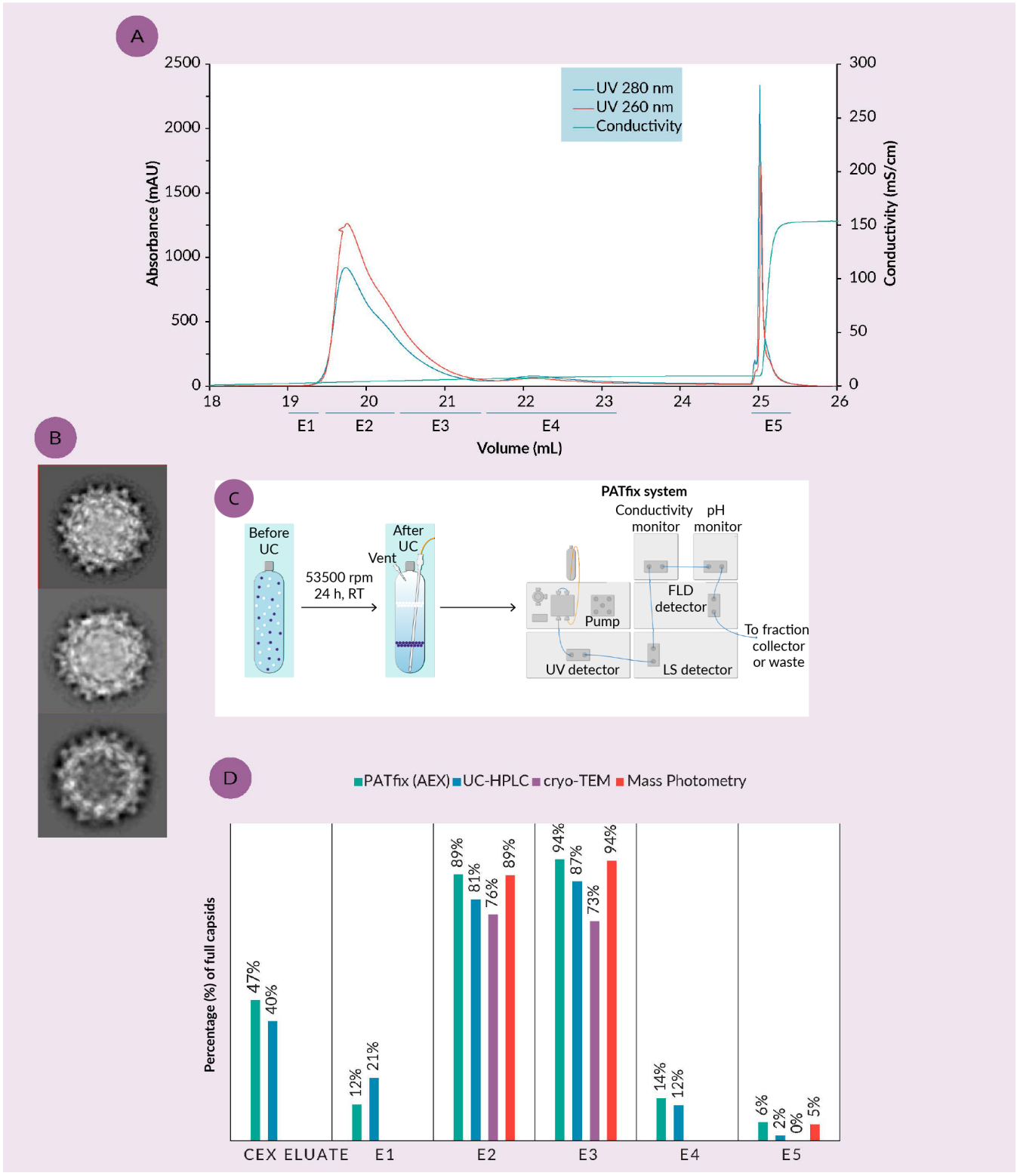
CIMmultus PrimaT is a second novel multimodal column which enables AAV sub-species separation. Sample heterogeneity poses a cumbersome challenge on downstream process to isolate and purify only the active drug substance. Accounting only on vector genome estimation might mislead and result in pooling not only potent intact full AAV capsids, but also product related impurities. Various factors such as AAV serotype; expression system which produces a combination of AAV subpopulation species; as well as glycosylation and phosphorylation of capsids, all contribute to slight charge variations. Relying only on charge differences, using ion exchange columns, results in diminished or absence of resolution between the capsids subspecies. To overcome limitations posed by charge separation, CIMmultus PrimaT was developed.

CIMmultus PrimaT funtions as a weak-anion exchanger with hydrogen bond properties and metal affinity coordination effect. Two different approaches are recommended for the elution of AAV capsids: first, a linear MgCl2 gradient where the full AAV particles elute and then a high salt wash step where most of the empty particles elute [21].

The PrimaS and PrimaT ligands are available on analytical scale as well.

FIGURE 8

A) Preparative chromatogram for CIMmultus PrimaT. E1 to E5 represent fractions taken and assessed by orthogonal analytics. B) Cryo-TEM micrographs for fractions E2, E3 and E5. C) The UC-HPLC system. D) Percentage of full AAV capsids based on PATfix HPLC AEX-FP-TRP, UC-HPLC, Cryo-TEM and mass photometry assays.



DOI: 10.18609/cgti.2022.192

ANALYTICS OF PRIMA T POLISHING

To test the ability of CIMmultus PrimaT to distinguish AAV subspecies, AAV 2/8 serotype sample captured by cation exchange chromatography (CIMmultus SO3), was buffer exchanged and loaded on CIMmultus PrimaT. SO3 eluate consisted of 47% full AAV capsids before the enrichment step. A novel column CIMmultus PrimaT provided significant evidence for ability of AAV subspecies separation (Figure 8A). Characterization of individual PrimaT fractions by orthogonal analytics revealed that there are at least three subpopulations of empty and two subpopulations of full AAV capsids, similar in both charge and density [22].

Additional analytics were performed to estimate the percentage of full AAV capsids in elution samples. One of them was UC-HPLC. During ultracentrifugation, capsid populations separate based on their density, with full capsids at the bottom and empty capsids at the top of the CsCl gradient [23]. Fractions can be collected after ultracentrifugation for analysis of UV260 and UV280 signals, tryptophan intrinsic fluorescence (FL) and Multi-Angle Light Scattering (MALS) (Figure 8C). HPLC analytics estimation using PATfix correlated well with mass photometry, cryo-TEM and UC results with average values of 83% ± 7%, 87% ± 10%, and 3% ± 78% in fractions E2, E3 and E5, respectively (Figure 8D). In case of cryo-TEM, only distinctive full capsids are shown not accounting uncertain species which represent additional 11.63% (E2) and 15.93% (E3) [22].

Our results show that novel monolithic column CIMmultus PrimaT is able to separate full AAV subspecies. In addition, novel ligands to improve full and partial AAV capsids separation are under development.

TRANSLATION INSIGHT

Although there have been many improvements in rAAV manufacture for gene therapy with licenced therapeutics already reaching the market, there are still some challenges manufacturers face to improve the final product purity. In the process of rAAV production, high viral titers must be achieved but attention should also be paid to impurities levels. Therefore, it is imporant to implement fast and robust analytical tools for process control to analyse such complex harvest samples. Optimizing chromatographic capture step to screen for optimal conditions is time-consuming and it can be refined by using high-throughput plates. Partially purified rAAV capsids are not only empty and full, but there are also multiple species of capsids. Since the evolution of new surface chemistries and methods for polishing step is still at its beginning, emphasis must be placed on optimization to achieve the adequate separation of the active product.

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DOI: 10.18609/cgti.2022.192

AUTHORSHIP & CONFLICT OF INTEREST

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

Acknowledgements: *We thank all colleagues who were involved in the research for this paper. They include Janja Merkelj Koren, Kaja Bažec, Valentina Novak, Petra Dekleva, Mirjam Krašna, Blaž Šimenc, Blaž Goričar, Ažbe Žnidaršič and Andrej Mihevc.*

Many thanks to Laboratory Scale Product Department at Sartorius BIA Separations for the development of CIM Monolithic 96-well plates, Research and Development Department for the development of novel CIMmultus PrimaS and CIMmultus PrimaT columns, and Refeyn Ltd for performing mass photometry analytics.

Disclosure and potential conflicts of interest: *All authors are long term employees of Sartorius BIA Separations. Portions of the work described in this publication will be presented on upcoming meetings and conferences. Participation costs will be covered by Sartorius BIA Separations. CIM is technology protected by patent. Following products might be subject to patent: CIM PrimaT, PATfix and CIM monolithinc 96-well plate.*

Funding declaration: *This manuscript is a result of general R&D work within Sartorius BIA Separations.*

ARTICLE & COPYRIGHT INFORMATION

Article source: *Invited; externally peer reviewed.*

Submitted for peer review: *Oct 24 2022;* ***Revised manuscript received:*** *Oct 27 2022;* ***Publication date:*** *Nov 17 2022.*

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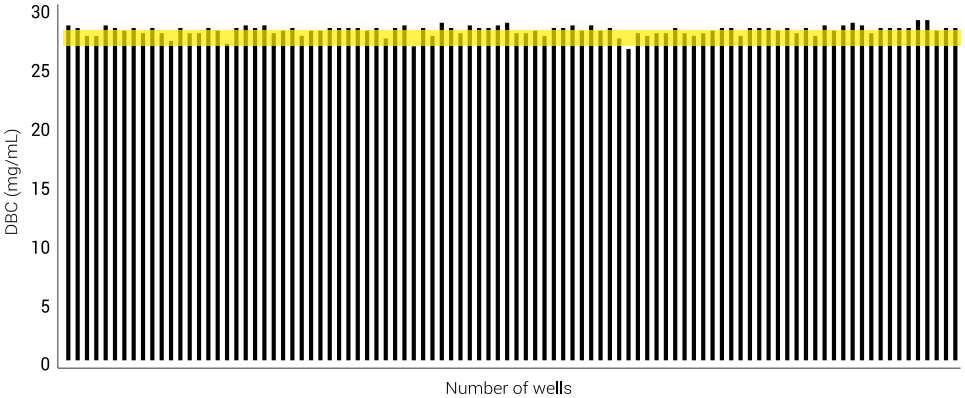

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IsoTag™AAV: an innovative, scalable & non-chromatographic method for streamlined AAV manufacturing

Jennifer Haley, JB Jones, Sophia Petraki, Melissa Callander, Shaleen Shrestha, Emily Springfield, Laura Adamson, Ashutosh Chilkoti, Michael J Dzuricky & Kelli M Luginbuhl

Demand for gene therapies capable of treating previously inaccessible targets has risen precipitously in the past decade. Adeno-associated viruses (AAVs) are the preferred vector for gene delivery because of their favorable safety profile and tissue tropism, but they have significant manufacturing challenges, with end-to-end yields as low as 10–30%. To combat these low yields, we developed IsoTag™AAV, a novel purification technology for AAV that is a departure from the chromatographic paradigm in downstream processing. This proprietary technology uses a self-scaffolding recombinant protein reagent that can improve manufacturing yields. It enables purification by cost-effective and scalable filtration processes and improves product quality with minimal optimization. Herein, we describe the development of IsoTag™AAV, provide a head-to-head comparison to industry-leading affinity chromatography (evaluation carried out through a joint research project with Capsida Biotherapeutics), and demonstrate how it can reduce cost of goods for a clinical AAV program by 25%.

Cell & Gene Therapy Insights 2022; 8(10), 1287–1300

DOI: 10.18609/cgti.2022.190

INTRODUCTION

The recent acceleration of the gene therapy industry has created hope of curing patients of once seemingly incurable diseases. However, these therapies rely on recombinant versions of naturally occurring viruses that have significant drawbacks in terms of their safety profile and manufacturability compared to the

previous generation of protein-based biologics [1–4]. Adeno-associated virus (AAV) is the most widely used vector for gene therapy because of its superior safety profile. However, low AAV expression titers, highly inefficient purification, and an evolving regulatory landscape are slowing development and casting doubt on the commercial feasibility of gene therapies. Regulatory and social developments are also increasing the pressure on gene therapy companies to deliver affordable products with additional attention to quality, safety, and efficacy. The industry is in desperate need of more efficient and scalable methods to accelerate development, alleviate manufacturing bottlenecks, and improve global access to these life-saving therapies.

The industry has taken several approaches to address the AAV bottleneck. By engineering cells, the industry hopes to improve viral titers, thereby reducing the reactor volume required for each dose [5–7]. Other researchers are seeking to improve the potency by engineering the capsid to have more desirable tissue tropism or engineering the promoter for higher or tissue-specific payload expression. Both solutions are attempts to reduce the required dose and thereby the respective production volume per dose. Finally, there has been a brute force effort worldwide to improve capacity by scaling out manufacturing capability.

New or expanded manufacturing facility announcements have frequented the headlines in the past several years. Billions of dollars have been invested in increased viral vector manufacturing capacity. Despite these efforts, the downstream bioprocessing segment has seen little improvement and a dearth of innovation. The lack of standardization and low yielding unit operations result in major inefficiencies and time-consuming development, leading to costly clinical campaigns [8–10]. Affinity chromatography – currently the dominant methodology to purify AAV – was invented for small molecules that are 10,000X smaller and less complex than AAV [11]. It is not well suited for large, complex, and fragile viruses under development today. This has contributed to the inefficiencies, supply-demand imbalance, and high cost of goods in the cell and gene therapy industry.

IsoTag™AAV is a technology that was developed with the unique needs of viral vectors in mind. The technology is a major departure from the current bioprocessing paradigm, leveraging the phenomenon of liquid-liquid phase separation that was evolved over billions of years ago by nature to perform complex cellular separations [12–13]. While traditional unit operations enable target biologic purification by either size or affinity interaction, the IsoTag™AAV technology consolidates both mechanisms into one simple, elegant process.

TECHNOLOGY

The IsoTag™AAV technology combines the principles of affinity capture with liquid-liquid phase separation. This is achieved with a proprietary fusion protein comprising an AAV-specific affinity ligand and a stimulus-responsive biopolymer fused together at the gene level and produced recombinantly as a single fusion protein. Stimulus-responsive polymers are a class of macromolecules that undergo liquid phase separation with an external environmental trigger, such as temperature, salt, or pH [14]. Thermally-responsive polymers include many families of materials that undergo a phase transition in response to changes in temperature and have been studied for their use in a variety of biomedical applications such as drug delivery and tissue engineering [15–19]. With scalable manufacturing of AAV in mind, the IsoTag™AAV reagent's biopolymer component has been engineered to undergo phase transition at ambient temperature with manipulation of salt concentration. The phase separated IsoTag™AAV reagent is fully reversible, returning to a small, fully soluble protein by lowering the temperature or lowering the salt concentration of the solution.

IsoTag™AAV reagents offer an innovative approach to AAV purification. Highly specific

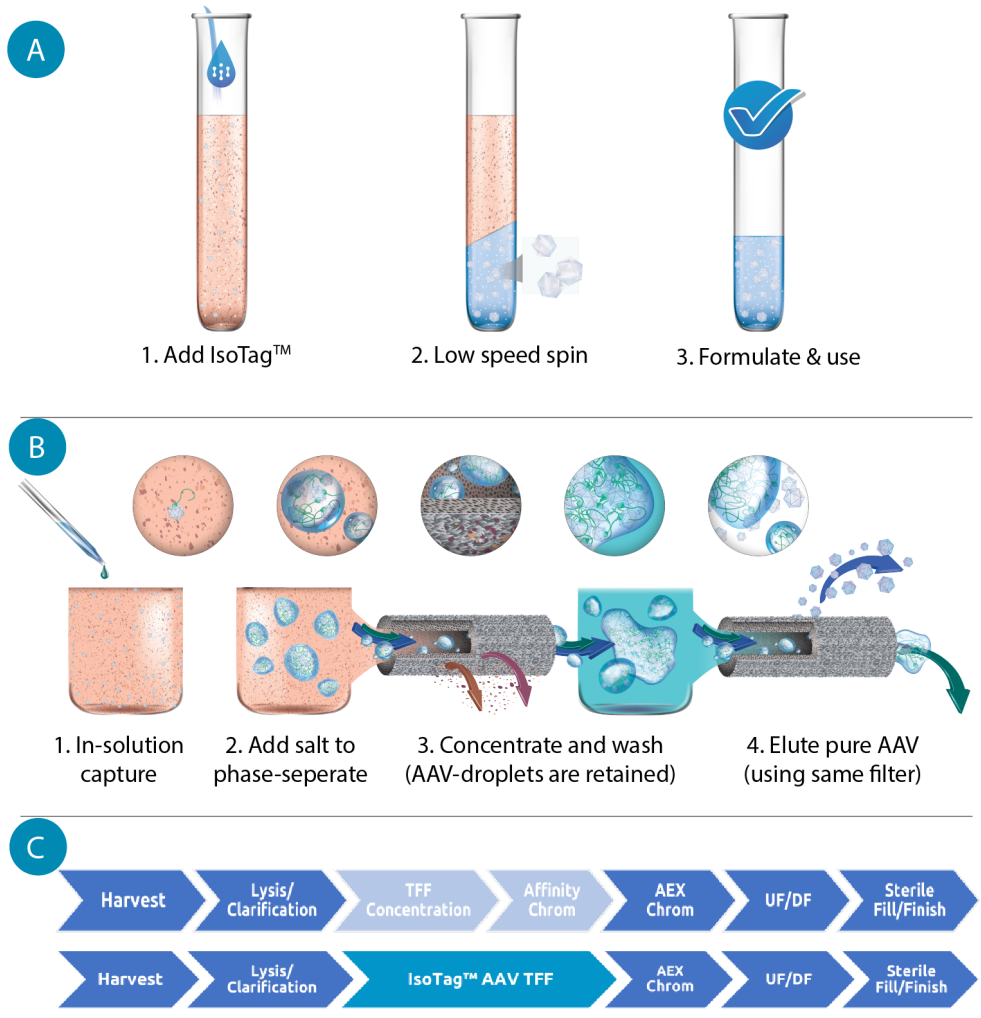
DOI: 10.18609/cgti.2022.190

affinity for AAV can be toggled on and off by manipulating solution conditions, such as pH and salt, that affect the ligand's binding affinity (Kd) to the AAV capsid. The effective size of the virus-IsoTag™ complex can be adjusted in situ by orders of magnitude (22 nm to >1000 nm) through the reversible phase separation phenomenon and its bound or unbound state.

Purification of AAV with this reagent is depicted in the schematics of Figure 1. When added to cell culture harvests, the affinity ligand portion of the IsoTag™AAV reagent specifically binds the AAV capsids. Salt is then added to the solution, triggering lower critical solution temperature (LCST) phase separation. In this state, the IsoTag™AAV forms large, micron-scale coacervates containing the bound AAV, sequestered and protected from contaminants. The AAV-containing coacervates can be separated from the bulk harvest through centrifugation by pelleting the dense,

FIGURE 1

(A) Schematic of the IsoTag™AAV process with density-mediated capture. Using centrifugation, the AAV is scavenged from the harvest media. (B) Schematic of the IsoTag™AAV process with size-mediated capture. Using microfiltration, the AAV can be captured and concentrated in the retentate of a TFF hollow fiber. Using buffer exchanges, the AAV can be purified in the retentate and then eluted from the IsoTag™ reagent and collected in the permeate. (C) Flow chart of steps in a traditional AAV downstream process compared to a downstream process using IsoTag™AAV technology. IsoTag™AAV streamlines purification, consolidating two unit operations into one step that is more efficient.



protein-rich droplets, or through microfiltration (TFF) by retaining the large droplets while harvest media and contaminants are eliminated as waste through the filter permeate. The pelleted or retained capsid-containing IsoTag™AAV droplets can then be resolubilized by lowering the solution's temperature or salinity. The AAV capsid is then dissociated from the IsoTag™AAV reagent by lowering the pH of the solution to disrupt the binding of the affinity ligand. The phase transition is once again triggered by the addition of salt; however, in this elution environment, the unbound AAV remains in solution and is not pulled into the coacervates. Now unbound, purified AAV can be separated from the IsoTag™AAV droplets using the same methods as the capture step.

The IsoTag™AAV approach to purification offers many benefits compared to existing affinity resins. The technology combines two unit operations typically present in downstream AAV purification – a TFF step for concentration and buffer exchange plus an affinity chromatography step. Our process also eliminates ancillary pain points associated with the current downstream structure, such as column packing and associated failures, long loading times, and scalability of TFF unit operations. In total, the IsoTag™AAV technology saves time and costs by improving total yield through unit operation reduction. Importantly, the IsoTag™AAV TFF process is based on harvest volume, not titer, and can therefore be scaled up linearly by simply increasing the filter surface area proportionally to feed volume. This allows for simple execution and scale-up across a variety of pipeline molecules. It also makes the process independent of upstream performance, allowing for significant flexibility while also accommodating improvements from upstream without changes to the downstream process.

IsoTag™AAV is a recombinant protein reagent that can capture AAV, followed by sequestration – and separation from other contaminants – in stable, surfactant-free droplets that are highly enriched in virus. This eliminates the requirement of a solid scaffold (e.g. resins, beads, membranes, monoliths) entirely, creating two important consequences: first, it eliminates the chemical conjugation step that is used to manufacture an affinity matrix, and second, it completely eliminates the mass transport factors that are intrinsic and limiting to heterogeneous separation processes such as affinity chromatography. Although technologies, such as membranes, fibers, and monoliths, avoid the mass transfer limitations of traditional packed beds, their adoption has been largely limited to flow through mode operations with ion exchange rather than affinity-mediated capture-elute mode.

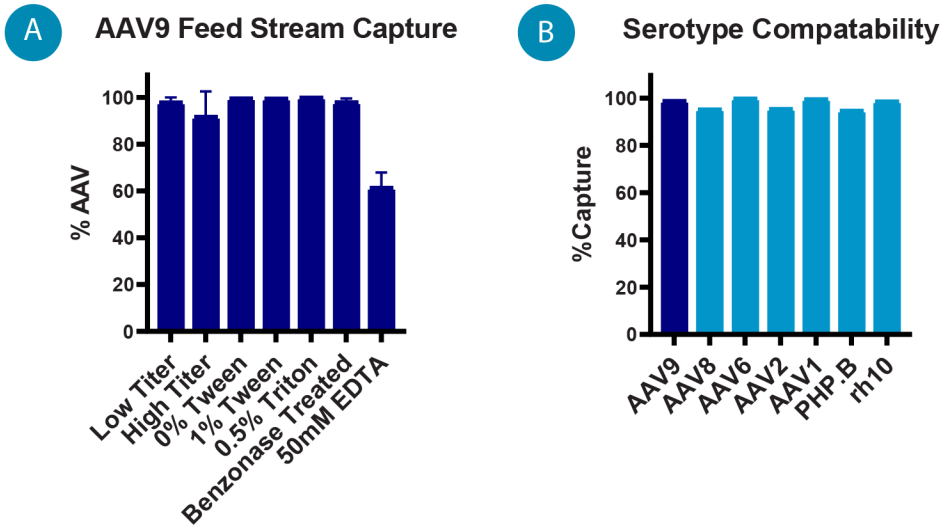
The IsoTag™AAV reagent can be produced in a single synthesis step in *E. coli* for fast, straightforward manufacturing. The use of this expression system allows us to leverage existing recombinant protein manufacturing infrastructure for simple production at various scales and various quality grades from research to GMP. As the recombinant protein reagent itself is <50 kDa, it can also be supplied as a fully sterile product in aqueous buffer, thereby eliminating the need for hazardous storage chemicals and opening up opportunities for fully closed processing.

The IsoTag™AAV process was first validated with the centrifugation format at small scale (1mL) to screen feed stream compatibility and to optimize process buffers. During this evaluation phase we determined basic operation parameters for the IsoTag™AAV process and confirmed broad serotype compatibility to AAV vectors of serotype 1, 2, 6, 8, 9, rH10 & PHP.B **(Figure 2B)**. The IsoTag™AAV capture and elution was then transitioned to a small-scale TFF process (0.2 L) using clarified AAV9 lysate. Next, the scalability of the process was evaluated with 1 L and 8 L batch volumes by increasing TFF filter area, but keeping all other parameters identical, including volumetric loading, shear rate, and flux. Finally, the performance of this process was evaluated by a leading gene therapy company by splitting a batch of cell culture harvest and running IsoTag™AAV TFF purification in parallel with their industry standard process. The two processes were compared

DOI: 10.18609/cgti.2022.190

FIGURE 2

(A) Percentage of AAV9 captured by IsoTag™AAV centrifugation process for various feed streams (n = 3). (B) Percent capture of different AAV serotypes using small-scale centrifugation process.



Error bars represent standard deviation of the mean.

head-to-head for productivity, yield, and quality. Based on these results, a detailed model was created to explore the value of IsoTag™AAV if it were implemented in larger scale clinical campaigns.

RESULTS

IsoTag™AAV reagent was successfully cloned, expressed in *E. coli* in a shake flask format and purified. After successful development of expression and purification methods in the shake flask format, the manufacturing was scaled to 2 L and 10 L bioreactors. Reagent purity was confirmed with SDS-PAGE (Figure 3).

Initial proof-of-concept for AAV9 capture and elution was performed in a small-scale centrifugation format. The capture for each sample was determined by comparing the AAV titer of the capture supernatant to the titer of the starting cell culture harvest to determine the percentage of AAV retained in the capture pellet. The elution percentage was determined by comparing the titer of the neutralized, eluted AAV to the titer of the starting cell culture harvest.

Repeated centrifugation testing with IsoTag™AAV demonstrated ~95% capture of the AAV9 present in cell culture harvest and resulted in an elution yield of ~80% (Figure 4). The same small-scale capture format was used

FIGURE 3

SDS-PAGE of IsoTag™AAV reagent expressed and purified from *E. coli*.

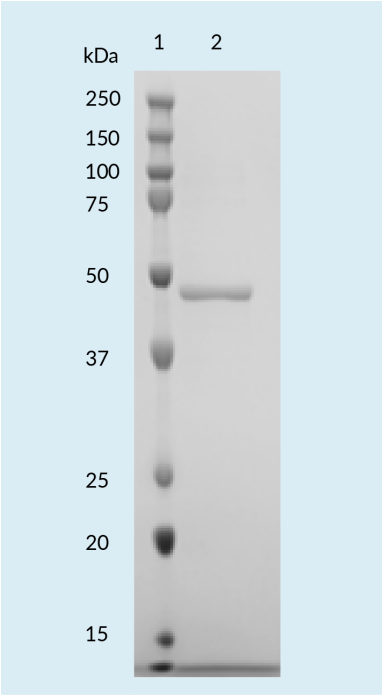
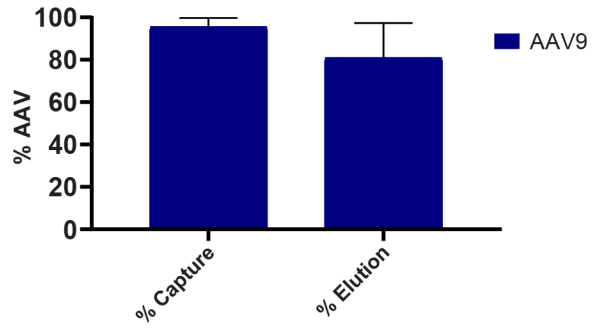


FIGURE 4

Percentage of AAV9 harvest captured and eluted using IsoTag™AAV centrifugation method by qPCR.



n = 3, error bars represent standard deviation of the mean.

to screen capture and elution from different AAV9 feed streams to determine compatibility of IsoTag™AAV with harvests of various titers, as well as samples that had undergone various lysis and clarification protocols (Figure 2A).

In the centrifugation format, IsoTag™AAV capture and elution was found to be compatible with starting titers ranging from 4 x 10^9 to 1 x 10^{13} vc/mL (Figure 2A). IsoTag™AAV performance was also evaluated with commonly used lysis buffer components and was found to be compatible with up to 1% Tween and 0.5% Triton X-100. However, addition of EDTA to the lysis buffer negatively impacted capture by IsoTag™AAV. In the centrifugation format, IsoTag™AAV capture was found to be compatible with material treated with benzonase concentrations ranging from 5 U/mL to 50 U/mL as well as harvest supernatant that had not undergone any nuclease treatment.

Based on these data, we expect optimization efforts for IsoTag™ capture conditions to be minimal, and any wash and elution buffer optimization to follow similar design of experiment models currently employed for chromatographic processes today to maximize yield and contaminant removal for each molecule.

The IsoTag™AAV capture and elution process was transitioned into a more scalable TFF format using Spectrum hollow fiber filters and a KrosFlo KR2i system (Repligen Corporation). Through evaluation of various hollow fiber options, filters with a 0.2-micron pore size, 0.5 mm lumen, and 20 cm effective length were found to yield efficient and reproducible capture. Although testing has not been exhaustive, initial screening experiments demonstrate that flat sheet formats also appear to be compatible, though may require some additional optimization to match the current hollow fiber performance detailed herein.

Additional TFF runs were completed to establish a baseline protocol, which employs 6000 sec^{-1} shear rate, ~50 LMH flux, and 6 wash buffer diavolumes (DVs). Evaluation of elution conditions identified a bulk addition step of cold elution buffer (100 mM glycine, pH 3.0) with the permeate valve closed. This resolubilizes the IsoTag™AAV droplets and releases the captured AAV, after which $MgCl_2$ is added to 0.6 M concentration to re-trigger the phase transition. A 10X concentration of the retentate followed by 8 elution DVs (100 mM glycine pH 3.0, 0.6 M $MgCl_2$) provided the highest yields. The total processing time for capture, purification, and elution was ~4 hours. Contaminant removal throughout the TFF process resulted in highly pure AAV that was recovered during elution with minimal loss during the flow-through and wash steps (Figure 5).

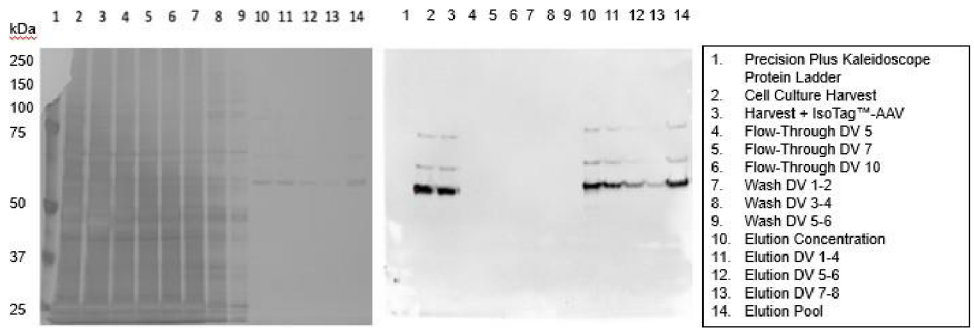
The IsoTag™AAV TFF process was scaled up from 0.2 L to 1 L and 8 L scales by linearly and proportionately increasing the filter area to keep volumetric loading of the feed constant. The permeate controlled flux was also kept constant at all volumes. The resulting runs had nearly identical processing times of ~3.5 hours.

As the process was scaled up with the same harvest material, the TMP profile improved, indicating that overall performance is improved as the process scales (Figure 6), hypothesized to be due to better flow paths and fluid dynamics across larger fiber bundles. The capture by IsoTag™AAV was consistent at each scale with average captures of 96.6% and 98.2% by qPCR and total capsid ELISA, respectively. The total elution yield for the process across each scale was 75.3% and 74.7% by qPCR and ELISA, respectively (Figure 7A). It is also worth noting that the

DOI: 10.18609/cgti.2022.190

FIGURE 5

SDS-PAGE gels of fractions taken throughout the TFF process to show: Left: purity of samples with silver staining and Right: presence of AAV capsids with an anti-AAV (VP1, VP2, VP3) immunoblot.



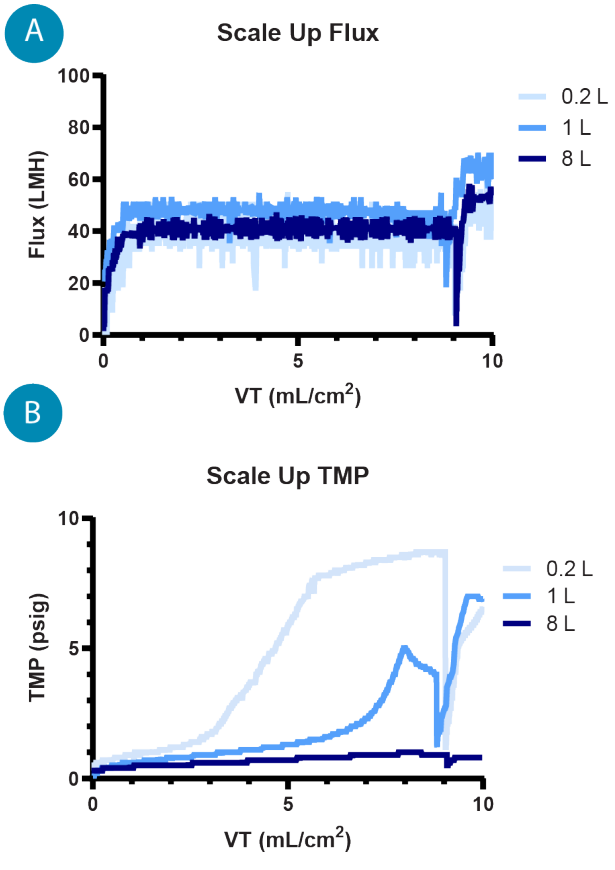
majority of the AAV that was not recovered during elution is found in the retentate. Losses to the permeate during capture and wash steps are approximately 5%, and additional yields of AAV may be achieved by further optimization of elution process parameters.

Log reduction values (LRVs) for host cell protein (HCP) and host cell DNA (HCD) were quantified using a HEK HCP ELISA (Cygnus) and Quant-iT PicoGreen dsDNA Assay (Thermo Scientific), respectively. HCP and HCD contaminants in the clarified lysate feed are significantly reduced by IsoTag™AAV at all scales, with LRVs averaging 4.1 for HCP and 3.8 for HCD (Figure 7B).

Next, to externally validate the technology, the performance of IsoTag™AAV was evaluated in a collaborative effort with Capsida Biotherapeutics, a gene therapy company that is engineering novel capsids for improved gene therapies. Capsida compared Isolere's baseline process to a standard AAV9-specific affinity chromatography method (Table 1). TFF-based purification with IsoTag™AAV resulted in 80.3% yield by ddPCR and ~100% yield by total capsid ELISA. By comparison, the affinity chromatography process yielded 61.2% by ddPCR and 62.6% by total capsid ELISA. As assessed by CE-SDS, the purity of AAV by Isolere's process was 99% whereas the AAV purity by the standard chromatography process was only 88%. Dynamic light scattering showed no appreciable differences in final product size or aggregation. Finally, AAV eluted from IsoTag™AAV compared to AAV purified by affinity chromatography had superior clearance of HCP and HCD. These results serve as external validation of

FIGURE 6

(A) Flux profiles for 0.2, 1, and 8 L TFF runs, the flux is controlled by the permeate pump to maintain a constant 40–50 LMH. (B) TMP profiles for 0.2 L, 1 L, and 8 L TFF runs where the TMP, measured throughout the run, is an indication of gel layer formation and filter performance.



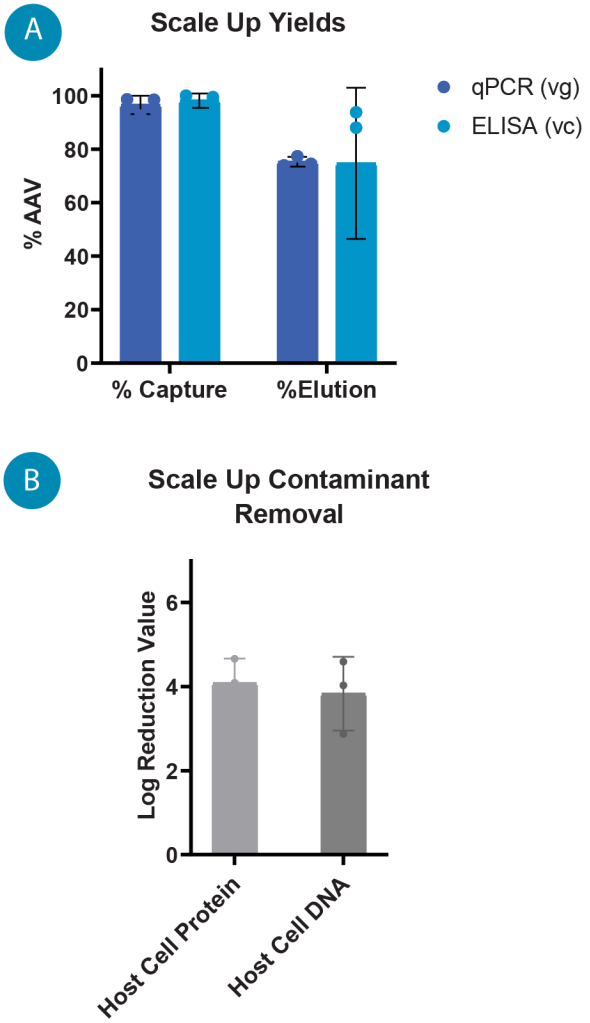
the IsoTag™AAV technology, showing similar results to those generated internally, as well as promising performance when compared to an industry leading process.

The promising results at 1 L scale with Capsida prompted the development of a highly sophisticated model in collaboration with Biopharm Services to better understand the potential impact of the IsoTag™AAV technology at clinical and commercial scales. Two process models were developed to holistically understand differences in end-to-end production of AAV with an industry standard process (TFF plus affinity chromatography) compared to one where both steps are replaced by IsoTag™AAV. Each process was modeled using a 200 L reactor volume, assuming a 12-month single bioreactor campaign, 1e11 vg/mL titer, 10% full capsid percentage, 1e17 cp/L resin capacity, a 1 x 10^{13} vg dose, and single use technology for all process and support equipment.

The modeling shows that IsoTag™AAV leads to ~28% lower cost of goods per dose of AAV purified. This is primarily driven by the increased yield of the IsoTag™AAV process compared to the affinity process and reduced time of operation, leading to lower labor costs (Figure 8). Even assuming 10X reuse of an affinity resin, which could be expected for commercial manufacturing, the IsoTag™AAV process is still cost competitive with single-use implementation, which serves to further streamline operations and reduce the risk and operational complexity that is incurred by resin reuse. Further cost reduction potential of the IsoTag™AAV technology through regeneration and reuse testing and validation is ongoing.

FIGURE 7

(A) AAV9 capture and elution percentages with IsoTag™AAV TFF across 0.2 L, 1 L and 8 L scales by qPCR (vg) and total capsid ELISA (cp). Percentages are based on total AAV9 present in cell culture harvest prior to TFF. (B) Log reduction values (LRVs) for host cell protein and host cell DNA contaminants using the IsoTag™AAV TFF process at 0.2 L, 1 L and 8 L scales.



CONCLUSIONS

As demonstrated herein, IsoTag™AAV is a promising technology for improving and streamlining the manufacturing of AAV. It has the potential to accelerate the development of therapies by reducing the time spent optimizing and scaling a process. Furthermore, this technology could profoundly impact the commercial feasibility of therapeutics by improving overall productivity per batch and harmonizing scale up from the bench to the clinic and beyond. Compared to industry-leading methods, IsoTag™AAV provided a more streamlined process with higher yields, higher purity, and equivalent or better contaminant removal. As a process that is performed on the basis of volume rather than titer, IsoTag™AAV has the ability to accommodate major improvements in upstream virus production and to also accommodate upstream changes between

DOI: 10.18609/cgti.2022.190

TABLE 1

Summary of key metrics from a head-to-head study conducted at Capsida Biotherapeutics, Inc. comparing AAV purification with IsoTag™AAV TFF versus TFF plus affinity chromatography.

Key metrics	IsoTag™AAV	Affinity chromatography
Process time (h)	3	5
Yield (vg %)	80%	61%
Yield (cp %)	118%	63%
Purity (CE-SDS %)	99%	88%
Diam (nm) / PDI / Peak %	28 nm / 0.05 / 100%	27 nm / 0.05/ 100%
TCID 50 (vg/IU)	3.6×10^3	9.3×10^3
HCP (LRV/dose)	4.9	4.1
HCD (LRV/dose)	4.3	3.7

batches or pipeline molecules with ease. While AAV is the first application of this technology, IsoTag™ has broad potential to become a highly enabling, first-in-class manufacturing solution for advanced therapeutics. Isolere is actively developing reagents for lentivirus and mRNA, among other complex biologic modalities.

METHODS

IsoTag™AAV production

The gene encoding the IsoTag™AAV fusion protein was cloned using recursive directional ligation by plasmid reconstruction (PRe-RDL), as described by [17]. The plasmid containing the gene of interest was then transformed into *E. coli* competent cells for expression and fermentation. Fusion protein was produced in shake flasks and purified to >90% purity for testing. 10–25X stock solutions of the reagent were made using both Beer's law calculation from their absorbance at 280 nm, as well as precise weights of lyophilized protein powders.

Centrifugation capture screening

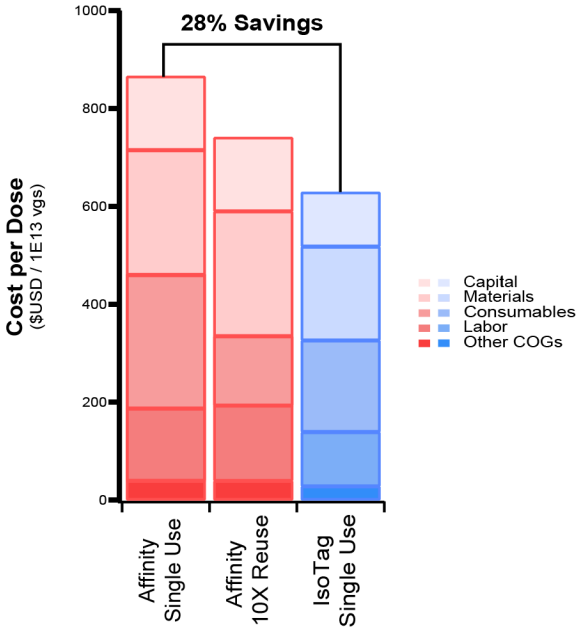
Small volume screening of IsoTag™AAV capture was conducted using a centrifugation format. These experiments were performed using 1 mL of cell culture harvest in triplicate for the following serotypes: AAV1, AAV2, AAV6, AAV8, AAV9, AAV PHP.B and AAV rh10. IsoTag™AAV was added to each 1 mL aliquot to a 1X concentration to bind the AAV. A 5 M sodium chloride stock was then added to a final concentration of 0.6 M, with mixing, to trigger the phase separation of the biopolymer. The samples were centrifuged at 13 krpm for 10 min at room temperature to pellet the AAV-containing IsoTag™ coacervates. The supernatant, containing soluble contaminants from the cell culture harvest and any uncaptured AAV, was removed and saved for analysis by ITR2 qPCR and total capsid ELISA. Elution buffer (100 mM Glycine, pH 3.0) was added to the pellets and left rotating in a cold room for ~1 h to resolubilize the IsoTag™AAV by reversing the phase transition, while the low pH buffer dissociates the AAV from the IsoTag™. Magnesium chloride was then added to a concentration of 0.6 M to trigger the phase separation of the IsoTag™. After a final centrifugation step at 13 krpm for 10 min at room temperature, the eluted AAV remains in the soluble fraction. This pure, eluted AAV was transferred to a new tube, neutralized with 1 M Tris pH 7.5, and saved for analysis.

AAV lysis & clarification

Harvest containing rAAV9 was produced by culturing HEK cells in suspension with

FIGURE 8

Cost breakdown per dose when implementing IsoTag™AAV process compared to an affinity-based process modeled after the Capsida comparison study.



The current IsoTag™AAV process could improve yield and lower cost per dose by >25% and this new process remains COGs competitive as a single use technology, even against 10X reuses of the affinity resin.

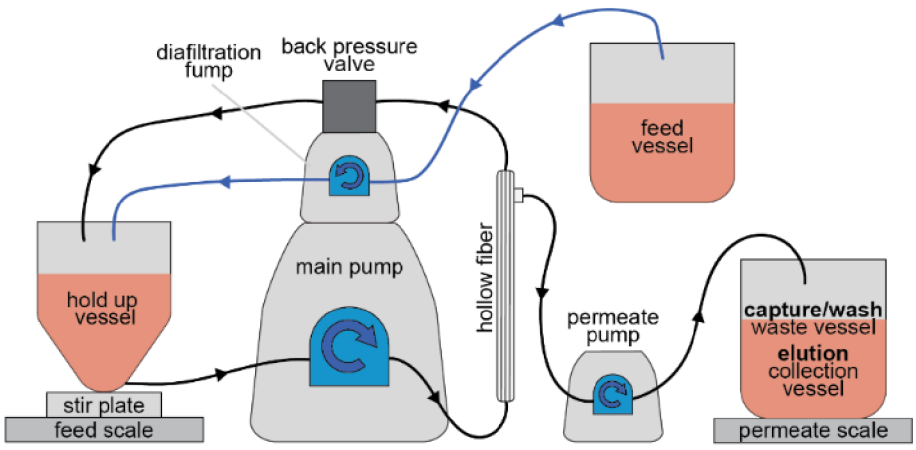
a triple plasmid transfection method. Clarified lysate was used for the process development and scale-up of the IsoTag™AAV TFF purification process. Frozen AAV9 harvest material was thawed and lysed using 0.5% Tween-20 and incubating for 15–30 min. The lysed harvest material was then nuclease treated by adding 2 mM $MgCl_2$ and 5 U/mL Benzonase and incubating at 37 °C for 1–2 h. Pluronic acid was then added to the lysate to a concentration of 0.01% to prevent AAV adsorption to tubing, containers and filter surfaces, and the lysate was then clarified using a MilliPore MilliStak D0SP uPod depth filter. Sodium chloride was then added to the clarified lysate to a concentration of 0.6 M and the material was filtered with a 0.2 µm bottle-top filter.

IsoTag™AAV TFF process development & scale up

The IsoTag™AAV TFF process was developed on a Repligen KrosFlo Kr2i system using Spectrum Hollow Fiber filters set up as shown in Figure 9. The process was run using a PendoTECH lab scale process vessel with a conical bottom and low point drain with a stir bar. The hold-up vessel was placed on a stir-plate on the feed scale of the Kr2i system. An auxiliary pump was used to run the system in permeate control mode at a set permeate rate while another auxiliary pump

FIGURE 9

Schematic for TFF system setup for IsoTag™AAV purification.



The setup is configured for permeate control mode with a hollow fiber filter and a stirred, bottom-fed retentate vessel.

DOI: 10.18609/cgti.2022.190

was used to feed bulk harvest material into the hold-up vessel at an equal rate to maintain a constant hold-up volume. A back pressure valve was used to maintain a constant pressure of 10 PSI on the retentate line. The cross-flow rate of the system was set to maintain a 6000 $^{s-1}$ shear rate and the permeate rate was set to maintain a 50–60 LMH flux rate.

For the 0.2 L process scale, a Spectrum C02-P20U-05-N (0.2 µm pore size, 0.5 mm ID, 23 cm total length, 28 cm^2 surface area) hollow fiber filter was used with size 16 tubing for the recirculation loop and size 13 tubing for the permeate line. The system was flushed with a 0.6 M NaCl buffer prior to filtration. A 25x concentrate solution of IsoTag™AAV was added to 10X final concentration in 20 mL of the clarified lysate, prepared as described above. IsoTag™AAV was added to a 1X concentration to the remaining lysate volume. The 20 mL of lysate combined with 10X IsoTag™AAV was added to the hold-up vessel, while the bulk harvest was placed in a feed reservoir connected to the hold-up vessel with size 16 tubing and an auxiliary pump. The system was run in a concentration-diafiltration (C-D) mode with a concentration factor (CF) of 1 (to maintain a constant hold-up volume) and 16 diafiltration volumes (DV) to process the clarified lysate in a continuous fed batch setup. The cross-flow rate was set to 45 mL/min and, once the backpressure valve had achieved a constant 10 PSI pressure on the retentate line, the permeate flow was started at a rate of 0.3 mL/min and slowly increased until a flux of 50–60 LMH was achieved. Once the entire volume of clarified lysate had been added to the hold-up vessel via the diafiltration auxiliary pump, a wash buffer containing 20 mM Tris pH 7.5, 0.6 M NaCl, 0.01% pluronic acid was added to the feed reservoir. The diafiltration process was continued until 6 DV of wash buffer had passed through the permeate line. Samples were taken from the capture flow-through and wash fractions for analytics, the remaining capture and wash permeate was discarded.

Following the capture and wash process, 80 mL of elution buffer (100 mM Glycine pH 3.0, 0.01% pluronic) that had been chilled on ice was added directly to the hold-up vessel containing the captured AAV and IsoTag™. The addition of the cold, low-salt elution buffer resolubilized the IsoTag™AAV, while the low pH dissociated the AAV from the ligand. The system was set to recirculate at 45 mL/min with the permeate line closed to allow thorough and complete solubilization and elution of the IsoTag™AAV. After 5 min of recirculation, 14 mL of 5 M $MgCl_2$ was added to the hold-up vessel to a final concentration of 0.6 M to trigger the phase separation of the IsoTag™ once more and the solution was allowed to recirculate for an additional 10 min. The permeate line was then reopened and the system was run in C-D mode with a CF of 10 and 8 DVs.

During the concentration step, the hold-up volume was concentrated 10X to a final volume of ~11 mL while unbound AAV in solution was passed through the permeate line. Following the elution concentration, 8 DV of elution buffer containing salt (100 mM Glycine pH 3, 0.6 M $MgCl_2$, 0.01% pluronic) was used to wash remaining free AAV into the permeate. The permeated elution material was neutralized by adding a volume of neutralization buffer (1 M Tris, pH 7.5) equal to 10% of the fraction volume. Neutralized elution fractions were collected and the bulk eluted material was pooled. The elution pool was concentrated and buffer exchanged into DPBS, 0.01% pluronic using Pierce PES Protein Concentrator tubes (Thermo Scientific).

The TFF process was scaled up to 1 L and 8 L using D02-P20U-05-N (140 cm^2) and S02-P20U-05-N (1000 cm^2) hollow fiber filters respectively (Repligen Corporation). Scale-up studies were performed using AAV9 material from the same 20 L cell culture harvest batch that was stored at -80 °C. The material was thawed and prepared using the lysis and clarification process listed above. The 1 L scale up run utilized size 16 tubing for the recirculation loop, permeate line and feed line.

The crossflow rate was set to 225 mL/min to maintain 6000 $^{s-1}$ shear rate and the permeate rate was started at 4.4 mL/min and slowly increased in 1 mL/min increments until a 50–60 LMH flux was achieved. The 8 L scale up run utilized size 18 tubing for the recirculation loop and size 16 tubing for the permeate and diafiltration lines. The crossflow rate was set to 1400 mL/min to maintain 6000 $^{s-1}$ shear rate and the permeate rate was started at 4.4 mL/min and slowly increased in 1 mL/min increments until a 50–60 LMH flux was achieved.

Purification of AAV harvest material at Capsida Biotherapeutics

AAV cell culture harvest material was lysed and clarified using Capsida's standard protocol and split for head-to-head comparison of IsoTag™AAV purification to affinity chromatography. One liter of clarified lysate was purified using the IsoTag™AAV TFF process described above and then buffer exchanged via TFF into a cesium chloride enrichment buffer. One liter of clarified lysate was prepared for affinity chromatography including a TFF concentration step prior to loading on an affinity chromatography column (Thermo resin). The AAV purified from both methods were run through the same platform downstream purification process including cesium chloride gradient ultracentrifugation and buffer exchange via dialysis. Process intermediate samples as well as final drug product from both purification streams were analyzed for yield using ddPCR and total capsid ELISA titers. Particle size and aggregation were analyzed using Unchained Lab's Stunner. AAV purity and capsid integrity were analyzed via SDS-PAGE with Coomassie staining, CE-SDS, host cell protein ELISA, host cell DNA and qPCR.

Isolere Bio AAV analytical methods

Process efficiencies were determined by comparing the total amount of AAV in individual process fractions to the total amount of AAV in the starting harvest. IsoTag™ capture efficiency was determined by subtracting the amount of AAV in the flow-through from the total starting AAV and dividing the resulting captured AAV by the total AAV in the harvest. The elution yield was determined by dividing the amount of AAV in elution fractions by the total starting AAV amount in the harvest. Total capsid titer was measured using an AAV9 capsid ELISA kit (Progen, PRAAV9) per manufacturer's protocol. Genome titer was determined via quantitative PCR assay using CFX Connect Real-Time PCR Detection System (Bio-Rad), AAV Universal ITR Primers (IDT) and SsoAdvanced SYBR Green Supermix (Bio-Rad).

Western blotting was also used to track AAV across process fractions. 10 uL samples from individual fractions were run in reducing conditions on 8% Bis-Tris Bolt 1.0 mm Mini Protein Gels (Invitrogen) and transferred onto PVDF using the Trans-Blot Turbo system (Bio-Rad) according to the manufacturer's recommendation. Blots were probed with mouse monoclonal anti-AAV (VP1, VP2, VP3) B1 antibody (1:400, ARP) as the primary antibody and goat anti-mouse HRP (1:6666, Invitrogen) as the secondary antibody. Blots were developed using SuperSignal West Pico PLUS chemiluminescent substrate (Thermo Scientific) and imaged using iBright imaging system (Thermo Scientific). AAV purity was determined using SDS-PAGE and host cell contaminant assays. SDS-PAGE was run on 8% Bis-Tris Bolt 1.0 mm Mini Protein Gels (Invitrogen) in 1X MOPS running buffer (Invitrogen) according to manufacturer's recommendation. Gels were then stained with the SilverXpress silver staining kit (Thermo Scientific) according to the manufacturer's protocol. Log reduction values (LRV) of host cell proteins (HCP) was determined using HEK 293 HCP ELISA kit (Cygnus). LRV of host cell DNA (HCD) was determined by assaying starting material and eluted material with Quant-iT PicoGreen dsDNA kit (Thermo Scientific).

DOI: 10.18609/cgti.2022.190

Capsida Biotherapeutics AAV analytical methods

Product recoveries from each process step were determined by digital droplet PCR (ddPCR; Biorad) and capsid ELISA (custom in-house AAV capsid ELISA). Purity of the elution and final products were assayed using SDS- PAGE/ Coomassie staining (NuPAGE 4–12% Bis-Tris, Invitrogen Novex SimplyBlue Safe Stain), capillary electrophoresis SDS (CE-SDS; PA800 Plus from AB Sciex), host-cell protein ELISA (Cygnus) and host-cell DNA quantitative PCR (qPCR-Thermo Scientific). Aggregation of the products was evaluated by dynamic light scattering using Unchained Lab's Stunner instrument. Final product *in vitro* infectivity was tested by Tissue Culture Infection Dose 50% (TCID50) assay using HeLa RC32 cells and Taq- Man qPCR.

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AUTHORSHIP & CONFLICT OF INTEREST

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

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *Haley J, Jones JB, Callander M, Shrestha S, Dzuricky MJ, and Luginbuhl KL were all employed by Isolere Bio, Inc. during their contributions to this work and they all own stock options in the company. Isolere Bio has received funding from Northpond Ventures and the North Carolina Biotech Center, as well as small business grants from NIH. Luginbuhl KM and Dzuricky MJ hold executive titles at Isolere Bio, Inc. Luginbuhl KM and Chilkoti A are founders of Isolere Bio and serve on the Board of Directors. Isolere Bio, Inc. has IP licensed from Duke University and Phase Bio Pharmaceuticals. Luginbuhl KM, Chilkoti A, and Dzuricky MJ are authors on patents licensed or owned by Isolere Bio, Inc. Only materials and information (no financial or other incentives) were exchanged by Capsida Therapeutics, Inc and Isolere Bio, Inc to carry out the comparison studies. The authors disclose no conflicts of interest.*

Funding declaration: *Research and development of the IsoTag™AAV process was in part funded by government grants from the NSF and NIH with grant numbers 1843074 and 1R44-GM139503-01A1 respectively. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or the National Science Foundation.*

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Article source: *Invited; externally peer reviewed.*

Submitted for peer review: *Sep 7 2022*; ***Revised manuscript received:*** *Oct 18 2022*; ***Publication date:*** *Nov 3 2022.*

DOI: 10.18609/cgti.2022.190

FASTFACTS

Driving the viral vector expressway: speeding through AAV manufacturing

Andi Ushijima, Scientist, Viral Vector Technology and Innovation, MilliporeSigma and **Henry George**, Head of Viral Vector Producer Cell Lines, MilliporeSigma

Optimized, scalable upstream production platforms are paramount in reducing the time it takes for gene therapies to reach patients. Here, we demonstrate how the VirusExpress® 293 AAV Production Platform can improve performance to achieve higher AAV titers overall, as well as the platform's ability to produce AAV2, AAV5, AAV6, and AAV9 specifically.

TRANSFECTION OPTIMIZATION FOR AAV2

As part of transfection optimization, a response surface design was used to generate a model to identify optimal transfection conditions **(Figure 1)**. This identified transfection conditions that resulted in a 60% increase in genome titers compared to baseline conditions. Additional optimization of transfection conditions was completed using alternate transfection reagents.

SCALING UP OPTIMAL AAV2 CONDITIONS

After completing transfection optimization in the Ambr 15 bioreactor, optimal conditions were scaled up to the Mobius® 3L bioreactor **(Figure 2)**. Increased genome titers were found in the Mobius 3L when compared to the Ambr 15, with a 3× improvement from the earlier baseline conditions.

TESTING OTHER SEROTYPES: AAV5, AAV6 AND AAV9

To demonstrate the applicability of the VirusExpress Production Platform to other AAV serotypes, transfection improvement learnings from AAV2 were applied to AAV5 and AAV6 production. This resulted in a 50–60% increase in genome titer **(Figure 3)**.

A process development project for a client was performed using AAV9 **(Figure 4)**. The mixture space of AAV9 plasmids was characterized using the Ambr 15 and the plasmid ratio that maximized genome titer was identified. A scale-up of the top conditions was then performed in the Mobius 3L bioreactor. As previously found with AAV2, increased genome titer was found in the Mobius 3L bioreactor when compared to the Ambr 15.

Figure 2. AAV2 production using the VirusExpress AAV platform in the Ambr 15 and Mobius 3L bioreactors.

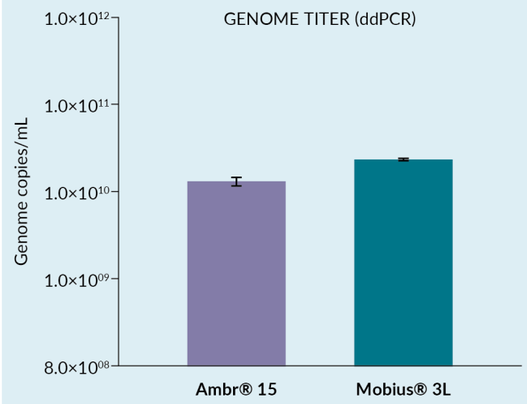


Figure 3. Application data of VirusExpress Platform for AAV5 and AAV6.

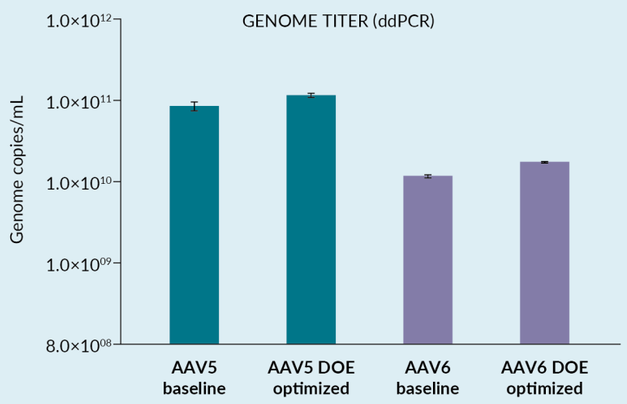


Figure 1. VirusExpress AAV Production Platform transfection optimization for AAV2 production in the Ambr® 15 bioreactor using response surface design of experiment (DOE).

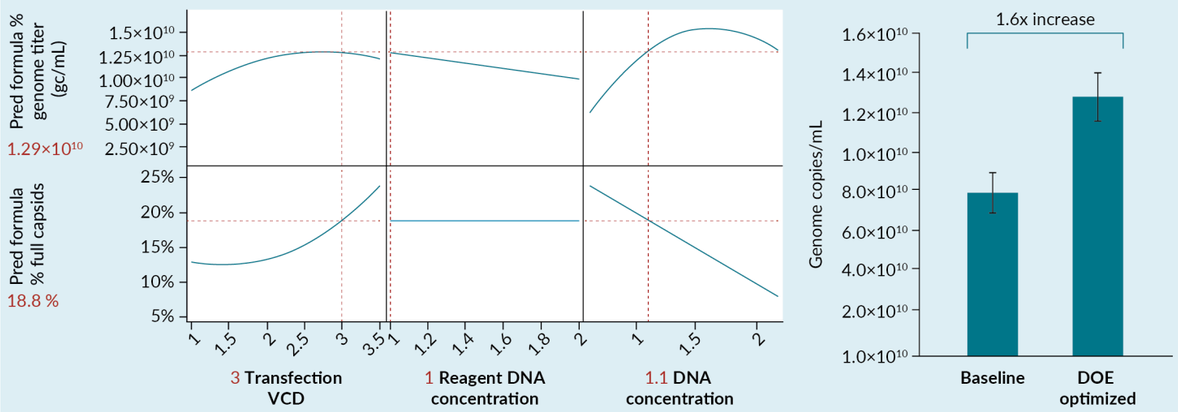
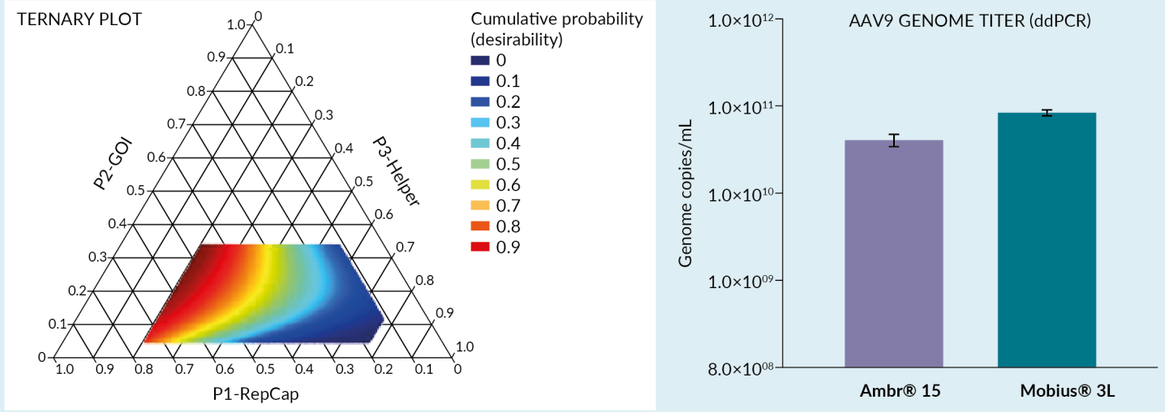


Figure 4. Application data of VirusExpress Platform for AAV9.



CELL & GENE THERAPY INSIGHTS

Cell & Gene Therapy Insights 2022; 8(10), 1371; DOI: 10.18609/cgti.2022.198

In partnership with: MilliporeSigma

Automated & scalable closed-system platform for cell isolation and activation

Hany Meås, R&D Scientist, Thermo Fisher Scientific

The cell therapy field is experiencing rapid growth with several recent regulatory approvals and further therapies in clinical testing. The Gibco™ CTS™ DynaCellect™ Magnetic Separation System and single-use kits have been designed for scalable and robust cell processing with the CTS Dynabeads™ platform. Using the Gibco CTS DynaCellect Cell Isolation Kit with CTS Dynabeads, >85% isolation efficiency of target cells with >95% purity is consistently achieved with no effect on cell viability.

Cell & Gene Therapy Insights 2022; 8(10), 1283 DOI: 10.18609/cgti.2022.188

CTS DYNACELLECT SYSTEM—AUTOMATED CELL ISOLATION & ACTIVATION

The DynaCellect instrument **(Figure 1)** utilizes an integrated magnet-rocker and a fluidics panel for target cell separation and subsequent bead removal in a closed system. The instrument is accommodated by single-use, fit-for-purpose, consumable kits for isolation and bead removal. DynaCellect software is 21 CFR Part 11 compliant, with suitable workflows for cell isolation, activation, and depletion. The automated system enables minimal handling and ease of use, allowing for operator-independent results.

Figure 1. The CTS DynaCellect instrument.

PROCESS SCALABILITY & PRECISION

The CTS DynaCellect is highly scalable, allowing up to 1 L of reaction volume for cell isolation with a throughput time of around 100 minutes. The seamless scale-up is demonstrated by consistent high viability, >90% recovery, and a minimal number of residual beads (below 100 beads per 3 million cells), as shown in **Figure 2**.

CELL ISOLATION

The high purity of isolated cells from cryopreserved leukopaks has been demonstrated using the CTS DynaCellect, in the isolation of 400 million T cells **(Figure 3)**. The healthy donor leukopaks were washed on the Gibco CTS Rotea™ before cell isolation using DynaCellect. Close to 97% purity was achieved after the 1-step isolation of CTS Dynabeads CD3/CD28 T cells and activation, with minimal cell contaminants compared to the starting material.

BEAD REMOVAL

Automated bead removal using the CTS DynaCellect Bead Removal Kit resulted in >85% target cell recovery. Bead removal is achieved through a continuous flow over the rocker-magnet to ensure rapid processing of volumes suitable for autologous and allogenic workflows while providing automation, modularity, flexibility, and scalability of cell therapy manufacturing.

SUMMARY

The new, automated, and scalable CTS DynaCellect Magnetic Separation System for closed cell isolation and activation is flexible, fast, and manufactured in-house to ensure supply security.

Figure 2. Viability, recovery, and residual beads after the bead removal process on the CTS DynaCellect at 1 L and 10 L scales.

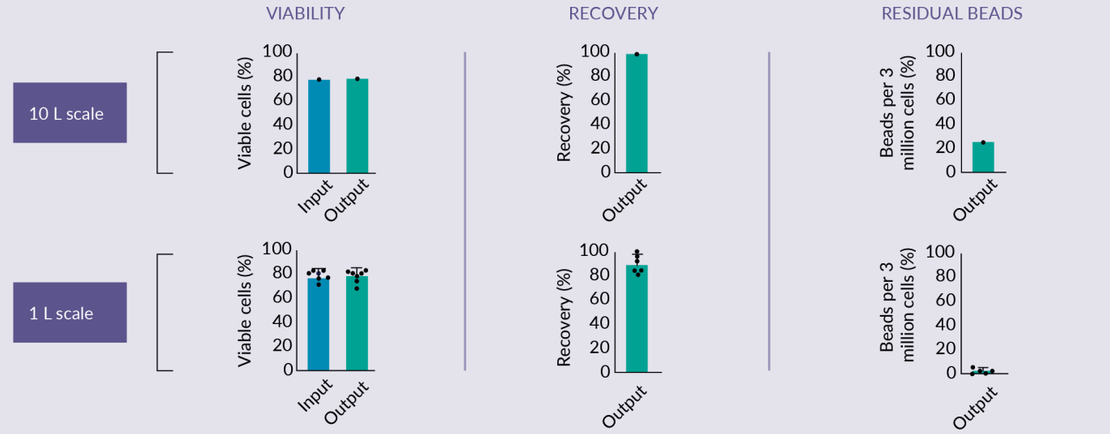
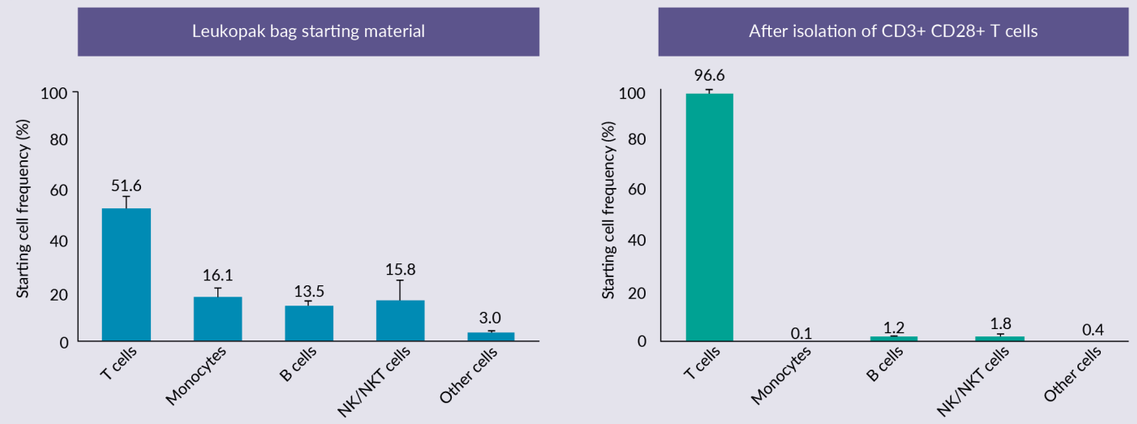


Figure 3. Phenotypes of cells in frozen leukopaks before and after isolation of CD3/CD28 T cells.



CELL & GENE THERAPY INSIGHTS

INTERVIEW

Shear ignorance? Think again: breaking the perception of shear within viral vector manufacturing

Charlotte Barker, *Editor, BioInsights,* speaks to Merck's **Ratish Krishnan,** Senior Strategy Consultant, Bioprocessing Strategy Operationalization and **Akshat Gupta,** Associate Director, Global Biopharma Center of Excellence.

Ratish Krishnan & **Akshat Gupta** (pictured from left to right)

Cell & Gene Therapy Insights 2022; 8(10), 1345–1352

DOI: 10.18609/cgti.2022.195

The tangential flow filtration (TFF) unit operation in viral vector manufacturing is a critical step on the path to commercialization. In this episode, Ratish Krishnan and Akshat Gupta discuss best practices – and common misconceptions – when establishing process conditions and utilizing different TFF device formats.

Q How and why is tangential flow filtration (TFF) used in viral vector manufacturing?

RK: TFF is a widely used unit operation in the biopharma industry for downstream processing applications. A typical TFF step employs membranes such as polyethersulfone (PES) or regenerated cellulose of varying molecular weight cut-off limits, to either concentrate a product of interest through volume reduction and/or perform buffer exchange through diafiltration.

Traditional TFF requires multiple passes through a system, using a pump to drive feed material through a filter, and then sending the retentate back to the feed tank for another pass through the entire system. The circuitry of operation is monitored using pressure and temperature gauges, to ensure avoidance of high pressures that can cause damage to the materials of construction of the membranes and the product of interest itself.

It is a very efficient method of separation of the product of interest for a diverse range of modalities, be it monoclonal antibodies, viral vectors such as adeno-associated viruses, lentiviruses, or different types of vaccine platforms that are used today. This step effectively removes undesirable contaminants, like cellular residues, and others, from the product of interest.

Zooming in on a standard viral vector manufacturing process, the TFF step is used a couple of times. Firstly, for volume reduction prior to a capture chromatography step; specifically, the loading step. And secondly, in a final concentration and diafiltration step prior to the final sterile filtration. This ensures the target concentration of the viral vector is reached, and the product of interest is in the desired drug substance matrix or buffer.

Q What are the main types of TFF formats and membranes used in viral vector purification?

RK: There's a lot to unpack in this question. If you're speaking about membranes, referring to the commonly used materials of construction, the answer would be either PES or its modified version or regenerated cellulose.

PES or regenerated cellulose membranes with a molecular weight cut-off of 30 or 100–300 kDa are generally recommended for viral vector production. The rule of thumb is to have a membrane with a molecular weight cut-off of about three-to-five-fold lower than the molecule of interest, which in this case, these cut-offs apply to AAV or lentivirus.

Another point to consider is that TFF filters are available both in single-use and reusable formats, and there are pros and cons associated with each type. When reusable formats are considered, comprehensive cleaning performance qualification and validation studies are required to ensure sanitization, regeneration, and consistent performance for the desired number of cycles that the membrane is intended for.

Single-use formats are more popular among bioprocessing and manufacturing groups, for obvious reasons.

DOI: 10.18609/cgti.2022.195

On the other hand, if you are talking about preferred filter formats then we have largely two options: flat sheet cassettes or capsules and hollow fibers.

> "...one aspect that we want to consider early on when we are approaching TFF is to keep the GMP considerations in mind...."
> - Akshat Gupta

AG: As Ratish mentioned, there are two prominent filter formats, which are widely used in the biopharmaceutical industry: hollow fibers and flat sheets.

Hollow fibers have been traditionally used for industrial and biomedical separations and have also been adopted for many biopharmaceutical applications. They are available with modified PES as well as mixed cellulose ester lumens. A hollow fiber filter can be selected based on lumen diameter, length and number.

On the other hand, flat sheet cassettes specifically designed for biopharmaceutical applications are very robust and offer efficient process performance and linear scalability in a compact format. These aspects are very critical for good manufacturing practice (GMP) manufacturing of biopharmaceuticals irrespective of modality.

A lot of research has gone into designing the feed channels of these cassettes and the appropriate feed channel geometry can be selected based on mass transfer, pressure drop, and shear rate requirements for a given application and modality. Cassettes are available with both modified PES and regenerated cellulose membranes.

Another thing to mention is that we recently introduced a new spiral wound format designed to provide high performance and linear scalability, which is an attractive alternative for single-use and closed applications.

Q What are the key factors and best practices when it comes to designing a TFF step?

AG: There are various considerations that need to be kept in mind when designing a TFF step. It starts with identifying the objective.

There are two key applications. The first one is if you are solely targeting the product concentration – this can be done to eliminate some of the processing bottlenecks downstream. The other application would be a typical formulation where the modality needs to be transferred into a specific diafiltration buffer or a formulation buffer and then concentrated to a predefined concentration. This is a step that is carried out at the end of the purification process.

Now, one aspect that we want to consider early on when we are approaching TFF is to keep the GMP considerations in mind, along with the scalability of systems and devices. It is critical to pick a system design and a device format that would be scalable, and the systems need to be characterized for at-scale performance. They should have the right turndown ratios, and you should be able to achieve the desired yields and capacities at the full scale.

The other aspect to keep in mind is that if you are targeting closed processing, both the system as well as the device should be designed for it. It is particularly helpful to have process performance and recovery data available for at-scale systems and at-scale devices.

From a modality standpoint, it is very important to have an idea about the size and the isoelectric point of the target molecule, and also how the key process variables like temperature, shear, and mixing affect the stability of the modality. That information can really come in handy when we are designing a TFF process.

Another aspect is how the impurities clear over the diafiltration. Here we are mainly targeting small molecule impurities, so that would be another consideration to keep in mind.

If we think in terms of process, Ratish introduced the concept that there are two key materials of construction of membranes, PES-based and regenerated cellulose-based, so it would be important to understand how the modality interacts with these materials of construction. Typically, regenerated cellulose has been widely used for applications requiring low protein binding. However, what kind of membranes work well with a given modality needs to be experimentally verified.

Another consideration to keep in mind is the buffer matrix, and how stable the modality is in a given buffer matrix. Sometimes the buffer matrix would be linked to a downstream unit operation, but again the excipients and the buffering system which keep the modality stable should be selected.

Q Thinking about the customers you work with, what are some of the common misconceptions regarding TFF for viral vector purification?

AG: Often when we are starting off with a novel modality, one challenge is that there is very little information available on-hand. That's something that we are seeing with a lot of new viral vector therapies. It's not atypical to make a selection based on certain fundamental observations, and some prevalent perceptions, regarding certain technologies.

One perception that is particularly prevalent in industry when it comes to viral vector TFF is around shear. To assume that envelope viruses and proteins are sensitive to shear is honestly not a bad assumption. But the challenge is there is very limited work that has been done to establish the thresholds for shear susceptibility for these modalities. This is something that needs to be understood, and these levels need to be defined, so we can have a better understanding of whether these perceptions are real or not.

Additionally, there's a generalized perception that hollow fibers introduce less shear stress to the modality, as compared to flat sheets. Shear rates truly depend on feed fluxes and feed channel design. To broadly suggest that hollow fibers would be introducing less shear may not be a correct statement. At the same time, we can also extend that and say that to consider that all flat sheets are the same or similar in terms of shear rates is also an inaccurate assumption.

As we move towards a better understanding of these modalities, their interaction with the physiochemical environment, and their susceptibility to shear, we can progress towards making better processes in the future.

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Q How are your teams working to overcome this perception about flat sheets in viral vector purification?

"...one of our main objectives is to solve our customers' toughest problems in bioprocessing, and we have been generating a large amount of data..."
- Ratish Krishnan

AG: There is a lot of ongoing work, and this is being done at various scales and with a broad spectrum of modalities.

One thing which cannot be ignored is the diversity of viral vector modalities. That diversity would require small-scale or rather ultra-small-scale systems, which can be used to characterize shear, and their interactions with various other process variables.

We are also working with our customers to understand how different materials of construction of membrane and device geometry are contributing towards the performance for processing of these viral vectors.

RK: Those are all great points – there isn't a cookie cutter approach to TFF. You also need to consider the uniqueness of the feed – that shouldn't be overlooked.

Process development scientists have the luxury of leveraging what has worked well in past projects, or relying on information that is publicly available to the bioprocessing community. But as with everything in science, the approach has to be data-driven. No data, no science, as a matter of fact.

Customers have engaged us in both simple and complex design of experiments with their process intermediates. As Akshat mentioned, this may be exploring membrane chemistry, cut-off, trans-membrane pressures, feed flux rate, operating temperature, and load ratio – just to summarize a few – for the intended TFF step.

The performance data with their feed material is then collected and packaged into a comprehensive report by our process development services and our MSAT teams. Our customers usually perform their own analytics after which they engage with us in a holistic understanding of the TFF step. We work together towards either optimization of parameters, scale up into a pilot plant, or a manufacturing facility, as necessary.

Sometimes the scope of our work with customers is to explore and evaluate a new product that is in alpha or beta testing phases. We really appreciate the support we get from our customers, who are instrumental in helping us in bettering an existing product or providing feedback for a new concept.

Oftentimes for the betterment of the scientific community, we author, co-author, or support manuscript preparation of technical articles with customers as well. A perfect example is a recently published article that looks at the scale-down model of a 30 kDa flat sheet cassette in a regenerated cellulose format for the popular serotypes of AAV 2 and 9.

In summary, one of our main objectives is to solve our customers' toughest problems in bioprocessing, and we have been generating a large amount of data – including best practices for TFF operations – to empower them and help them design processes firmly based on data.

We are excited to continue to partner with our customers in their journeys of developing potentially curative solutions for patients using these viral vectors.

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We hope you enjoyed reading this interview.
You can also listen to the recorded podcast here:

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DOI: 10.18609/cgti.2022.195

AUTHORSHIP & CONFLICT OF INTEREST

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

Acknowledgements: *None.*

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

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

ARTICLE & COPYRIGHT INFORMATION

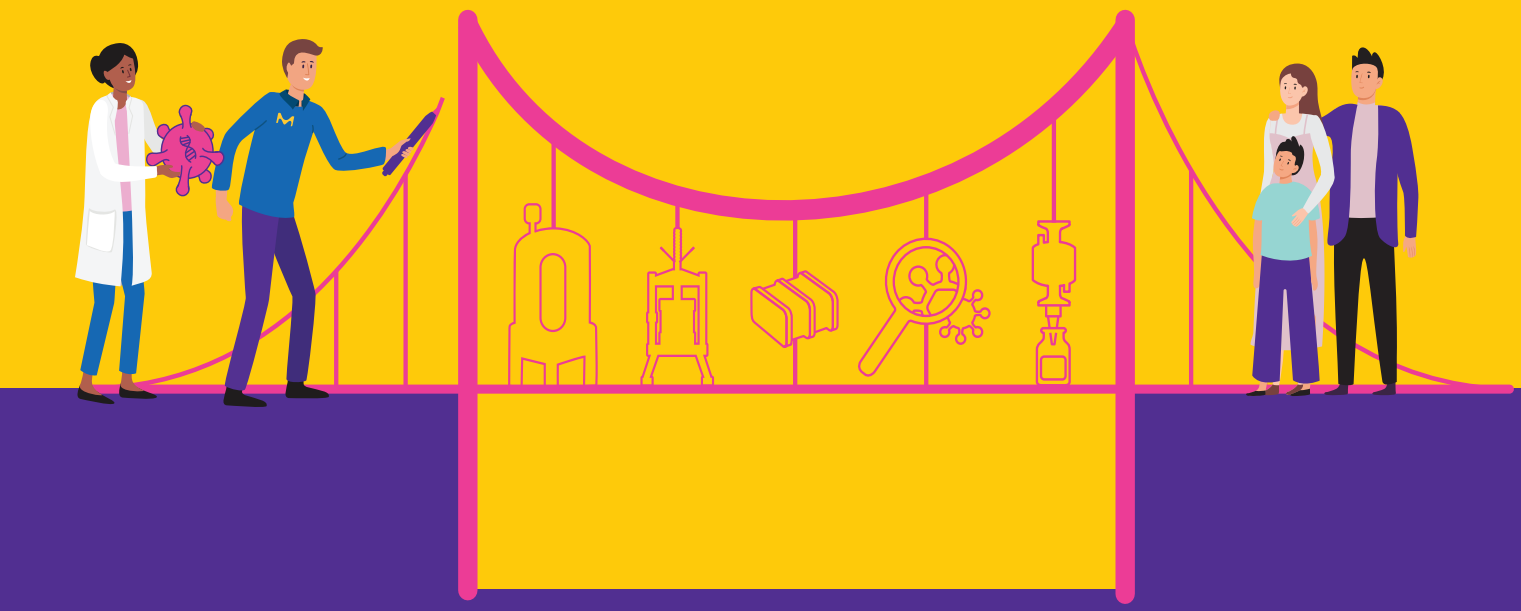
Article source: *This article is based on a Podcast which took place on Aug 23 2022.*

Podcast conducted on: *Aug 23 2022; Revised manuscript received: Oct 26 2022;* ***Publication date:*** *Nov 9 2022.*

Draw on our experience

To bring your gene therapy to life

Products – Testing – Manufacturing



Developing a new gene therapy is rewarding but there are unique challenges ahead. Lack of process templates, scale-up challenges and evolving regulatory guidelines can slow your progress.

We're here to help. With 30+ years of gene therapy experience we bring expertise in viral vector manufacturing and testing as well as a global organization to integrate regulatory, safety, and process development to fit your needs.

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FASTFACTS

A closed, modular approach to autologous CAR-T cell therapy manufacturing

Jason Isaacson, Scientist, Thermo Fisher Scientific

The complex, multi-step process of generating functional CAR-T cells includes cell isolation, activation, modification, expansion and finally cryopreservation. Introducing closed processes to replace manual manipulations can reduce contamination, errors, and variability. Closed, modular, automatable instrumentation for specific unit operations within the workflow can improve upon consistency, purity and safety of the final CAR-T product. Additionally, scalable and compliant platforms support the transition from early discovery to commercial scale manufacturing.

This demonstration provides an in-depth look into Thermo Fisher Scientific's GMP-compliant manufacturing platforms.

CELL PROCESSING

CTS™ Rotea™ Counterflow Centrifugation System

The CTS Rotea system separates cells using the principle of counterflow centrifugation. The system suspends cells in a fluidized bed by exerting a constant flow force against centrifugal forces. The fluid-suspended cells are gently concentrated without ever forming a pellet in the cone and washed efficiently at unmatched recovery rates.

CELL ISOLATION

CTS™ DynaMag™ Magnet

This versatile magnetic device is designed for medium- to large-scale sterile cell isolation and removal of beads prior to, during and after expansion in translational research. The DynaMag CTS magnet is intended for use with Dynabeads® CTS™ products, but can be used with all Dynabeads cell separation products. Scale-up capacity is 50–330 mL in static systems, and >10 L in continuous-flow systems.

CELL EXPANSION

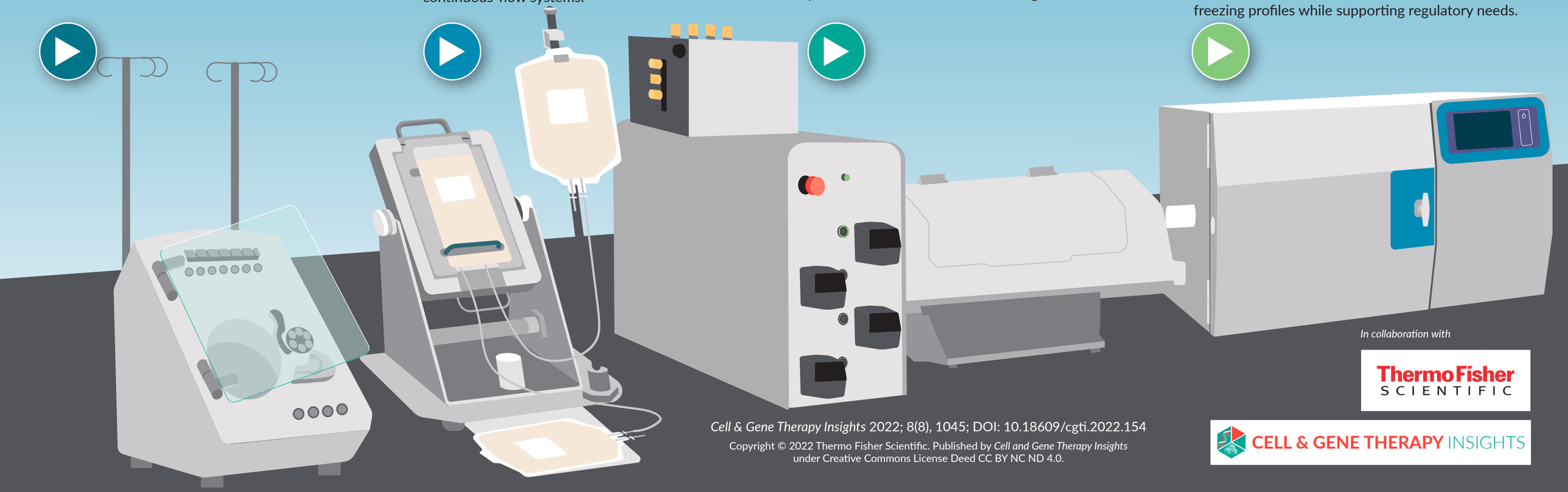
HyPerforma™ G3Lab Controller & HyPerforma™ Rocker Bioreactor

The HyPerforma Rocker Bioreactor is controlled by a HyPerforma G3Lab Controller and TruBio software, providing a complete solution for research, process development, or seed train production applications. The rocker is compatible with a 10 L, 20 L or 50 L Thermo Scientific Rock-it BioProcess Container (BPC), which provides from 5 L to 25 L of working volume.

CRYOPRESERVATION

CryoMed™ Controlled-Rate Freezer

For cell therapy manufacturers, CryoMed with OPC UA provides precise, repeatable freezing results that protect samples from intracellular freezing. The CryoMed features OPC UA serial communication (ethernet) capabilities, comes with standard factory certifications, provides enhanced data traceability via a touch screen user interface and offers customizable freezing profiles while supporting regulatory needs.



Cell & Gene Therapy Insights 2022; 8(8), 1045; DOI: 10.18609/cgti.2022.154

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CELL & GENE THERAPY INSIGHTS

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INNOVATOR INSIGHT

Introducing Lonza's AAV suspension transient transfection platform to de-risk your path to clinic

Suparna Sanyal

Adeno-associated viral vector (AAV) is the most common delivery vehicle for potentially curative *in vivo* gene therapy. Following a couple of landmark approvals, this field has experienced accelerated pipeline growth and investor interest in the past 5 years. Currently, AAV therapies are predominantly targeting rare genetic disorders for which the patient populations are often limited. As a result, drug developers feel immense pressure to be the first to market and commercialize their therapies.

In this article, we will focus on a robust, streamlined platform process for rapid production of AAV using Lonza's suspension-adapted HEK293 cell line and proprietary plasmids to ensure high productivity, and in-process analytics enabling enhanced full-to-empty capsid ratio. The approach for building a reliable, de-risked path to the clinic to avoid unforeseen costs and compliance-related delays will be discussed.

Cell & Gene Therapy Insights 2022; 8(10), 1177–1185

DOI: 10.18609/cgti.2022.175

THE CURRENT STATE OF THE AAV-BASED THERAPY FIELD

Adeno-associated virus (AAV)-based gene therapies represent a fast-paced, rapidly growing market, where almost 80% of pipeline products are in the preclinical phase. To date, only two AAV-based gene therapies are approved by the regulatory authorities, namely Luxturna and Zolgensma. However, AAV continues to be the vector of choice for

in vivo gene therapy and constitutes almost 60% of overall gene therapy pipeline products in development. AAV pipeline products have demonstrated a compound annual growth rate of almost 27% over the last 5 years. Although there is an expectation for fast approval of these therapies, there is also heightened regulatory scrutiny to ensure that the therapies are safe. One element that has been highlighted is toxicity due to process residuals, as well as empty and partially filled AAV capsids.

AAV gene therapy development constitutes a highly competitive market as many of the targeted therapies are for rare indications with small patient populations. This, coupled with the fact that AAV is often intended to be curative, means that being first to market is critical for developers. There is also a requirement for flexible operational models for the ability to increase, and subsequently decrease, production requirements to match the needs of these therapies.

Key requirements for successful AAV gene therapy development can be distilled into three key areas: speed to market, commercial viability, and regulatory approval. Safety and efficacy are key focus areas for developers, whilst manufacturers focus on having a robust, de-risked, and scalable platform process. For AAV, this also entails having a high-productivity system with a high titer of AAV, high overall yield, and a high percentage of full capsids containing the transgene of interest.

In recent years, there has been a shift in addressing scalability, which is now largely accomplished through a transfer from adherent, open, manual processes towards the utilization of bioreactors for developing closed, scalable, and suspension-based processes.

The requirement for a robust platform process continues to be a challenge for AAV therapies. The components are highly variable (including the host cell line, AAV serotypes, and capsids) and the purification process for the AAV can be impacted by the starting components, impacting the overall yield and function of the product. As a result, a single platform process typically cannot be used for all these different starting components of AAV, and often will require some degree of optimization to obtain final overall commercial viability.

LONZA'S APPROACH TO A STREAMLINED PRODUCTION PROCESSS

To address these nuances of AAV therapy development, Lonza is utilizing a new product introduction process. This process has a holistic, standardized, systematic approach from beginning to end, and from preclinical to commercialization stages.

The streamlined process is accomplished through the utilization of pre-qualified assays, off-the-shelf optimized unit operations, and ready-to-go tech transfer documentation, saving months of development time. Commercial viability is attained through using production processes that utilize proprietary starting materials, such as an established suspension cell line and proprietary rep/cap and helper plasmids, thus leading to higher productivity and yield of AAV. Established analytics as well as quality systems and checkpoints at each milestone ensure that all quality and regulatory standards for commercialization are met. This streamlined process helps to reduce time to market, including the time required for assay development and qualification, as well as the technology transfer to good manufacturing practice (GMP).

Whilst this is a platform process that is streamlined and with a significant reduction in the timeline, only a certain number of AAV serotypes and GOI combinations will be able to utilize these efficiencies without requiring any optimization. To encompass a larger number of AAV therapies, often some degree of optimization will be required. While some AAV therapies can be well suited for a plug-and-play platform process, many therapies may have a realistic

DOI: 10.18609/cgti.2022.175

timeline of 14–16 months, with some degree of optimization built in.

THE LONZA AAV PLATFORM

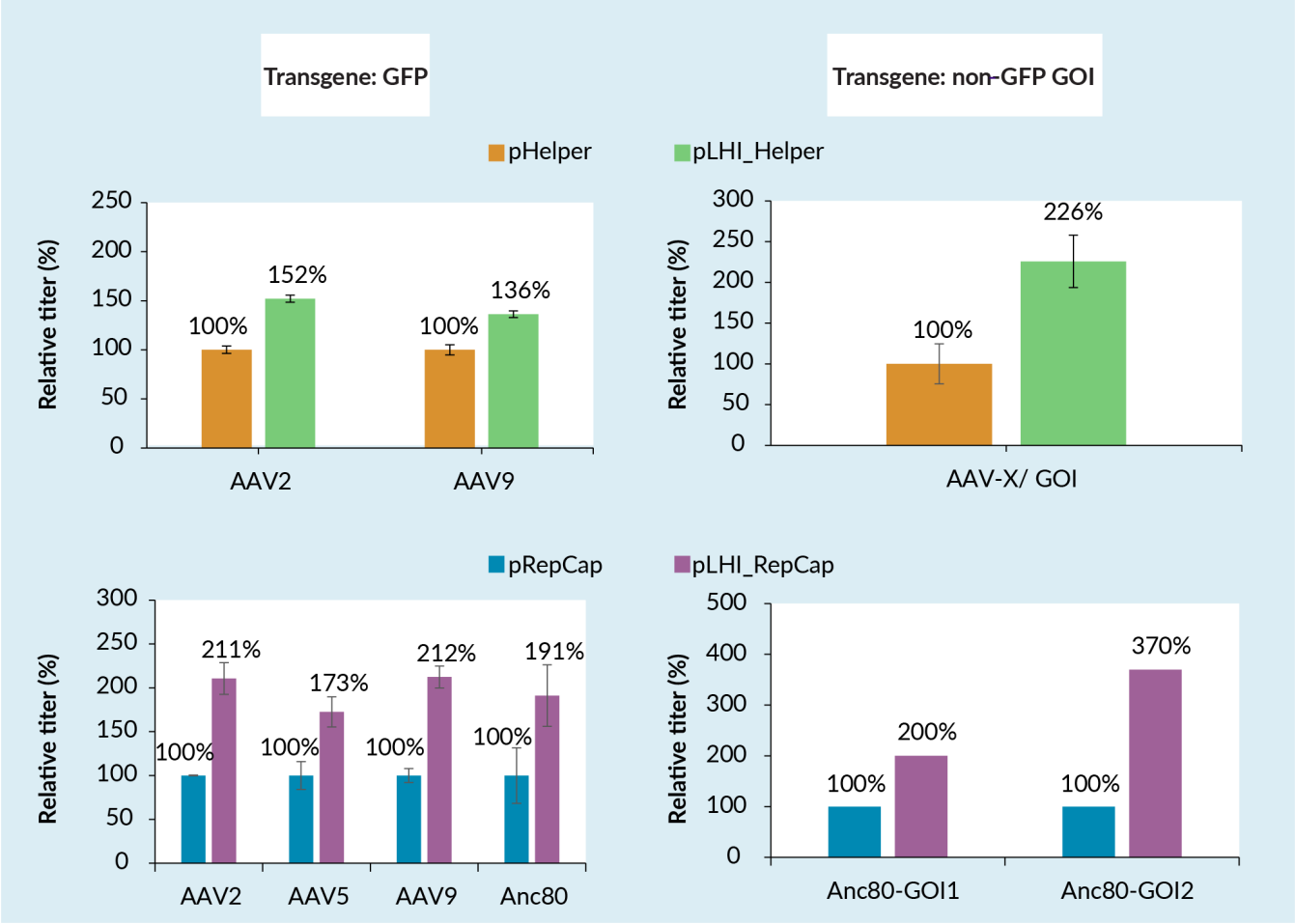
The Lonza AAV platform rests on three cornerstones for success. The first is high productivity and yield, achieved by a proprietary suspension clonal cell line, as well as some proprietary plasmids. The second is speed and reliability, which is attained by a pre-qualified assay library, optimized unit operations, and ready-to-go tech transfer documentation. The final cornerstone is Lonza's viral vector expertise with quality and regulatory support, from research and development to product development, assay development, and manufacturing (both clinical and commercial).

Lonza's proprietary cell line and plasmids increase AAV productivity. The 5B8 Suspension Cell Line is a HEK293 cell line that has been adapted to suspension culture in animal component-free media. It has proven scalability in AAV production from small-scale shaker flasks to 50 L production of green fluorescent protein (GFP). Production of AAV with non-GFP GOI has been demonstrated at a scale of up to 250 L. The cell line has also been shown to have high AAV productivity for multiple serotypes and GOIs, full traceability for regulatory compliance, and released GMP cell banks available for customer use.

Lonza also offers proprietary AAV production plasmids, including pLHI Helper plasmid and pLHI promoter for balanced rep/cap expression, to increase AAV productivity

FIGURE 1

The relative titer of Lonza's proprietary AAV production plasmids compared to standard plasmids in Lonza's 5B8 cell line.



and titer for both AAV2 and AAV9 serotypes (Figure 1). For multiple AAV serotypes, the Lonza cell line and pLHI rep/cap boosts productivity compared to a standard rep/cap by almost 200%. A full/empty AAV capsid ratio that is comparable to conventional production plasmids is observed when using these plasmids.

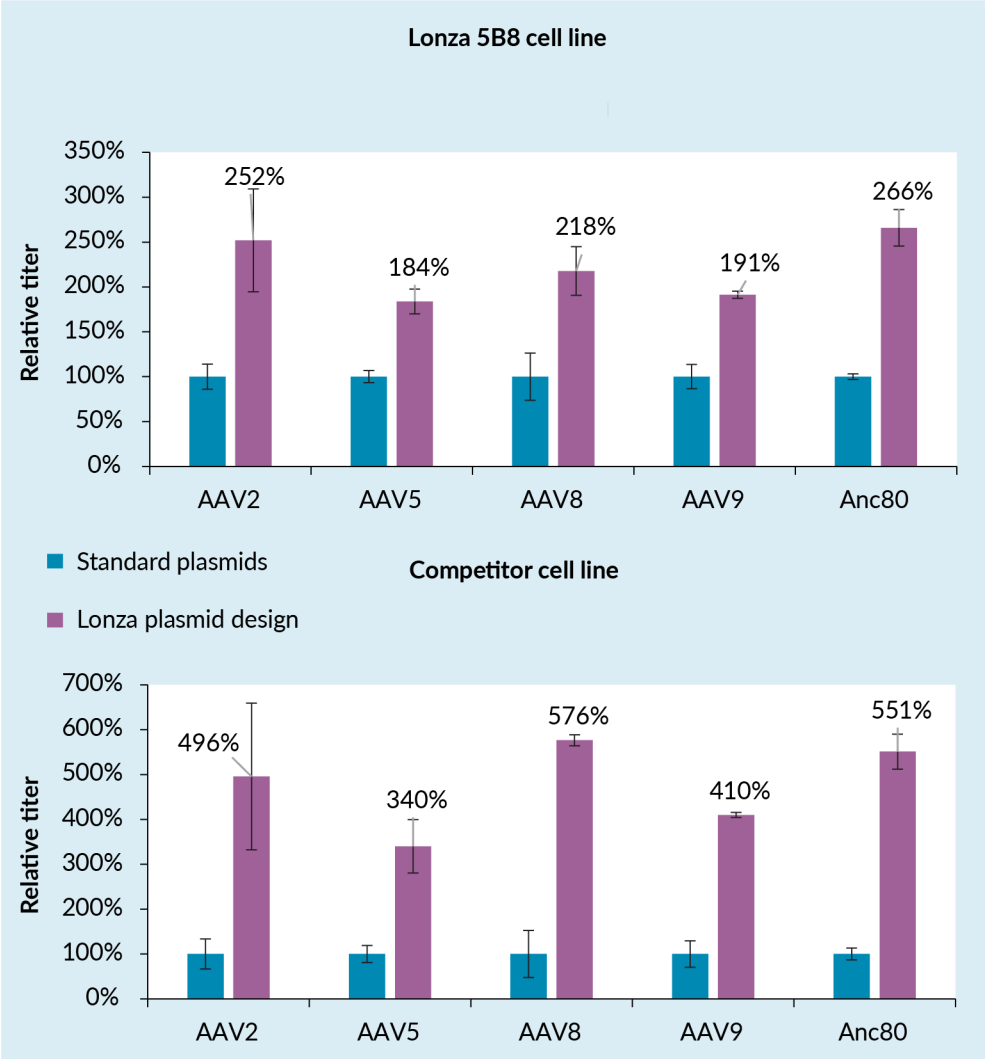
Lonza plasmids combined with the customer's transgene plasmid increases AAV productivity by almost two-fold for multiple serotypes, as shown in Figure 2. The efficiency of the Lonza system for AAV production ranged from two-fold to nine-fold depending on the specific AAV serotype used. Lonza's proprietary plasmids, either used alone or in combination, provided a high degree of enhancement for AAV productivity for both Lonza cell lines and key competitor cell lines, for multiple serotypes and GOIs.

FULL VERSUS EMPTY ANALYSIS

The optimization of the full/empty capsid ratio is critical for drug product quality and has implications for clinical efficacy and

FIGURE 2

Performance of Lonza's AAV production plasmid system in different HEK293 cell lines.



Transgene = GFP
Lonza plasmid design includes combined use of LHI pHelper + LHI Rep/Cap

DOI: 10.18609/cgti.2022.175

regulatory approval. Size exclusion chromatography (SEC) using multi-angle light scattering (MALS) is an assay that can be used to measure full/empty capsid ratio by determining the molecular weight and size of an analyte in solution. This enables optimization of % full AAV capsids at all stages and scales of process development. SEC MALS methodology shows a good correlation with analytical ultracentrifugation (AUC) for multiple GOIs and AAV serotypes (Figure 3), has a fast turnaround time and requires low sampling volumes. This assay is suitable for all stages and scales of process development, enabling efficient process optimization.

AN ESTABLISHED PARTNER FOR VIRAL VECTOR SERVICES

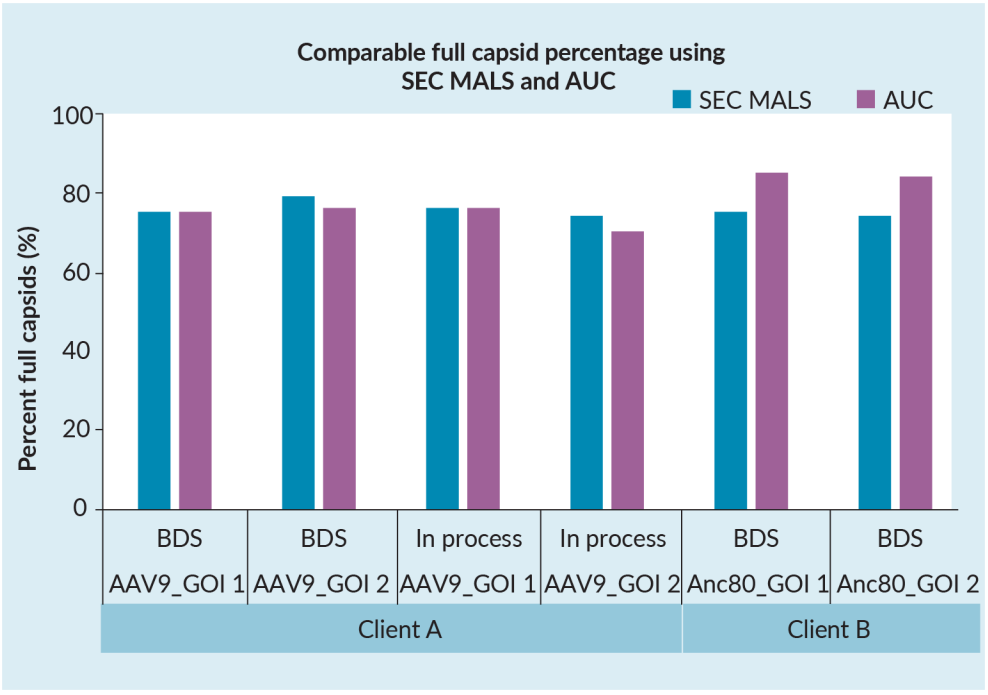
Lonza is a long-standing established partner for viral vector services, with 20 years of experience in viral vector manufacturing, over 70 projects currently in process development, and a track record of serving more than 50 viral vector customers.

Lonza was one of the first Contract Development and Manufacturing Organizations (CDMOs) to manufacture AAV at a 2000-L scale using the AAV producer cell line method. Lonza's Houston facility is certified for commercial manufacturing and has passed regulatory agency inspections for viral vector manufacturing for cell and gene therapies. They have over five projects in the late phase and commercial stage.

Lonza follows a streamlined path to the clinic and the market by utilizing prequalified assay libraries, optimized unit operations for upstream and downstream production, and ready-to-go technology transfer documentation. Commercial viability is ensured from early on by utilizing propriety starting materials with licensing options, namely a suspension cell line for AAV production, and Lonza propriety plasmids, both of which enable higher AAV productivity and yield. An SEC MALS analytical method is used for enhancing the percent full AAV capsids containing the customer's GOI.

FIGURE 3

Percentage full capsids of varying GOI and AAV serotypes as measured by SEC MALS and AUC.



Q&A

David McCall, Editor, BioInsights
speaks to (pictured) **Suparna Sanyal, Head of Viral Vectors Commercial Development, Lonza Cell and Gene Technologies**

Q Are the Lonza proprietary plasmids a part of the platform, or can customers use their own plasmids with Lonza's process?

SS: Customers' plasmids can be used with our platform process.

The proprietary plasmids are intended for customers who are looking to boost the productivity of their AAV. If there is a certain serotype or GOI that starts out with poor productivity or needs a boost, then these plasmids are available for optimization. Our customers can also choose to use them right from the get-go.

Q Is there a licensing fee for using Lonza's proprietary cell line or plasmids?

SS: If customers or developers are utilizing these materials in the Lonza process and doing the development and manufacturing work at Lonza, all licensing fees are waived.

If they should decide to take these proprietary elements outside of Lonza, then there are licensing fees.

Q With regards to your proprietary plasmids, what is the approach to navigating the legal landscapes surrounding serotype-specific rep/cap plasmids requiring usage licenses?

SS: Our rep/cap plasmid is essentially a sequence and a promoter, where we drop in the customer's choice of rep/cap to capitalize on the efficiencies.

It is the developer's responsibility to figure out any licensing implications with the rep/cap sequences. Their rep/cap can then be utilized and inserted into the plasmid that we have.

DOI: 10.18609/cgti.2022.175

Q What is a typical yield from a 3 L batch of AAV using Lonza's platform?

SS: This varies, depending on the serotype and GOI. If we were to take a 3 L bioreactor where our working volume is close to 2.5 L, we get anywhere from 1×10^{11} to 2×10^{12} at harvest. The overall yield that we achieve could be somewhere between 1×10^{13} to 5×10^{14}, or even higher.

Q Are titers measured using a quantitative polymerase chain reaction (qPCR) or a Droplet Digital PCR (ddPCR) method?

SS: They are measured using a ddPCR method.

Q The AUC results mentioned suggesting 80% full capsids. Are these values from the samples after purification and removal of empty capsids, or is it in the accrued lysate?

SS: Most of these results were not at harvest, when there is accrued lysate, and were instead measured later in the purification process. Some were in-process, and others were after full purification. The values that are closer to 80% are typically not what you see at harvest, which tends to be lower.

BIOGRAPHY

DR SUPARNA SANYAL is Head of Viral Vectors Commercial Development at Lonza Cell and Gene Technologies where she works with their global business, R&D and operations teams to enable strategic development and commercialization of their viral vector manufacturing services. Suparna's background is in Neuroscience and she earned her PhD from the University of Toronto in Neuropharmacology. She has over 15 years of R&D, drug discovery, CDMO and cell and gene therapy experience.

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Lonza
Cell & Gene

Learn more about Lonza's Viral Vector development and manufacturing service offering.

AUTHORSHIP & CONFLICT OF INTEREST

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

Acknowledgements: *None.*

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

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

ARTICLE & COPYRIGHT INFORMATION

Article source: *This article is a transcript of a webinar, which can be found here.*

Webinar recorded: *Feb 24 2022;* ***Revised manuscript received:*** *Oct 03 2022;* ***Publication date:*** *Oct 25 2022.*

DOI: 10.18609/cgti.2022.175

Strategic partnering to enable cell therapy commercialization

An ArsenalBio case study

Jenessa Smith, Director of Process Development, ArsenalBio

As emerging cell therapies move towards the commercialization phase, focus has been placed on establishing scalable and reproducible manufacturing processes and incorporating innovations to streamline cell therapy manufacturing. Strategic partnering between biotech and pharma companies can facilitate the challenging transition of moving cell therapies through the commercialization pipeline.

Cell & Gene Therapy Insights 2022; 8(9), 925
DOI: 10.18609/cgti.2022.138

Live 30

ArsenalBio and Thermo Fisher Scientific are working together in a mutually beneficial relationship, allowing both companies to leverage partner expertise and accelerate development of their technologies and therapeutic candidates. A critical evaluation timeline for this continued collaboration is detailed in **Figure 1**.

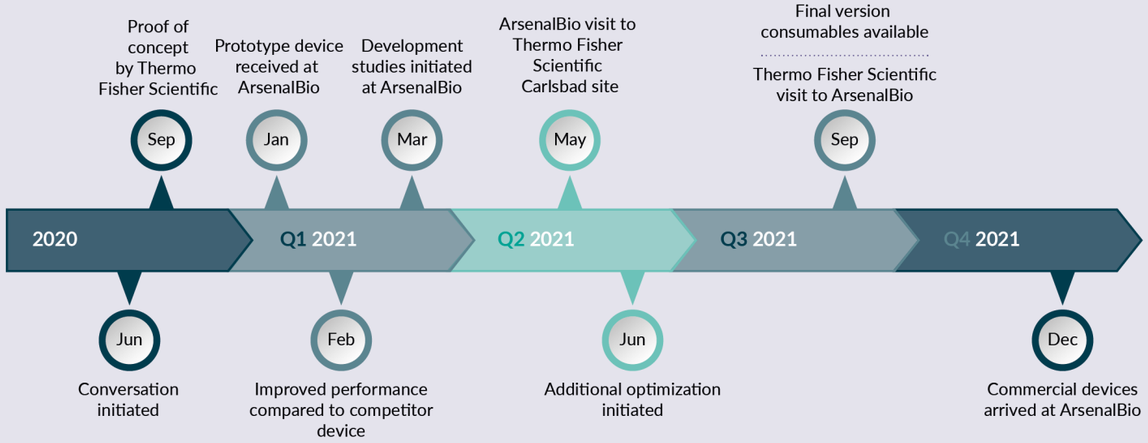
ArsenalBio's primary product is an autologous integrated circuit T cell (ICT) for chemotherapy resistant ovarian cancer. Working within a partnership gave ArsenalBio early access to novel technologies, as well as the opportunity to co-develop processes and integrated workflows **(Figure 2)**. Multiple unit operations, of the cell therapy manufacturing workflow have been evaluated within the collaboration.

Based on this work 1 mM Citrate, pH 6.0 would be the recommended buffer as an alternative elution buffer to RNase-free water for various mRNA sizes. The addition of EDTA to the citrate buffer resulted in a larger elution pool with similar recoveries.

"From our collaboration with Thermo Fisher Scientific, we have gained insight into not only the electroporation, but the entirety of the end-to-end process development. Thermo Fisher Scientific has many other solutions we have investigated. We not only gained insight into the unit operations, but of how Thermo Fisher was thinking about developing and prioritizing internally. Our relationship evolved from information sharing and thinking through troubleshooting in a collaborative way."

—Jenessa Smith, Director of Process Development, ArsenalBio

Figure 1. Case study collaboration timeline.



LESSONS LEARNED FROM A PRODUCTIVE COLLABORATION

Developing a productive relationship takes time and personal connection is the cornerstone of effective teamwork across both management and subject matter experts. Stating goals at the outset is helpful to ensure expectations are met. Shared, honest, open, and constructive communication is key.

For more information, watch the webinar or read the full article:

WATCH THE WEBINAR

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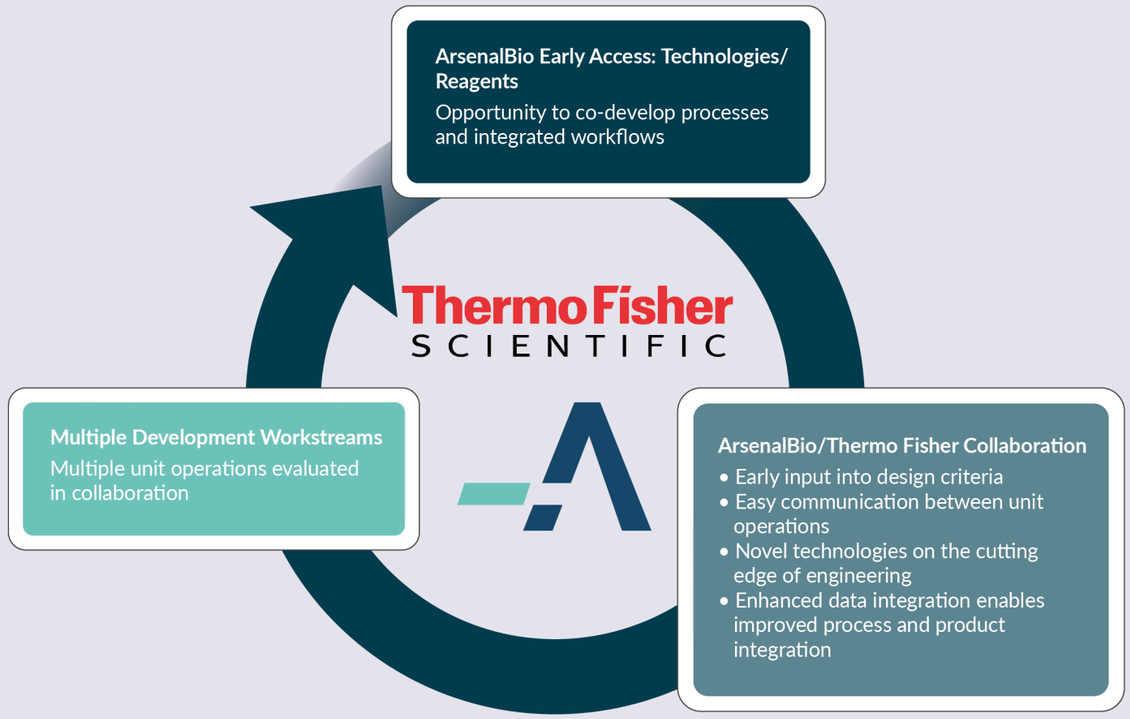
Figure 2. Mutually beneficial relationship between ArsenalBio and Thermo Fisher Scientific.



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