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## SPOTLIGHT ON
Induced pluripotent stem cells (iPSCS)

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CELL & GENE THERAPY INSIGHTS

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INDUCED PLURIPOTENT STEM CELLS (iPSCS) SPOTLIGHT

EXPERT INSIGHT

# Genetic stability of hPSCs and considerations for cell therapy

**Fabian Zanella and Raluca Marcu**

Continuous advances in the isolation, reprogramming, and gene-editing, as well as their scalable and efficient culture and differentiation for commercialization, have positioned human pluripotent stem cells as an attractive starting material for cell therapy. Nonetheless, continuous culture can predispose human pluripotent stem cells to genetic alterations at the chromosomal, region, copy number, and single nucleotide levels. The detection of those alterations is challenging, as no single assay can detect all potential genetic abnormalities in human pluripotent stem cells. However, combinations of approaches can provide a clearer assessment of human pluripotent stem cell lines, and when executed at key process steps can help mitigate costly expansion of genomically-compromised cells and the potential risks that those cells may pose to patients. Encouragingly, while the appearance of some genetic alterations may be concerning, in comparison with other cellular models, human pluripotent stem cells are not inherently prone to genetic instability. Furthermore, emerging strategies to mitigate the appearance and propagation of those abnormalities have shown encouraging results. Close partnerships between human pluripotent stem cell-derived cell therapy developers and regulators will help define genetic stability risks, best practices on testing pipelines, acceptance criteria, and robust guidelines in order to deliver these transformative therapies to patients in need.

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## INTRODUCTION

Human pluripotent stem cells (hPSCs) have held promise in regenerative medicine since the establishment of the first human embryonic stem cells [1]. The development of technologies to generate human induced pluripotent stem cells (hiPSCs) [2–7] marked a new era in the field, and the continuous development of ever more precise and accessible gene editing strategies have generated tremendous excitement towards the perspective of using these cells to manufacture life-saving cell

## TABLE 1

**Assays currently available to assess genetic instability in hPSCs.**

| Assay | Analyte | Target | Resolution | Scale | Estimated lowest level of mosaicism detected | Turn-around time | Cost |
|---|---|---|---|---|---|---|---|
| DNA index analysis by flow cytometry | Individual nuclei | Total DNA | Gain or loss of ≥5 chromosomes | Whole genome | 2–3% | ⌛ | $ |
| G-band karyotyping | Individual nuclei | Individual chromosomes and regions | 5–10Mb | Whole genome | Typically, 5–15%, depending on number of cells analyzed | ⌛⌛ | $$ |
| aCGH, SNP arrays | Bulk genomic DNA | Chromosome regions | 10–100 kb | Whole genome | 10–20% | ⌛⌛⌛ | $$$ |
| FISH | Individual nuclei | Individual regions, genes | 100kb–1Mb | Target sequences | 5% | ⌛⌛ | $$ |
| iCS-digital™ PSC 24-probe (ddPCR) | Bulk genomic DNA | Individual regions, genes, SNPs | >1bp | 24 specific sequences | 20% | ⌛ | $ |
| Stem-Seq+™, OncoPlex (targeted NGS) | Bulk genomic DNA | Individual regions, genes, SNPs | >1bp | 361, 350 genes | 1–3% | ⌛⌛⌛ | $$$$ |
| WES (NGS) | Bulk RNA | Individual transcripts, SNPs | >1bp | Whole transcrip-tome | 1–3% | ⌛⌛⌛⌛ | $$$$ |
| WGS (NGS) | Bulk genomic DNA | Individual regions, genes, SNPs | >1bp | Whole genome | 1–3% | ⌛⌛⌛⌛ | $$$$ |

aCGH: array comparative genomic hybridization; FISH: fluorescence *in situ* hybridization; NGS: next-generation sequencing; ddPCR: digital-droplet PCR; SNP: single nucleotide polymorphism; WES: whole exome sequencing; WGS: whole genome sequencing.

therapies. Altogether, their virtually infinite propagation and differentiation potentials combined with novel genetic edits make them an ideal starting material with broad opportunity for treating unmet medical needs.

In spite of remarkable advances and innovations in process development tailored for large-scale hPSC culture, gene-editing, and differentiation, the cell growth and differentiation paradigms can be aggressive, fomenting a selection process favoring the outgrowth of cells that may have genetic instabilities and acquired undesirable genetic mutations.

Genetic instability has been well documented for hPSC culture and differentiation [8–24]. Furthermore, continuous propagation of hPSCs has been seen to drive genetic instability at the three fundamental levels of organization of DNA:

- Chromosomal stability, where whole chromosomes or large regions are lost or amplified;

- Copy number variations, where smaller regions containing clusters of genes and regulatory elements are amplified or lost; and
- Single nucleotide polymorphisms (SNPs), where individual nucleotides impacting the expression of specific genes are altered.

These genetic changes can alter the phenotype and function of hPSCs and/or their differentiated progenies, and therefore assessing genetic stability at all stages of the hPSC-derived cell therapy manufacturing process is critical for successful commercialization and patient delivery.

## ASSESSING GENETIC INSTABILITY IN hPSCS

A number of test methods are available to assess and characterize the genetic integrity of hPSCs, summarized in Table 1. These technologies include flow cytometry [25],G-band karyotyping [26], array comparative genomic hybridization (aCGH) and SNP arrays, Fluorescence *in situ* hybridization (FISH), droplet digital (dd)PCR [27], and next-generation sequencing (NGS)-based assays [20,23,24,28]. When defining the testing strategy for genetic characterization of hPSCs the assay target and the type of genetic abnormalities detected, the assay resolution, and the assay sensitivity with respect to the level of variant mosaicism should all be carefully considered. It is important to note that currently, no single assay is able to detect all levels of genetic instability simultaneously, and at a satisfactorily low limit of detection. Nonetheless, these testing strategies can be complementary to each other when deployed at critical stages of the hPSC-derived cell therapy product manufacturing: donor cell characterization, iPSC reprogramming and seed bank generation, gene-editing of seed bank, and creation of the master and working cell banks [10,17,29,30]. Together, these tests can help build a narrative towards go/no-go decisions (Figure 1). As a practical example, G-band karyotyping can provide information on the number of chromosomes found, and on their structure (absence of duplications, translocations, etc.) but it cannot detect smaller region amplifications and deletions which can be detected by aCGH; while the latter cannot detect translocations that do not cause clear loss of heterozygosity. Similarly, G-band karyotyping will not be able to provide insights on mutations in specific genes, which can be detected by targeted, NGS-based assays, albeit the latter will not be able to detect translocations, duplications, and other larger scale rearrangements with no clear impact in individual gene sequences.

Importantly, except for whole exome sequencing (WES) and whole genome sequencing (WGS), all other assays can be provided as a service and include a report that can be used as a basis for assessing the potential pathogenicity of the findings. Although extremely rich in information, WES and WGS data typically require somewhat intensive bioinformatics analyses, adding complexity and timing requirements to the assays, however once those advanced assays and analyzes are performed, they can also be included in quality control of hPSCs.

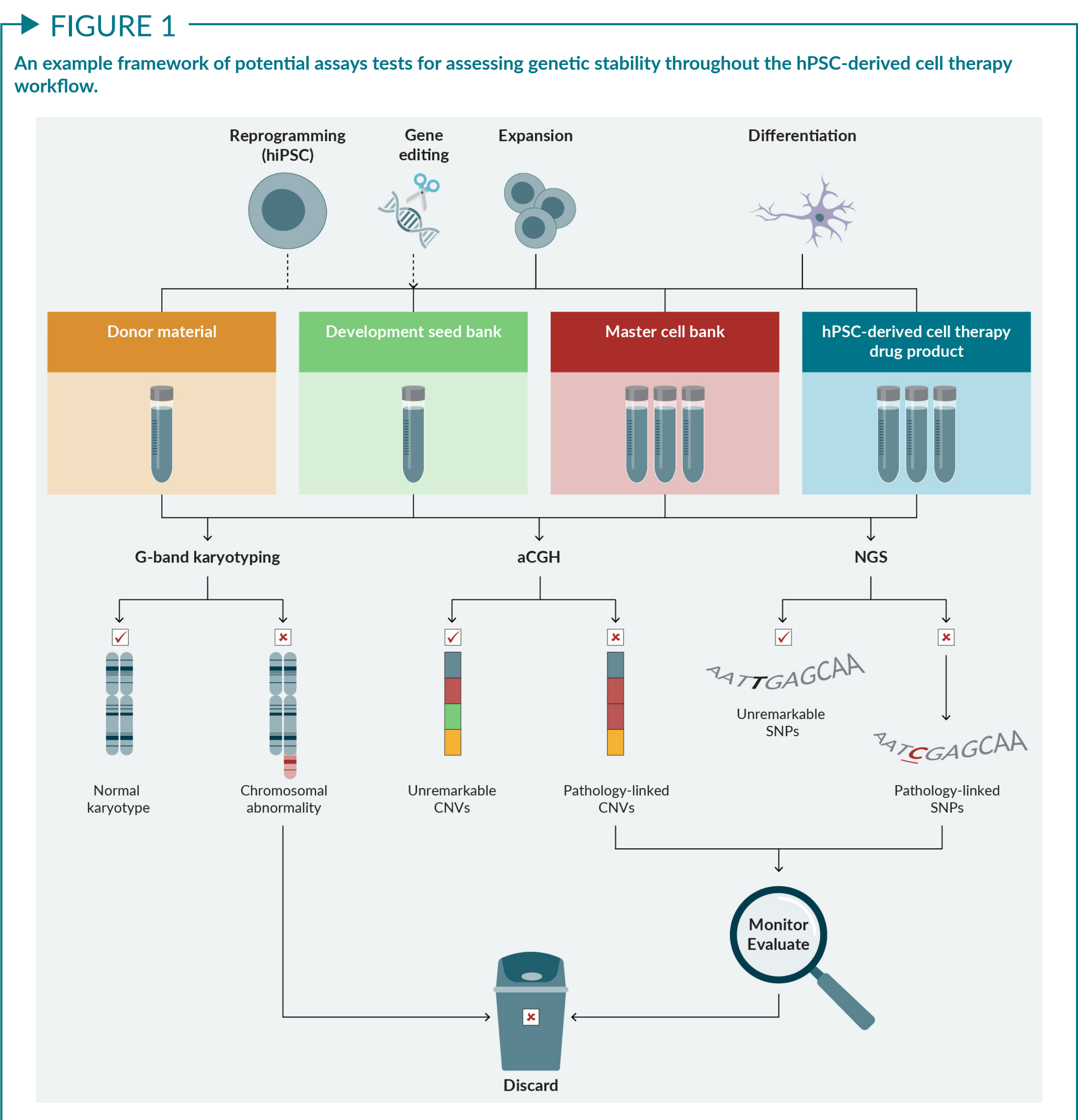
## ALTERED GENETIC PROFILES IN hPSCS: WHAT DO WE KNOW?

At a high level, genetic instability can impact cells destined for therapy by disrupting the expression of key genes involved in the cell physiology, leading to potentially oncogenic, transformed phenotypes, or negatively impacting stemness and differentiation ability. Several chromosomal, regional, and gene-level abnormalities have been reported in hPSCs.

The breakthrough demonstration that the pluripotent state can be induced in differentiated cells by overexpression of a defined set of transcription factors [31] opened the avenue for generating iPSC by reprogramming a wide range of somatic cells (skin fibroblasts, peripheral blood and cord blood

FIGURE 1

**An example framework of potential assays tests for assessing genetic stability throughout the hPSC-derived cell therapy workflow.**




aCGH: array comparative genomic hybridization; FISH: fluorescence in situ hybridization; NGS: next-generation sequencing; ddPCR: digital-droplet PCR; SNP: single nucleotide polymorphism; WES: whole exome sequencing; WGS: whole genome sequencing.

cells, keratinocytes, urine cells and others). Considering the genetic background of the starting material, the reduced efficiency and clonogenic nature of the reprogramming process, and the complexity of epigenetic changes associated with reverting cellular identity during reprogramming (reviewed in [32]), particular attention should be paid towards careful genetic characterization of both starting material and reprogrammed material when manufacturing iPSCs. The choice of the starting material's source and donor age, need to be carefully considered, as well as the selection of the reprogramming method (non-integrating: Sendai-virus, episomal and mRNA-based versus integrating viral

vectors), in order to minimize the mutational burden of the resulting iPSC line. Several studies reported chromosomal aberrations, mutations and genetic variants associated with both parent somatic cells and the reprogramming process, in addition to culture-acquired genetic changes [33–36]. Identifying pre-existing and *de novo* acquired mutations and genetic variants is key to ensure safety of the manufactured cell therapy product.

At the chromosome level, gains of regions of the long arms of chromosomes 1, 17 and 20 or the short arm of chromosome 12, or gain of a whole chromosome (trisomy 1, trisomy 20), as well as losses of regions on chromosomes 10 and 18 are common and recurrent abnormalities in hPSCs [11,17,18,27]. Most of these large chromosomal abnormalities can be detected, with varied sensitivity, by several methods, including G-band karyotyping, ddPCR, aCGH, and FISH.

At a sub-chromosomal level, aCGH and SNP arrays have brought to light copy number variations and loss of heterozygosity in hPSCs [15–17]. Furthermore, the occurrence and propagation of those alterations seem to have a dynamic behavior across reprogramming and hPSC culture [14]. Given the complexity of the analysis and interpretation of results in these studies, alterations observed outside of genomic regions known to cause clear consequences to cell behavior must be dissected carefully, and their relevance may need to be weighed against the cell therapy niche.

At the level of individual genes, three examples of alterations that gained attention include the tumor suppressors Tumor protein 53 (TP53) [20,23,24] and B-cell lymphoma 6 (BCL6) corepressor (BCOR) [20], as well as the oncogene Bcl-2-like protein 1 (BCL2L1) [12,13,37]. Although different in nature, alterations in these three genes seem to provide selective advantage to the affected cells, as their representation increases rapidly with culture and propagation. TP53, BCOR and BCL2L1 genetic variants are typically identified through next-generation sequencing methods (targeted-NGS, WES, and WGS).

Located on chromosome 17, TP53 is a well-established tumor suppressor gene, functioning to shepherd cells that display key genetic abnormalities through apoptosis. Additionally, TP53 has been implicated in regulation of cellular stemness, differentiation and reprogramming. Noteworthy, several studies reported substantial increase in the reprogramming efficiency of somatic cells to iPSCs by inhibition or inactivation of the p53 pathway, likely through mechanisms related to promotion of cell cycle progression [38–40]. Mutations in TP53 have been detected and analyzed at length in hPSCs [8,19,22–24]. A common finding in these studies is the higher prevalence of dominant-negative TP53 mutations in hiPSCs generated from skin fibroblasts, which have been linked to exposure of parental fibroblast cells to DNA damage caused by UV radiation [20,23]. However, *de novo* TP53 mutations have been observed to occur in hPSCs that either were not derived from skin fibroblasts, or whose parental skin fibroblasts did not display detectable levels of mutations in the gene [23]. The variant allele frequencies of these mutations were generally seen to be amplified by sequential culture and through differentiation processes. Remarkably, although in a smaller number of cases, the allelic fraction of those mutations exceeded 50%, suggesting additional selective advantage resulting from the loss of heterozygosity at the TP53 locus [23]. Surprisingly, mutations in TP53 do not seem to impact the differentiation potential of affected cells towards gut epithelial cells [41] neuroepithelial cells [42] and pancreatic polyhormonal cells [43]. However, given the prominent role of TP53 in cancer, mutations in this gene have deservingly raised concerns amongst cell therapy developers [23]. Therefore, the mutational status of TP53 should be thoroughly characterized at the donor level, after reprogramming for hiPSCs, through hPSC expansion and differentiation and in the final differentiated hPSC-derived cell therapy drug product.

BCOR is a BCL-6 interacting corepressor, ubiquitously expressed, located in the X chromosome. When overexpressed, BCOR can potentiate BCL-6 repression, leading to evasion from apoptosis [44]. BCOR has been implicated in embryogenesis regulation, modulation of mesenchymal stem cell function, hematopoiesis, and lymphoid development. Somatic gene mutations of BCOR and its homolog BCORL1 have been detected in several hematologic malignancies and aplastic anemia [45]. Additionally, BCOR mutations have been linked to oculofaciocardiodental syndrome [46], highlighting its potentially complex roles in early development. Interestingly, it has been reported that blood-derived hiPSCs display lower mutational burden from UV damage compared to skin fibroblast-derived hiPSCs, but a high prevalence of acquired BCOR mutations [20]. Furthermore, BCOR mutations seem to predominantly arise *in vitro*, enabling strong selective pressure towards the altered cells. The role of BCOR mutations in hPSCs deserve further investigation: on one hand BCOR mutant cells have been reported to have impaired differentiation into ectoderm, mesoderm, and downstream hematopoietic lineages [47], while on the other hand deficiencies in forming neural stem cells and terminally differentiated neurons were observed and attributed to a bias towards mesoderm and endoderm priming [20].

BCL2L1 resides in chromosome 20q11.21, a genomic region frequently amplified in hPSCs [11–13]. BCL-XL is an anti-apoptotic protein isoform that results from the expression of BCL2L1, and which is observed to be upregulated in several human cancers [48]. Independent studies have linked the amplification and overexpression of BCL2L1 to increased resilience and growth advantage of the affected cells in hPSC cultures [12,13,19,21]. Interestingly, BCL2L1 amplification appears to favor the survival of pancreatic progenitors at crucial steps during differentiation by favorably modulating glycolysis and oxidative phosphorylation pathways [37].

## MITIGATING GENETIC INSTABILITY OF hPSC-CELL THERAPIES

As more sophisticated detection methods for assessing genetic instability develop, a tighter control of starting materials and culture conditions may be able to prevent the emergence or delay the expansion of genetically abnormal cells, should they already exist below current limits of detection in starting cell lines, or develop through different culture landmarks.

Comprehensive testing of the starting material for hiPSCS will establish the genetic profile of the source material and provide an early screen for donor materials which may present abnormal genetic profiles. Alternative sources of starting material should be explored, such as bone marrow-derived mesenchymal stromal cells, which, given their internal localization in the body, are likely more protected from UV-driven TP53 mutations commonly occurring in skin fibroblasts. Given their favorable growth properties, bone marrow-derived mesenchymal stromal cells may represent a viable alternative as starting material for reprogramming to hiPSCs that would reduce the risk of UV-driven TP53 mutations.

Parallelly, evolving cell culture paradigms can be adopted to reduce the selective pressure of cells carrying alterations through culture bottlenecks including reprogramming, recovery following electroporation and low density seeding after gene editing, and adaptation to suspension culture for large scale expansion. While it may not be possible to fully prevent the appearance of genetic alterations, different strategies can be employed to delay the expansion of altered cells. These strategies may include novel and richer hPSC culture media, designed for enhanced cell tolerance of culture bottlenecks, the addition of pro-survival Rho-associated kinaseinhibitors such as Y-27632 [49] or Thiosivivin [50], or the more encompassing CEPT cocktail [51]. Other approaches, including culture under hypoxic conditions to lower oxidative stress-facilitated DNA damage [52] and the

inclusion of nucleosides into the cell culture medium to compensate for the deficiency of DNA precursors in rapidly dividing cells [53], constitute interesting avenues to be explored, and hinge upon relatively simple manipulations of hPSC cultures. Regardless of the strategy employed, comprehensive genetic testing should be performed at all critical stages in iPSC manufacturing to detect abnormalities associated with culture bottlenecks, different culture conditions and large-scale expansion.

Clustered regularly interspaced short palindromic repeat (CRISPR) technology [54] has become a central tool for gene editing in hPSCs. Further iterations of the technology enable temporal silencing of gene expression [55], as well as changes in methylation patterns [56]. Forward-looking, as more and better defined genomic hot spots impacted by hPSC culture are identified, perhaps these different modalities of CRISPR technology can be used to (i) reduce the expression of target genes that are often mutated, thus diminishing the selective advantage of mutated clones or (ii) modulate methylation patterns around fragile DNA areas to protect them from erosion and alteration.

## TRANSLATION INSIGHT

hPSCs will play a central role in numerous upcoming cell therapies that aim to revolutionize treatment options and bring renewed hope for patients suffering from devastating and incurable diseases. The demonstrated safety of these cell therapies, including the genetic stability of hPSC-derived cell therapy drug products, will be crucial to their success.

Even as we conclude this manuscript, we recognize the current paucity of robust studies evaluating the implications of genetic stability of hPSCs that are statistically well-powered, while simultaneously using rigorously controlled and defined, animal component-free cell hPSC culture conditions and reagents that have a clear path towards the clinic. More work needs to be done under comparable and unified cell culture and testing paradigms to ensure translatability of results. Recent guidelines for stem cell-based research have produced a relevant framework of best practices [30], however, more work needs to be carried out to define tighter culturing and testing criteria for the safety of developing hPSC-cell therapies. At a minimum, cells should be evaluated for the presence of genetic changes when a new line is derived (by reprogramming or gene editing), when master and working cell banks are manufactured, and ideally, throughout the timespan of experiments [30].

Key to the continuing advancement of the field is higher genetic testing sensitivity, producing results that are clearer and with faster test turnaround times. Lowering the limits of detection for abnormal variants and shortening turnaround time are critical to avoid costly manufacturing of genetically abnormal cells. Next generation sequencing technologies may continue to play a prominent role here. Ideally, assays that would enable the detection of unknown mosaicism at a threshold below 1% are strongly desired, as it may be feasible to eliminate low-recurrence mutations, for example, by subcloning, with the clear caveat that this approach in itself introduces bottleneck events that may lead to similar or additional genetic alterations.

Given that cell therapy is still a relatively new treatment modality that targets several diseases for which there are no curative treatments, it will be important that therapeutic developers and regulatory bodies work closely together to more clearly define genetic stability acceptance criteria for these promising therapies. On one hand, patients need to be protected from dangerous unintended outgrowths, while on the other hand, regulations should accommodate risks deemed acceptable for patients who desperately need those therapies and have no alternative options.

Central to those discussions will continue to be evolving notions on which types of genetic instability may disqualify a cell therapy, and which gene mutations have deleterious consequences. Outlining a roadmap to

approach *de novo*, uncharacterized mutations for which patient or animal model data are not available will also be important. Closer partnership between hPSC-derived cell therapy developers, clinical geneticists, bioinformaticians, and protein engineers will be key to understanding the potential functional consequences of those mutations.

Similarly, it will also be important to factor in the role of the local niche targeted by the cell therapy. More work needs to be carried out to dissect situations where, for example, a genetic alteration impacts a gene perceived to be implicated in malignancy, however the niche or tissue where the therapy will be delivered does not favor expression of that same gene. Supporting this view are several documented examples of mutations in genes linked to cancer in specific tissues that either have no effect or cause benign conditions in other tissues [57].

Finally, while acknowledging the presence of genetic instability in hPSCs causes discomfort, the acquisition of genetic changes in cultured hPSCs is inevitable but manageable when recognized timely. It is important to acknowledge that cell culture environments are generally harsh from a biochemical perspective, with stresses thought to favor better fit, frequently altered populations. hPSCs rely on different mechanisms than somatic cells to maintain genomic fidelity, and while they do show a propensity to acquire recurrent genomic changes, when compared to other cellular models, such as cancer cell lines, these cells are not inherently genetically unstable [19], and thus, they remain an attractive starting material for cell therapies.

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## AFFILIATIONS


**Fabian Zanella**
Pluristyx, Inc.,
201 Elliott Ave., Suite 290,
Seattle WA 98119, USA

**Raluca Marcu**
Pluristyx, Inc.,
201 Elliott Ave., Suite 290,
Seattle WA 98119, USA

AUTHORSHIP & CONFLICT OF INTEREST

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**CELL & GENE THERAPY** INSIGHTS

INDUCED PLURIPOTENT STEM CELLS (iPSCS)

SPOTLIGHT

# Autologous induced pluripotent stem cell (iPSC)-derived therapies: a realistic solution for advancing regenerative medicine

**Jarett Anderson and Timothy Nelson**
HeartWorks, Inc.

"Many patients, if given the choice, would likely prefer to receive their own cells."

VIEWPOINT

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An ongoing debate in the regenerative medicine space today is whether development of donor-specific (autologous) iPSC-derived products is practical. Common criticisms of the autologous approach are that patient-specific product manufacturing is too time consuming, too costly and cannot be scaled. Additionally, some believe that inherent variability in starting material and final products may present challenges from a licensing perspective. While these claims may bear some truth, they alone are not sufficient to discount autologous products as they may offer a reasonable alternative for some patients/conditions.

There are currently at least four 'authorized' Investigational New Drug (IND) Applications in the USA utilizing autologous iPSC-derived technologies. Indications for these IND's include macular degeneration, Parkinson's disease, and congenital heart defects. Two of these programs have clinical studies which are actively recruiting and listed on ClinicalTrials.gov: NCT05647213 (Sponsor: HeartWorks) and NCT04339764 (Sponsor: National Eye Institute). Numerous groups are actively pursuing clinical development plans in the autologous space, with the real likelihood of several additional clinical studies in the next 1–3 years. Recently, we participated in a forum of over 50 independent experts working on a variety of autologous iPSC-derived therapies gathered in a first of its kind event focused on collaboration to speed development and promote growth in the autologous iPSC field. This demonstrates the significant effort and investment being directed towards personalizing next-generation therapies.

Clinical-grade products derived from iPSC, whether autologous or allogeneic, are indeed time-consuming and costly to manufacture. Multiple factors influence the overall production time and cost, including starting material, iPSC reprogramming method, method of expansion (2D vs 3D), and scale of batch size. While allogeneic product manufacturing may allow for larger number of doses in a single run and can be made available as an off-the-shelf product, there are several considerations with regard to impact on patients and populations. These include:

- **Potential for allo-immunization:** given the relative infancy of this field, some patients receiving iPSC-derived therapies may still become transplant candidates for their current or alternate indications. Exposure to allogeneic tissue may increase the risk of allo-immunization even with the use of immunosuppression;

- **Immunosuppression cost and side effects:** immunosuppression costs can range from $1,000–3,000 per month or more; These costs would likely be passed to the patient/payor rather than the iPSC drug manufacturer and adds to the overall cost of treatment with the allogeneic product. Additionally, there are numerous side effects/risks associated with both short- and long-term use of immunosuppression. Patients with reasonably good quality of life may not be willing to tolerate the significant side effects, and the risk of lymphoproliferative disorders associated with long-term immunosuppression is well documented. In the absence (or failure) of immunosuppression the durability of allogeneic products is compromised;

- **Overall risk to public health:** a single batch of allogeneic product used across multiple patients poses risks that are not fully understood at this early stage of iPSC clinical application;

- **Health equity:** allogeneic cell lines may have limited compatibility with some ethnic backgrounds. Diversity in the population will inherently place some individuals at a disadvantage.

Genome editing approaches are being utilized to develop 'non-immunogenic' iPSC which may address some

TABLE 1

**Considerations associated with autologous and allogeneic iPSC-derived products.**

| | Autologous | Allogeneic |
|---|---|---|
| Pros | ▶ No immunosuppression<br>▶ Reduced risk of allo-immunization<br>▶ Health equity | ▶ Cost-efficiency per batch/dose<br>▶ Product consistency<br>▶ Off-the-shelf availability |
| Cons | ▶ Manufacturing cost<br>▶ Not conducive to urgent administration<br>▶ Batch variability | ▶ Immunosuppression: cost and side effects<br>▶ Alloimmunization<br>▶ Greater number of patients at risk from unknown factors within a single batch |

issues relative to health equity and immunosuppression/alloimmunization associated with allogeneic products. However, these approaches can involve multiple rounds of gene editing which may add unknown safety risks.

The patient's voice is heard now more than ever regarding healthcare delivery and options. Many patients, if given the choice, would likely prefer to receive their own cells. This societal factor must also be considered in terms of differing approaches to product development. A case in point is within the congenital heart disease community. Infants born with severe forms of congenital heart defects will require palliative surgery to extend the longevity of their heart muscle. These surgeries are palliative because they do not offer a curative approach and the likelihood of these individuals needing a heart transplant are very high despite the best care available. Additionally, the multiple surgeries and multiple blood products these individuals require significantly increases the risk of developing allo-antibodies that may eliminate heart transplant as an option. Therefore, developing an autologous product that offers a durable engraftment of new heart muscle and minimizes any risk of exacerbating allo-antibody formation is clearly an ideal product for these individuals. Furthermore, this patient population has a predictable natural history of heart failure which gives time to anticipate and prepare for an autologous product to be manufactured. Thus, there are clinical situations where the investment in an autologous approach can justify the resources needed to make this option possible. As these products become more scalable, feasible, and clinically effective, we anticipate the value proposition of autologous iPSC products will continue to expand into new clinical indications that are currently not being considered solely because of the perceived financial constraints.

The 'autologous versus allogeneic' approach will continue to be debated; both have merits as well as drawbacks (Table 1), and the truth is there is a time and place for both. Continued development of autologous iPSC-derived therapies is reasonable in the current landscape of regenerative medicine.

## BIOGRAPHIES

**TIMOTHY NELSON,** a physician/scientist trained in medicine and cardiac developmental biology, has been driven to innovate with available technology with the goal of new product development. Congenital heart disease provides the optimal mix of challenges and opportunities to allow cross-functional teams to succeed and grow a whole new industry. The industry is the sustainable and growing academic research fueled by impact philanthropy

that can de-risk product development and enable corporate-partners to provide the lowest-cost solutions to medical institutions- patients benefit and purpose-built team expand. Nelson describes HeartWorks' purpose as "to inspire biomedical innovation teams and solve real-world medical problems with a focus on products that will obsolete the traditional processes that define yesterday's healthcare experience."

**JARETT ANDERSON** is the Chief Regulatory Officer for HeartWorks, a not-for-profit 503(c)(3) foundation focused on bringing congenital heart disease scientific discovery out of the lab and into the clinic. His primary responsibility is to build a culture of quality and regulatory compliance in all aspects of the cross-functional teams engaged in product development and clinical trial execution. Anderson has over 20 years experience in translation of basic research protocols into clinical application, in-cluding development of associated regulatory, quality, and training materials. He has collaborated on multiple IND submis-sions for a variety of cellular therapy products, including pancreatic islets, mesenchymal stromal cells, dendritic cells, mono-nuclear cells (from cord blood and bone marrow), and induced pluripotent stem cells. This experience has given Jarett an in-depth understanding of the regulatory requirements associated with investigational product manufacturing, interaction with regulatory bodies, and execution of both pre-clinical animal studies and early-phase/first-in-human clinical trials. Jarett earned his BSc in Medical Technology from North Dakota State University in Fargo, ND, and is a certified Medical Technologist (American Society of Clinical Pathology, ASCP).

## AFFILIATIONS

**Jarett Anderson**
Chief Regulatory Officer
HeartWorks, Inc.,
Rochester,
Minnesota, USA

**Timothy Nelson, MD, PhD**
Founder and CEO,
HeartWorks, Inc.,
Rochester,
Minnesota, USA

### 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.

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INDUCED PLURIPOTENT STEM CELLS (iPSCS)

SPOTLIGHT

REVIEW

# AI and iPSCs: current applications for characterization and quality control

**Jonathan Dack**

Induced pluripotent stem cells are a type of pluripotent stem cell generated by reprogramming adult cells to an embryonic state. Similar to embryonic stem cells in terms of morphology and pluripotent marker expression, induced pluripotent stem cells have been found to retain epigenetic memory, including DNA methylation and histone modifications, of the cell type from which they are created [1]. Induced pluripotent stem cells are also susceptible to genetic anomalies arising from the reprogramming process and prolonged *in vitro* cultivation [2]. Somatic cell reprogramming involves a series of genetic manipulations, potentially inducing mutations, copy number variations, or chromosomal abnormalities. Prolonged culture amplifies the risk of instability [3], leading to unintended alterations in the induced pluripotent stem cell genome, which could compromise their therapeutic utility. In the pursuit of safe and efficacious clinical applications, a thorough assessment of induced pluripotent stem cell-derived cell products across a range of culture stages is imperative. Current methods are characterized by their labor-intensive nature and susceptibility to subjective biases, relying on manual steps to discern induced pluripotent stem cell induction and maturation stages [4]. To overcome these limitations, researchers are exploring the utility of AI and machine learning for greater objectivity and efficiency in the evaluation process. Proposed strategies encompass the identification of specific biomarkers coupled with quantitative gene expression analyses [5], leveraging advances in the field of micro-spectroscopy for precise characterization [6], the adoption of automated imaging systems integrated with analytical algorithms [7], and single-cell analyses to discern heterogeneity [8]. This article outlines the emergence of AI-integration in stem cell research and offers translational insight on the future of induced pluripotent stem cells in regenerative medicine.

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## BACKGROUND

### Reprogramming mechanisms

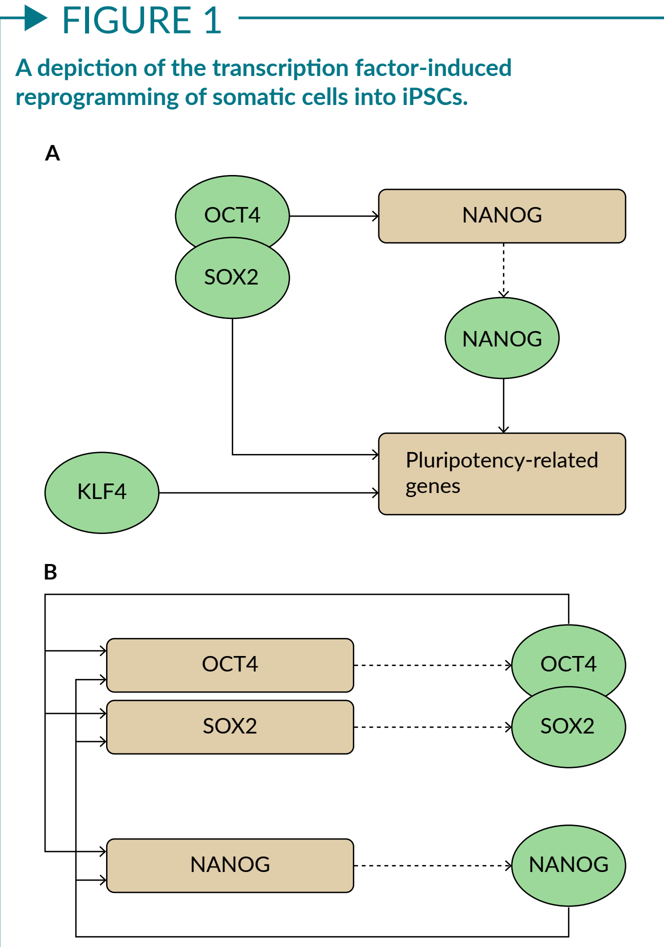
Induced pluripotent stem cells (iPSCs) are created when adult somatic cells are subjected to a reprogramming protocol, designed to revive embryonic characteristics. The process is typically induced through the introduction of specific transcription factors, selected for their ability to reengage drivers of pluripotency within the cell nucleus [9]. Through the initiation of endogenous mechanisms, components of the intracellular environment (including certain chromatin-modifying enzymes, regulatory RNA molecules, and signal-transduction pathways) revert the treated cells to an embryonic state. The key mechanism is an autoregulatory loop that is initiated by OCT4, SOX2, and NANOG taking up positions on their own promotor genes to drive expression of their endogenous counterparts (Figure 1) [10]. This cycle represents the foundation of a transcription network that drives the creation of genes linked to pluripotency (such as Fbx15) and inhibits genes that cause differentiation (such as Pax6 and Dlx5) [11]. To demonstrate that reprogramming has been successful, the resulting cells must exhibit certain properties to be classified as iPSCs. These characteristics include the expression of embryonic stem cell (ESC) markers (including SSEA-3, SSEA-4, tumor-related antigen (TRA)-1–60, and TRA-1–81), unique morphology and phenotype, self-renewal capacity, the ability to give rise to teratomas containing cell types from all three germ layers, and the capacity to form specialized tissue [12]. In his seminal paper from 2006, Yamanaka demonstrated the plasticity of his invention by growing iPSC-derived tissue from the brain and heart [9].

### Cell sources

While some suggest that iPSCs are functionally equivalent to ESCs, recent studies have highlighted genomic differences that compromise iPSCs' long-term stability in culture. These differences necessitate careful consideration when utilizing iPSCs in research and clinical applications. Factors such as genetic stability may impact various aspects of iPSC characteristics [45]. For example, aneuploidies, sub-chromosomal copy number variations, and single nucleotide variations [13] have all been observed in iPSC populations, hampering differentiation potential and also raising biosafety concerns. These genetic variations stem from a number of sources, including the population of starting cells used to make iPSCs [14]. For

**FIGURE 1**

**A depiction of the transcription factor-induced reprogramming of somatic cells into iPSCs.**




(A) Illustrated regulatory circuitry of pluripotency in iPSCs, delineating the transcription factors KLF4, OCT4, SOX2, and NANOG. (B) Autoregulatory positive loop highlighting the interactions among OCT4, SOX2, and NANOG. In the diagram, green circles represent transcriptional activators, and rectangles symbolize gene promoters. The binding of regulators (depicted as green ovals) to promoters (light brown boxes) is indicated by solid arrows, while gene expression is represented by dashed arrows [44]. iPSCs: induced pluripotent stem cells.

example, iPSCs made using skin cells are susceptible to chromosomal abnormalities caused by lifetime ultra-violet light exposure [15]. If the starting cells carry a genetic abnormality that is conducive to the reprogramming process, such mutation will be preferentially selected in the derived iPSCs. This means that the reprogramming process is likely to propagate genetic variations and increase the expression of potentially harmful mutant proteins [16].

## Factor delivery

The delivery vehicles used during the refinement process, specifically to introduce external transcription factors into the starting cells, contribute to perceived instability and safety concerns associate with iPSCs [17]. The choice of factor delivery method has the potential to influence genomic makeup, with certain methods linked to sub-karyotypic and sub-chromosomal mutations, loss of heterozygosity, and integration of foreign DNA [18]. In early iPSC research, lentiviruses were commonly used to deliver 'Yamanaka factors'—OCT3/4, SOX2, KLF4, and c-Myc. However, retroviral vectors, like those from the lentivirus, are known to integrate into the genome of cells, posing a risk of insertional mutagenesis [19].

Over the past two decades, alongside retroviral-mediated delivery, other methods for introducing exogenous reprogramming factors have emerged, aimed at enhancing reprogramming efficiency and generating transgene-free iPSCs for therapeutic applications. These methods encompass a range of reprogramming techniques, including non-integrating viral approaches and non-viral methods such as episomal vectors, DNA vectors, synthetic mRNAs, and recombinant cell-penetrating proteins [20]. Among the non-integrating viral systems, the Sendai virus provides long-lasting transgene expression while mitigating the risk of unwanted integration. However, even this option demands careful monitoring in a clinical setting, with screening required for traces of Sendai virus backbone or transcript [21].

Episomal vector-based reprogramming, another commonly employed method, offers advantages such as safety and reduced risk of genomic integration. By utilizing episomal vectors, researchers can achieve reprogramming without the permanent alteration of the host genome, which is a significant concern with integrating viral vectors. However, episomal vector-based reprogramming may suffer from lower efficiency compared to viral methods, requiring optimization for robust and consistent results [46].

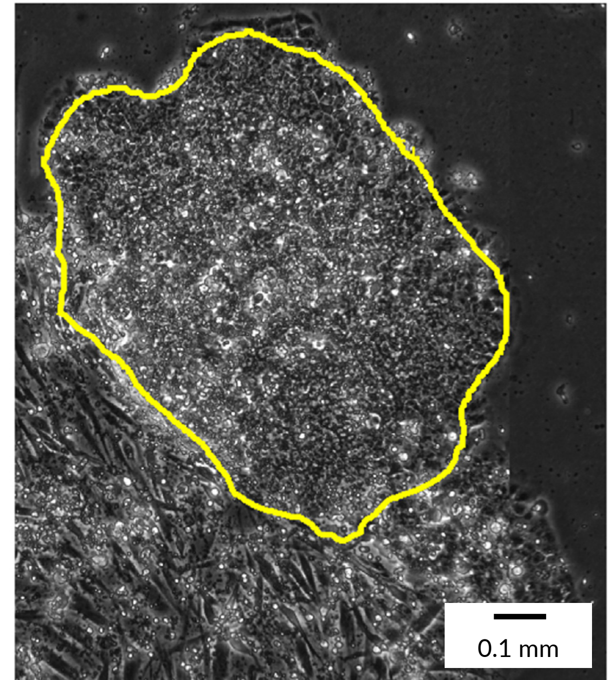
A safer, transgene-free approach for regenerative medicine may prove to be direct transfection, such as endocytosis within a cationic carrier, with mRNAs encoding pluripotency factors to form mRNA-induced pluripotent stem cells [22]. However, this approach also presents challenges in terms of efficiency and scalability for clinical applications. Ultimately, the choice of delivery vehicle remains a compromise, with clinicians often sacrificing transduction efficiency for safer methods. It is imperative for researchers and clinicians to weigh the benefits and limitations of each method carefully, considering factors such as efficiency, safety, and scalability, to determine the most suitable approach for their specific applications.

## Somatic memory

Notwithstanding improvements in the quality of source cells and delivery methods, a challenge persists in the form of somatic memory and the enduring presence of epigenetic features that survive the reprogramming process. To highlight the complex epigenetic profile of iPSCs, Shijun Hu's laboratory compared two cell types to determine if cell source impacts differentiation, *in vivo* behavior, and the expression signature of reprogrammed iPSCs [23]. Endothelial cells were compared with dermal fibroblasts, showing that iPSCs derived from the former had greater endothelial cell differentiation tendencies and

FIGURE 2

**An illustrative example of manual annotation masking where only hiPSC cells within a colony were outlined [47].**




hiPSC: human induced pluripotent stem cells.

were more likely to exhibit origin-specific gene expression markers *in vivo* (for example, PECAM1, KDR, and ICAM). As the two cell types were harvested from the same individual, Hu's findings are a strong indicator that somatic memory does impact the linage differentiation propensity of iPSCs, however incomplete or inefficient reprogramming methods cannot be ruled out. Although epigenetic memory may not necessarily impact gene expression patterns in iPSCs due to missing transcription factors, it does represent a limitation of the technology and increases the likelihood of undesired clinical outcomes [24]. These epigenetic leftovers underscore the need for systematic screening to confirm full and comprehensive reprogramming, thereby guaranteeing the complete erasure of original epigenetic marks. AI and machine learning have the potential to make these checks more efficient and accurate. Furthermore, AI-based models can be shared, reused, and developed over time, lending themselves to the challenge of standardizing certain aspects of iPSC research.

## AI AND iPSCS

Advances in our understanding and control over stem cell biology are progressing on two fronts:

- the ability to collect and monitor data pertaining to the inner workings of cells has improved with developments in microscopy; and
- the refinement of new imaging techniques [25].

In tandem, the capacity to interpret vast datasets is growing with mastery and integration of AI models in cellular research [26]. In this section, we explore how researchers are currently using AI and in particular, deep structured learning (DL), to demonstrate the quality and safety of iPSCs, and to interpret internal processes. We also investigate the potential impact of AI on the future of stem cell research and its anticipated role in regenerative medicine.

### Image recognition and processing

Routine microscopic examination of cultured iPSCs is standard laboratory practice, typically conducted using a contrast microscope at varying magnifications. Advanced methodologies involve the automated extraction of crucial data, including parameters such as cell confluence, cell-free areas, dead cells, and atypical cell morphology [27]. However, further screening in a clinical setting is essential to ensure the safety of therapeutic applications. This includes meticulous assessment for potential genetic mutations, foreign DNA integration, and comprehensive validation of the iPSCs' genomic stability before clinical use [28]. Manual execution of these tasks not only exhibits low accuracy but also demands rapid assessment to ensure adequate throughput (Figure 2) [29].

Computational image analysis has emerged as a transformative tool for characterizing and monitoring iPSCs more efficiently [7]. The process involves the automatic extraction and interpretation of quantitative data from digital images of molecular structures, with verifiable and repeatable results (Figure 3). The applications of DL and other types of machine learning in computational image analysis provide for unbiased annotation of process markers, predictions of differentiation trajectories, and accurate classification of stem cell identity [30]. Turning to an illustrative example, the XGBoost algorithm was recently employed in research to forecast the induction and differentiation of iPSCs using 'computer vision'—a field of AI that enables computers to derive meaningful information from digital images and other visual input [31]. The study tracked treated colonies through the early stages of reprogramming, leveraging time-lapse images to capture morphological features and motion patterns of the cells. A live cell imaging system recorded the reprogramming process 48 hours post-infection, with retrospective labelling of iPSCs and feeder fibroblasts between 3 to 5 days. The AI successfully tracked and analyzed eleven cell morphological and motion features, such as size, area, sphericity, ellipsoid-prolate, ellipsoid-oblate, nucleus-cytoplasm volume ratio, displacement, and velocity, showing utility in charactering iPSC progenitors.

By selecting other pertinent examples from the literature, we see that a diverse range of AI-based analytical methods and imaging techniques have already been applied after the reprogramming phase. Fischbacher *et al.*

### FIGURE 3

**Utilizing AI for image analysis.**

A

0.1 mm

B

0.1 mm

C

0.1 mm

Three colonies of different sizes were analyzed (A–C). The provided phase-contrast images were extracted from a complete FOV image captured using the CellX™ system [47]. Notably, AI-driven segmentation has automatically outlined hiPSC colonies in yellow, while reviewer-selected picks are highlighted in green, and automated picks are depicted in light blue. The images showcase colonies of varying sizes derived from different patient lines and wells, demonstrating the versatility and precision of AI-assisted analysis in discerning subtle variations across samples. FOV: field-of-view.

[32] recently evaluated iPSC monoclonality (a trait that is often attributed to high-grade cell lines) using three distinct AI systems (Monoqlo, RetinaNet, and ResNet) on a considerable dataset of around 30,000 images. Their focus on the automatic detection of colony presence and clonality identification using the PerkinElmer High-Content Screening system showcases how AI can be used for quality control. Imamura *et al.* [33] undertook the significant endeavor of constructing an amyotrophic lateral sclerosis (ALS) prediction model using healthy control subject- and ALS patient-iPSCs. They compared the results of a convolutional neural network deep learning algorithm with cellular analysis carried out by 10 well-trained cell biologists, and found that the convolutional neural network demonstrated a much higher level of performance (assessed through the accuracy of its classifications and predictions) than its human counterparts. The clinical relevance and challenging nature of predicting ALS adds significance to this notable contribution. Joy *et al.* [34] went to great lengths by training five different DL-based neural networks (FCRN-A, FCRN-B, U-Net, Residual U-Net, and Count-ception). The comprehensive approach aimed to localize individual cell nuclei within a human-iPSC colony, generating precise longitudinal measures of cellular properties and demonstrating versatility through the use of multiple models in parallel. Finally, Orita *et al.* [35] trained VGG16 with bright-field images of cultured human iPSC-derived cardiomyocytes. With a dataset comprising 14,000 images for training, 2000 for validation, and 2000 for testing, this study highlights the utility of AI in categorizing specific cell types, offering valuable insights into cardiac-related applications of iPSC technology.

At single-cell level, Hsu *et al.* employed Raman micro-spectroscopy, a label-free technique grounded in the inelastic scattering of light, to unravel the biochemical intricacies of iPSCs at different developmental stages [6]. This innovative approach involved the collection of 8774 single-cell Raman spectra across three distinct human iPSC lines and their neural derivatives at various stages of differentiation. The Raman spectral analysis distinguished between cells originating from multiple donors and classified groups within clinically relevant human neural systems. To make sense of this extensive dataset, the team turned to a machine learning classification model, leveraging the power of t-distributed stochastic neighbor embedding-enhanced ensemble stacking. This analytical strategy demonstrated exceptional accuracy, achieving a classification rate of 97.5% in categorizing iPSCs by their developmental stage and predicting differentiation trajectory. Given the imperative to prevent the transplantation of undifferentiated iPSCs into the human body for safety reasons, the results of this study showcase the critical role that AI, as exemplified by t-distributed stochastic neighbor embedding-enhanced ensemble stacking model, can play as a robust quality control system.

## Other applications for AI

Advances in image recognition technology have been the main driver of recent AI-related innovation in stem cell research, however, machine learning has also been applied outside of image processing, including in the analysis of gene expression and phenotypic profiling [36]. As discussed in previous sections, iPSC refinement methods, the source and quality of starting cells, and other external factors may contribute to instability, senescence, and tumorigenesis *in vitro*. These concerns are magnified by a poor understanding of the biological mechanisms that underpin key stem cell processes, and could be alleviated (at least in part) through accurate phenotypic and genotypic profiling [37]. Experimental approaches based on these types of profiling activity are limited due to their expensive and time-consuming nature. However, with increasing mastery of machine learning technologies, it is hoped that these limitations will soon be overcome.

Datasets generated from experiments to quantify molecular variables related to stem cell biosafety and bio-efficacy, such as gene and protein interactions, present a complex network of molecular intricacies demanding sophisticated analysis. Danter *et al.* pioneered the introduction of DeepNEU, an unsupervised deep machine learning technology devised to tackle the complex genomic landscape of iPSCs [38]. Their approach simulates artificial iPSC systems through the strategic utilization of a predefined set of reprogramming transcription factors. By implementing a sophisticated, fully connected, and recurrent neural network architecture, DeepNEU can be used to improve our comprehension of gene and pathway regulation *in silico*. Notably, the system can be used to identify genes and molecules that are critical for iPSC generation, concurrently facilitating the discernment of unnecessary and potentially problematic cellular components. In another initiative aimed at addressing instability and enhancing our understanding of iPSCs at a molecular level, Bardy *et al.* harnessed machine learning techniques to extract biologically relevant transcriptomic and epigenetic signatures from next-generation sequencing data [39]. Their development of an extremely randomized trees model enabled the classification of functional states in human iPSC-derived neurons by analyzing transcriptomic data from 56 single cells and electrophysiological information.

Similarly, Wu *et al.* employed next-generation sequencing and machine learning to scrutinize a library of 6107 synthetic promoters with enhanced cell-state specificity (SPECS) [40]. This strategic approach led to the identification of multiple SPECS displaying distinctive spatio-temporal activity during iPSC differentiation. The ability to control and manipulate gene expression patterns with SPECS provides a means to override or suppress any lingering somatic memory in iPSCs, which is highly significant. This precision in regulating the expression of genes during differentiation ensures that iPSCs more effectively attain a pluripotent state without retaining unwanted characteristics from their somatic cell origins. In this way, the research by Wu and his team contributes to the optimization of iPSCs for therapeutic applications by enhancing the reliability of these cells in various clinical situations. To scan for specific genetic mutations, namely heterozygous loss-of-function NOTCH1 mutations, another study leveraged iPSC and machine-learning technologies for network-based screening in the context of aortic valve disease [48].

Together, these findings exhibit how AI technology could be used in the future to screen for known complications associated with iPSC production and maintenance, or in conjunction with iPSC technology as a diagnostic tool. They not only contribute to understanding of the biological mechanisms that underpin stem cell dynamics, but also lead us down a path to safer and more efficacious stem cell transplants in humans. A parallel can be drawn with cancer research, where machine learning has proven valuable in identifying and classifying cancer cells [41]. Similar AI-based methods could be applied in stem cell research, for example to reduce the prevalence of iPSC-associated tumorigenesis.

## TRANSLATION INSIGHT

Imagining the trajectory of iPSC research towards therapeutic applications, the integration of recent advances in AI technology have built-on years of machine learning research to enhance applications in cell therapy. However, a number of significant challenges remain, from both a computational and biological perspective, demanding interdisciplinary collaboration to achieve this goal. The primary challenges lie not only in the acquisition of extensive, labeled datasets but also in the subsequent data trending and analysis necessary for effective deep learning applications [42]. AI methodologies have the potential to significantly aid in data trending and analysis by employing appropriate

simulations and models. These AI-driven approaches can assist in discerning patterns, correlations, and predictive insights from complex datasets, thereby enhancing the interpretation and utilization of data in stem cell research. However, as the research cited in the foregoing section illustrates, greater accuracy and efficiency through AI relies heavily on the curation of robust datasets to train models. The inherent complexity of stem cell biology, with its diversity of cell types and states, amplifies the difficulty of assembling comprehensive libraries. Furthermore, capturing high-definition images at a molecular level, as will be required to identify, classify, and make sense of intricate details in stem cell dynamics, not only presents a technological challenge but also underscores the critical need for advances in other fields [43].

Current applications of AI in stem cell research have enhanced our understanding of complex biological systems, particularly disease progression and mechanisms. AI and iPSC-based technologies are also being put to use in drug discovery, with numerous high-profile collaborations announced between the tech and pharma sectors [36]. However, the real prize lies in regenerative medicine, where machine learning algorithms have already proven useful in combating biosafety risks associated with iPSCs. In the future, AI may have the potential to revolutionize personalized medicine, tailoring autologous iPSC therapies to individual patients based on their unique biological profiles.

## CONCLUSION

In conclusion, the integration of AI and machine learning has emerged as a transformative avenue in iPSC research. The susceptibility of these pluripotent stem cells to genomic instability, whether caused by external factors during reprogramming or by their intrinsic nature, highlights a safety risk that requires great attention in the context of their potential for clinical applications. Recognizing the limitations of current labor-intensive and subjective screening methods, researchers have shown that AI-driven approaches may provide the solution. Despite upfront investment—for example, in relation to the time it takes to curate datasets and train new models—AI systems provide many efficiencies once the initial work has been done. Eventually, established models (that have been honed and validated) will be shared between research groups, paving the way for standardization of key protocols and large-scale production of iPSC-derived products.

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## AFFILIATION

**Jonathan Dack**
Senior Associate,
Arnold & Porter

## AUTHORSHIP & CONFLICT OF INTEREST

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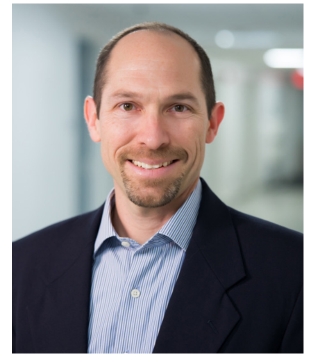
INTERVIEW

# Blazing a trail for iPSC-derived cell therapy in the hair loss space



In the less than two decades since their discovery, induced pluripotent stem cells have proven transformational for the cell therapy field. In this interview **David McCall**, Senior Editor, *Cell & Gene Therapy Insights*, and **Kevin D'Amour**, Chief Scientific Officer, Stemson Therapeutics, explore the inexorable rise of induced pluripotent stem cells, their current applications in hair loss, and the future evolution of the space.

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## What are you working on right now?

**KD:** I joined Stemson Therapeutics as CSO a year ago. The company is focused on a cell therapy solution for hair loss—we are creating a tissue engineered product that is designed to be a nascent follicular unit for the *de novo* formation of new hair follicles for any type and stage of this ailment. This would be a breakthrough therapy to address major unmet needs in the hair loss market, as no other therapy has been able to generate a new source for hair follicles.

## Can you tell us more about your background in the stem cell therapy space, and in particular, how you have experienced the rise of induced pluripotent stem cells (iPSCs) over the past 17 years?

**KD:** I trained as a stem cell biologist during my PhD and have been working for 22 years in industry since then, with my primary focus being pluripotent-derived cell therapies. My graduate training was at the Salk Institute with Professor Fred 'Rusty' Gage. Other than in the hematopoietic field, I'm probably one of the first scientists that really got my base training in stem cells. I joined a neuroscience lab that was on the cusp of transitioning into stem cells, and found myself entering a fledgling field.

I consider myself very fortunate to have been in at the ground floor—my training and career began concurrently with the first published description of human embryonic stem cells in 1998, almost a decade before the first reports of iPSCs. I recall being in Australia at the 2007 International Society for Stem Cell Research meeting when both Drs Yamanaka and Thompson reported successfully deriving human iPSCs, about a year after the Dr Yamanaka report of doing the same in mouse cells. Embryonic stem cells, as much as they were and still are a useful avenue for some therapeutics, don't offer the ability to create an autologous embryonic stem cell. Nuclear transfer was thought to be possible, but at this time had already proven very difficult. The fact that iPSCs could be reliably made from adult cell types through a reprogramming that didn't involve enucleated oocytes completely solved the ethical conundrum of dealing with early embryos for deriving stem cell lines.

I remember the field being completely abuzz—this was transformational. Since then, the impact of research using iPSC has been immense, and the future is even more promising. A testament to this fact is the sharing of the Nobel Prize by Dr Yamanaka a mere 8 years after his initial iPSC discoveries.

In those early years I remember attending annual International Society for Stem Cell Research meetings and hearing about the next dozen or so ways in which one could reprogram cells. This aspect of the field settled down, which was important because while there are a variety of approaches that can be used, it wasn't going to serve the field to completely reinvent what factors you use to do reprogramming every few years. There has been an important standardization down to a core of six or fewer factors that are commonly used. Since then, the speed at which iPSCs have been picked up and developed into therapies—mostly by companies but also academic groups such as such as Dr Kapil Bharti's at NIH—has been astounding to watch.

Being in this field since the nascent years has been so exciting. Back when only embryonic stem cells existed, you could count on one hand the number of people who were really seriously considering creating therapeutics with them. I keep track of the space and there's now upwards of 40 or 50 corporate therapeutic developers dealing with pluripotent cells. Around 90% of them are focused on iPSCs, and for those that have embryonic stem cell programs such as ViaCyte/Vertex, it's probably just a matter of time before those are converted over to iPSC-derived programs.

Finally, a field that I'm not as close to but which has been really important for iPSCs is disease modeling. We now have the ability to go to patient groups, derive pluripotent cells, and model *in vitro*. This greatly aids in understanding the fundamental biology behind the

"We now have the ability to go to patient groups, derive pluripotent cells, and model *in vitro*. This greatly aids in understanding the fundamental biology behind the pathology of cells in certain genetic diseases—even complex, polygenic diseases."

pathology of cells in certain genetic diseases—even complex, polygenic diseases. This is an area I think we will see bear fruit, if not quite yet then certainly in the future.

Stemson Therapeutics is blazing a trail in applying iPSC-derived cell therapy in the hair loss area—what are some of the benefits/advantages of their application in this particular space?

**KD:** The only time we make hair follicles is during fetal development. We are all born with every hair follicle our bodies will ever have. They don't all have hair shafts—that changes and wanes over time—but we have all of our follicles. The fact that folliculogenesis is exclusively a fetal biological mechanism lends itself very conveniently to iPSC-derived therapy, as iPSC-derived cells are typically fetal-like in nature. Additionally, we are focused on autologous iPSC both because of the issue of immune acceptance of the cells, and also because many important aesthetic attributes of our product may have genetic determinants, such as hair curl, color, texture, and so on.

Can you take us through the manufacturing process?

**KD:** This is an autologous iPSC-derived product, so we begin with donor cells. We have long thought that these will be blood cells because of the existing infrastructure for phlebotomy and shipping blood at ambient temperatures. However, the company is further evaluating this. We are making a hair follicle and dermal cells, and it now seems to be a fairly conclusive aspect of reprogramming that there is some epigenetic memory. One might take steps to erase that memory, or work within it. It is not yet known whether a dermal starting cell type might be different from blood in its ability to reproducibly generate dermal and epidermal folliculogenic cells. That is an active area of investigation for the company right now.

Once we have selected a donor cell, we take that donor cell, put it in culture, and reprogram. However, at Stemson we don't intend to become reprogramming experts. There are a lot of people out there doing it, and we don't want to reinvent the wheel; we want a manufacturing partner that has an established track record on this. However, there are not many CMDOs interested in doing so from an autologous perspective so far, and I think that's why developers such as Aspen Neuroscience and others have so far been relying on internal manufacturing programs for this work.

We are exploring the space to externalize the reprogramming, which encompasses donor cell collection, reprogramming, and through to banking the iPSC clones. After you bank them, we think there should be multiple different clones for each patient, and then you want to have a robust QC process to select one of those clones to advance to cell product manufacturing.

When it comes to the cell product manufacturing itself, we are actually making two individual cell products. Our two different types of drug substance would be independently differentiated from the iPSCs to folliculogenic dermal cells akin to a dermal papilla, as well as a folliculogenic and multi-potent epithelial cell—what we would call an epithelial stem cell or a hair follicle stem cell. These two cell types are independently derived through separate manufacturing programs and cryopreserved, and that's the drug substance. You would then do your

QC processes, release that drug substance bank, and then at the end, those two independent cell types are thawed and formulated into the final drug product.

This is not a mature hair follicle, but an organized collection of the two cell types with a biomaterial and a hydrogel to form into what we call a follicular unit. This makes the cells handleable, and the design of the follicular unit is meant to ensure directional hair follicle growth and hair shaft outgrowth through the skin, as we don't want to make ingrown hairs.

There are a lot of important design elements to that tissue-engineered product to get the final product to perform appropriately, as well as to fit into the very robust clinical and commercial industry of hair transplant. One of the things that struck me, and one of the reasons I came to Stemson, was the fact that there are more hair transplants than any other cell therapy or organ transplant out there, at least when considering living cell transplants. There are quite a few acellular grafts, but looking at cellular or organ grafts, if you were to add up every solid organ transplant and every cell therapy, you might come to 100,000 transplants a year in the US. There are 200,000 hair transplants every year in the US, which is already twice as many procedures as every other form of transplant combined.

## What are some of the key challenges in working with iPSCs in this particular field, and how does Stemson seek to address them?

**KD:** Focusing on the autologous field for iPSCs, there are two major challenges. One is manufacturing timelines for making the iPSC banks themselves. The majority of the therapeutic developers in the field right now are using the Sendai virus method of delivering reprogramming factors. Some other viruses are hamstrung in how much payload they can bring forward, so Sendai is convenient. However, the lengthy culture time required is a challenge. There are alternative methods such as mRNA reprogramming, but the robustness is perhaps not there yet.

The other key aspect is differentiation, reliability, and effectiveness from clone to clone or donor to donor. Having robust differentiation procedures, and ones that are efficient in generating your target cell types, is really important. This is where I am able to leverage my 20 years of experience in directed differentiation and focus in on that very first fate commitment from pluripotent cell to nascent ectoderm lineage. There isn't a more important step in the process of trying to get to a hair follicle than that very first 24–48 hours. Making sure these directed differentiation processes are efficient is crucial. You need high-purity intermediate cell types, and you need to understand what those intermediate cell types are, so that you can fine-tune their generation from both an efficiency and a QC/manufacturing perspective.

## What are some of the chief considerations as you approach the clinical translation of Stemson's product candidates—and are there any learnings you take from your previous experience in translating another first-of-its-kind approach at ViaCyte?

**KD:** Clinical translation overall can be problematic for cell therapies. If you are not using a hematopoietic cell where you can deliver into the vein, you are likely to have clinical delivery challenges. For example, I know ViaCyte and the islet cell field in general have really struggled

with this, in part because the preclinical models are just not very good at predicting the human condition and the human tissue.

Stemson has invested a lot of time into our preclinical modeling program, and one thing we are really excited about is our humanized mouse model. It came about by necessity, as rodent skin is about one tenth the thickness of human skin. It is impossible to robustly make a human hair follicle in a 200 µm-thick rodent skin, so we created a rodent model in nude mice where we make a reconstituted human skin graft. Using dermal and epidermal human cells you can create a skin graft that is physiologically very similar to adult human skin. We then implant our tissue-engineered follicles into this now 2 mm-thick skin pad.

This model is proving very valuable—it is one of the best preclinical models I have come across with respect to the ability to be predictive of future clinical testing. However, it is entirely custom and hence, we need to run it internally. It is also labor intensive, so scaling that model for our IND-enabling studies in future is a challenge we are focused on overcoming.

What do you view as the current technological state-of-the-art in the iPSC banking and differentiation fields? And where is further innovation most required?

**KD:** I have touched on one area already, which is reducing manufacturing timelines for reprogramming into a usable iPSC clone and bank. One thing that dovetails into that is how we define 'usable'. QC, especially QC for genomic integrity, is going to become very important. Autologous is a mixed bag with regulators: if a product goes wrong, it only affects one patient, which is a benefit, but you need robust QC processes for manufacturing because you are going to be doing it from different starting material each and every time.

Next-generation sequencing technologies are certainly powerful, but they need to be complemented by other types of genomic integrity assessment. When we consider the macro structural elements of the chromosomes, whether inversions or larger rearrangements, next-generation sequencing workflows are not well designed to detect those events. One option might be optical genome mapping. There is a local company here in San Diego, Bionano Genomics, that focuses on that kind of technology.

Robust QC of the iPSC bank, as well as the ultimate product cells, is an area where the field is still fairly nascent and primed to grow. Similar to how I described those early years where we had so many different ways to reprogram a cell but ended up settling on just a few, one of the next things may be settling on how you actually define high-quality iPSC clone and bank genetics. Considering epigenetics might be another point of investigation.

Looking to the future, what do you anticipate in the way of 'platforming' opportunities and likely market evolution in general in the iPSC space, and how can cell therapy developers capitalize?

**KD:** Over the last 3–4 years we have seen a lot of contract manufacturers take an interest in iPSC technologies and build up their internal experience with them. I anticipate the iPSC field will see this mature in a similar fashion to what has happened with hematopoietic cell manufacturing, where there are robust opportunities for developers to leverage CDMO

> "There isn't a more important step in the process of trying to get to a hair follicle than that very first 24–48 hours. Making sure these directed differentiation processes are efficient is crucial."

partners. Most developers don't necessarily want to be the manufacturer, as transitioning into that manufacturing environment requires a totally different set of skills and a totally different set of professionals. This is an area where I would expect to see this field maturing into centralized manufacturing and expertise, certainly for the iPSCs themselves. I would then predict a second phase of development as CDMO partners try to become the experts in differentiating iPSCs to everybody's individual cell product. This second phase will take some time to manifest as every developer is going to have their own unique process.

In the autologous field, I also wonder if there is a way to leverage one cell line to create multiple therapies. Ultimately, a patient could potentially benefit from several different therapeutics over their lifetime that are all based on one cell line. Again, that would probably have to come down to some understanding of a broad-based quality metric in manufacturing to make a cell line that can work into any therapeutic developers' particular cell type and therapy. However, I don't know how the field will handle that. Who will pay for the initial banking of someone's iPSC line?

I spend a lot of time talking about autologous because that's what I am doing now but previously, at ViaCyte, I spent the majority of my time thinking about genetic immune evasion and allogeneic cell lines. That is an area for the future. There are a lot of people doing it but mainly for hematopoietic cells, with relatively few people talking about doing it for non-hematopoietic cells. I do expect different genetic immune evasion strategies to be employed across different types of programs. For example, in type 1 diabetes you have not just alloimmunity, but autoimmunity to contend with. Does that create a system where you have a different set of genes and factors you need to manipulate, either by knocking out or knocking in?

It's the same for us at Stemson—the skin is one of our barriers to the outside world, so it contains a lot of immune cells such as macrophages in high abundance. Will that create a different need for an immune-evasive type of therapy? Over the next decade we will have an emerging story around genetic immune evasion and whether it is something we can rely upon for, if not permanent, certainly very long-lasting cell persistence. Most cell therapies will require long-lasting persistence.

Then there are some related sub-questions therein: if a patient happens to 'escape' immune evasion and reject a graft, is that approach off the table due to immune memory aspects, and now do you have to come up with a brand-new strategy for a second retransplant? These questions are more theoretical or hypothetical, but this is an area I expect the iPSC field to mature in over the next 5–10 years.

## Lastly, can you sum some key goals and priorities that you have for Stemson Therapeutics over the foreseeable future?

**KD:** I described the basis of our humanized preclinical model, but we are still doing it at small-scale. With IND-enabling and safety studies being planned that potentially leverage this

model, we need to scale it up, increase its robustness, and increase the banks of primary cells with which we can execute this model reliably.

Another focus is that at some point in the future, we want to be able to generate 10,000 tissue engineered units per patient, across all patients. We are in the process of leveraging bioprinting for manufacturing these small units at high-scale and high-throughput. This is a big focus for the company. Being pragmatic and keeping it simple enough is a crucial balance to strike—you don't want to make it any more complex than is necessary to make it efficacious, in order to ensure manufacturing can ultimately be successful in the future.

## BIOGRAPHY

**KEVIN A D'AMOUR** is the Chief Scientific Officer at Stemson Therapeutics. He has over 20 years' experience in cell therapy R&D, with specific background in stem cell biology, stem cell differentiation, gene editing, immunology, and cell therapy development. Prior to joining Stemson, Kevin was at ViaCyte from 2002 until 2021, and served as the company's Chief Scientific Officer from 2011–2021. At ViaCyte, he led the development of the first stem cell-derived cell therapies designed to replace pancreatic β cells for the treatment of type 1 diabetes. Under Kevin's leadership, ViaCyte took three cell therapy products into the clinic. After his time at ViaCyte, Kevin was Chief Scientific Officer of Brooklyn ImmunoTherapeutics, where he focused on developing cell therapies using mRNA-based cell engineering technology. Kevin is a co-author of high-profile publications in renowned scientific journals and an inventor on over 100 patents in the fields of stem cells, cell engineering, and cell therapy. Kevin holds a BSc in Animal Science from the University of New Hampshire, and a PhD in Biology from the University of California, San Diego.

## AFFILIATION

**Kevin A D'Amour PhD**
Chief Scientific Officer,
Stemson Therapeutics

## AUTHORSHIP & CONFLICT OF INTEREST

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**CELL & GENE THERAPY** INSIGHTS

INDUCED PLURIPOTENT STEM CELLS (iPSCS)

SPOTLIGHT

# Following the (mAb) leader: leveraging monoclonal antibody cell line development & banking CMC strategies for iPSC-derived cell therapies

**Damien Fink**
Analytical Development and Quality Control, Century Therapeutics

"For iPSC-derived cell therapies, the establishment of a master cell bank enables the application of GFI CMC and ICHQ5D principles."

VIEWPOINT

*Cell & Gene Therapy Insights* 2024; 10(1), 29–33

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Cell therapies represent a class of therapeutics that are substantially more complex than traditional small molecule and biologic therapeutics in terms of mechanism(s) of action, manufacturing, and control. While there have been a number of cell therapies approved, to date there is no prescribed CMC strategy based on the general heterogeneity of the class of therapies. As such, quality strategies must be established, refined, and tailored to the therapy based on a number of factors including, but not limited to, donor origin (autologous vs allogenic), cell source (primary cell derived vs stem cell derived), genomic engineering, and manufacturing process. This does not take into account that cell therapies are often lumped into a larger class of gene and cell therapies, further complicating an option for a prescribed CMC strategy as guidance documents typically attempt to be inclusive of all therapies in class.

Understanding the nature of the therapeutic as well as similarities/differences between the novel therapeutic and existing therapies is key to developing a robust, focused CMC strategy. Quality assessments and regulatory strategies established by classic therapeutics can be used as a framework for cell therapies. However, care should be taken to ensure the CMC strategy is both applicable to the novel therapeutic and the strategy is comprehensive to sufficiently demonstrate quality across all applicable quality assessments. Scientific judgement and justification should be used to include or exclude such assessments. There may be additional, non-compendial or hereto undescribed measures that are required to ensure product quality. Here, we describe a way to leverage a substantial portion of established CMC quality strategy from a monoclonal antibody drug for an induced pluripotent stem cell (iPSC)-derived cell therapy.

Monoclonal antibody production requires a cell line engineered to secrete the antibody of interest. Monoclonal antibody CMC framework for Investigational New Drug (IND) submission was formally established in 1996 with the Guidance for Industry: For the Submission of Chemistry, Manufacturing, and Controls Information for a Therapeutic Recombinant DNA-Derived Product or a Monoclonal Antibody Product for *In Vivo* Use [1] release (henceforth known as GFI CMC). In this guidance, the definitions of a drug substance (DS), drug product (DP), master cell bank (MCB), working cell bank (WCB), and end of production (EOP) cells are established in the context of a biological therapeutic. Additionally, expectations for all manufacturing stages from raw materials (including testing) through final DP presentation are outlined in this guidance. Interestingly, this guidance document was written and finalized (1997) many years prior to the first approved cell therapy. Cell usage in the pharmaceutical landscape at the time was limited to using cells as the 'factories' to manufacture the resultant drug substance. In fact, the first approved cell therapy came in 2010 with the approval of sipuleucel-T. Subsequent guidance documents that directly or tangentially apply to cell therapy CMC filing strategy are summarized in previous work [2].

Established in 1990, the International Council on Harmonisation (ICH; formerly International Conference for Harmonisation) champions a number of working groups and guidelines covering quality, safety, efficacy, and multidisciplinary considerations. Included in the Guidelines series, ICHQ5D details the derivation of and characterization of cell substrates [3]. Taken in combination with ICHM4Q(R1) [4], the consortium gives a clear roadmap to address all quality sections of the common technical document (CTD) for regulatory submission. Similar to the FDA guidance [5], these documents use consistent definitions for MCB, DS, and DP; and were approved prior to the first cell therapy approval (ICHQ5D in 1997, ICHM4Q[R1] in 2002). These guidance frameworks have been used in combination to successfully launch monoclonal antibody therapies in the USA for over two decades.

Previous work [2] has demonstrated some key differences between iPSC-derived cell

therapies and autologous or donor-derived allogenic cell therapies. iPSC-derived cell therapies offer the ability to have a sustainable source of a fully engineered, clonal cell line. Unlike other non-iPSC derived cell therapies, engineering and cloning of these therapeutic cells is expected to happen only once. The authors quite nicely lay out a comprehensive quality testing strategy for cell banks to enable the use of a MCB strategy as described in the aforementioned guidance documents. In doing so, they establish a strategy to create a well characterized and qualified MCB; differentiating iPSC-derived cell therapies from other cell therapy products and opening additional CMC strategy avenues.

For iPSC-derived cell therapies, the establishment of a MCB enables the application of GFI CMC and ICHQ5D principles. The entire sourcing and engineering strategy for an iPSC-derived cell therapy resembles the cell line engineering strategy for a monoclonal antibody. Based on this, it stands to reason that a similar CMC strategy for this type of cell therapy could follow a similar path. In Table 1, the relevant sections of ICHQ5D are cited alongside the corresponding analogous iPSC-derived cell therapy application.

## SUMMARY

In establishing a true MCB, a CMC strategy for an iPSC-derived cell therapy closely mimics that of a monoclonal antibody. To be clear, these strategies are not identical. However, drawing the parallels between both cell line

### TABLE 1

**Cell line development.**

| Designation | Reference | Definition | Analogous iPSC-derived cell therapy step |
|---|---|---|---|
| **Cell source** | | | |
| Donor material | ICHQ5D 2.1.2<br>GFI CMC II.3.b.i.A.I | Source of cells from which the cell substrate was derived | Donor cells are obtained and sorted/selected; donor cells reprogrammed through viral or non-viral vectors |
| Parental cell line | ICHQ5D 2.1.3 | Cell line used to generate cell substrate | Reprogrammed cells expanded into parental iPSC bank |
| **Cell line development** | | | |
| Cell substrate | ICHQ5D 2.1.3<br>GFI CMC II.3.b.i.A.V | Fully engineered cells cloned from a common cell progenitor | Cells bulk engineered (may include multiple rounds); single-cell cloning and small-scale expansion for clonal selection; further expansion to a preliminary cell bank(s) in anticipation of clonal selection |
| **Manufacturing** | | | |
| Master cell bank | ICHQ5D 2.2.1<br>GFI CMC II.3.b.ii.A | Characterized, common starting source material for production | Full expansion of selected preliminary cell bank in compliance with GMP principles |
| Working cell bank | ICHQ5D 2.2.1<br>GFI CMC II.3.b.ii.B | Optional further expansion of MCB | Two-tiered banking strategy with additional WCBs manufactured from the MCB can be used if additional supply of cells required to support full lifetime of product |
| End of production | ICHQ5D 2.3.3<br>GFI CMC II.3.b.ii.C | Analysis of cells at the end of production campaign to assess for phenotypic and genotypic changes | Extended culture of MCB and subsequent expansion and differentiation of cells. Ensures phenotypic and genotypic stability. Also enables use of WCB |
| Drug substance | GFI CMC Section II | Unformulated active substance which may be subsequently formulated with excipients to produce the drug product | Final, fully expanded and differentiated cells prior to final formulation |
| Drug product | GFI CMC Section III | Final formulated drug | Differentiated cells filled and finished. Material undergoes cryopreservation at end of DP production |

development processes enables using the monoclonal antibody framework as the basis for an iPSC-derived cell therapy CMC strategy. Refinement of the monoclonal antibody strategy is required to accurately describe the therapy and this new strategy will contain aspects that are unique to cell therapies. The benefit of such a strategy is enabling the use of a proven CMC framework used to approve hundreds of therapeutics. Additionally, both the sponsor and regulatory agencies are familiar with the monoclonal antibody CMC framework and language, further simplifying the submission and approval process.

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## BIOGRAPHY

**DAMIEN FINK** currently leads the Analytical Development and Analytical Science and Technology groups at Century Therapeutics. He's a graduate (and avid fan) of Pennsylvania State University and Villanova University. His experience in analytical development stems from his tenure at Merck Manufacturing Division (4+ years) and Janssen R&D (16+ years) where he worked extensively on assay development and validation efforts in both the GMP and GLP space relating to products such as Recombivax HB®, Vaqta®, Gardasil®, golimumab, amivantamab, teclistamab, talquetamab, and ciltacabtagene autoleucel among many others. In his current role, he leads the development, validation, and assay lifecycle management of release and characterization assays ensuring the quality of iPSC-derived allogenic cell therapies as well as serving as CMC lead on current collaboration projects.

## AFFILIATION


**Damien Fink**
Analytical Development and Quality Control,
Century Therapeutics

AUTHORSHIP & CONFLICT OF INTEREST

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INDUCED PLURIPOTENT STEM CELLS (iPSCS)

SPOTLIGHT

COMMENTARY

# Unlocking the full potential of human induced pluripotent stem cells from haplo-selected cord blood samples—the how and the why

**Begoña Aran, David Morrow, Ester Rodriguez, and Anna Veiga**

There is a critical need worldwide for tissue for transplantation in patients with organ failure and with degenerative diseases with no treatments available. Cell therapy can represent an alternative to organ transplantation and for the treatment of degenerative diseases (such as heart failure, macular degeneration, type 1 diabetes, or Parkinson's disease, among others). The generation of human induced pluripotent stem cells offers a unique opportunity to obtain an unlimited supply of specialized cells. The use of patient's cells for the generation of human induced pluripotent stem cells and their derivatives for treatment ensures immunological compatibility and minimizes the risk of rejection. However, the time and cost necessary to produce customized human induced pluripotent stem cell lines and their derivatives in GMP conditions are excessively high.

An alternative to the use of patient-specific human induced pluripotent stem cells would be an human induced pluripotent stem cell collection from allogeneic healthy donors that could be expanded and differentiated to treat different patients. This collection should comprise lines with enough diverse and compatible homozygous human leukocyte antigen to reduce the risk of immune rejection in a high percentage of the population. Homozygous human leukocyte antigen-matched iPSC lines suitable for a wide variety of homozygous human leukocyte antigen genotypes would be valuable for significant numbers of patients and will allow delivery of off-the-shelf cells for the manufacturing of cell therapy products for multiple diseases by reducing time and costs.

HAPLO-iPS aims to create a collaborative network to provide a framework for human induced pluripotent stem cell generation of human induced pluripotent stem cells homozygous for frequent homozygous human leukocyte antigen haplotypes, compatible with a

significant percentage of the population to be used for cell therapy clinical trials, and to collect a data collection system for such lines and all the associated data.

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## INTRODUCTION

There is a critical need worldwide for cell and tissue for transplantation in patients with organ failure and an increasing impact of degenerative age-related human diseases for which there are very limited or no treatments available [1]. Cell therapy can constitute a future alternative to organ transplantation and for the treatment of degenerative diseases (such as macular degeneration, Parkinson's disease, heart failure, type I diabetes, or spinal cord injuries, to name a few) [2,3]. The generation of human induced pluripotent stem cells (hiPSC) from somatic cells offers a unique opportunity to obtain a virtually unlimited supply of a broad spectrum of specialized cells [4,5]. iPSC-derived differentiated cells have great potential for cell replacement therapy even though the clinical relevance of such treatments is still to be clinically realized in the form of licensed cell-based medicines. The reason for this is that the time and costs required for the production of customized hiPSC lines and their derivatives that would be suitable for use in humans is prohibitively high. For a large-scale therapeutic landscape, immune- homozygous human leukocyte antigen (HLA) matched iPSC lines suitable for a wide variety of HLA genotypes would be valuable for significant numbers of patients. An alternative to the use of patient-specific hiPSC would be a hiPSC collection from allogeneic healthy donors that could be expanded and differentiated to treat different patients. To reduce the risk of immune rejection, this allogeneic hiPSC collection should comprise lines with sufficiently diverse and compatible homozygous HLA haplotypes to ensure maximum possible population coverage. Manufacturing of scalable unique cell standardized final products from haplo-selected hiPSCs suitable for various types of diseases and multiple clinical indications, should in addition reduce the cost of the final products and patient immune-suppression. Moreover, cell derivatives from HLA-matched hiPSC banks will allow delivery of off-the-shelf cell therapy products, easily accessible for critical acute or subacute diseases and for new emerging diseases such as the current pandemic SARS-Cov-2-induced inflammatory disorders and cancer. To achieve this goal a new initiative, the HAPLO-iPS project, led by the Bellvitge Biomedical Research Institute in Barcelona, and supported by the European Research Infrastructure for Translational Medicine, the European Research Infrastructure for Translational Medicine, has created a first of its kind collaborative network through a recently funded European Cooperation in Science and Technology (COST) action across 30 EU countries and beyond [6]. The aim of this multistakeholder network is to provide for the first time a framework for hiPSC generation of hiPSC homozygous for frequent HLA haplotypes, compatible with a significant percentage of the population to be used for cell therapy clinical trials and to create a data collection system (REGISTRY) for such lines. This network will pioneer new approaches that will foster the progress of a haplo-selected hiPSC generation of therapeutics by the development, implementation, and exploitation of a registry with all the information required for the benefit of patients.

## UNDERSTANDING THE CHALLENGES

Currently, some registries of available hPSCs do exist. The most prominent is hPSCreg,

which was created as a registry of European human embryonic stem cells (hESC) lines, but that now involves hESC and hiPSC lines from over the world [7]. Although individual approaches for using hiPSCs for therapeutic applications already exist [3] and also for setting up the first haplo-banks throughout Europe, these initiatives are not yet working as a coordinated community and only have the capacity to provide cell lines for a limited number of common HLA haplotypes. To date, these approaches, which are still at the proof-of-principle stage, will at best cover only a limited percentage of the population in need. With their limited resources, they result in a social-economic imbalance and even exclusion of certain population groups from future medical possibilities. Furthermore, there are key scientific and regulatory discussions yet to be resolved to achieve a European consensus on essential issues that must be tackled to progress such haplo-registry/bank resources to a clinical reality. The use of patient's cells for the generation of hiPSC and subsequent differentiation to the desired cell type for treatment ensures immunological compatibility and minimizes the risk of rejection. However, the time and cost necessary for the production of customized hiPSC lines and their derivatives that would be suitable for use in humans is prohibitively high. For a large-scale therapeutic landscape, immune-HLA matched iPSC lines suitable for a wide variety of HLA genotypes would be valuable for significant numbers of patients. An alternative to the use of patient-specific hiPSC would be a hiPSC collection from allogeneic healthy donors that could be expanded and differentiated to treat different patients. To reduce the risk of immune rejection, this allogeneic hiPSC collection should comprise lines with sufficiently diverse and compatible homozygous HLA haplotypes to ensure maximum possible population coverage. Manufacturing of scalable unique cell standardized final products from haplo-selected hiPSCs suitable for various types of diseases and multiple clinical indications can reduce the cost of the final products and patient immune-suppression. This idea was already proposed by Bradley *et al.* and Taylor *et al.* for hESC. hiPSC technology facilitates the prospective selection of interesting donors based on their particular HLA haplotypes [8].

The selection of homozygous donors for common HLA haplotypes for the generation of hiPSC can facilitate compatibility with potential recipients. Nakatsuji *et al.* calculated that 30 carefully selected hiPSC lines would provide coverage to 82.2% of the Japanese population coinciding in the three loci (HLAA, HLA-B and HLA-DR), and 90.7% of the population would be covered with 50 hiPSC lines [8]. However, identifying these 50 potential donors would necessitate studying the HLA system of 24,000 individuals. Okita *et al.* calculated that 140 homozygous donors for HLA haplotypes would cover 90% of the Japanese population, requiring the screening of 160,000 potential donors [9]. Similarly, Gourraud *et al.* calculated that 26,000 donors of European-American ancestry and 110,000 donors of African American ancestry would need to be screened to obtain hiPSC representing the 20 most frequent HLA haplotypes, and that these lines would provide coverage to 50% and 22% of these populations, respectively [10]. All of this confirms that relatively few donors, if very carefully selected, would allow the generation of hiPSC lines with a strong potential for clinical utility. Similar calculations have been established for Korean population in comparison with China, Japan, and the West [11]. Alvarez-Palomo *et al* calculated that ten cord blood units from homozygous donors stored in the Spanish cord blood banks can provide matching for 28.23% of the Spanish population [12]. Abberton *et al* and Clancy *et al* have calculated similar estimations with Australia and Finnish populations respectively [13,14]. The estimated number of hiPSC lines needed to coverage several populations is shown in Table 1.

The collaboration of multiple centers worldwide is therefore necessary to perform

TABLE 1

**Estimated number of hiPSC lines needed to coverage several populations**

| Author | Number hiPSC lines | Coverage (%) | Population | Potential donors |
|---|---|---|---|---|
| Nakajutsi *et al.*, 2008 | 30 | 82.2 | Japanese | 24.000 |
| | 50 | 90.7 | Japanese | |
| Okita *et al*, 2011 | 140 | 90 | Japanese | 160.000 |
| Gourraud *et al*, 2012 | 20 | 50 | European-American | 26.000 |
| | 20 | 22 | African-American | 110.000 |
| Lee *et al*, 2018 | 10 | 41.1 | Korean | 4.200 |
| Alvarez-Palomo *et al*, 2021 | 10 | 28.2 | Spanish | 30.000 |
| Abberton *et al*, 2022 | 33 | 50 | Australian | 13.679 |
| Clancy *et al*, 2022 | 41 | 69.3 | Finnish | 20.737 |

hiPSC: human induced pluripotent stem cells.

the screening and identifying individuals among the large number of potential donors [15].

One feasible possibility is to prospectively search for potential donors in registries/banks of bone marrow and cord blood (CB), since these donations are already typed for elements of the HLA system. There are several reasons why CB cells are the cell type of choice to generate homozygous HLA haplotype hiPSC collections for clinical translation:

- There is no risk for either the mother or the newborn at collection;
- CB units, preserved in CB banks, are already HLA typed, which facilitates donor screening;
- Cells in the CB are less likely to have accumulated genetic or epigenetic risks compared to adult and differentiated cells; and
- hiPSC generation methodology with CB samples is well established [9,16].

The use of CB-hiPSC as an alternative to the use of patient-specific hiPSC would minimize the time and cost necessary for the production of customized hiPSC and their derivatives. Moreover, although CB samples are designated for clinical application for hematological pathologies, many CB banks keep surplus samples sufficient to generate hiPSC lines and CB samples with an insufficient number of hematological progenitors not suitable for transplantation might also be used. Methodology for GMP-grade $CD34^+$ selection from HLA-homozygous CB units has been reported [17]. Lee *et al.* described the generation of hiPSC lines with the ten most frequent HLA-homozygous haplotypes, which can match 41.07% of the Korean population. Comparative HLA analysis indicates that the lines are relevant to other Asian populations, such as Japan, with some limited utility in ethnically diverse populations, such as the UK. Similarly, Rim *et al.*, report the generation of 13 homozygous GMP-grade hiPSC lines from blood and CB cells with selected homozygous HLA types from the Catholic Hematopoietic Stem Cell Bank of Korea [18].

The World Marrow Donor Association estimates 256,006 CB units preserved in the CB banks in Europe and 798,372 units in the world [19]. Bone marrow registries represent an alternative to CB banks as potential providers of samples for hiPSC generation, but both the availability, lower invasiveness, and the easy access to samples in the latter are obvious advantages to be considered.

There are 37,346,669 bone marrow donors registered in the World Marrow Donor Association registry [19].

Another option to be considered to make hiPSCs compatible with a significant percentage of the population is the use of genetic modification techniques in hiPSC or hESC to knock-out or down-regulate HLA genes to generate 'universal' donor cells. Xu *et al.*, described two genome-editing strategies for making immunocompatible donor hiPSCs [20]. First, they generated HLA pseudo-homozygous hiPSCs with allele-specific editing of HLA heterozygous hiPSCs. Second, they generated HLA-C-retained hiPSCs by disrupting both HLA-A and -B alleles to suppress the natural killer cell response while maintaining antigen presentation. HLA-C-retained hiPSCs could evade T cells and natural killer cells *in vitro* and *in vivo*. The authors estimated that 12 lines of HLA-C-retained hiPSCs combined with HLA-class II knockout are immunologically compatible with over 90% of the world's population, greatly facilitating hiPSC-based regenerative medicine applications. Other publications also report encouraging results using similar or RNA silencing techniques as well as cell-based immunomodulation strategies genetic ablation of HLA molecules from hiPSC combined with gene transduction of several immunoregulatory molecules [21–22]. These 'universal' hypo-immunogenic strategies could be valuable for rare haplotype cells, and in relevant clinical applications such as hematopoietic cell transplantation (where HLA mismatches profoundly affect engraftment) and in autoimmune diseases (where autoantigen presentation would cause side effects). Non-HLA minor histocompatibility antigens from Y chromosome genes and single-nucleotide polymorphism profiling should also be taken in account. However, genome editing could induce a risk of off-target modifications that must be extensively controlled, and such modifications can enhance the complexity of safety evaluation and regulatory delay. Both of these non-exclusive models will be enriched by variant models, and innovative strategies will evolve as a step towards complete immune-matched hiPSC lines with fully personalized therapy.

Advantages and disadvantages of different approaches for hiPSC generation are shown in Table 2.

Few commercialized allogeneic clinical-grade hiPSC lines are currently available

**TABLE 2**

**Advantages and disadvantages of different strategies for hiPSC generation for clinical application.**

| | Autologous therapies | Allogenic therapies | Allogenic haplo-matched hiPSC therapies | Allogenic gene edited hiPSC therapies |
|---|---|---|---|---|
| Immunosuppression | No or low immunosuppression required | Immunosuppression required | No or low immunosuppression required | No or low immunosuppression required<br>Few lines, high compatibility |
| Safety | Quality control (genetic stability, genome integrity, and tumorigenicity) required for each line | Quality control performed during characterization | Quality control performed during characterization | Risk of off-target modifications |
| Time required | Long time (individual generation and characterization) | Short time (the line is already generated and characterized) | Short time (the line is already generated and characterized) | Short time (the line is already generated and characterized) |
| Costs | Expensive | Less expensive | Less expensive | Gene edition costs to be covered |

hiPSC: human induced pluripotent stem cells.

from private companies (e.g. Fuji-CDI, in Wisconsin, USA, from the five most common HLA types matching to 35% of US population), with non-exclusive license fee and restriction rights to develop and commercialize a product. Very few allogeneic hiPSC lines for cell therapy are provided by public research organizations such the NIH through RUCDR Infinite Biologics, Korea HLA-Typed iPSC Banking, and the Center for iPS Cell Research and Application. The Center for iPS Cell Research and Application has generated a total of 27 hiPSC lines made from seven donors (four peripheral blood and three CB) who are homozygous for four of the most frequent HLA types in Japan. These lines cover approximately 40% of the Japanese population [23]. Rim *et al.* published the generation of 13 homozygous GMP-hiPSC lines from blood and CB cells from the Catholic Hematopoetic Stem Cell Bank of Korea [19]. Kim *et al.*, recently reported 22 GMP-compliant homozygous HLA-type iPSC lines, which cover HLA haplo-type matching for 51% of the Korean population [25]. Kuebler *et al.* have generated seven iPSC lines from HLA-homozygous CB samples covering 21.37% of the Spanish population [26]. These lines have been banked in GMP conditions and are ready to be used for cell therapy. Table 3 shows the number of existing hiPSC lines generated in GMP conditions from homozygous HLA types.

The different lines give versatility in HLA typing and differentiation capacity for the treatment of different diseases. Some of these cell lines have already been used in hiPSC cell-based clinical cell therapies. A European hiPSC collection to manufacture cell therapy products needs to be developed within a global organization to face emerging scientific medical and industrial needs.

The feasibility of hiPSC large-scale expansion in existing bioreactor systems under cGMP has been tested for many authors and reviewed by Rivera-Ordaz *et al.* [27]. Relating the quality of hiPSC-based products to critical features and process parameters of existing bioreactors appears the best approach for the future development of hiPSC-tailored culture systems and manufacturing processes. Cell lines for use in human therapy need to be established in GMP conditions in facilities with a relevant product manufacturing license under strict quality assurance. These lines must also be generated with all ethical and legal requirements [28]. Use of hiPSC lines as a starting material for the manufacture of cell

### TABLE 3

**GMP-hiPSC lines generated from homozygous HLA types**

| Center | Number of hiPSC lines | Number of haplotypes | Coverage (%) | Population |
|---|---|---|---|---|
| Fuji-CDI | 5 | 5 | 35 | USA |
| Center for iPS Cell Research and Application (Yoshida *et al.*, 2023) | 27 | 4 | 40 | Japanese |
| Pochon CHA University (Lee *et al.*, 2018) | 10 | 10 | 41.07 | Korean |
| Catholic Hematopoietic Stem Cell Bank of Korea (Rim *et al.*, 2018) | 13 | 13 | | Korean |
| Korea National Stem Cell Bank (Kim *et al.*, 2021) | 22 | 22 | 51 | Korean |
| Banc de Sang i Teixits/IDIBELL (Kuebler *et al.*, 2023) | 7 | 7 | 21.37 | Spanish |

hiPSC: human induced pluripotent stem cells; HLA: human leukocyte antigen.

FIGURE 1

Addressing the right challenges with the right stakeholders.

| FOCUS AREA | STAKEHOLDERS REQUIRED |
|---|---|
| Sample selection | CB banks, hiPSC generators |
| hiPSC production | hiPSC generators, cell therapy products manufacturers |
| Quality control, biobanking, and regulation of cell lines | hiPSC generators, cell therapy products manufacturers, biobanks, regulatory bodies, hPSCreg |
| Safety and regulation | Immunologists, regulatory bodies, cell therapy products, manufacturers |
| Data handling and ethics | CB banks, geneticists, experts in data handling and protection, ethical experts |
| Training | CB banks, hiPSC generators, immunologists, experts in data handling |
| Clinical application | Regulatory bodies, cell therapy products manufacturers, clinical researchers, immunologists, hiPSCreg |

therapy products requires demonstration of comparability of lines derived from different individuals and in different facilities. This needs agreement on the quality attributes of such lines and the assays that should be used [29–31].

## CREATING THE RIGHT FRAMEWORK FOR hiPSC GENERATION TO BE USED FOR CELL THERAPY

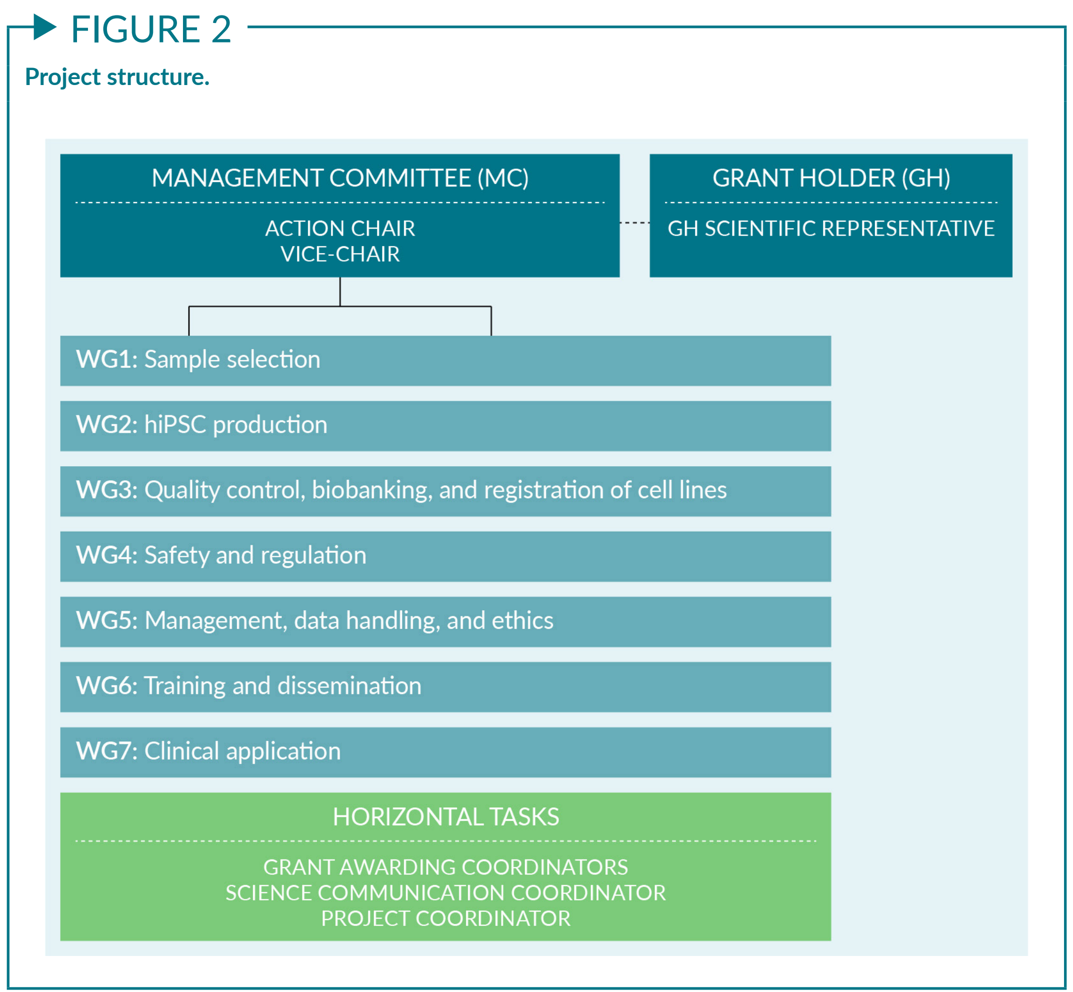
The size of this challenge becomes clear with some numbers. It is estimated that 405 theoretical HLA homozygous combinations are sufficient to cover 100% of the UK Caucasian population—based on a sample of 10.000 real persons to be matched. The hurdle to be overcome is that of these 405, only 236 existed in a pool of 17 million registered donors. Therefore, far more than 17 million need to be screened to find all required combinations. Currently, approximately 22 million HLA-mapped donors are registered worldwide.

One aspect of the HAPLO-iPS network is therefore to elucidate strategies to identify the best possible approach to access donor pools. HAPLO-iPS is now striving to develop a strategic framework using CB sample donations as a source as these provide the most accessible source for hiPSC generation. The framework can be expanded to other HLA typed sources such as bone marrow registries and HLA modified cells, as described above. This network includes all the relevant stakeholders (Figure 1) including: hiPSC generation and banking centers, CB banks that will supply CB units, manufacturing centers complying with GMPs and CMCs to produce stem cell derivatives for cell therapy (advanced therapy medicinal product experts), clinicians, and clinical centers involved or aiming to get involved in cell therapy using hiPSC derivatives, and regulators such as national agencies that supervise compliance with the regulations in the different countries. Ethics experts for the correct handling of samples and adequate data confidentiality and sharing sample procedures are also critical to this network. Immunology experts are also key to ensure an optimal selection of the CB samples.

HAPLO-iPS is managed by the management committee led by a chair and a vice chair. The management committee is the decision-making body. It is responsible for the coordination, implementation and management of the Action activities. The grant holder provides administrative support to the management committee. Seven working groups with working group leaders and co-leaders are in charge of developing the scientific activities. Other key positions

FIGURE 2

Project structure.




are the Grant Awarding Coordinators, the Science Communication Coordinator and the Project Coordinator **(Figure 2)**.

The overall aims of the HAPLO-iPS network also benefits significantly from broader international interactions that are facilitated through established international stem cell networks. Even so, it must be considered that producing hiPSC that are suitable for manufacture of therapeutic products involves more than quality standards and key cell line characteristics must also be addressed for their impact on safety and efficacy of the final products.

The use of hiPSC derivatives for cell therapy requires special attention not only to quality control processes but also with respect to assessment of the differentiation properties, tumorigenicity, and genome integrity, as well as guidelines for ethics and regulatory advice/contacts (such as license landscape), registration and 'look up' systems (available manufacturing capacities), and strategic roadmap including other possible source materials. In addition, the utility of haplo-banks and registries of hiPSC lines to make a single product type will require special attention to establish appropriate comparability studies to assure that multiple cell lines can generate an equivalent product.

## THE FUTURE AIMS OF THE HAPLO-iPS NETWORK

The future aim of HAPLO-iPS is to set the basis for an inclusive approach, making stem cell therapies accessible and affordable for the broadest possible EU population in need. This will be achieved by considering the broad range of haplotypes needed to serve that community. The challenges now being addressed by this collaborative network

require an international approach rather than a national or local one, given the magnitude and complexity of the proposed goals, the rarity of individuals with homozygous HLA haplotypes and the diversity of skills and resources required. Central to this is to consider the pluralistic nature across Europe on the ethical, legal, and socioeconomic levels and the different stages of preclinical and clinical advances. The problem to be solved does not only affect a local or national community, not even a European one, instead it is a global concern for those working in the field. Therefore, the time is ripe to combine the current efforts in one place, to set up a fully coordinated and state-of-the-art European haplo-registry from which to do the groundwork for future patient matched cell-based medicines. Necessary technologies are already described and the first hiPSC-based clinical trial in Europe is progressing (Cynata, UK) and others are moving forward worldwide [2]. Putting in place a first of its kind EU haplo-registry will ensure strict data and procedural standards and harmonization of the procedures used in the different centers involved throughout Europe, together with the definition of the required rigorous standards and regulatory acceptability regarding cell quality and safety. Comparability of the efficiency and safety of different hiPSC lines for therapeutic applications will also be essential. Legal and ethical issues will have to be aligned throughout the different European countries and decentralized GMP manufacturing centers and biobanking hubs will also have to be established with a smoothly working logistic network. Furthermore, to reach the highest quality standards, traceability, and automation solutions, all with effective standardization measures in place must be proactively developed. This is crucial at all stages of cell production, characterization and biobanking. However, hiPSC biobanking procedures are currently being developed largely within individual projects for GMP manufacture of cell-based products and the nature of the haplo-banking challenge means there are huge benefits to be realized from greater co-ordination between device developers, current users, future product developers and regulators at national, European, and international levels. A sophisticated combination of decentralized and centralized facilities for cell production, quality control and distribution are likely to be needed to serve the broad range of hiPSC cell-based medicines under development. This will involve an extended quality control and auditing process to assure the same baseline for quality and safety in all participating resource centers throughout Europe. The HAPLO-iPS network already has a wide geographical distribution among many EU countries in order to achieve this. Currently, there are 42 members in the management committee from 25 countries, and 133 working group members from 32 countries. Moreover, it can increase because COST Actions are open during all the lifespan of the Action. HAPLO-iPS is now well placed to coordinate with broader international haplo-banking activity in Asia and the USA to further increase impact in this regard.

HAPLO-iPS will add value to existing efforts at both the European and International level because it has all the prerequisites to provide a sustainable solution to avoid faulted workflow design and fragmented implementation of hiPSC derived products for cell therapy. HAPLO-iPS brings together an unprecedented pool of experts from CB banks, reprogramming centres, companies, clinicians, regulators, and ethics experts in the relevant science domains. Furthermore, this network aims to update and educate the researchers in the concepts and technologies necessary to further advance this field to deliver a cohort of consistently trained scientists and clinicians fit to engage in effective translational research and the development of future cell-based medicines.

The ultimate goal of the HAPLO-iPS network is to utilize the power of innovative stem cell technology to provide every European citizen with a safety-assured, perfectly characterized, and immunologically

matched stem cell line. This will allow much wider access for European patients to treatment with future cell therapies and regenerative medicine without a long-lasting or even unsuccessful search for compatible cell donors and the need for significant immunosuppression of recipient patients. Creating the right network of stakeholders is only the first step, creating a sustainable ecosystem and the resources to do so in the EU to support the clinical application of hiPSCs, will be the challenge for the next years to come.

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## AFFILIATIONS

**Begoña Aran PhD**
Stem Cell Bank Coordinator,
Barcelona Stem Cell Bank,
and
Regenerative Medicine Program,
Bellvitge Biomedical Research Institute

**David Morrow PhD**
Senior Scientific Program Manager,
European Research Infrastructure for
Translational Medicine

**Ester Rodriguez PhD**
Head of Projects,
Regenerative Medicine Program,
Bellvitge Biomedical Research Institute

**Anna Veiga PhD**
Director,
Barcelona Stem Cell Bank,
and
Group Leader,
Regenerative Medicine Program,
Bellvitge Biomedical Research Institute


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INTERVIEW

# Exploring the frontiers of iPSC research, clinical application, and standardization

**David McCall**, Senior Editor, *Cell & Gene Therapy Insights*, speaks to **Julie Allickson**, Director of Mayo Clinic, Center for Regenerative Biotherapeutics, about the transformative potential of iPSCs across their potential application areas, including as the foundation for novel cell therapies and *in vitro* models for drug discovery. Dr Allickson also discusses goals and priorities for driving both technological innovation and standardization in the field.

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## What are you working on right now?

**JA:** The Center for Regenerative Biotherapeutics at Mayo Clinic recently shifted its focus to biomanufacturing, which entails a robust manufacturing process led by industry-experienced experts. There is currently a team specializing in process development, manufacturing, quality assurance, and quality control. This team prioritizes the clinical needs of patients at the Mayo Clinic whilst also considering broader applications for all patients, and exploring commercial potential. Our current efforts involve assessing market viability and partnership potential.

> "...we are exploring a platform technology that combines CAR-T with an oncolytic virus for solid tumors."

One of our primary focuses is cancer-related therapies—in particular, CAR-Ts for targeting solid tumors and specifically, investigating applications for thyroid cancer. These proprietary technologies are currently in the process development stage within Mayo Clinic. Additionally, we are exploring a platform technology that combines CAR-T with an oncolytic virus for solid tumors. The plan is for this research to be extended to various tumor types once positive results have been observed.

Beyond immunotherapy, there is a big focus in the field of 3D printing for laryngeal and tracheal bioproducts. We are currently printing out defects of the trachea and larynx tailored to individual patient requirements. These are initially printed as a mold, which is then utilized as a scaffold and implanted into patients. Ongoing development focuses on enhancing the effectiveness of these products by incorporating cells or exosomes. Another aspect of our research involves exploring the use of induced pluripotent stem cells (iPSC) in these products—we are assessing whether these cells can serve either as a replacement for or as a contributor to tissue engineering.

One crucial project in development involves addressing type 1 diabetes using iPSC, differentiating them into beta cells and other islet cells. Our goal is to modify or match these cells in a way that eliminates the need for immune suppression in patients.

## Can you give a brief overview of your own background in the iPSC space?

**JA:** I have been in the stem cell space for almost 30 years. I initially started my career in bone marrow transplant, playing a pivotal role in establishing the first transplant manufacturing unit at the University of Miami, where my focus was on cells and cell therapy. This involved the exploration of mobilized peripheral blood implants, transplants, and cord blood.

Subsequently, I moved to the Diabetes Research Institute, where my expertise extended to islet cell transplants and vertebral bone marrow for tolerance for solid organs, along with islet cell transplants. My career then led me to neonatal cells, delving into the use of cord blood and perinatal cells as a cell therapy for various application, including potential use in cancer and other types of regenerative applications. I relocated to Wake Forest Institute for Regenerative Medicine to build a team focusing on several DOD-funded tissue engineering products to accelerate to FDA-approved in early phase clinical trials.

Upon joining Mayo Clinic, my research interest included the use of iPSCs as a biotherapeutic. Mayo Clinic, along with BioTrust, has established a foundation over many years, enabling physicians to collect fibroblasts and other types of cells that can be reprogrammed

into iPSCs for the purposes of research, drug development, and enhancing understanding of disease.

## Tell us about the work of the ISCT iPSC-focused subgroup you are a member of—why was it formed, and what are its specific goals and outputs?

**JA:** The International Society of Cell and Gene Therapy (ISCT), of which I am a founding member, has a rich history spanning more than three decades. Originally rooted in academia, it evolved into a collaborative effort that now encompasses an even split between industry and academia. The ISCT includes an industry committee focused on advancing cell therapy and gene therapy and related applications.

Recognizing the need for efficiency and acceleration in bringing therapies forward, the ISCT formed various committees. In my role within the industry committee (despite being based in an academic healthcare facility) I was involved in the creation of a subgroup called Emerging Regenerative Medicine Technologies (ERMT), which aimed to harness the collective expertise of industry professionals and accelerate advancements in the field.

The initial focus of the ERMT subgroup was iPSCs due to the numerous challenges and lack of standards in the field, particularly in relation to automation. We collaborated to produce a white paper addressing the manufacturing of GMP master cell banks for iPSCs, which was recently submitted to *Cytotherapy*, marking the completion of our first phase.

Moving forward, our second paper will focus on gene editing, exploring its promises, potential challenges, and considerations. Beyond process development and manufacturing, our efforts extend to issues related to supplies, reagents, and intellectual property in the field. Securing licenses from various areas for commercialization is a particular challenge—however, there are groups addressing the complexities associated with business models and cost of goods.

As progress in the technologies are made, emphasis lies more and more on the importance of considering the cost of technologies and how to mitigate that through automation. iPSCs, being a relatively nascent field compared to other cell types, requires some catching up in terms of standardization and commercialization. The ISCT actively contributes to these efforts and facilitates discussions through roundtable sessions, fostering a collaborative environment for questioning, answering, and knowledge exchange within the community.

## Can you tell us more about your iPSC-related work at Mayo Clinic?

**JA:** Our primary focus is on building resources for the BioTrust where physicians provide either biopsies or fibroblast samples, allowing us to reprogram cells for the assessment of

rare and complex diseases. Simultaneously, we are developing master cell banks to support various investigators.

Approximately eight investigators here are currently exploring various diseases, including type 1 diabetes, macular degeneration, cardiovascular regeneration, multiple sclerosis, and liver regeneration. The cardiovascular work, led by Dr Tim Nelson, has already progressed to clinical trials, where we are actively assisting him with GMP fibroblast collections.

The overarching goal is to effectively de-risk the technology, guide it through the initial phases of clinical trials, and set it up for success, streamlining its transition into industry. We strive to incorporate automation, reagents, and supplies to minimize costs, recognizing the crucial need for patient access to therapies.

In the pursuit of developing the master cell banks, we are also exploring automation technology. While the Sendai virus is commonly used for reprogramming cells, we are also developing in-house proprietary techniques in the hope of using those for a variety of cell sources. The overall goal is to generate master cell banks using multiple technologies, possibly incorporating the Sendai virus and our proprietary method, and in collaboration with institutions like the NIH and the CiRA Foundation in Japan.

Our engagement with the CiRA Foundation is particularly promising, as they are not only focused on iPSCs but are also investigating gene editing to prevent cell rejection during expansion. While this is in the early stages, it aligns with our goal of expanding and differentiating cells for replacement therapy without triggering rejection.

Collaborative grant applications are ongoing and reflect the excitement surrounding the therapeutic applications, disease monitoring, and potential uses of organoids. We have also collaborated with the University of Minnesota on cryopreservation of organoids, incorporating iPSCs for varied applications, and we aim to continue and expand upon this.

## What do you view as the current technological state-of-the-art in the iPSC field currently? For example, where is automation being actively applied?

**JA:** There are various types of automation in the context of producing clones, and the challenge lies in selecting the right clones. While there isn't a one-size-fits-all solution, there are several technologies available.

Expanding cells is a well-understood process—however, iPSCs are somewhat different to other cells we have expanded. Various automation technologies, such as hollow fiber bioreactors, are currently being used in different applications for iPSC expansion and it is something that has a growing importance. The paper we wrote for the ISCT focuses on selecting clones, manufacturing the product, and achieving GMP, as well as addressing the challenging aspect of differentiation.

One significant challenge is obtaining the appropriate reagents and supplies—an issue that the field has yet to fully resolve. Expansion, on the other hand, seems to be already in place,



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> "Looking at the genetic stability and pluripotency thoroughly is challenging, but essential. As we examine different clones, rigorous characterization and quality control become a top priority."

with several technologies being employed (although none have emerged as the definitive best option). As the field is still relatively new, there is ongoing learning, with only a few technologies in clinical use, such as retinal epithelial cells at the NIH and in congenital heart disease here at Mayo Clinic. So, while there is progress, the market is not yet flooded with clinical trials involving iPSCs.

## Q Where would you like to see further technological innovation enabling this space?

**JA:** A comprehensive assessment of characterization is crucial, as it holds the key to advancement with the US FDA and eventual commercialization of these products. Looking at the genetic stability and pluripotency thoroughly is challenging, but essential. As we examine different clones, rigorous characterization and quality control become a top priority.

The second priority is the cryopreservation of these cells. This becomes particularly challenging when incorporating iPSCs into organoids, especially when aiming for their use in drug and product development. Additionally, the cryopreservation process needs to align with the demands of utilizing organoids effectively.

Another critical aspect is the regulatory framework. Over the decades, I have witnessed and been involved with the US FDA's substantial contributions to the field, particularly in the realm of immunotherapy. Similar in-depth research is needed for iPSCs and while it is anticipated to be on the horizon, the sooner it arrives for those actively working in the field, the better.

## What do we really know about stability and variability of IPSCs and how can we optimize around these aspects?

**JA:** The primary concern lies in the risk associated with tumorigenicity and immunogenicity, especially during the differentiation process of iPSCs. Ensuring thorough differentiation or selectively extracting non-differentiated cells is crucial. The reprogramming and differentiation procedures pose a current risk until we can confidently eliminate undifferentiated cells.

Tumorigenicity stands out as a top consideration, and addressing the best approaches to manage immunosuppression is essential. Questions arise about whether matching HLA, minimizing rejection through editing, or exploring other opportunities can effectively mitigate the risk.

## What is your take on recent advancements and current lingering concerns in terms of multiplex iPSC editing approaches?

**JA:** The recent breakthroughs with CRISPR technology, particularly in treating sickle cell disease, hold the potential to cure numerous patients. CRISPR-Cas9 is one of the pioneering technologies in this realm and holds the capability to edit multiple genes, offering great flexibility for therapeutic applications. However, caution relating to the associated risks should be considered.

While base editing is still in early stages of discovery, it presents significant potential by allowing the modification of specific nucleotides within the genome without introducing double-strand breaks. Compared to CRISPR-Cas9, I believe base editing holds substantial promise for iPSCs.

Synthetic biology approaches, such as TALENs and zinc finger nucleases (ZFNs), also play a role, but the current focus seems to be on CRISPR-Cas9, which is progressing rapidly. CRISPR-Cas9 and base editing are pivotal choices in advancing iPSCs with multiple genetic modifications. These technologies are likely to be advantageous as research continues into the potential of iPSCs and their applications in genetic modification.

## What would you identify as the main priorities in terms of standardization in the field, and who is addressing these?

**JA:** In terms of standardization, a key focus is on cell characterization and ensuring a consistent and reliable approach. The National Institute of Standards and Technology (NIST) has been involved in cell therapy, although not specifically in iPSC therapy as yet. They are currently looking at providing guidance on processes like cell counting as executed in ISO standards. Standards Coordinating Body (SCB) a 501C3 organization plays a significant role in leading the drafting of these standards. Standardizing these methodologies will help to facilitate meaningful data comparison across different research groups.

Cell characterization stands out as a major priority, and various organizations are actively contributing to the automation efforts here. The NIH has a significant role, with efforts such as the human pluripotent stem cell registry, which serves as a valuable resource for researchers seeking cell line and quality standards.

The International Society for Stem Cell Research (ISSCR) has been involved significantly in promoting knowledge exchange, collaboration in the field, guidelines, and ethical

considerations of iPSCs. Additionally, there are international stem cell banking initiatives that are focused on standardization.

However, a significant driving force for standardization lies with the US FDA. The guidance documents and feedback provided by the FDA, especially during the submission of pre-IND and IND applications, play a crucial role in shaping the standards for the field. This has previously been done in the immunotherapy space. The collaborative efforts of all the aforementioned organizations, together with FDA guidance, are needed for accelerating advancements and standardizing the rapidly evolving field of iPSC-derived therapy.

## Q Looking to the future, what do you anticipate in the way of platforming opportunities and likely market evolution in the iPSC space?

**JA:** As mentioned earlier, a key area for future development is disease modeling and drug discovery using iPSCs from patients, facilitating personalized medicine—that will form a substantial market. The applications in regenerative medicine and various cell therapies, particularly in tissue engineering and organ development, hold a noteworthy place in the future of iPSCs, too. As there are various cells required for tissue-engineering organs, it would be difficult to generate them in any other way. Tissue engineered organs could revolutionize healthcare, although scalability remains a challenge.

The potential for smaller organs and tissues, as well as the replacement of cells, presents opportunities. For instance, iPSCs are making strides in diabetes treatment, and ongoing research targets in neurodegenerative and heart diseases. The prospect of patient-specific treatments and precision medicine holds promise, potentially revolutionizing individualized patient care.

Additionally, there is a role for iPSCs in biobanking and cell line repositories, enhancing research and development capabilities. As the field progresses, collaboration between in-house developments and outsourced efforts, along with the use of key cell lines, will likely do much to shape the evolving landscape of the iPSC space.

## Can you summarize one or two key goals and priorities for your work in the foreseeable future?

**JA:** My primary goals involve using iPSCs to differentiate cells for tissue engineered organs, focusing on complex structures such as the trachea and larynx, and the replacement of cells or islets for type one diabetes. There is roughly $450 billion spent on healthcare for diabetes in the US alone, and that figure is from 2 years ago now—however, it is not just about reducing costs; it is also about enhancing quality of life. Having worked with patients who

underwent islet cell transplants, their life-changing stories highlight the impact on both quality of life and healthcare economics. These are the key priorities guiding my work.

## BIOGRAPHY

**JULIE ALLICKSON** is the Michael S and Mary Sue Shannon director of Mayo Clinic's Center for Regenerative Biotherapeutics, the Otto Bremer Trust director of Biomanufacturing and Product Development, Center for Regenerative Biotherapeutics, and Associate Professor of Regenerative Medicine. Dr Allickson is leading the next phase of development of the Center for Regenerative Biotherapeutics as it delivers on innovations that cure, connect and transform patient care in alignment with Mayo Clinic's 2030 vision. She directs the enterprise-wide biomanufacturing strategy that aspires to introduce new regenerative therapeutics into the practice and establish Mayo Clinic as a category of one in regenerative medicine for rare and complex conditions. Dr Allickson provides strategic leadership for all center activities and operations across the Mayo Clinic Enterprise. The Center for Regenerative Biotherapeutics has over 25 clinical trials in cell and gene therapy underway. With more than 25 years of experience in clinical translation of cellular therapies and regenerative medicine products, Dr Allickson has expertise in business management, regulatory affairs, strategic planning, project management and team building. She has been in industry and academic healthcare facilities. She has served as an executive officer of a publicly traded company that builds services for cellular banking, including licensure of technology with international affiliates. Mayo Clinic, Center for Regenerative Biotherapeutics is building a team of industry experienced leaders to frame a successful structure for Biomanufacturing including cellular therapies, gene and viral therapies, and tissue engineering and bioprinting technology.

## AFFILIATION

**Julie Allickson PhD**
Director of Regenerative Biotherapeutics,
Mayo Clinic


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CELL & GENE THERAPY INSIGHTS

SUPPLY CHAIN CHANNEL EDITION

# Cryopreservation and Cold Chain

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Barry Fuller, Glyn Stacey, and Roland Fleck

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CELL & GENE THERAPY INSIGHTS

SUPPLY CHAIN EDITION:
CRYOPRESERVATION & COLD CHAIN

COMMENTARY

# Innovation in cryopreservation & cold chain management

Barry Fuller, Roland Fleck & Glyn Stacey

Successful cryopreservation depends to a large extent on how the cell water compartments respond to ultra-low temperature cooling, which in itself is necessary to inhibit all molecular interactions for long-term biopreservation. Biophysical principles dictate that water will undergo ice nucleation during cooling, which will cause severe cell injury in a number of complex ways, which can be mitigated by how the cryo-cooling is undertaken. The ice burden can be reduced by adding appropriate biocompatible solutes, called cryoprotectants (CPA), which act in a colligative fashion to interfere with the water-to-ice transition as deep cooling progresses, until the temperature range where the whole mixture enters a low temperature 'glassy' state (Tg) whence all other molecular interactions are inhibited. Optimisation of cell survival can also be achieved by controlling the kinetics of both cooling and warming rates during cryopreservation, which limit ice crystal growth until final melting temperatures are reached and normal cell biology can resume in the liquid aqueous state.

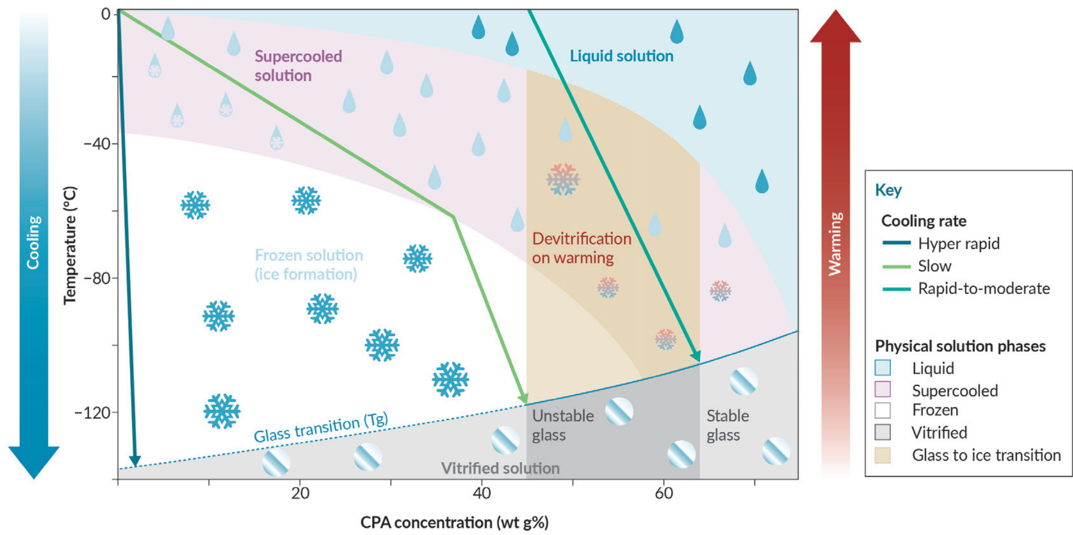


FIGURE 1: Processes during temperature transitions in cryopreservation.

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The interest in and application of biologics (biological products derived from cells, agents, and small biomolecules) continues unabated in the current decade. These all require a range of integrated technologies, one of which is the ability to control biological time, both within production schedules and for distribution of validated products to end users. This is the so-called cold chain whereby unwanted molecular processes can be halted either for short periods (liquid storage: hours up to a few days) or for greatly extended time (cryopreservation: weeks, progressing forward to years) depending how 'cold' is defined and applied. True long storage by cryopreservation can offer significant advantages such as matching supply and demand of cell therapies, which is often dictated by patient disease and treatment course, and availability of specialist staff facilities. Cryopreservation can also avoid wastage and facilitate timely batch testing to meet quality assurance and release criteria. The current understanding of the scientific principles underpinning both approaches have been reported recently but of course, the clear difference between the methods is that cryopreservation requires cooling to deep subzero temperatures where the inescapable property of aqueous solutions (including the intracellular environment) to nucleate ice (Elliot-Fuller) dominates cellular damage beyond other adverse effects associated with low temperatures. The often-reported limited stability of cell therapies such as chimeric antigen receptor (CAR)-T cell products additionally points to benefits of cryopreservation. Currently, amongst the growing portfolio of cell therapies, many CAR-T cell products which have progressed to clinical application have relied on the cryo-cold chain. Although there have been as yet few studies comparing delivery of fresh versus frozen CAR-T cell products, the evidence supports the concept that thawed cryopreserved products perform with comparable efficacy to fresh cell products from the same production batches.

The debate about the suitability of cryopreservation strategies often centers around the undeniable fact that there are losses in cell numbers, reductions in early post-thaw viability indices, and in some cases, a delay in patient overall response rates. The discussions can become further complicated where cryopreservation is used at different stages in the production pathway, and/or for the batch banking of the starting materials (e.g., peripheral blood mononuclear cells [PBMC]) and subsequent transduced cell products ready for patient delivery. The focus of our current review is therefore an assessment of the current understanding of the cellular impacts of the various biophysical stresses of cryopreservation, and what novel ideas are being proposed to mitigate these.

## CURRENT UNDERSTANDING & LIMITATIONS OF CRYOPRESERVATION

It is of course well known that for stable long-term biopreservation, the temperature range needs to be pushed below −40 °C, and into deep cryo-cooling beyond −80 °C [9,10]. In some ways, the temperature range for storage is dictated by the widespread availability of a suitable cryogenic environment; specialist electrical freezers can operate down to about −135 °C, whilst vapor phase or liquid phase of nitrogen provides temperature control from about −170 °C to −196 °C (depending on the working phase chosen) [2,11,12]. Many recent reviews have discussed the biophysical aspects of cryopreservation [11,13] but it is worth outlining some of these to set them in the context of new approaches to cryopreservation. Biological stability is crucial, along with the equally-important

need to recover the full range of cellular and molecular functions upon return to normal physiological temperatures, which for most clinical and biotechnological applications will be around 37°C [14–16]. However, we should remember that in other scenarios such as aquatic species biopreservation, recovery to physiological temperature will be to about 10–18°C [17] depending on the species. For any cell to survive cryopreservation, the supreme question is how to deal with ice formation within cellular aqueous compartments. Ice crystals, and the localized associated dehydration as water molecules join growing ice crystal fronts, cause catastrophic, multi-focused injuries [18], which can disrupt the plasma membrane, intracellular organelles, endoplasmic reticulum, and other membrane-bound compartments. Consistent biological survival almost invariably requires addition of chemical agents termed cryoprotectants (CPA) [19], which stabilize molecular and ultrastructural moieties within the cells when the water relationships are severely perturbed by the growing ice phase [20] during slow cooling freezing, which is commonly used. Equally, but in contrast to slow freezing, with the cryogenic storage technique of vitrification (where cooling rates are high and ice formation is deliberately suppressed [21,22]), survival depends upon stabilizing properties of high concentrations of cryoprotectants [23]. The injuries resulting from the biophysical changes both during cryopreservation and warming can become additive, leading to progressive and delayed onset cell death in the early post-thaw phase [24]. The combination of all of these processes constitutes the science of cryobiology [25,26].

Figure 1 depicts what happens during the temperature transitions of cryopreservation. Liquid water in the aqueous compartments is shown in blue droplets at the top of the image. Cooling temperatures are depicted on the X axis, and the concentration of added CPA are shown on the Y axis. Three different kinetic approaches to cooling (hyper rapid, slow, and slow-to-moderate) are shown which are pragmatic descriptions dictated by available procedures for cryopreservation depending largely by sample size and methods for applying the cryogens needed for cooling.

As cooling proceeds the ice nucleation temperatures for a particular mixture are reached and water enters a super-cooled state (represented by the pink zone) containing potential ice nuclei shown as pale clear droplets. During slow cooling (middle green curve) the ice burden increases significantly shown by ice crystals in the white zone. The stable 'glassy' state in the grey zone is reached once the Tg threshold has been passed, and thereafter long-term biopreservation is assured. As the concentration of added CPAs are increased (Y axis), the Tg is shifted upwards, and Tg can be reached with a lesser ice burden from the cooling process. Controlled slow cooling with moderate added CPA concentrations (between 5–20 wt g%) has been one of the traditional approaches to cell cryopreservation, an approach widely termed 'two-step cooling.

Hyper-rapid cooling (dark green curve) is an experimental approach to achieve Tg using very specific approaches to handling the cryogens used for cooling. It is currently being refined for more user-friendly approaches.

Rapid to moderate cooling (light green curve) is the other main current approach, which depends for success on the use of relatively high added CPA concentrations (about 40–60 wt g%) and rapid cooling approaches. This is the approach widely termed 'vitrification' and has found widespread applications where small bio-specimens are being preserved (such as mammalian embryos or plant shoot tips). The Tg range is elevated and can avoid cell injury by the speed of attaining the 'glassy' state (grey zone). Truly stable 'glassy' states can only usually be achieved with addition of very high CPA concentrations (>80 wt g%) but these are often toxic to cells.

Given that in most cases cryopreserved cells end up within the unstable 'glassy' range (middle of lower grey section), control of the warming processes is also essential. The temperature transition out of the Tg range

## FIGURE 2

A visual representation of the different approaches to cryo-cooling, the sizes and containers used, the kinetics of cooling and warming relevant to each, and the biological impacts on cells and their environment. The traffic light scheme depicts which are in most widespread usage at the moment. The limitations of each approach are also described. Given the pace of research into cryopreservation, there will likely be mitigation for some of these challenges in the next few years.

TWO-STEP APPROACH | VITRIFICATION | HYPER-KINETIC VITRIFICATION

### 1 Applications

Genetic conservation and cell banking

Reproductive biology

Organ transplant/ embryology

VOLUME

SAMPLE SIZE

### 2 Tools/equipment

Vials, straws, bags

Vials, straws

Bags, vials

### 3 Cooling/warming

Cooling/warming rate (ºC.min⁻¹)

10 — Slow

1,000 — Rapid

1,000,000 — Hyper rapid

### 4 Cellular cooling

$H_2O$

Ice crystal

CPA

Slow-cooling, low volume dehydration

Fast-cooling, low volume vitrification

Hyper-kinetic cooling, high volume vitrification

### 5 Warming

Established technology

Emerging technology

Theoretical technology

Rapid warming (water bath)

Rapid warming (water bath or dry warmer)

Very rapid warming (nanoparticles/induction loops)

Hyper rapid warming (large damage risk, tech not ready)

### 6 Limitations

Two-step approach:

- Sample-size dependent
- Cells on periphery over dehydrated (cells killed)
- Cells in the core/middle insufficiently dehydrated (cells killed)
- Damage/cell death during CPA removal after thawing
- May have limitations for complex cell mixtures/mixed cell organoids where permeability varies between different cell populations

Vitrification:

- High CPA concentration needed
- Potential for CPA toxicity
- Sample size/surface area to volume critical
- Intracellular ice formation

- Extremely high rates of cooling and warming
- Commercial technology needed
- Clinical approval needed

Hyper-kinetic vitrification:

- Technology limited
- How do you achieve sufficiently fast cooling?
- How do you warm the middle as well as the outside?
- Intracellular ice formation on warming

Traffic Lights: Green—very close or in use in certain areas; Amber—background science established but requiring some further development (3–5 years); Red—basic science principles requiring further in-depth evaluation, enhanced equipment technologies and regulatory approval needed (5–8 years). CPA: Cryoprotectant.

can result in a process called devitrification (buff coloured zone), where water molecules start to become mobile and can aggregate on pre-existing ice crystals. Thus the ice burden can increase (so-called freezing during warming) which can also add to the total injury profiles of the preserved cells. On this basis, warming is usually applied to be as fast as logistically achievable.

Whilst cryopreservation has become almost routine over the past four decades to promote cold chain logistics across a wide range of applications in medicine and biology, there are widely acknowledged limitations to it as it is currently employed (Figures 2 & 3) [27,28].

Current biobanking standards address the need for strategic management of known and adventitious microbial contamination hazards, requiring emergency planning and procedures to be in place to manage integrity, cleanliness, and biosecurity of long-term storage systems for frozen viable material [29]. Emergency response to vessel failure is also crucial to prevent total loss of frozen biological resources [30], as well as strategic approaches to avoid risk including planning, cleaning, and maintenance to assure stability and avoid spread of microbial contaminants into experimental work. Unfortunately, it is not uncommon for institutions to lack cleaning regimes for storage vessels, and it has been recognized for some time that there is a need for vessels that are more readily decontaminated or are designed to reduce or remove contamination [11].

## THE NON-FROZEN COOL CHAIN—ITS RELEVANCE IN THE OVERALL PROCESS

Non-frozen cool chains have often been actively pursued by cell therapy manufacturers based on the significant effectiveness of this approach from historical tissue shipment practices [31,32]. This approach is simple and inexpensive, relying on maintenance of cells in the liquid state at any convenient temperature above the freezing point, but is hampered by the short useful shelf-life of only about 2–3 days. Currently, across the cell therapy sector, there is still a general lack of understanding, expertise, and investment in necessary infrastructure for global application of cryo-technologies which is challenging for routine application of cryopreservation. The non-frozen cool chain can allow early startups to move more easily from laboratory settings into the clinic, or allow a simpler 'hub and spoke' delivery process for cell shipment from a central manufacturing process to end-user clinics nearby—for example, to linked hospital groups in major cities. However, growth of microbial contamination may not be fully inhibited in the chilled liquid state and thus, the serious potential hazard of patient infection must be managed. Such short shelf lives also create difficulties for completion for industry standard sterility and mycoplasma testing, which cannot be fully completed within the use-by date of the products. Accordingly, there has been much effort to establish guidance to facilitate rapid test methods for non-frozen products, which is now being implemented in European regulatory guidance for Good Manufacturing Practice [33] and an ISO standard for method selection and validation is under development [34]. Short shelf life is also very challenging for healthcare providers to ensure patient availability in readiness to receive the products within validated shelf life, especially when patients must be conditioned for treatment as in CAR T treatment. Failure to assure such fine coordination can mean increased cost and delayed therapy. A range of technologies for shipment of non-frozen materials are under development including gels (e.g., agarose, alginate) and liquid cell suspensions liquids (e.g., Hypothermasol™, Wisconsin solution) some of which have been used in delivery of cell therapy preparations [35–37]. However, extending the maximum storage/shipment times beyond a few days has proven to be challenging. Further improvements in such approaches may be achieved through the use

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of apoptotic inhibitors [38, 39], but use of such supplementary storage excipients will require additional regulatory approval. It is likely that over the next 5–10 years, many liquid storage delivery platforms will be replaced by cryogenic preservation platforms.

## HORIZON SCANNING TO IMPROVE CRYOPRESERVATION OUTCOMES

As outlined above, the collective understanding of the essential biophysical controls which are needed to support cell recovery from the cryogenic excursions has improved in the past decade [1,2,9], but there are still many gaps in the fine details. These can be subdivided here for the purpose of this current commentary.

### (i) Manipulating ice nucleation, crystal growth & total fraction

Addition of traditional CPA prior to cooling impacts the total ice fraction, but we are beginning to understand there are additional manipulations which can be beneficial. Ice re-crystallization inhibitors (IRI) are molecules of specific composition and structure which can interfere with water molecules joining growing ice fronts. These can be synthetic agents which have been developed based on knowledge of freeze avoidance or survival in the natural world [40–43]. The agents modify ice fractions in the mixtures by this limiting water molecule binding to ice on a kinetic basis, but are particularly important in the rewarming phase, where water molecules in the frozen matrix regain molecular mobility as temperatures reach about $-40o^{C}$ and warmer. This ice re-crystallization can induce additional injuries, which may be mitigated by effective IRI but in some cases the IRI molecules may confer additional protection mechanisms [44].

The physical events following the water–ice transition include an increase in volume. Since most cryopreservation procedures are performed in vials or bags with free head spaces, these volume changes are without significant consequences. Increasing pressure during cryogenic cooling can itself inhibit water molecules joining the ice mass. However, this property can be manipulated by isochoric cryopreservation by which the volume expansion is constrained, increasing pressure and facilitating the ‘glassy’ transition in the sample. The challenge is to control the effect reproducibly by developing novel technologies, with promising initial results [45].

The mobility of water molecules can be modified by oscillating and high magnetic field strengths [46], similar in concept to manipulation of water in other fields such as magnetic resonance imaging. This approach has been studied for application of magnetic fields during cryo-cooling as low-frequency oscillating electric and magnetic field cryopreservation [46,47]. The fundamental principles of this which might enhance cell survival remain a matter of debate [48], but commercial cryo-coolers have been built and tested based on this principle (ABI Corporation, Chiba, Japan). As more knowledge is accumulated, this may become a helpful technology in improving the cold chain.

### (ii) Cryoprotectants & their acceptability in cell therapies

The selection, efficacy, and limitations of both cell permeating and extracellular molecules which enhance cell survival have been reviewed in the cryobiology field [19,20]. As a generalization, cell cryo-survival requires some degree of intracellular distribution of cell permeating agents such as dimethyl sulfoxide (DMSO), or ethylene glycol, glycerol (to name a few). Other classes of compounds (e.g., sugars, oligosaccharides, or polymers) may modify water-ice interactions but to a large extent remain in the extracellular fluid, and are considered secondary or adjunct CPAs. There are always exceptions to these definitions where successful recoveries of specific cell types have been reported [49,50], but these are often

## FIGURE 3

Translational status (on the basis of closeness to widespread applicability) of the main current fields of innovation in cryobiology.

| Field | Innovation | Requirements |
|---|---|---|
| TRANSLATIONAL SCIENCE | 1 Low-frequency oscillating electric and magnetic field cryopreservation [46,47,48] | 1 Requires application across wider cell types and multi-user trials |
| | 2 Non-linear cryopreservation [12,13] | 2 Requires application across wider cell types and multi-user trialling |
| | 1 Isochoric preservation [45] | 1 Requires commerical equipment availability and multi-user trialling |
| | 2 EMR warming and supplements [58,64,92] | 2 Needs availability of commercial and regulatory evaluated materials, plus multi-lab trialling in a range of cells |
| | 3 Ice nucleation and supplements [12,87] | 3 Needs availability of commercial devices with multi-lab trials and regulatory assessment of nano-supplements |
| | 4 Vitrification (basic principles understood [12,21,22,90] | 4 Need for scale-up to larger volumes, other cell types, specialist equipment, automation and sample holders, multi-user trials and regulatory evaluation |
| CRYOPROTECTIVE AND CRYOSURVIVAL AGENTS | Managing CPA toxicity [80,81] | DSMO toxicity now managed by mechanised washing; further development needed for widespread uptake |
| | 1 Ice recystallisation inhibitors [82,83] | 1 Identified inhibitor molecules are moving to early-phase trials, but need commercial manufacture and regulatory approval |
| | 2 Novel cryoprotectants [49,50,53,54,84] | 2 Need for objective evaluation against more traditional CPAs (toxicities will require comparability); further mechanistic studies required and assessment in different cell types and clear regulatory path |
| COLD CHAIN LOGISTICS | 1 Monitoring of samples (temperatures and locations) [73,74,75,76] | 1 Need for wider adoption of barcoding, RFID, etc, and guidance from regulatots on acceptability |
| | 2 Storage, shipment reagents, devices [11,75] | 2 Existing reagents need testing across wider range of cell therapies; also require readily available product stability and sterility monitoring system for quality assurance |
| | New generation improved cryogeneic shipment vessels and technologies [82,83] | Dry-shipper vessel cleaning protocols not straightforward or fully effective; wider testing of electrically-powered systems and regulatory acceptance needed |
| | New generation cryogenic storage vessels [11,67] | Novel designs needed which limit contamination and sustain vial temperatures during manipulation |

Traffic Lights: Green—very close or in use in certain areas; Amber—background science established but requiring some further development (3–5 years); Red—basic science principles requiring further in-depth evaluation, enhanced equipment technologies and regulatory approval needed (5–8 years).

associated with other manipulations of the physics of the cooling processes, which may not be easily applied to routine cell therapy applications. DMSO has been widely used for hematopoietic stem cell cryopreservation for several decades with good efficacy, whilst acknowledging potential limitations concerning adverse patient events [1]. The toxicity of the agent itself to cryopreserved cell populations is generally low and can be mitigated by careful handling protocols, including time and temperature of cell exposure to DMSO and washing away the residual CPA before patient delivery the cells [51,52]. Novel approaches to formulate DMSO-free CPA are areas of intensive study [53,54] but as yet, these have not been widely applied in the current cohort of cell therapy products and will need to meet patient-orientated regulatory requirements. At present, it is often possible to reduce the concentration of DMSO used in cryopreservation by introducing secondary CPA such as polymers or oligosaccharides [55,56].

## (iii) Improved cryopreservation by vitrification

Vitrification (VF) methodologies, in contrast to those of slow freezing, attempt to vitrify both the surrounding bulk media and the cell/tissue components. A vitreous state is one where water reaches an amorphous, glassy, metastable state. Vitrification is a promising approach for cryopreservation (CP) of biological materials as it is simple, robust, and cell agnostic. In its simplest form, vitrification relies on rapid single-step sample cooling by direct immersion into liquid cryogen (e.g., liquid nitrogen)—accurately described as kinetic vitrification (K-VF). However, it is challenging to achieve in practice with pure water requiring cooling rates in the order of –107 °C/s [21,22]. In practice, the critical cooling rate (CCR) for K-VF achieves the 'glassy state' throughout the entire sample volume. Unfortunately, the high CCR often limits the volume of material which can be cryopreserved, e.g., oocytes, embryos, sperm, and human embryonic stem cells limited to very small (in the 0.5–10 μL range) sample volumes [57]. Moderation of CCR can be achieved by addition of high CPA concentrations (more than 4 M) but the toxicity profiles need careful attention [57]. Nevertheless, successful protocols are currently being developed, e.g., for pancreatic Islets of Langerhans [58].

Other technical challenges to K-VF include the propensity of liquid nitrogen to vaporize and 'boil' around the plunged sample (known as the Leidenfrost effect), which reduces the effective cooling rate [59] below CCR. Additionally, vitrified samples need to be stored below the effective glass transition temperature range (Tg), which for most biologicals is below approximately 120 °C. During sample recovery and warming, once Tg has been passed, ice nuclei within the sample can rapidly promote injurious ice crystal growth even at intermediate subzero temperatures. Thus, warming must also be rapid, introducing the concept of a critical warming rate (CWR). Sample storage and transport also need to be stable and below Tg to avoid devitrification [57]. As a proof of principle for K-VF, some studies have been reported for CPA-free systems, but so far are only amenable to very small sample volumes of near single cell thickness [60].

Attempts to increase sample volume whilst maintaining low vitrification CPA concentrations have largely focused on achieving higher rates of cooling than can be achieved by direct plunge into LN. Use of intermediate cryogens (e.g., propane or ethane) with large temperature differences (more than 90 °C) between solidification and boiling points prevent the Leidenfrost effect and thus, increase cooling rate [59]. Employing specialist cell supports which allow multiple individually separated cells to be plunge frozen in LN as small volumes (droplets) has been successful in reaching both CCR and CWR. Pancreatic islets supported on a nylon mesh, with excess CPA

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wicked away prior to freezing, increased cooling and warming rates by roughly an order of magnitude [58]. A comparable droplet approach is also supported by successful vitrification of plant genetic resources [61]. Hyper-kinetic VF can be achieved with rapid (jet) delivery of pressurized LN, potentially allowing volumes up to 4000 µL containing 15% glycerol CPA solution to be cooled at a CCR of up to 10,000 °C/s [62]. Comparable approaches to achieve vitrification are routinely employed in electron microscopy to preserve cell and tissue ultrastructure [63], but application to cell therapies will require significant technological development.

Novel approaches to increase the rate of warming are also being actively developed to meet CWR requirements not achievable by simple surface warming. With joule heating–based platform technology, biosystems are rapidly rewarmed by contact with an electrical conductor [64]. Other approaches use radiofrequency-excitable iron oxide nanoparticles in the CPA mix to provide uniform and fast rates of warming throughout large vitrified volumes (up to 80 mL), which reduces thermal mechanical stress and prevents rewarming phase ice crystallization [65].

## (iv) The move to centralized cryopreservation facilities for greater efficiency in the cold chain

In some ways, the cold chain for cell therapies in the 2020s has built upon the expertise which evolved over decades into a robust patient treatment using cryopreserved bone marrow and associated progenitor cells. Highly specialized hematology units provided their own in-house cryo-expertise. Today, as commercialized or regionalized health service groupings grow at pace in cell therapy production, it is inevitable that there will be increasing pressure to move towards a contract development and manufacturing organization scheme as a hub and spoke model for the cryopreservation process. Such schemes are starting to evolve; for example, a centralized production and cryopreservation organization for bone marrow in the organ donor pathway is now functional in the USA [66]. There will be advantages, including a cost reduction at scale, with focused safety and regulatory oversight, an ability to train and accredit specialist translational cryobiologists, and harmonize protocol and data management. The potential disadvantages include a need for improved delivery options for cryopreserved product with high traceability, safety, and training of the receiving institutions who may not have deep cryobiology expertise but who need to store (in some cases for short periods), thaw, and manipulate the product in robust and traceable ways for patient delivery. There may also be advantages at scale for specialist off-site cryo-storage utilization. As is currently happening in many countries, this requires collaborations and information exchange between cell therapy producers and the end-user units, two-way conversations with the relevant regulatory bodies, and a higher-level organizational network. This is very much a work in progress for the coming years.

## (v) Improved reproducibility & traceability in the cold chain

Wider adoption of novel cell therapies will trigger requirements to meet the types of stringent health standards already in place in other treatment pathways—for example, those in hematopoietic stem cell transplants and associated cryopreservation. In the UK, as one example, these activities are licensed by the Human Tissue Authority [HTA], and are accredited and registered by Joint Accreditation Committee ISCT-Europe & EBMT (JACIE) [67]. The agencies will differ in different countries, but will function with a broadly similar remit.

Automation and closed system manipulation in cell therapy pathways are acknowledged as important steps to permit

product consistency, traceability, and regulatory-compliant release [68]. The multiple steps involved in cryopreservation, storage, and recovery have proven a challenge to automation, but these hurdles are gradually being surmounted [69,70], and the importance of these approaches is likely to grow in future. Best practice guidelines for storage and handling of cryopreserved cells are increasingly being updated [71]. Traceability is being enhanced by use of RFID labeling (supplied by commercial companies) e.g., [72, 73] for cryo-vials or product bags. Remote cloud-based monitoring of samples in cryogenic storage and associated artificial intelligence medical enhancements are becoming an expected standard [74,75]; the development of electrically powered non-nitrogen-based cryogenic transport systems [75] will likely enhance this, alongside automated monitoring of dry thawing systems [76–78].

One area of concern in current cryopreservation pathways has been the continued application of protocols developed in some cases more than 20 years ago. Such protocols have enabled successful cryopreservation applications in a number of areas such as stem cell cryo-banking and have been accepted by relevant regulatory bodies as standard. However, whilst promising new technology may appear even after extensive uptake in research laboratories, the path to implementation can be fraught with challenges. In translation to public health practice, there may be a reluctance to consider a new technology. In part, this may simply be because hard-pressed and resource-stretched service staff may not wish to contemplate the time and expense that will be needed to develop the validation including patient data required for regulatory acceptability. This concern is not unfounded; however, some public health systems are beginning to provide support for development and adoption of novel medical technology (e.g., NIHR UK) and some also look to the use of such technologies in the veterinary field, where early adoption may be possible in association with experimental treatments [79].

The cost of implementing new approaches and technologies must also be seriously considered and planned in order to ensure appropriate validation plus careful and efficient 'change control' during the transition to new technology. Where there are historical data that can be utilized, validation can be based on collaborations of multiple labs working in consort. This was exemplified by collaborative multicenter studies such as those run to enable a switch to alternative preservation methods to reduce the DMSO load in therapeutic cellular products [80,81].

## TRANSLATIONAL INSIGHT—WHAT'S COMING SOON

The importance of the cryogenic cold chain for delivery of the relevant functional biomass in cell and tissue therapies will continue to grow in order to control and stop biological time—i.e. time to select cells at their optimal functional status phenotype for the particular therapy, time to avoid wastage when product cannot be immediately used, time to validate and provide batches against release criteria, and time to deliver to patient cohorts at distance and to dovetail with the clinical logistics for treatment. However, improvements in functional recovery are still clearly needed, which will depend on better cryo-technologies. One area for improvement which seems within grasp is the avoidance of injury from ice recrystallization and redistribution during transit upon warming through higher sub-zero temperatures [82] using novel synthetic agents such as ice recrystallization inhibitors, antifreeze proteins, or polyampholytes [83–85]. Some success has also been achieved in vitrification techniques without addition of any CPA, but this has only been achieved using extremely high cooling rates and very small samples [86] with a specific cell type (spermatozoa). Wider application will require further cryo-technological developments.

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Similarly, the ability to control the ice nucleation temperature and avoid supercooling may have a more widespread application in the future [12]. Development of inert crystalline materials as catalysts for ice nucleation have recently been investigated for this purpose [87].

Use of water-modifying CPA which can better stabilize the ice/residual aqueous and glassy states has led to investigation of natural deep eutectic solutes (NADES [88,89]) which may limit ice associated cryo-injury. The combination of these solutes can promote high depression of aqueous melting points compared to those recorded for individual solutes, through co-operative molecular interactions which enhance the 'glassy' transition at higher sub-zero temperatures during cry-cooling. Similar application of a better understanding of fundamental cryobiology is leading to the reappraisal of older concepts such as super-cooling (biopreservation at high subzero temperatures without ice nucleation), high subzero freezing (preservation in the presence of a stabilized ice/aqueous mix), and isochoric freezing (in which the biophysical principle of volume expansion following the water/ice transition can be channeled to increase pressure in a closed system and favor transition to the 'glassy' state [90]). Stable vitrification of larger samples may also be possible by a process known as liquidus tracking, where higher conditions of CPA can be progressively added during the cooling process, thus limiting the exposure of the cells to these higher, toxic CPA levels [91]. However, this is not yet close to routine cell therapy application. All or any of these approaches may make valuable future contributions to the cryo-based cold chain.

In a different direction, cryobiology is already merging with nanotechnology to yield better warming control by modulating heating by oscillating magnetic fields via radio frequency induction [64,92]. Significant further development will be needed to move this into general applications, but the ability to consistently warm products of various sizes with defined monitoring will be a bonus. All of these improvements will require investment in research and development. They will be driven not only by enhanced end-product function but also by the need to demonstrate and deliver better regulatory oversight of cryopreservation, which is likely to grow in demand as more cell therapies reach the clinic.

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## AFFILIATIONS

**Barry Fuller**
Division of Surgery & Interventional Science,
UCL Medical School,
Royal Free Hospital Campus,
London, UK

**Roland Fleck**
Fleck Centre for Ultrastructural Imaging,
King's College London, London, UK

**Glyn Stacey**
International Stem Cell Banking Initiative, Barley, UK
and
National Stem Cell Resource Centre,
Institute of Zoology,
Chinese Academy of Sciences, Beijing, China
and
Innovation Academy for Stem Cell and Regeneration,
Chinese Academy of Sciences, Beijing, China

## AUTHORSHIP & CONFLICT OF INTEREST

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FASTFACTS

# Improving cell viability through controlled freezing

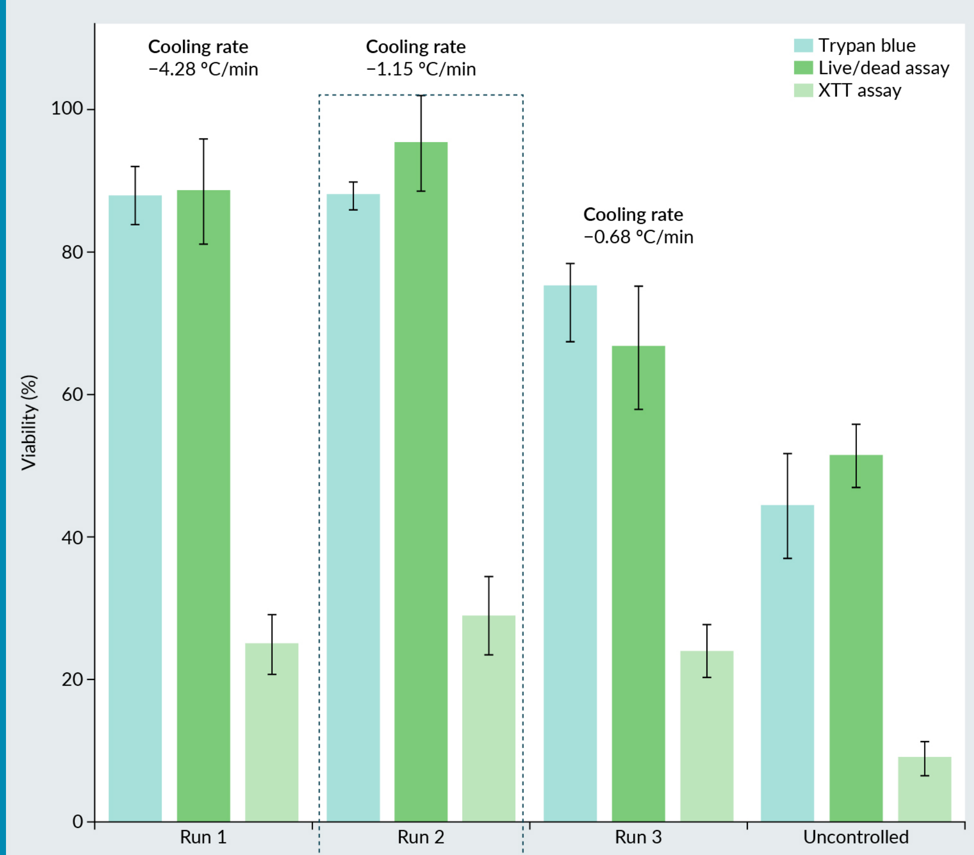
**Brian Moloney**, Director, New Products & Innovation, Single Use Support

**Uncontrolled freezing of cell and gene therapies can significantly reduce cell viability after thawing. This poster presents data demonstrating the importance of innovative solutions for improving cell viability through controlled freezing of mammalian cell lines.**

## OPTIMIZATION OF CELL FREEZING RATE

Defined freezing protocols for specific cell lines and cryopreservation setups can advance cryopreservation techniques for advanced therapy medicinal products (ATMPs) and other biotechnological applications. It is beneficial to determine the optimal cell freezing rate for a particular project, although 1 °C/min is generally accepted to be an average optimal freezing rate for cell-based therapies.

**Figure 1.** Comparison of optimal freezing rate results.




Cell freezing that is too slow can result in cell death, cryoconcentration (separation and non-uniformity of salts, water, or cryoprotectants in the frozen product), or ice crystallization. Cell freezing that is too fast can also lead to cell death, due to osmotic stress and intracellular ice formation.

## CASE STUDY: FINDING AN OPTIMAL FREEZING RATE

To find an optimal freezing rate for a particular project, a study with a cryogenic controlled rate freezer with temperatures down to −150 °C was performed. CHO-K1 cells were frozen in 250 mL bags in a RoSS.KSET protective shell within a controlled-rate liquid nitrogen freezer. Three freezing runs at varying freezing rates were performed in addition to an uncontrolled freezing run.

As shown in **Figure 1**, significantly higher cell viability and recovery were achieved with controlled rate freezing compared to uncontrolled freezing. Cell viability within the uncontrolled freezing sample was around 50%, possibly due to cell lysis. The optimal freezing rate was determined to be −1 °C/min, which resulted in cell viability of >90%.

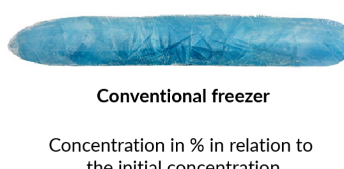
## ACHIEVING FREEZING HOMOGENEITY

Blast freezers have previously been shown to lead to inconsistent freezing results and lower homogeneity within the final cell product. This is because the internal design of the unit impacts its performance, with inherent hot and cold spots within the chamber. To regulate the exposure of liquid nitrogen, the controlled rate liquid nitrogen freezer controls the freezing rate according to recipes.

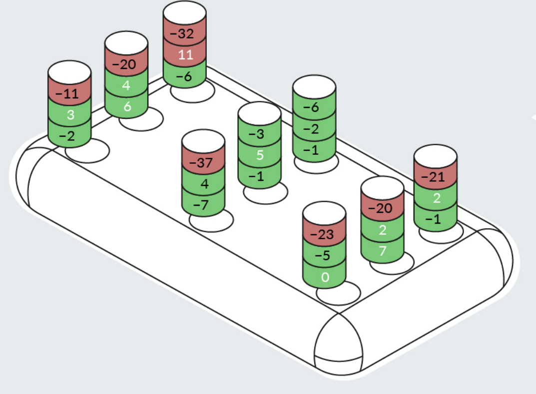
As an alternative to blast freezing, plate freezing uses direct surface contact and control to enhance cell survival and homogeneity of freezing. Ice front growth from the bottom and top prevents the effect of cryoconcentration and direct cold exposure speeds up freezing, with no air between the plate and the bag.

**Figure 2.** Comparison of conventional static freezer versus advanced plate freezer.

10 L single-use bag in the static freezer




10 L single-use bag in the plate freezer



A comparison analysis of blast freezing and plate freezing was performed. Results are shown in **Figure 2**, in which the dark blue spots indicate a higher cryoconcentration seen in the product frozen within the conventional blast freezer. This shows that fast and controlled freezing with a plate freezer enables a homogenous freezing result with less cryoconcentration.

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In partnership with:

# CRYOPRESERVATION: Best Practices for Cell-Based Products

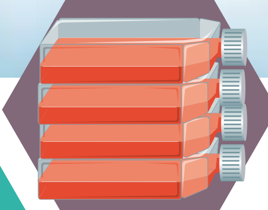
**Cell-based products originate from biological starting material, such as cells from tissue biopsies, blood, and bone marrow. These cells can be developed into clinical products *ex vivo*. However, they require specialized processes to remain viable and functional throughout manufacturing, storage, and transport.**

**Optimizing cryopreservation processes is essential for maximizing product efficacy and process efficiencies. Suboptimal cryopreservation can lower the potency of the final product, as well as greatly increase batch-to-batch variability.**

## 1 Sample isolation & cell processing

Retrieve donor sample at clinical site, package material, and ship to manufacturer with scheduled processing time and set development parameters.

Add a cryoprotectant like HypoThermosol® FRS or CryoStor® CS10 directly to the apheresis material. This may extend sample shelf life and allow for the time it takes the sample to reach the manufacturing facility [1].

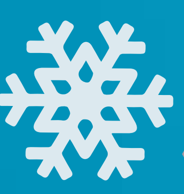


Performance is different for a cryopresered apheresis pack versus fresh. Compare the risks of fresh vs frozen, including post-thaw performance and resource allocation for shipping and manufacturing. How much flex do you have in your schedule, and what does the freeze/thaw process look like at the apheresis centers?

## 2 Formulation

Perform purification and concentration steps to leave only what will remain in the final delivered drug product. The reduced cell culture is suspended or formulated in cryopreservation media.

Closed-system formulation can reduce contamination risks. Choose a system that provides process flexibility and can grow according to your requirements.



Select a commercially-ready, proven, high-quality media solution, early in development for success at scale. CryoStor® CS10 is the gold standard for cryopreservation. It is supported by extensive scientific evidence, ensures a sustainable supply chain, and will ensure that your production complies with cGMP standards. Good selections made early empower scaled success.

## 3 Fill

Aliquot formulated product and fill primary containers. This can be done with different commercially-available fluid transfer devices; either automated or manually.

Cell viability clock starts once cryopreservation media is added. Consider how an automated, or semi-automated filling system may facilitate this step compared with a manual approach. Qualify and validate this process against each cell type because holding time will vary.

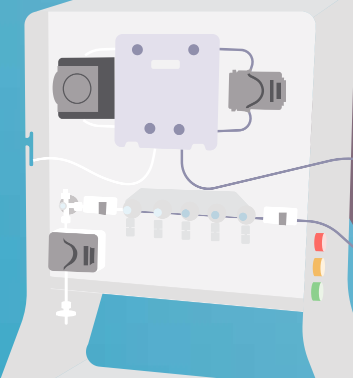
The fill steps demand speed, accuracy and precision. Maintaining solution agitation throughout this step will help ensure homogeneity and avoid sinking cells or coagulation.

When selecting primary containers, consider that your selection meets performance, quality and regulatory requirements. Review for container enclosure integrity, extractables and leachables profile, protection against particulates, and system flexibility for scaling up downstream processes.

## 4 Freezing the sample

Utilize a controlled-rate freeze to apply a 'seeding' or ice-nucleation step to the sample. This should reduce the risk of a supercooling event and improve sample consistency and process reproducibility.

When qualifying the freeze protocol, define the operational space. Understand the smallest and largest payload volume and how it relates to the sample temperature in a certain freeze profile. Vials, bags, cassettes, solution volumes, or even differing container materials will change the freezing profile.



Signata CT-5™ is a flexible, automated and closed fluid-management system capable of formulating and filling drug product. It works with vials, bags or bottles without limiting the number of containers filled in one batch. It incorporates passive cooling and controlled agitation to support product consistency and process efficiency.

Consider how to limit the difference between QC sample containers, retention samples, and final product. Varying materials may change the freeze profile.

Optimize and qualify a cooling rate according to cell type. Use −1°C per minute as a starting point only.

Temperature °C (0, -196) vs Time: 2 — Cryopreservation solution choice; Cryoprotectant concentration; Rate of cryoprotectant addition; Temperature of cryoprotectant addition. 4 — Temperature of ice nucleation; Cooling rate. 5 — Storage temperature. 7 — Cryoprotectant removal, duilution and/or inclusion in cell product; Warming rate.

## 5 Storing the sample

Storage (temperature/humidity) criteria should be defined and qualified to maintain sample integrity and viability throughout the duration of storage and transport.

Partner with a biostorage services company like BioLife Solutions, to meet growing and changing sample storage needs with added cold chain logistic planning and local sample pickup and delivery, free of charge.

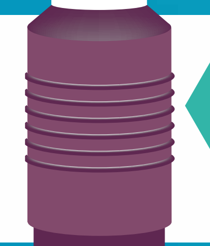
Clinical site storage capabilities vary. Determine distribution plan early and revisit often. Partnering with a long-term biostorage partner may ease clinical site storage anomolies and provide product 'just-in-time' for administration. If relying on clinical site storage, understand how individual site SOPs may alter the product.

If the master cell bank location is prone to natural disasters, (earthquakes, hurricanes, tornadoes, etc.) create a mirror bank with a cGMP biostorage partner in another, less active spot that allows regular sample access without miscellaneous fees.

-196°C -20°C

## 6 Shipping the sample

Secure transport of biologic material demands shipping solutions with uncompromised thermal integrity and real-time payload visibility.

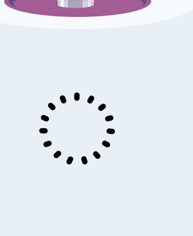


A shipper such as the evo® DV10 provides up to 15 days of cryogenic protection, with monitored visibility, while limiting handling mistakes.

The manufacturing, distribution, and warehousing strategy needs to be developed early and revisited often. When establishing the plan, consider these questions: How far will the product be shipped from collection to administration? Will a hub-spoke storage and distribution model meet geographical targets? Who is the courier partner to arrange regular cold chain movements? How long will a shipper protect the specific sample type?

## 7 Thawing the sample

A thawing program must follow a consistent warming algorithm across the sample and be reproducible at any clinical site.



Once the sample has left the manufacturing site, the manufacturer has lost control of the product. Ensure each individual clinic has qualified the appropriate thaw equipment and are amenable to adapting their protocol to meet your sample requirements.

Selecting an automated, mechanical thawing system over a water bath, bead bath, or thermal block limits site variability.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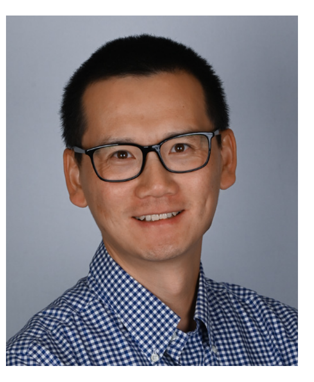
**CELL & GENE THERAPY** INSIGHTS

CRYOPRESERVATION AND COLD CHAIN

INTERVIEW

# Developing a robust ultra-cold chain for cell therapy



**Abi Pinchbeck,** Assistant Editor, *Cell & Gene Therapy Insights*, speaks to **Lindong Weng,** Principal Scientist, Formulation, Novo Nordisk. They discuss the guiding principles for success in cell therapy cryopreservation and the pressing need for automated and standardized ultra-low temperature cold chains in the space.

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## What are you working on right now?

**LW:** I am a Principal Scientist in formulation at Novo Nordisk. I currently lead a team consisting of scientists and engineers to develop formulations that can enable the storage, transportation, and clinical delivery of cellular therapy products.

## Can you outline the current best practices and guiding principles for success in cell therapy cryopreservation?

**LW:** The cryopreservation-based drug product process for cell therapy begins with the identification of a suitable formulation that can protect cells from freezing-related damages

without posing significant toxicity to the cells themselves. There are several commercially available and clinically used DMSO-based cryopreservation media. It is important to develop a process that can introduce the formulation solution to the cells and meanwhile, minimize the physical stress to them.

The formulated cell suspension will be distributed into a proper container closure system during the filling process that yields a drug product (DP) batch for freezing. Controlled-rate freezing is currently the mainstay for cell therapy cryopreservation. There are several things that we should pay attention to here because the majority of damage can occur during this step. Freezing the batch uniformly is critical, especially for allogenic products that are off-the-shelf and have relatively large batches. Controlling ice nucleation is important, but the current approaches are still not 100% deterministic.

After cryogenic storage and transportation, an optimized and uniform thawing process is also important. The optimal rewarming rate can be affected by the specific freezing process history and the cell nature. It is generally accepted that the faster, the better, though this may not always be true. A dose can be prepared with or without washing or media exchange at the clinical site. It in part depends on the toxicity profile of the cryopreservation formulation used.

One of the guiding principles I follow is to look at the cryopreservation process holistically, meaning recognizing the interdependency between the steps. We usually evaluate the cryopreservation outcome based on the post-thaw samples, which is the very end of the process. This can make the troubleshooting a bit complicated.

## Q What recent advances in technologies in the cryopreservation space stand out to you?

**LW:** First of all, the cryopreservation research space is a dynamic area. I am excited about technological advancements beyond slow freezing-based cryopreservation. These new advancements offer potential applications in the field of cell therapy. For example, the cryomesh system was developed by a group at the University of Minnesota for the vitrification of more complex biological systems than isolated cell suspensions. This is a combination of creatively designed and systematically optimized techniques that eventually enable the successful vitrification and recovery of pancreatic islets.

Vitrification is an ice-free cryopreservation method that was originally demonstrated in the mid-1980s. However, it has been mostly used with a small sample size of tens or hundreds of microliters and a high and potentially toxic concentration of cryoprotectants. The cryomesh system suggests new ways to overcome these limitations. There are also new cryopreservation media that are free of DMSO. There remains debate about the toxicity and benefits of DMSO, but DMSO-free formulations have the potential to simplify, for example, the dose preparation step if they are demonstrated to be safer for the cell product and the patient.

## In your view, what should be the next critical steps in cold chain R&D and technology development? Where do the innovation gaps remain in the space?

**LW:** The cold chain topic was relatively a sleepy corner within the healthcare industry until recently. The COVID-19 pandemic taught us a lot of things, and one of these learnings was the importance of the cold chain for extending the shelf life of mRNA-based vaccines. However, in that case, we are only talking about −70 °C. For cell therapy, we are looking at −196 °C (the boiling temperature of liquid nitrogen). This highlights the unique challenge of developing a robust ultra-low temperature cold chain for cell therapy.

Automation and integration can be the two critical areas in advancing this ultra-low temperature cold chain development. The biggest concern is temperature deviation as it affects drug product stability. Automation can improve standardization and reduce human manipulation or intervention, which can help minimize temperature excursions. It is also critical to provide an end-to-end, integrated solution for cold chain development. For example, how can the frozen DP batch be transferred quickly and safely from a controlled-rate freezer into a liquid nitrogen storage system? How can the frozen DP units be handled at the clinical sites more effortlessly? These areas are still the gaps remaining in the workflow, though a couple of specialized companies are offering solutions to address them. The availability of ultra-low temperature cold chains can greatly impact patient access to life-saving cell therapy products.

## How can standardized cryopreservation approaches be applied in the cell therapy space to reduce human error and improve patient outcomes?

**LW:** Like any other processes in the industry, we need to develop and implement standard operating procedures, providing clear and specific instructions for each step of the process. Proper training and documentation are equally important. Automation is one of the technologies to leverage to promote standardization but that being said, we also need to apply sensitive QC testing to capture any deviations when they occur. In this regard, reliable analytical assays are paramount for cell therapy QC.

We may have to tailor the entire cryopreservation process for each specific cell product under development. We all must acknowledge, at least at this stage, the importance of finding a balance between standardization and individualization.

## How can we work towards bridging the cold chain knowledge gap between cryobiologists, therapy developers, and clinicians?

**LW:** In an ideal world, all the key stakeholders would work together seamlessly, and the technologies would translate effectively from the lab benches to the clinic. To reach this goal, cell and gene therapy developers must acknowledge the scientific rigor and technical complexity within cryopreservation and the maintenance of cryopreserved products. It is far more complex than putting cells in a 10% DMSO solution and freezing them in a Mr. Frosty

device. For cryobiologists working in research labs, it is critical to understand and materialize the scalability and robustness of the technology under development.

Manufacturing and supplying drugs raise the bar, not only for drug product quality but also for patient safety. We must learn from clinicians about operational feasibility and the user experience. Their feedback on receiving, preparing, and administering the drug product is critical for us to develop cryopreserved products and processes that can help maximize benefits for patients.

**Q** What will the temperature-controlled vein-to-vein cell therapy supply chain look like in a decade?

**LW:** I look forward to an ultra-cold supply chain for cell therapy that is more automated, more integrated, more operator-friendly, and more accessible. Cryopreservation is not the only approach to extend the stability of biological materials, at least in the research space. Other non-cryogenic supply chain approaches such as hypothermic storage, and even freeze-drying should also be explored. These may help us bring innovative cellular medicines to more patients faster.

How do you see the space evolving in the next 12 to 24 months? What are your own goals over this time?

**LW:** In general, there is an increasing recognition of the importance of cryopreservation in the field. Many cell and gene therapy companies are investing heavily in this area because cryopreservation affects the commercial value, quality, as well as safety of these products.

My goal is to leverage the interdisciplinary expertise of the team to develop the formulations and the related systems and processes to extend the shelf life of cell therapy products. We want to fill the gap in time and space related to the logistics of cell therapy products.

## BIOGRAPHY

**LINDONG WENG** currently serves as the Principal Scientist in Clinical Formulation at Novo Nordisk, contributing his expertise to the field of cell and gene therapy. Previously, he held the role of Director of Drug Product Process Development at Sana Biotechnology. Weng holds a PhD in Mechanical Engineering from Dalian University of Technology and an MBA from the University of Illinois Urbana-Champaign. His academic journey includes impactful postdoctoral research at UNC Charlotte and Massachusetts General Hospital and Harvard Medical School. With over 15 years of experience spanning formulation and cryopreservation, microfluidic device engineering, biomaterials, and mathematical modeling and molecular simulation, his work has been navigating the intersection of biology, physical chemistry, materials, and thermodynamics. In both academic and industrial settings, Weng has contributed to the scientific community with over 40 peer-reviewed journal articles, ten international patent applications, and presentations at over a dozen international conferences and symposiums.

AFFILIATION

**Lindong Weng PhD**
Principal Scientist,
Formulation,
Novo Nordisk

AUTHORSHIP & CONFLICT OF INTEREST

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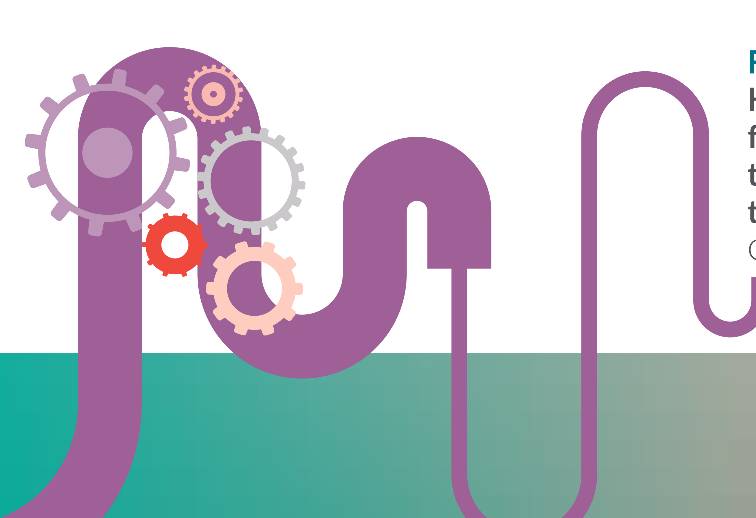

# Empowering the tissue engineered product field

**David McCall**, Senior Editor, *Cell & Gene Therapy Insights*, speaks to the Alliance for Regenerative Medicine's **Michael Lehmicke**, Senior Vice President, Science and Industry Affairs, and **Natalie Fekete**, Manager, Science and Industry Affairs, about the aims and outcomes of the Alliance for Regenerative Medicine's recent Tissue Engineering and Therapeutics Workshop. In light of recently renewed interest in the space, could tissue engineered products be on the verge of fulfilling their long-held potential?

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## What are you working on right now?

**ML:** I am the Senior Vice President of Science and Industry Affairs at the Alliance for Regenerative Medicine (ARM). We currently have over 400 corporate members who are primarily developers of cell and gene therapies and tissue-engineered products (TEPs). Alongside my team, including Natalie, we work on their behalf to advocate for an appropriate regulatory environment in the US and Europe, as well as globally, to drive towards getting these therapies to patients who desperately need them.

How would you sum up the current state of play in the tissue engineered product space, particularly from the industry perspective?

**ML:** Out of the several areas that we at ARM are focused on, TEP is the smallest. This has been the case since ARM's inception—TEPs have not attracted as much funding as some of the other areas in cell and gene therapy. However, it is key to note that in general, the entire field is currently being affected by a downturn in funding, which is a cyclical thing and not specific to this industry.

In our Tissue Engineering and Therapeutics Workshop, held in September 2023, the idea that the TEP field is changing and evolving was highlighted. One significant change can be seen by looking at the approved products to date globally—with a few notable exceptions (including Rethymic, which was fairly recent) all previous approvals of TEPs were for wound care and burns. However, today, very few are pursuing those indications anymore.

At the workshop, we heard about work on a range of other indications, including complex diseases—liver disease, organ transplantation, retinal applications such as age-related macular degeneration, and metabolic diseases like diabetes. It is often argued that TEPs have a unique opportunity to affect a positive impact on some of the more complex diseases in larger patient populations—this is now being pursued in earnest.

What were the drivers behind the Tissue Engineering and Therapeutics Workshop? What did it set out to achieve, specifically?

**ML:** We had not previously held a workshop that was solely focused on TEPs. In part, we chose to do so because of a request from our members for more attention on the area. Additionally, we are now seeing a turning point in the industry and the development of some of these products being reached. In larger and more complex indications, companies are now getting to the IND-enabling studies stage, and some are actually filing INDs for TEPs. There has been a relatively small number of these products in clinical trials consistently over the last five years, and we see the potential for this to change quite dramatically over the next five years. We thought it was a good time to talk about the potential for these products, and hopefully bring more information on what these products are capable of to the attention to the investment community.

What were some of the key points of discussion and takeaways from the workshop, firstly relating to development progress and challenges in specific indication/therapeutic areas?

**ML:** One general challenge that many companies that are pursuing TEPs are struggling with is the complexity of dealing with products with applications in various indications. For example, when a product works to repair function or restore tissue at the tissue or organ level, such as a bone replacement product like EpiBone, or Humacyte's vascular replacement product, it can have applications in multiple different disease states. That can complicate the

> "It is often argued that TEPs have a unique opportunity to affect a positive impact on some of the more complex diseases in larger patient populations—this is now being pursued in earnest."

development process from a regulatory standpoint, because it is difficult under current regulatory paradigms to pursue an approved indication in more than one disease using the same study.

## ...and in terms of product development and CMC considerations?

**ML:** Many of the CMC, scaling, and manufacturing issues are similar to those in gene therapy, and certainly in cell therapy. In my opinion, all TEPs are, by definition, cell therapy products, but with additional complexity from the scaffold and the interaction with that scaffold.

It is difficult, if not impossible, to find CDMOs in this area, which does distinguish it from cell and gene therapies in general. Many cell and gene therapy companies rely on CDMOs for manufacturing at various points in development, depending on their business plan. This is not an option for tissue engineering companies, whose processes are largely bespoke and developed in-house. This brings with it many unique CMC challenges. Developing the experience in-house often means that the development and manufacturing of these products is slower relative to other biological products, including cell and gene therapies.

**NF:** One of the complexities surrounds the CMC specifically, because both the cell and the scaffold need to be considered when performing studies. Typically, the US FDA asks for the combination effect of both the cells and the scaffold product together. We are happy to see that there has been a reorganization within the Office of Therapeutic Products that captures the TEP part of the sector, and that there are dedicated agency staff now looking at TEPs specifically.

There is some regulatory guidance available that pertains to TEPs specifically, but there is a need for clarification in some instances on how far that guidance can be applied to the products at hand. These products are multifaceted and complex, so it can be difficult to give a one-size-fits-all answer.

**ML:** With TEPs, the regulatory pathway depends on the nature of the product, how it is used, and how it is applied at the point of care. Questions of whether there is a surgical procedure or device involved must be answered. Is the product effectively a combination of a device and a biologic? Today, it is not always the case from a regulatory standpoint that TEPs are combination products. Most tissue engineering developers have to struggle with that question early on in development when they are determining how the product will be used.

> "Each patient's eyes or cardiovascular heart tissue will be different from the next, making TEPs personal, bespoke applications."

## What were the key challenges and takeaways regarding manufacturing and scalability?

**ML:** The additional challenge here is that many of the processes are bespoke and still highly manual. Some developers at the workshop discussed platform processes that can facilitate scaling and automation—for example, 3D printing. The field is moving towards developing process modalities and platforms that can be automated to the appropriate extent, which should then facilitate scaling. However, not much of this has been put into practice yet. In part, the problem is the funding situation because automation needs a certain amount of upfront capital. Thus, many people are still in the early days process-wise, and many of the processes are still custom and manual.

**NF:** Many of the indications, like cardiovascular bone repair and eye tissue repair, are intended for large patient populations. These are large, prevalent indications to be treated, but one size does not fit all. Each patient's eyes or cardiovascular heart tissue will be different from the next, making TEPs personal, bespoke applications. The scaling question is interesting because a balance between treating a large patient population and ensuring the TEP is bespoke and custom enough to be fit for purpose must be found. Then, there is the question of how much you can automate some of these applications, because of the breadth and diversity of the products that we see in this field.

## What about regulatory pathway development?

**ML:** It is critical to understand what your end indication is and what a reasonable strategy for pursuing that in the clinic looks like. It is also true that for tissue engineering specifically, there is no definitive clinical guidance. Much of the other guidance does apply, but for instance, there have been some interesting examples in terms of clinical and regulatory development where, as a de-risking strategy, some developers have looked at the scaffold first.

The first question developers must answer here is: does the scaffold have some clinical utility by itself? If developers can demonstrate that it does and that it can be used as a product or a device in another way, they can not only demonstrate that it is safe and effective, but they have an additional source of revenue.

The next step is to figure out how to combine cells with the scaffold to achieve the ultimate end goal. A good example of this is the approach that Miromatrix has taken for liver replacement, which is a stepwise approach to mitigate risk from a regulatory standpoint, a clinical standpoint, and a business standpoint.

**NF:** This captures the stepwise increase of complexity and risk of the process that the FDA does appreciate. It is a learning process on both sides; both the developers and the regulatory agencies are learning with and from each other, and from the experience now being gathered.

Lastly, what were the outcomes of the workshop relating to the current funding and business environment in the tissue engineering space?

**ML:** We received some good feedback from investors in a panel discussion, which will be detailed in a white paper that we are soon to release. The key for the business environment is in a way similar to the key in working with investors in cell and gene therapy; building a relationship is critical. Finding an investor who understands and believes in what the product can do can be even more challenging than the tissue engineering itself.

There are also different types of investment: dilutive and non-dilutive funding. We hear more about non-dilutive funding in the tissue engineering space. Numerous companies at the workshop have had very successful Series A rounds, and some have identified a successful path to increasing their valuation, allowing them to move on to Series B and Series C, and in some cases, to being acquired. For example, Miromatrix, one of the companies at the workshop, was recently acquired by United Therapeutics.

Although the magnitude of the financing is not huge compared to some other areas in biopharma, we are seeing some notable success stories. As we come out of the cyclical downturn in funding affecting the entire field, and as some TEPs start to get INDs approved and move into the clinic, we will see a lot more interest from the investment community. Having actual clinical data will help here.

**NF:** Part of the workshop discussion was regarding the state-driven mechanisms for funding. Both the State of Maryland and the National Institute of Health federal funding were present. They can provide funding until or even beyond the clinical stage. Clinical data is the key to success for many companies and reaching this stage means that you can take advantage of some of the available funding. It may be surprising to some biotech companies that there is this mechanism available.

As we have discussed, clinical development and commercialization of TEPs and associated technologies has lagged behind other areas of the advanced therapies space over the past couple of decades. Looking to the future, what will be some strategic keys to reversing this trend?

**ML:** We hope that as we and others support the growth in knowledge about the potential of these therapies, the further investment needed will be established. Additional investment is one of the things that drove cell and gene therapies forward, and this has not yet happened for TEPs. That investment could come from state funding, non-dilutive funding, venture capitalists who develop a greater interest in the field, or through partnerships and company acquisitions as in the case of United Therapeutics and Miromatrix.

While the FDA is taking some good strides forward by reorganizing the Office of Therapeutic Products, which now has specific resources and a focus on tissue engineering, there is additional work that needs to be done in terms of the gaps in regulation. This was—and still is, to an extent—true with cell and gene therapies as well, but they have come a lot further in the

past couple of years in terms of regulatory clarity. As we start to see clinical data and clinical success, that will ultimately drive the field forward.

When we think about organ replacement, for example, there is no other good solution. The status quo today has many patients on waiting lists and in some cases dying when they cannot receive a transplant, but there is no magic cell or gene therapy solution for some of these problems. If we can see clinical success with TEPs in areas like organ transplantation, that will drive this part of the field.

**NF:** Education is key, and regulatory pathways need to be clear. Approved products pave the way for other products to follow. That is much needed in this field in particular.

## What will be the next steps for ARM in this area?

**NF:** The initial next step is to publish the summary white paper of the Tissue Engineering and Therapeutics Workshop, in February 2024. One of the takeaways from the workshop was that this field is interested in defining itself more to understand what 'tissue therapeutics' means, and what exactly is inside and outside of the scope. One of our activities moving forward will be to capture that definition more precisely.

### BIOGRAPHIES

**MICHAEL LEHMICKE** joined ARM in 2018 as its first Director of Science and Industry Affairs. Michael has over 20 years of R&D experience in biomaterials, medical devices, and regenerative medicine. He has led product development teams for class II devices, human cell and tissue-based products, and drug/device combination products. He is a creator and an inventor with multiple US patents to his name. Michael has a MSc in Biomedical Engineering, with a focus on tissue engineering, from Drexel University. Michaels areas of expertise include cell-based tissue engineering, bioceramics, biodegradable polymers, project management, strategic pipeline development, and business development. He is passionate about regenerative medicine and believes that it represents our best hope for meeting many unmet clinical needs, thereby changing patient's lives for the better.

**NATALIE FEKETE** joined ARM in 2022 as a Manager for Science and Industry Affairs. She brings over 10 years of experience in regenerative medicine, having worked both in academia and the pri-vate sector. At ARM, Natalie is building bridges among developers, manufacturers, and regulators. An immunologist by training, Natalie is experienced with GMP-compliant cell therapy manu-facturing. She holds a PhD from the University of Ulm, and conducted a postdoctoral fellow-ship at the University of Enschede (the Netherlands), where she researched the impact of surface topographies on cell fate. She then continued her research as a postdoctoral fellow at McGill University (Canada) and developed assays to characterize the interaction of human cells with materials typically used in tissue culture for regenerative applications. Before joining ARM, Natalie worked at Saint-Gobain, where she established the Life Sciences Laboratory dedicated to the company's Cell and Gene Therapy product portfolio and managed R&D activities focused on improving bioprocessing workflows and cell performance. Born in Germany, Natalie has lived in the Netherlands and Canada, and is currently based in the Boston area.

## AFFILIATIONS

**Michael Lehmicke**
Director,
Science and Industry Affairs,
Alliance for Regenerative Medicine

**Natalie Fekete**
Manager,
Science and Industry Affairs,
Alliance for Regenerative Medicine



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CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

# De-risking cell therapy product development: strategies for commercial success

Alex Sargent and Chad Anderson

As the field of cell and gene-modified cell therapies continues to rapidly advance, manufacturing processes and analytics must be de-risked in development before entering the clinic to mitigate technology transfer and clinical development setbacks. Thus, a scalable and robust framework for new products is critical to repeatably and reliably guide cross-functional teams through the product lifecycle. In this article, experts from Charles River Laboratories discuss FLEX Platforms in addition to the new product introduction framework and how this approach is applied to streamline the complex journey to the clinic and beyond. Central to the project inception of new products is the implementation of effective commercial readiness risk management strategies from the early stages of development. By identifying potential risks early and proactively addressing them, a more efficient progression through the entire process can be ensured.

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## PROCESS DEVELOPMENT: CLINICAL TO COMMERCIAL

Process development in cell therapy is ideally rapid, robust, and results-driven. Developers want to streamline process development and eliminate any hurdles to move as fast as possible, whilst also ensuring a robust and reproducible manufacturing workflow. A good process is replicable from lab-to-lab and site-to-site. The field is also evolving beyond the adage "the process is the product", and instead is moving towards an environment in which the desired product now shapes the process. Starting with the desired cell type or therapy and then working backward is the results-driven approach more commonly taken today.

To shorten timelines in process development, it is key to not attempt to reinvent the wheel. Instead, it is possible to pick the existing 'wheel' that works best for a particular

product. There are many existing technologies and platforms designed with cell therapy manufacturing in mind to automate and close the process and make it reproducible, efficient, and seamless. One can systematically evaluate available cell therapy platforms to establish the best technology—or wheel—for a process.

By utilizing a platform-based approach to process development, a number of benefits are seen, particularly around cost saving. By using an automated platform, reductions in labor, space, and overall COGs are seen. It also minimizes risk and improves process robustness with closed processing and reduced subjectivity and variability. Process

## FIGURE 1

**The use of automated platforms for cell therapy development can reduce overall costs.**

Manual (baseline) | Partially automated | Fully automated

USD: Capital, Materials, Consumables, Labor, Other

Manual (baseline): Capital 18%, Materials 25%, Consumables 1%, Labor 50%, Other 6%

Partially automated: Capital 47%, Materials 18%, Consumables 3%, Labor 18%, Other 14%

Fully automated: Capital 41%, Materials 16%, Consumables 5%, Labor 26%, Other 12%

| Parameters | Manual (baseline) | Partially automated | Fully automated |
|---|---|---|---|
| Total installed capital (million USD) | 1.39 | 10.6 | 6.31 |
| Total yield (%) | 28 | 38 | 38 |
| Batch failure rate (%) | 10 | 3 | 3 |
| Cost per million cells (USD) | 94 | 68 | 77 |
| Throughput (batches/year) | 26 | 84 | 50 |

Lopes AG *et al.* *Bioprocess International* (2018)

Cost: Depreciation, Operating resources, Personnel, Investment

Manual: Depreciation 501,717 € (7.3%); Operating resources 1,846,838 € (26.87%); Personnel 4,023,506 € (58.53%); Investment 501,717 € (7.30%)

Automated: Depreciation 1,005,000 € (21.68%); Operating resources 1,478,703 € (35.09%); Personnel 632,527 € (15.54%); Investment 1,005,000 € (24.69%)

Nießing B *et al* *Processes* (2021)

control and analytical technologies enable process decisions in real-time.

Figure 1 compares two publications automating different cell therapy processes. These examples show that partially or fully automating a process can yield significant savings in the overall costs. This does however vary based on the type of product such as whether the therapy is allogeneic versus autologous. Generally, some degree of automation in a process will significantly lower costs, especially when scaling up.

Automating processes and moving to more closed platforms and systems helps to improve process robustness. This removes the overall subjectivity and variability seen when humans perform operations.

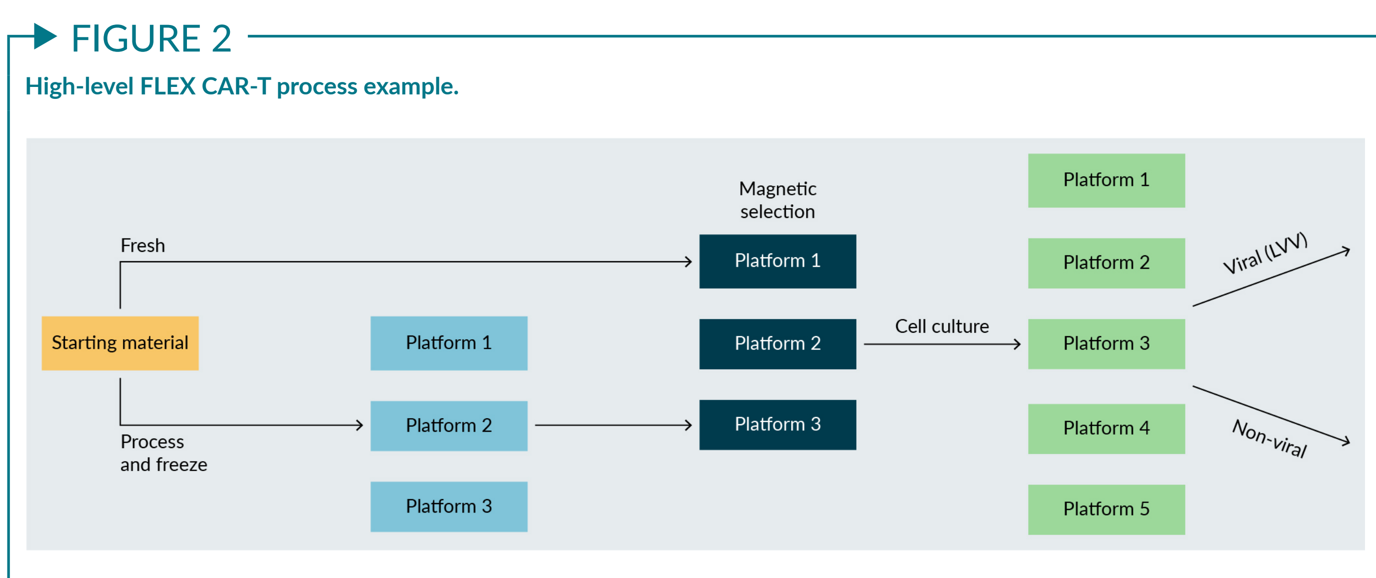
Another advantage of these automated platforms and technologies is that they often include some degree of inline analytical capabilities and continuous process monitoring of factors such as temperature, dissolved oxygen, pH, and inline metabolic sensors. This helps to ensure a process that is robust and reproducible from batch to batch. Inline analytics also help users to make process decisions in real-time. Biological variability, such as from differing donors, can be measured via inline analytics, allowing a process to respond to changes and helping to overcome inherent biological variability.

## LEVERAGING TECHNOLOGIES FROM A CDMO

At Charles River, FLEX Platforms are being employed to help significantly speed up process development timelines. These fully automated and closed cell processing platforms are flexible depending on the product and quality attributes in a plug-and-play fashion. The platforms involve a series of developed and defined unit operations designed to meet process targets that can be interchanged depending on the product. These include ready-to-use platforms and protocols from cell selection, expansion, electroporation, wash/concentration, and fill/finish. FLEX Platforms have been validated with a variety of cell types, including T cells, natural killer cells, mesenchymal stem cells, and induced pluripotent stem cells. An example of how a high-level FLEX CAR-T process could work is given in Figure 2. There are several platforms available at each stage to fit individual needs. Since the number of platforms is limited, standard operating procedures can be built for each platform to enable the selection of the ideal candidate. New technologies are continuously being developed and implemented to expand options. With this FLEX Platform offering, the process development timeline can be shortened from months to weeks.

## FIGURE 2

**High-level FLEX CAR-T process example.**



## ESTABLISHING EFFICIENT ANALYTICAL DEVELOPMENT

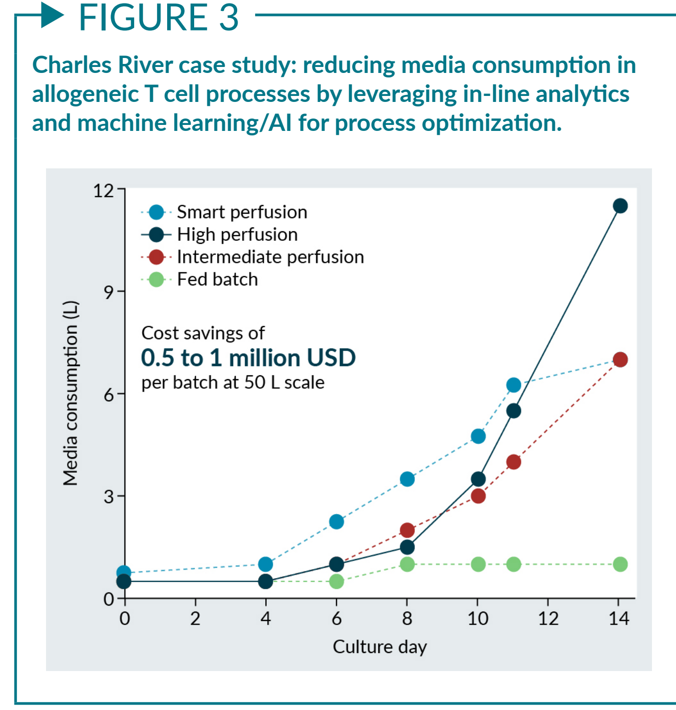
Efficient and effective analytical development can be achieved via a stage gate approach. First, the definition and availability of product and test materials must be established, before identifying the suitable controls for each analytical method to be used. Then, the transferability of the methods must be considered, before establishing the analytical method development timeline.

Challenges within the definition of product material can stem from the in-process or final product material critical parameters not yet being identified or defined as well as a lack of material availability. The solution is to leverage the material being produced early on, such as that used in process development runs, and rigorously test that material to ensure the methods are robust. The use of characterized cell material produced using standardized protocols can also significantly help in methods development.

Another challenge lies within the transferability of the method. The method needs to be robust and reproducible to persist in the QC environment, with an understandable and phase-appropriate process. A common stumbling block in transferability is potency and functionality assays, which must be easy to perform and reproducible at large scales. To overcome these roadblocks, using DoE can ensure assay parameters are robust. It is also necessary to build in appropriate controls, whilst keeping the method simple, clear, and repeatable. To ensure assays are ready to leave development, a fit-for-purpose test should be established and performed.

### FIGURE 3

**Charles River case study: reducing media consumption in allogeneic T cell processes by leveraging in-line analytics and machine learning/AI for process optimization.**




As a part of analytics, it is important to have in-line capabilities, especially when utilizing automated platforms. In-line monitoring of pH, dissolved oxygen, cellular metabolites, and cell health/viability can be essential to maintain critical quality attributes and compensate for donor and process variability.

Leveraging machine learning and AI for process optimization can help to make the most of the data acquired from in-line analytics. Figure 3 shows the results from a case study in which media consumption in allogeneic T cell processes was reduced by using in-line analytics. Data for metabolite levels, glucose consumption, and lactate accumulation were put into a novel AI/machine-learning program to develop smart perfusion, through which media consumption in the process was significantly lowered whilst maintaining high T cell densities in the bioreactor. This translates to savings of US$500,000–1 million for each 50 L scale batch.

## CELL THERAPY NEW PRODUCT INTRODUCTION

A robust new product introduction (NPI) process is needed to ensure that processes are developed and transferred into the clinical manufacturing space in a standardized and streamlined way. De-risking the manufacturing process as early as possible, essentially in process development labs, gives the greatest opportunity for manufacturing success. Early involvement from all stakeholders ensures that roadblocks can be avoided or mitigated. It is also critical to have an NPI

roadmap established for all partners. Cell and gene therapies are life-saving, so it is important to have standardized and robust processes in place for the therapies to reach the clinic as soon as possible.

A scalable and robust NPI process de-risks cell therapy programs from day one, from project inception through process development and GMP, helping developers navigate the complex path to clinic and beyond. For a successful NPI process, the program needs to be clear, unambiguous, and understandable. Roles and responsibilities must be defined early and constantly reevaluated. A templated, repeatable, well-communicated process will ensure alignment between multiple team members. The Charles River NPI program is robust in that it describes the roadmap for transfers, whilst also maintaining flexibility to allow for the nuances of each individual program or process. Speed to clinic is also paramount due to the life-saving nature of these therapies, and the NPI program was designed with this in mind. The framework of the NPI allows developers to be nimble and forward-looking to de-risk many of the steps, ultimately leading to quicker transfers.

The NPI program is broken down into three phases—capture, planning, and execution—as a program progresses from project inception to project closeout. Each of these phases is equally important to the overall success of a program. First, the capture phase oversees the contract signing and proposal development process. The NPI form is completed by incoming clients and the business development team during this stage. The tech transfer team identifies any risks before the kick-off meetings occur to expedite and de-risk processes moving forward. The second phase is the planning phase, which encompasses all activities for early planning for long lead items and scope of work finalization. A solid NPI project plan contains realistic timelines and deliverables to be shared with all stakeholders. The final phase is the execution

FIGURE 4

Project plan to de-risk onboarding and tech transfer to GMP.

phase, which is inclusive of all actionable development, tech transfer, and manufacturing activities following project kick-off.

Within NPI, every functional department has its own role and responsibilities. A standardized project plan to monitor actions, such as the one shown in Figure 4, is critical to the success of the program. Many of the actions do not need to be completed sequentially. To go from Phase 1 to clinical manufacturing as quickly as possible, numerous opportunities to condense the timeline by performing actions in parallel were identified. A good example of this is starting the tech transfer work activities in conjunction with the process development work. This requires a strong partnership between manufacturing science and technology and process development teams. The NPI change control system is used to list all the completed work and the appropriate documentation to ensure that the process being brought into the GMP facility is compliant with GMP standards.

Measurements for success for the NPI involve four main key performance indicators (KPIs). The first is on-time delivery, meaning delivering and meeting internal and external customer team needs and project requirements. Additionally, project timeline adherence ensures the process meets project dates through effective planning and governance of project activities. Another KPI is process flexibility, meaning the ability to adapt business practices as the industry and customer requirements change. The final KPI is effective transfers, measuring how effective planning and cross-functional communication enable seamless transfers.

## SUMMARY

To accelerate cell therapy development, leveraging existing experience and innovation is key to reducing time and costs. Delivering process robustness through analytical capabilities to characterize the process and define the product can help speed up the timeline and overcome analytical development roadblocks. Keeping the product and patient in mind can help developers bypass the common roadblocks in new product development, as can leveraging the offerings and experience of a CDMO.

A standardized NPI process de-risks and streamlines the path from development to clinical manufacturing. The NPI process lays out a robust cell therapy tech transfer framework that incorporates risk management strategies from day one, with a standardized approach to documentation to simplify backtrack regeneration and integrated analytical development services and QC testing.

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# Q&A

Alex Sargent and Chad Anderson

## Q How do you incorporate non-viral gene editing into an efficient process development workflow?

**AS:** Non-viral gene editing is one of the more challenging unit operations when it comes to process development. To use a recent example, Casgevy was freshly approved in Europe as the first commercially approved therapy that uses CRISPR. How do you approach that from a process development perspective?

One of the most important considerations is off-target effects. Whether you are using CRISPR, a transposon system, or another method, it is critical to understand that an appropriate design is required to avoid off-target effects and erroneous insertions or deletions. This must be considered before establishing the actual process of making the therapy from a manufacturing perspective.

The other key consideration is utilizing scale-down models. The primary route for getting these systems into the cell is electroporation. All large GMP electroporator systems tend to have scale-down components that can be used for process optimization and DoE to establish the best electroporation conditions for your targets and modality. However, do be aware that when you scale up into larger systems, findings will not always correlate directly, and time for further optimization may be required. All systems have recommended settings for different cell types and modalities, but you should take into account some optimization for your specific process and gene of interest.

## What is the average time that it takes to move a product from the lab to clinical batches?

**CA:** This is dependent on several factors, such as the complexity of the process and how defined it is. If the process that we are transferring is highly complex and requires a lot of work in process development, that will impact the overall timeline. If it is a standard well-defined

process, such as for a CAR-T product, we aim to get it through process development and into engineering rounds in 9–12 months.

## At what stage of clinical development should process automation be considered?

**AS:** At the very beginning, even before Phase 1, there are many advantages to considering automated, closed processing systems. For smaller biotechs, the initial investment here can be burdensome as this equipment and these systems can be expensive especially if they require product optimization.

That is where partnering with a CDMO with the systems and experience in-house can be advantageous in terms of cost and time. Chances are, unless your indication is a rare orphan disease and you will only treat a few patients per year, you will need an automated solution in order to scale to meet the demand for your therapy.

Even in the case of rare diseases, more clients are considering the benefits of automation and adopting this pre-Phase 1. The reduction in risk in terms of contamination events and tech transfer is greatly reduced with fully automated off-the-shelf solutions. Understanding some of the cost constraints to bring in that automation technology pre-Phase 1 is a good idea.

## How can we ensure effective client-CDMO communication?

**CA:** One of the reasons we introduced our NPI form, which is a document used to capture the process specifics during the capture phase, is to communicate before we even start. The form is only two or three pages long, but it hits on all of the high-level potential roadblocks that we have seen in the past so that we can get in front of those before we even start.

It has helped tremendously since its introduction to give a better understanding of the process and the specifics before we even have our first technical discussions. When we have those first technical discussions, everyone already knows what the process looks like and if there are any roadblocks to overcome. This means we can act a lot quicker and it helps us navigate through those early phases a lot easier. This is something that we have done to streamline the process to help both the client and our internal team better align before we even start.

### BIOGRAPHIES

**ALEX SARGENT**—better known as "Sarge"—is currently the Director of Process Development at Charles River Laboratories. He obtained his PhD from Case Western Reserve University in Cleveland Ohio, where he studied the challenges and promises of stem cell biology, neuroimmunology, and Cleveland sports teams. After a brief stint in academia at the Cleveland Clinic, he left Cleveland to pursue his industry career in cell therapy. During his many years in this industry, he has had the honor of working at several esteemed enterprises on new technologies and approaches for cell therapy scale up, automation, and gene editing. He is especially passionate about the challenge of curing cancer, working on CAR-T and CAR-NK

cell therapy process and analytical development from discovery, through regulatory submission, manufacturing, and pivotal clinical trials. He wakes up each day excited to help advance cell and gene therapy to treat and cure disease, with the steadfast goal of improving human lives.

**CHAD ANDERSON BIO NEEDED**


## AFFILIATION

**Alex Sargent**
Director,
Process Development,
Charles River Laboratories

**Chad Anderson**
Associate Director,
Manufacturing Sciences and Technology,
Charles River Laboratories



## AUTHORSHIP & CONFLICT OF INTEREST

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EXPERT ROUNDTABLE

# Smooth cell therapy analytical assay translation from analytical development to QC

**Hadar H Adams, Ilya Shestopalov, Kitman Yeung, and Takele Teklemariam**

In this Expert Roundtable, four experienced industry professionals, **Hadar H Adams**, **Ilya Shestopalov**, **Kitman Yeung**, and **Takele Teklemariam** (left to right), discuss challenges and strategies for effective assay translation in the cell therapy space, with a particular focus on flow cytometry assays. The panelists share insights on effective communication between analytical development and QC teams, risk mitigation, and regulatory considerations.

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## Q What are the unique challenges of developing quality assays, in particular flow cytometry assays, for a cellular therapy?

**IS:** Flow cytometry assays are integral to cell therapy, in the same way mass spectrometry is to biologics or nuclear magnetic resonance is to small molecules. Over the last decade in

> "The most common mistake is that when the assay is translated to QC, the foundations of statistically relevant data are frequently lacking, which compromises robust analytical procedures."

the space, I have seen many of the historic challenges in developing flow cytometry assays being resolved. We previously struggled with the variability of reagents, difficult-to-use instruments that lacked standardization, and software compliance, but these are no longer challenges today.

There are still three primary areas in which challenges exist. One area is strategic and relates to how assays are designed. For example, many companies design panels that aim to measure identity, purity, and viability in a single flow cytometry panel, using four to five colors. This is fantastic for working towards an IND submission and is highly efficient, but it may cause lifecycle management issues later. If you want to switch to a different viability modality, and your validated panel has viability markers, it might not be so useful. If one of the reagents used to validate your panel becomes unavailable, the whole assay goes offline. For product release, the decision of whether to use one multicolor flow panel or multiple smaller panels must be made early on. Smaller panels will lead to fewer compensation issues and easier lifecycle management. This can be hard to align strategically within companies.

The second area is positive assay controls. Often, a positive control is needed to know if instruments are performing properly. This could take the form of a mimic or something with equivalent fluorescence to the population being measured. There are great reagents out there for some cell types, but not all.

The third area that must be considered is assay transfer during manufacturing site transfer. In many cell therapies, flow cytometry is performed with fresh samples during manufacturing at the QC lab within the manufacturing site. Later, you may want to open another manufacturing site or switch sites. Once products have been manufactured at those new sites, at the stage of applying for Biologics License Application (BLA) or Market Authorization Application (MAA), you must demonstrate comparability of the products across manufacturing sites. To demonstrate comparability, you first must demonstrate assay equivalence. The gold standard is testing the same sample at both the old and new QC labs. If the old lab is already closed, this will be impossible. If you are working with fresh material, it may become impossible if you miss the opportunity to do that during site transfer. Comparability studies can become difficult if you no longer have access to the old QC site to perform site-to-site equivalence.

**KY:** One key challenge in assay transfer is the controls. As a CDMO business, we have many clients sending us their R&D-stage assays. When an assay comes in, they often do not have sample-accepting criteria. In some unique cases, they do not even have system suitability set up. Other cases may have a positive control, but it may lack robustness, and a negative control may not exist. Having robust controls and the relevant data to back them up is critical.

We also encounter issues wherein the client may have no analytical target profile (ATP) in place, meaning that they have not identified critical reagents or alternatives. This means that if a reagent ran out, they would have no bridging study.

**TT:** One of the challenges especially in the CDMO business is balancing instrument availability. Currently, there are many kinds of available instruments from various companies. It is critical to ensure the release assay is as simple as possible. Companies offer fluorochromes

of eight or more which can become difficult to manage. Minimizing the number of fluorochromes and focusing on the target is critical.

Currently, for cell and gene therapies specifically, there are no standard commercial positive controls available. You must establish a standard from the beginning, make a cell bank that works well, and characterize it to demonstrate comparability. You do not need to go through the entire manufacturing process to show comparability. You can use a well-characterized positive control. Having a well-established method from the beginning is key.

**HA:** At the beginning of development, one of the challenges can be material. Usually, the process development and analytical development (AD) are happening side by side and it can be hard to get representative samples. The process is continuously changing, so the samples are changing, which can be a challenge for assay development. Working closely with the process development team to understand the changes and how impactful they are on the final sample, and risk analysis for the samples, is important.

## What are the key challenges (or most common mistakes) in translating assays from analytical development to QC teams?

**KY:** The most common mistake is that when the assay is translated to QC, the foundations of statistically relevant data are frequently lacking, which compromises robust analytical procedures. In the early development phase, a lot of material used is not representative, and thus it is hard to accumulate statistically relevant data. Later on, that data will be important to setting system suitability and certain criteria as you enter qualification and validation. To solve this, have the ATP in place as early on during development as possible, and carry out a gap assessment.

As a CDMO, we often deal with rapid timelines. We need clients to understand that when dealing with IND filing, less is sometimes more. Many assays are not necessary as release assays. These can instead be classed as categorization assays, for example, potency assays. Adding things such as release assays early on can cause problems when entering Phase 2 or 3, as it can be hard to justify to the US FDA why a release assay is being changed.

**HA:** Equipment, instrument configuration, and workflow capabilities are all different between AD and QC labs. There are many more things in place in the QC lab and there is a longer checklist to consider. For example, in an AD lab, you may be able to use a research-use-only antibody, but the QC lab may not permit that. These elements must be discussed in advance of the transfer so that the AD lab can ensure that they are developing the right kind of assay that can easily be transferred into the QC lab.

Another element related to that is reagent traceability. We have talked about controls and system suitability, which will come from the AD lab. The documentation and traceability within the two settings can be different. The QC lab might not accept reagents that the AD lab routinely uses if there is not enough traceability, documentation, and characterization of those reagents.

**IS:** A common situation that can cause issues is AD providing the QC team with only a template for an instrument. This can be a mistake—you must have both a template and a well-described gating strategy in the standard operating procedure (SOP). This should include

descriptions of what you should see at each gate, with representative examples and some flexibility to adjust parts of the gate. It is acceptable to not have an entirely rigid strategy.

**TT:** Provide a range for critical steps, such as staining times as a range. Robustness, in terms of the hold time, the number of sets, and the instrument, must be shown so that there is no variability. Robustness in terms of time is a critical aspect of translating AD to QC.

## What practical steps can teams take to minimize risk in assay translation?

**HA:** The number one step that teams can take is to have well-characterized assays. Robustness in ranges for incubation times and understanding the reagent requirements must be considered. Small elements can become problematic if they are overlooked during transfer. Do you thaw a reagent at room temperature or on ice? How long before you must use it? Each of those small steps can make a big difference when transferring an assay from one lab to another and they are often overlooked.

Identifying the most critical steps is key. In some steps, you can pipette differently. Other steps may require pipetting in a very specific way, such as reverse pipetting. This must be outlined and highlighted both in the SOP and during training.

Another key step is gap analysis. At the beginning of the transfer process, a conversation between the teams should be held, looking at every step of the procedure, including all the instruments, reagents, and capabilities of each lab to identify any key differences. Any differences must be looked at more deeply to understand if these can be worked around and proceed accordingly.

Another key consideration is staffing and training. If you lack a skilled person in the QC lab, then you will need to provide much more information in the SOP about gating. Problems can arise from an incompatibility in training in the different labs. For the QC lab to be aware of all the development and robustness work that the AD lab has done, being able to communicate freely and often is important.

Finally, hands-on training is critical. Training should involve being in the transfer lab and seeing how processes are performed. Knowledge of the workflow and even the orientation of equipment can be important, and seeing how that might be different in the QC lab is critical to identifying any possible challenges. For example, we had a situation where we had two very similar cell counters from the same manufacturer, but one was high-throughput, and one was not. This was fortunately identified during training, as this could have added a significant amount of time if the cell counts had to be performed one at a time.

**TT:** I agree that training and follow-up communications are critical. One of the reasons for this is that assay development at the AD stage is usually performed using a healthy donor. At QC, this is done using patient samples that sometimes behave differently. The patient sample may not match up to the analysis in the SOP. Having follow-up communication between the two groups is critical to ensure all data is being interpreted in the same way.

**KY:** To solve the problem of instrument transfer, at Miltenyi Bioindustry, we use the MACSQuant® System with Smart Gain technology to align instrument settings when transferring assays from site to site. Using the Smart Gain function can minimize those problems.

> "For the QC lab to be aware of all the development and robustness work that the AD lab has done, being able to communicate freely and often is important."

Teams like Analytical Science and Technology (ASAT) are still required to draft all the details on the gating, but this technology can help a lot.

## Can you suggest strategies for effective communication between teams during assay translation?

**KY:** We all need a collaborative mindset, including the client and the CDMO. Early on, we perform a gap assessment. We do a paperwork exercise including a detailed and comprehensive Program Scope Development in place to work through all the details to identify the must-haves and the good-to-haves.

I also recommend leaning heavily on the project manager (PM). A PM should have a satisfactory technical background, because sometimes when we speak about these technologies and platforms in meetings it can sound like a foreign language to people without a cell and gene therapy background or even a science background. A PM should orchestrate all meetings, understanding who needs to be in each meeting and involved in each decision. For example, if a decision impacts the QC team, a QC representative needs to be involved in the decision-making.

**IS:** AD must have a say in the transfer. As the originating lab, once the assay is within QC, the AD team should still be involved in solving problems. AD probably has more experience with the assay, so when things go wrong, AD should have access to the raw data (FCS files) and trending. In an ideal situation, this should go as far as allowing AD to have a part in training new analysts in QC. Another approach is that AD can provide training videos. An SOP only goes so far; a recording of someone doing a procedure can be highly effective for assay transfer to QC.

**TT:** The AD team's communication across all stakeholders is critical. Once the assay is transferred to the QC team, there may be unforeseen outcomes such as an invalid assay. The subject matter expert from the AD team will have the expertise to help. From the technical perspective, it makes sense to maintain communication with the originator for backup information. Assays sometimes need improvement in the latter phases so help from the originator is critical during clinical testing.

**HA:** Face-to-face communication is critical. Things get lost or misinterpreted in emails so I would not rely heavily on these. Having frequent face-to-face check-ins for the QC and AD labs to get questions answered and check on minor things is essential. If things are slightly misinterpreted, it can make a big difference.

Before initiating the transfer, have all the documentation handed over from AD to QC so that they can review the development work and the procedure thoroughly. This ensures they

> "AD must have a say in the [assay] transfer. As the originating lab, once the assay is within QC, the AD team should still be involved in solving problems."

are as informed as possible and ready to ask questions at the transfer stage. It is important to have the analysts who are actually in the lab as part of this conversation, not just managers and supervisors.

## What are the impacts on communication when running QC internally versus externally?

**IS:** I have seen both models, and they can both work well and poorly.

Often, even with internal QC, the QC teams tend to silo themselves away to manage the risk of GMP non-compliance. They do not want AD walking in and watching over their shoulders. This is valid, but we want the QC to be inclusive to allow effective communication. If QC becomes too isolated, you cannot effectively have face-to-face meetings or proper training.

One of the differentiating factors that makes QC work, whether internal or external, is its mission. If the QC team is invested in the success of your product, they will behave differently in terms of how they communicate with you. You must work hard to establish that mission-driven approach, whether your QC is external or internal.

**KY:** Being inclusive, even very early on, is key. At Miltenyi Bioindustry, we include our QC team in the Program Scope Development and ATP planning. The QC team is on the front line holding the pipette, so they should know what assays are going to come into their lab.

**HA:** It is a common misconception that internal QC is better and external is harder. It is important to understand the differences between the AD and QC settings It may be easier to get information from an internal QC in terms of processes, workflows, and systems, but this is not always true. One of the things that can be more challenging with an external QC is coordinating the onsite training. This must be considered early in the agreements between the two companies. Factors such as how many onsite visits are permitted should be pre-decided. It is important to include the AD and QC personnel in those early conversations because hands-on training is critical.

**TT:** For Mitenyi Biotec's San Jose site, currently, most of the analytical methods are in-house QC testing, but we do have some safety tests performed in an external lab. We have biweekly meetings to follow up on progress with this external lab. For instance, if we send samples for lot release, we have a follow-up meeting so they can be prepared to test the sample on time and ensure they have the resources to do so. This meeting is critical, whether it is done with internal or external QC.

**IS:** It is helpful to have a common SharePoint® set up with external QC labs, so any preliminary results and raw data can be placed there. This cuts down on the number of emails

needed to check how samples are going. If an assay has been run, it will appear in SharePoint® way before a certificate of testing is received.

## Which technological advances can smooth the transition from assay development to QC—now and in the future?

**HA:** Regarding flow cytometry assays, the biggest challenge is the gating. Flow cytometry is highly automated, but the gating can be very manual. There is a lot of work being done on this, which is fantastic. Having simple assays can also help here, as this enables simpler gating. If you have many different colors and populations, things can get more complicated as automated gating is not an option. To minimize the variability seen in flow assays, automation is key.

Another consideration is having good controls. Reference standards are not always available, but having good positive controls will assist in troubleshooting. If there is an issue in the QC lab, good controls will help pinpoint the cause of the issue.

**TT:** Miltenyi Biotec's Express Mode automated flow cytometry software is beneficial here. However, when clients come from AD, they may not see the benefit of this as they are used to manually adjusting gating. In addition to making the assays simpler for analysis, the software is also good for regulatory impact. It removes any subjectivity within flow cytometry. Although the result might not vary much between analysts, in the case of getting results quickly for manufacturing processes to continue, automation is critical. The MACSQuant Analyzer's automation system, specifically Express Mode, can be adapted for different kinds of assays.

**IS:** Automated gating has a lot of potential. The danger is that you may validate an assay, for example using Express Mode version 3, but if the vendor releases Express Mode version 4 a year later, you may have to revalidate that assay. If you are planning to use an automated approach, you need to have an understanding with the provider that you will be able to continue using that automated module without any updates in perpetuity. If a new version comes out that automates slightly differently, it could pose issues. This is a common challenge when talking about technology that is constantly evolving.

The other technological piece that I find intriguing is microfluidic-based flow cytometry systems, in which staining is automated and all reagents are preloaded. This parallels how cell counting has evolved, where, 5 years ago, almost everyone was doing manual cell counting with trypan blue. Now, the industry standard has become single-use fluorescent chips. I believe flow cytometry will move that way once the technology matures to accommodate slightly more complex gating. With any evolving technology, you need to have a lockdown version that exists in perpetuity, to prevent issues arising in lifecycle management.

**KY:** Standardization is one of the ways that we can transfer assays from AD to QC more smoothly. At Miltenyi Bioindustry, we have the standard Express Mode and all-in-one reagents in a dried form. During AD, we use the StainExpress™ Cocktail to collect data for QC. We have already collected a lot of data using Express Mode and a standardization gating strategy. We are also developing a robotic system to help standardize the workflow.

> "Most small companies do qualify their assays, particularly release assays. There is some confusion from companies who believe every assay needs to be qualified, but that is not the case."

## Q What are some of the key regulatory considerations (e.g., validation, qualification, regional differences)?

**TT:** The terms assay validation and qualification are sometimes used interchangeably. In general, both assay qualification and assay validation are performed to check how fit for purpose a method will be down the line. Typically, ICH Q2(R1) is the guideline used for both assay qualification and validation.

However, there are some differences between assay qualification and validation, particularly in terms of the study depth, as well as the phase in which they are completed. In assay qualification, the attributes that are being evaluated are similar to those in assay validation. However, assay qualification generally happens at an earlier stage in development, during Phase 1. Typically, during assay qualification, a single representative lot can be evaluated to demonstrate that any assay is fit for purpose, particularly a release assay. There are no clear written regulatory requirements to qualify an assay from region to region. However, it is in the best interest of the sponsor to qualify an assay to demonstrate that the method can be validated in the future. In the simplest terms, qualification is an early stage of demonstrating the method can perform as intended in the future. It also helps to establish acceptance criteria in the future.

However, for validation, there are clear requirements from the regulatory agencies, particularly in the late phase of IND application or BLA submission. Qualifying an assay at the beginning will help to validate a method. The acceptance criteria are typically similar to those in qualification, but they can be stricter. During validation, you must validate not only your final product but also the intermediate controls.

In general, from a sponsor point of view, qualifying a method early in development, even in Phase 1 before IND submission, is critical. In general, the requirements for validation are clear, but for qualification there are none. Most small companies do qualify their assays, particularly release assays. There is some confusion from companies who believe every assay needs to be qualified, but that is not the case. There will be some characterization assays down the line that are critical in decision-making, but that do not need to be qualified. When data is available, particularly patient data, this can be critical to evaluate those kinds of qualification methods.

**IS:** In the US, at the BLA stage, there is greater scrutiny of robustness than is needed earlier on in development. After you validate your assay, during your pivotal trial, make sure you invest in robustness studies, or this can become a post-approval commitment.

With these assays, we are typically aiming to capture some precision during qualification. That is the acceptance criteria for validation. The precision might be good, but it must be considered that there is a certain temporal variation in flow cytometry that is not captured by precision. Over a few years, you might see that the precision of your assay changes dramatically. This should be factored into how specifications are set.



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**HA:** One thing that needs to be discussed early on with a QC partner is where the qualification needs to happen. Everybody understands validation needs to happen in the QC lab, but in the early phases, oftentimes AD does the qualification, and then they transfer the method. Some QC labs want qualification performed in their lab. This needs to be understood and agreed upon in advance. I have even seen co-qualifications shared between the two labs.

The idea that characterization assays do not necessarily need to be qualified was mentioned. New potency guidelines came out recently describing how critical potency assays are. From my reading, it seemed that the recommendation was that potency assays should be qualified. It did cover characterization versus release, but my interpretation was that because potency assays are so critical, having the assurance that the assay is performing robustly and well is important.

**KY:** Statistics are also key here. I think there should be standardized 'magic numbers' in terms of the number of replicates needed for qualification.

## BIOGRAPHIES

**HADAR H ADAMS** is the Director of Analytical Development at Atara Biotherapeutics, an allogeneic T cell immunotherapy company. She has over 10 years of experience in development, optimization, troubleshooting, qualification/validation, and transfer of analytical methods for diagnostic testing, release testing, stability, and characterization of biological and cell therapy drug products. In her current role, Hadar leads a team focused on developing and qualifying a wide range of assays to support an IND for a CAR-T drug product.

**ILYA SHESTOPALOV** is currently the Vice President of Analytical Development and Analytical Product Lead at bluebird bio. His research focuses on development of cell-based assays for hematopoietic stem/progenitor products, CAR-T products, and lentiviral vectors. Prior to bluebird bio, Shestopalov was a postdoctoral fellow in stem cell biology at Boston Children's Hospital and Harvard University working with zebrafish hematopoietic stem cells.

**KITMAN YEUNG** is a biopharmaceutical specialist at Miltenyi Bioindustry, a division of Miltenyi Biotec, with 15 years of experience in cell and gene therapies. Her expertise in technology transfer, analytical development, and quality control testing supports the production of Phase 1/2 GMP cell and gene therapies. Yeung is currently an MSAT Analytical Manager at Miltenyi Bioindustry, where she leads technical and operational teams in developing, characterizing, and qualifying analytical tools for cell and gene therapies across multiple modalities.

**TAKELE TEKLEMARIAM** is a Quality Control Professional at Miltenyi Biotec with 15 years of experience in cell and gene therapy. He has broad experience in QC assay design and development, assay transfer, and qualification/validation. Currently, Takele is Associate Director of Analytical Development and QC Assay Transfer at Miltenyi Biotec, where he leads QC assay development and transfer, assay qualification and validation, stability studies, and QC in-process and release testing in cell and gene therapy for multiple clients.

## AFFILIATIONS

**Hadar H Adams PhD**
Director of Analytical Development,
Atara Biotherapeutics

**Ilya Shestopalov PhD**
Vice President,
Analytical Development, and
Analytical Product Lead,
bluebird bio

**Kitman Yeung MS**
MSAT Analytical Manager,
Miltenyi Bioindustry,
Miltenyi Biotec

**Takele Teklemariam MS**
Quality Control Professional,
Miltenyi Biotec

## AUTHORSHIP & CONFLICT OF INTEREST

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INNOVATOR INSIGHT

# Considerations for platform AAV affinity capture

Jett Appel

From discovery to the clinic, as the quest to produce new, high-quality AAV molecules intensifies, an efficient and scalable platform purification process for many AAV serotypes is advantageous for both drug developers and CDMOs. In this poster, the considerations for evaluating a platform affinity capture step involving the industry leading POROS™ CaptureSelect™ AAV affinity resin for the purification of AAV vectors are explored.

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## BROAD SPECIFICITY BINDING

Considerations for developing an AAV affinity platform capture step include the ability to have broad specificity to different AAV serotypes. A resin that can recognize both native and engineered capsids is ideal. Data presented in **Figure 1** was generated by researchers using POROS™ CaptureSelect™ AAVX resin. A static binding experiment was performed with a mix of AAV serotypes with AAVX resin. The AAV was quantified by qPCR. All serotypes tested were able to demonstrate binding to the AAVX resin.

## SCALABILITY CONSIDERATIONS

Scale-up is another important consideration. As **Figure 2** demonstrates, binding capacities of over $>1 \times 10^{15}$ capsids/mL at residence times ≥0.5 min. for AAV2 can be achieved with POROS CaptureSelect AAVX resin. Process calculations suggest that for large bioreactor volumes (e.g. 2,000 L) and high titers (e.g., 6E11 vg/mL) columns of 20–30 cm diameter meet typical processing limits while maximizing resin utilization.

FIGURE 1

**POROS CaptureSelect AAVX resin: broad specificity binding data.**

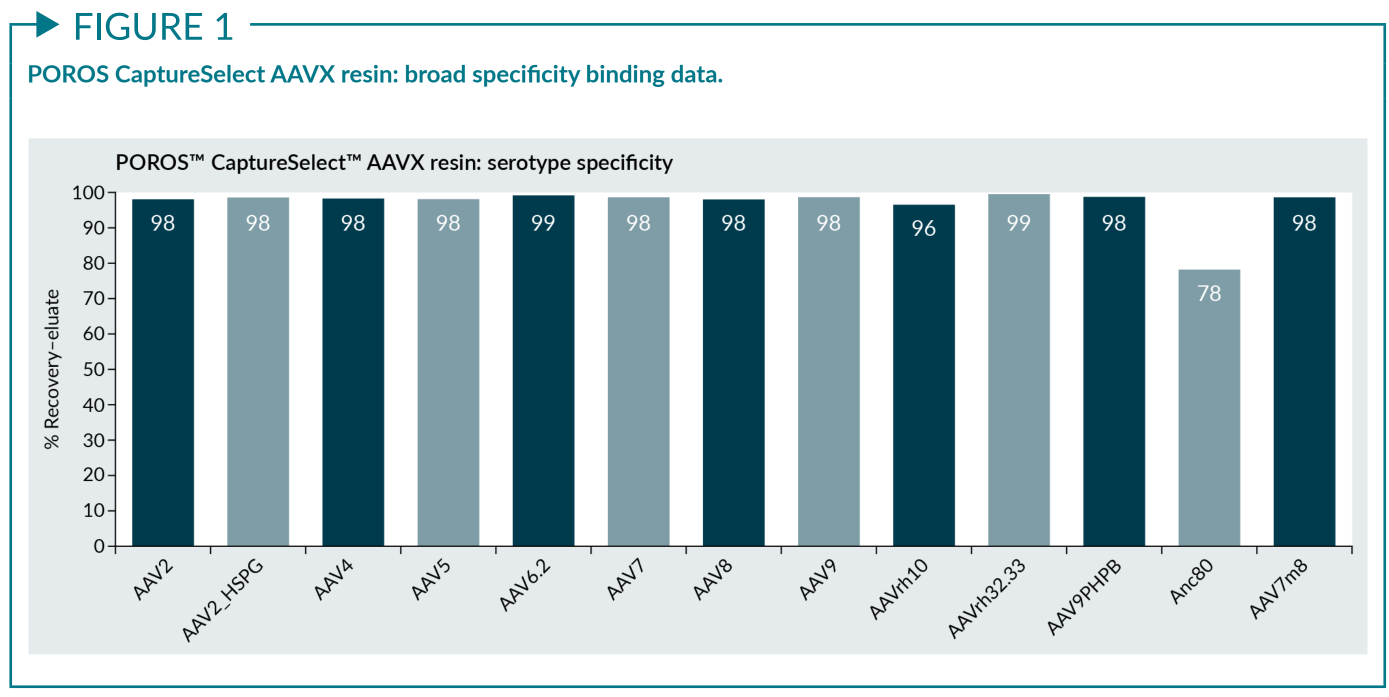
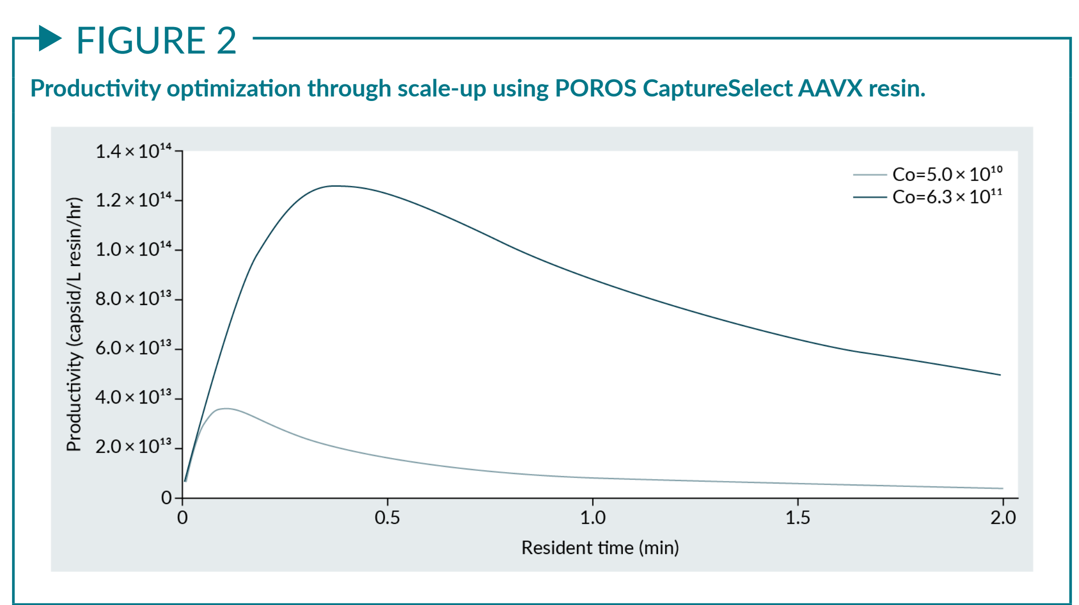



FIGURE 2

**Productivity optimization through scale-up using POROS CaptureSelect AAVX resin.**




## DYNAMIC BINDING CAPACITY

Having a high dynamic binding capacity is key to reducing column size requirements and maximizing productivity. Data in **Figure 3** demonstrates that POROS CaptureSelect AAVX resin has high dynamic binding capacity for multiple serotypes, including at short residence times.

## ELUTION OPTIMIZATION

High purity and recovery after an affinity step are desirable and can be further improved by elution optimization. An internal elution optimization study using the AAVX resin shows that buffer composition (pH and excipients) can be optimized to maximize AAV recovery, specific to each molecule and process.

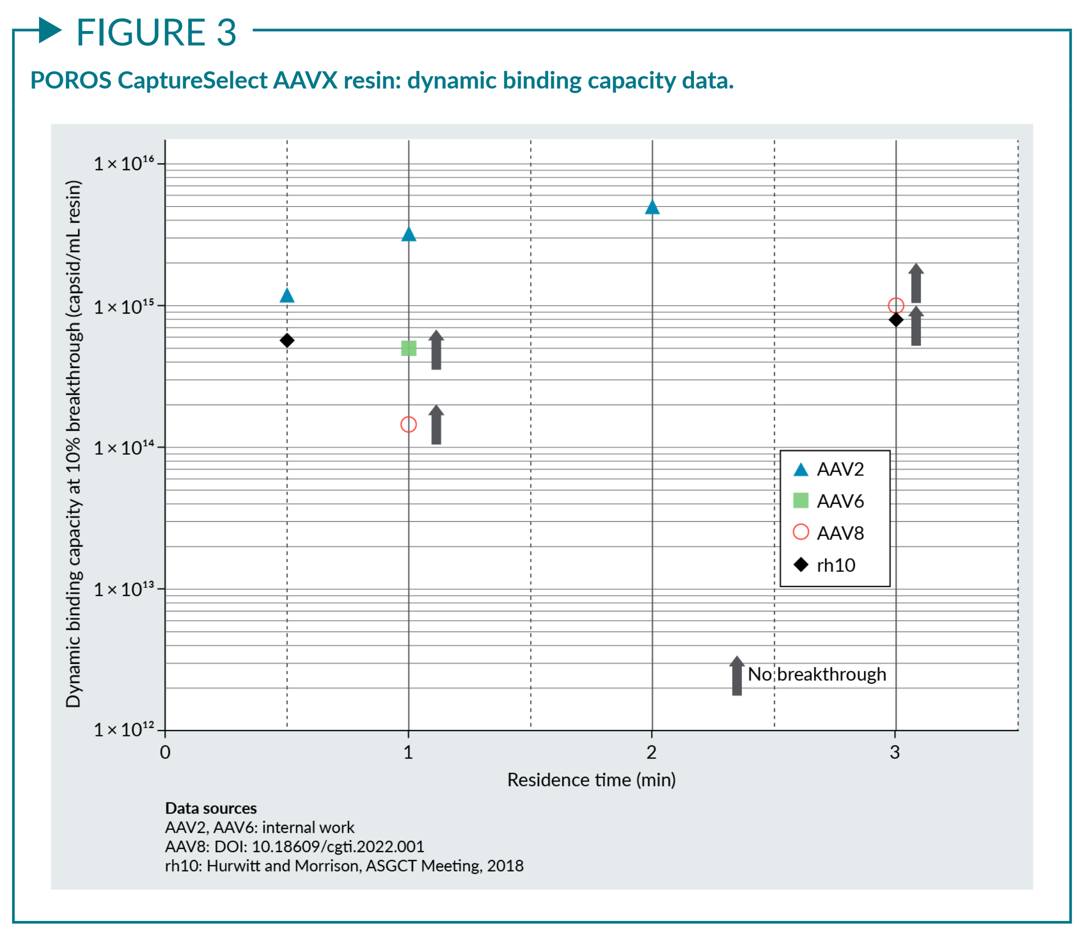



FIGURE 3

**POROS CaptureSelect AAVX resin: dynamic binding capacity data.**

## WASH OPTIMIZATION

Wash optimization can also improve purity and recovery after an affinity step. A wash optimization study for AAV6 was performed, using wash buffers with variable salt concentrations, pH, and inclusion of arginine. Intermediate wash optimization results in improved clearance of process-related impurities. Regardless of the wash conditions evaluated, 80% recovery was achieved for all the conditions tested.

## REUSE AND CARRYOVER

Reusability helps reduce cost of goods and the risk of carryover. The AAVX resins exhibit little to no carryover after 14 cycles using 0.1 mL phosphoric acid and further cleaned using 6 mL guanidine hydrochloride.

## VIRAL CLEARANCE CONSIDERATIONS

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

## AFFILIATION

**Jett Appel**
Purification Field Applications Scientist,
Thermo Fisher Scientific

ThermoFisher SCIENTIFIC

## AUTHORSHIP & CONFLICT OF INTEREST

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INNOVATOR INSIGHT

# Dear lentivirus: we haven't forgotten about you and how to improve your polishing recovery

Åsa Hagner-McWhirter

Navigating the landscape of lentivirus production poses a multitude of challenges, demanding innovative strategies to enhance recovery rates and address inherent stability concerns. To maximize physical and infectious titer, it is important to carefully tune the conditions of each downstream step with these challenges in mind. This Innovator Insight explores a strategy to refine the downstream process by optimizing capture from clarified feeds using weak anion exchange with Capto™ DEAE resin. A subsequent Capto Core 700 resin polishing step utilizing different buffer pH values and flow rates is also evaluated.

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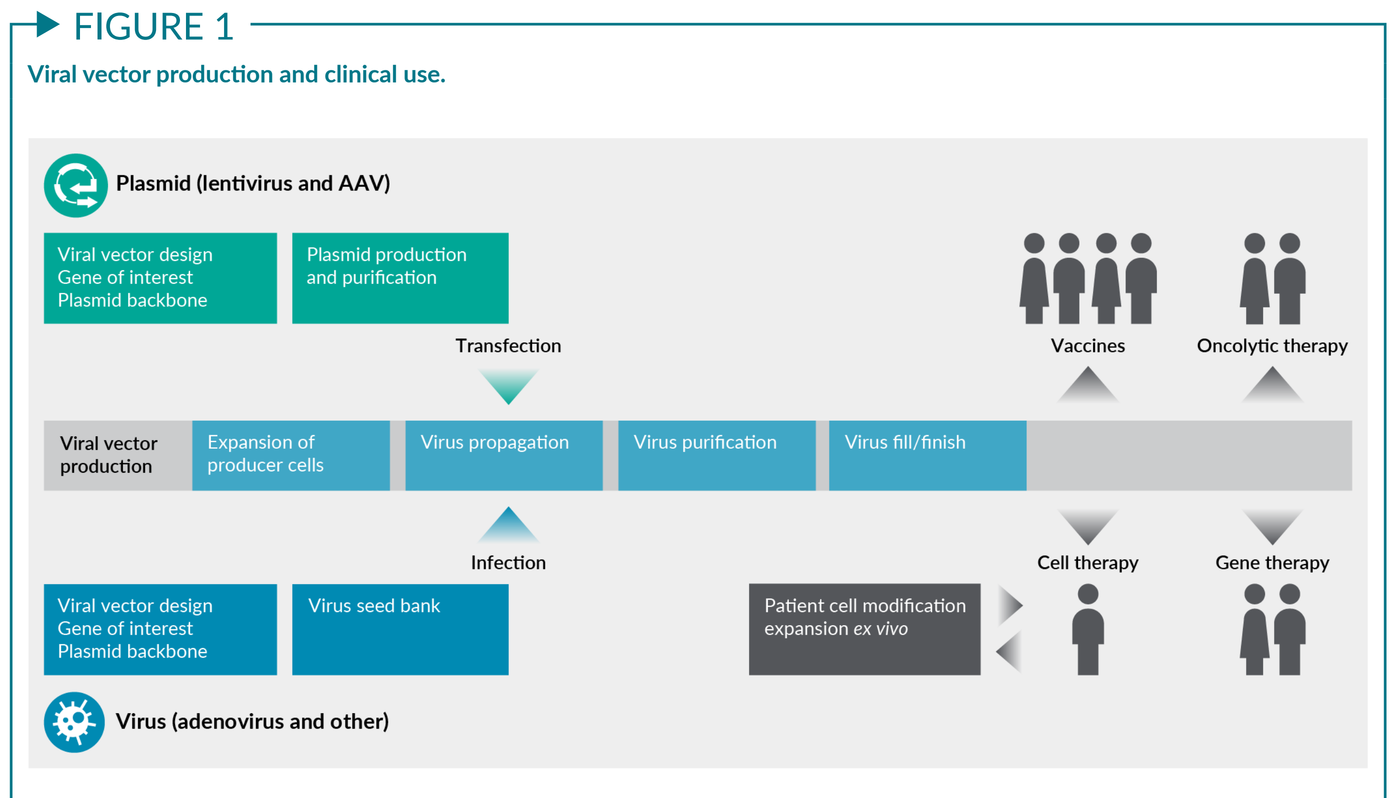
## LV PRODUCTION

Lentivirus (LV) is an enveloped virus, consisting of an outer membrane structure and an inner capsid made from p24 protein. Transfection of LV typically involves between three and five plasmids. The production process is based on either adherent or suspension HEK293 or HEK293-variant cell lines.

Different viral vectors are generally produced in a similar way. As shown in Figure 1, the viruses are propagated in the host cells, purified, and then used for different therapeutic applications. The main point of variation in the production of the different viral vectors is that the virus is introduced and propagated in two different ways: transfection using plasmids or infection with virus seed banks. There is also the option to develop either an inducible producer cell line, which doesn't require plasmid transfection, or a packaging cell line that only requires transfection of the plasmid containing the gene of interest. Transfected cells are used in LV vector production for *ex vivo* cell therapy applications.

FIGURE 1

Viral vector production and clinical use.




LV can be used in autologous and allogeneic CAR-T cell therapies, both of which utilize *ex vivo* transduction. There is also an ongoing effort to use LV for *in vivo* gene therapy applications.

## PROCESSING CHALLENGES

Several challenges are associated with LV processing, including a low recovery rate for infectious vector and variability. These challenges are largely caused by the low stability of the virus. Purification via binding and elution can inactivate or inhibit the virus depending on the elution conditions used, since LV is sensitive to low pH, high salt and temperature, and shear forces. The result is that typical overall process recoveries often fall within the range of 20–30%.

To address these challenges, it is crucial to reduce the number of process steps, optimizing conditions like pH, salt concentrations, and the use of stabilizers. Strict sample handling procedures during analysis, side-by-side analysis of the sample to be compared, along with control or reference samples, are also necessary.

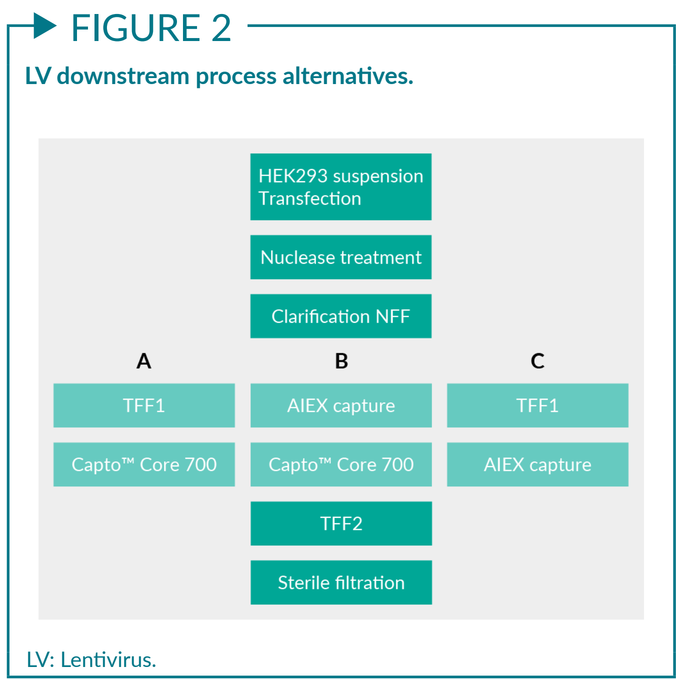
## CHROMATOGRAPHY CONSIDERATIONS FOR LV PURIFICATION

Chromatography purification of large entities such as viruses involves specific considerations. Since the virus size limits the access to ligand inside pores of the chromatography support, maximizing the surface area available for virus binding is essential to maximize binding capacity. This is achieved by using smaller resin beads or membrane formats. The choice of resin chromatography approach (e.g., bind/elute or flow-through) depends on the size of the virus. For viruses up to 80–100 nm, bind/elute chromatography is recommended. For larger viruses, a flow-through approach is recommended.

Figure 2 illustrates LV processing alternatives. The choice of LV processing steps and methods is influenced by differences in purity demands—for instance, between *in vivo* and *ex vivo* applications. The number of processing steps also plays a critical role in this choice. To maximize overall infectious titer recovery, the number of processing

FIGURE 2

LV downstream process alternatives.




LV: Lentivirus.

steps must be minimized. In Figure 2, following harvest and clarification, Alternative A involves a tangential flow filtration (TFF) step directly followed by Capto Core 700 polishing. Alternative B involves anion exchange (AIEX) capture followed by a polishing step with Capto Core 700. Alternative C involves a TFF step, then ion exchange capture, followed by Capto Core 700 polishing. Alternative B or C may be required for *in vivo* application with higher purity demands.

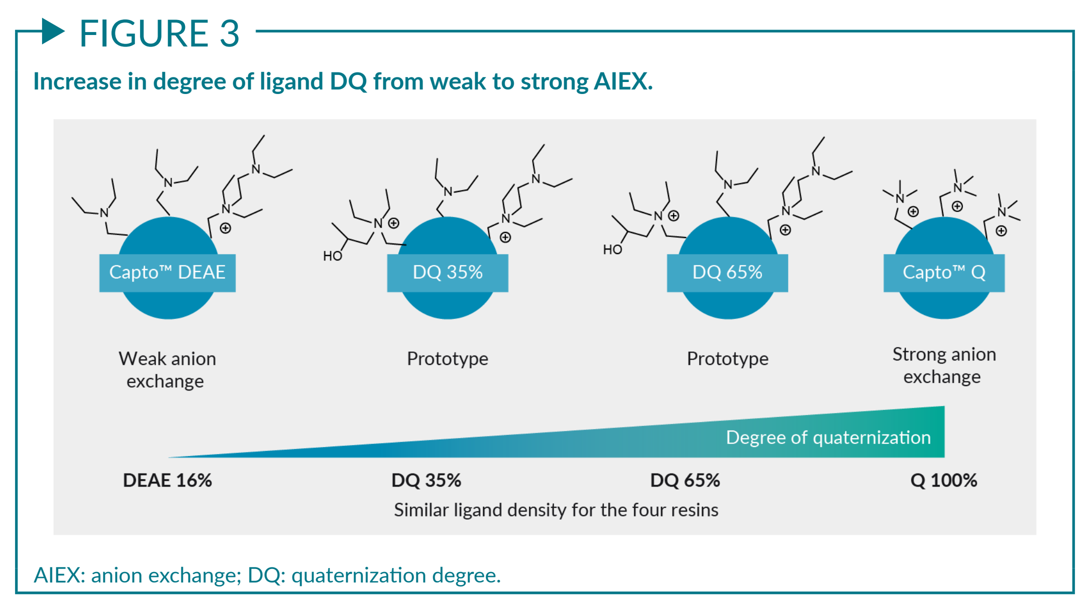
## ANION EXCHANGE CAPTURE: A STUDY OVERVIEW

For a study of AIEX capture, LV expressing green fluorescence protein (GFP) was produced in HEK293 suspension cells by transfection with four plasmids. The vector was harvested and treated with nuclease before clarification. The harvest titers used were $10^9$ viral particles (VP)/mL and $\geq 10^6$ transducing units/mL. Four different types of anion exchange were then tested, from weak to strong. The capacity was approximately $10^{10}$–$10^{11}$ VP/mL. Following this, a polishing step using Capto Core 700 was employed, which was evaluated using different pH and residence times. The load capacity in this step was approximately 25–30 column volumes (CV), depending on the level of remaining impurities in the AIEX eluate. p24 ELISA was used for analysis, together with a transduction assay, total protein analysis with Micro BCA, and total DNA analysis with PicoGreen.

To determine if the strength of the anion exchanger, as demonstrated by ligand quaternization degree (DQ) or degree of positive charges, would affect the LV capture performance, different resins with similar ligand densities were compared. As demonstrated in Figure 3, a weak

FIGURE 3

Increase in degree of ligand DQ from weak to strong AIEX.




AIEX: anion exchange; DQ: quaternization degree.

## TABLE 1

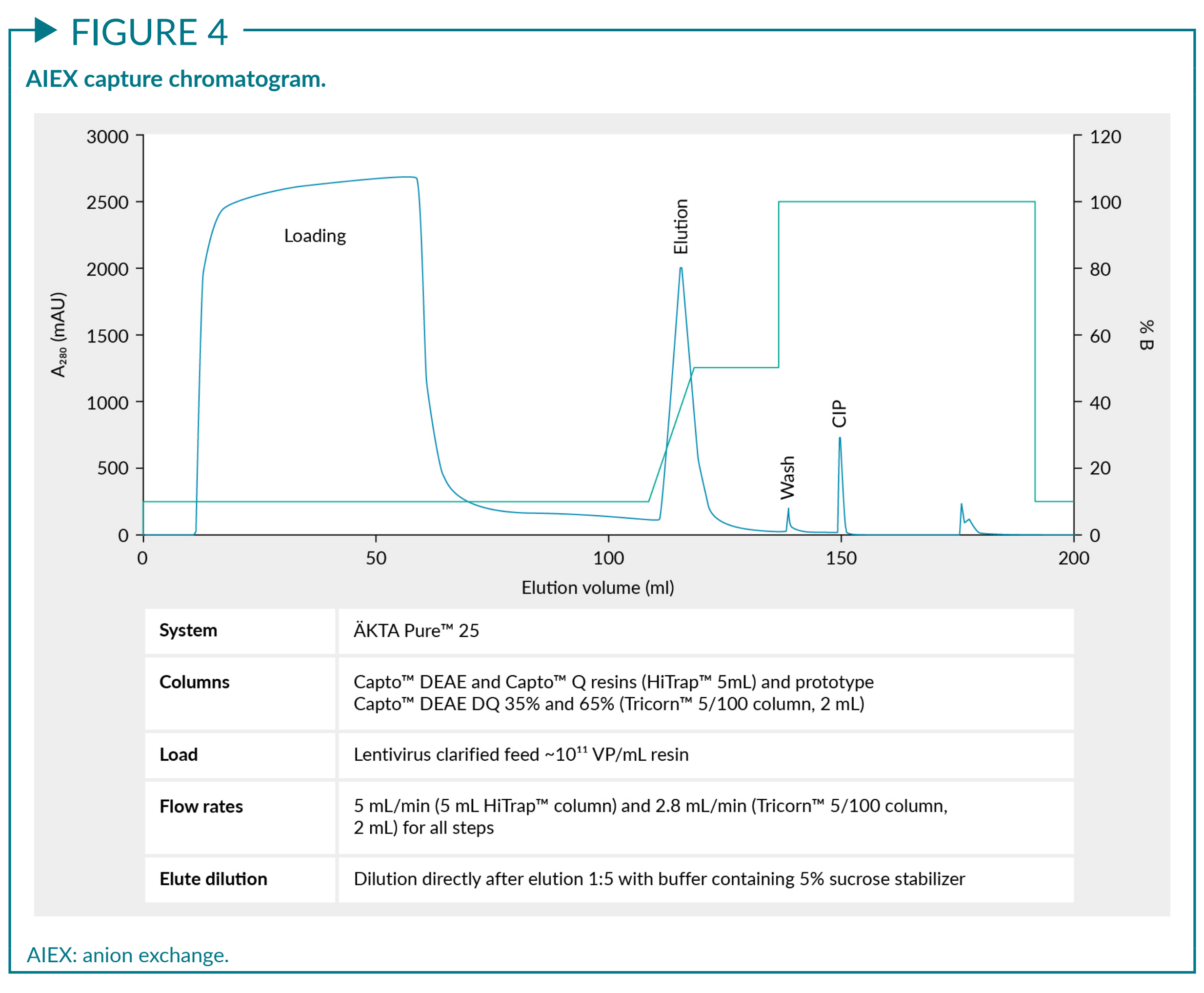
**AIEX capture method**

| Phase | Composition | Volume |
|---|---|---|
| Equilibration | 50 mM Tris-HCl, pH 8.0, 130 mM NaCl | 3–10 CV |
| Sample loading | LV feed (load ~E11 VP/mL resin) | 48–120 mL |
| Wash | 50 mM Tris-HCl, pH 8.0, 130 mM NaCl | 10 CV |
| Elution | 50 mM Tris-HCl, pH 8.0, 130–650 mM NaCl | 25 mL |
| Wash | 50 mM Tris-HCl, pH 8.0, 1.3 M NaCl | 10 CV |
| CIP | 1 M NaOH | 15 CV |
| Wash | 50 mM Tris-HCl, pH 8.0, 1.3 M NaCl | 15 CV |
| Re-equilibration | 50 mM Tris-HCl, pH 8.0, 130 mM NaCl | 10 CV |

AIEX: anion exchange; CV: column volume; CIP: clean in place; VP: viral particles.

## FIGURE 4

**AIEX capture chromatogram.**



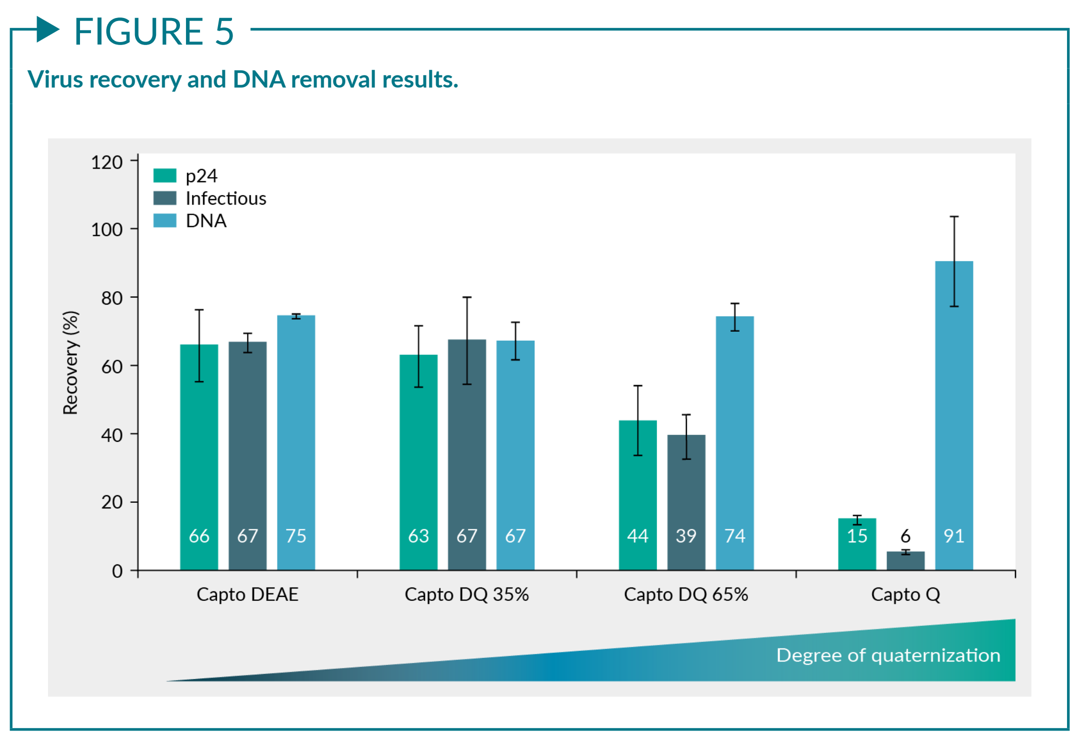

| | |
|---|---|
| **System** | ÄKTA Pure™ 25 |
| **Columns** | Capto™ DEAE and Capto™ Q resins (HiTrap™ 5mL) and prototype Capto™ DEAE DQ 35% and 65% (Tricorn™ 5/100 column, 2 mL) |
| **Load** | Lentivirus clarified feed ~$10^{11}$ VP/mL resin |
| **Flow rates** | 5 mL/min (5 mL HiTrap™ column) and 2.8 mL/min (Tricorn™ 5/100 column, 2 mL) for all steps |
| **Elute dilution** | Dilution directly after elution 1:5 with buffer containing 5% sucrose stabilizer |

AIEX: anion exchange.

AIEX with Capto diethylaminoethyl (DEAE), which contained approximately 16% DQ, was used initially. On the opposite end of the study, a strong AIEX ligand (the Q ligand, which has 100% DQ,) was investigated. In between, two additional prototype resins, with 35% and 65%

## FIGURE 5

**Virus recovery and DNA removal results.**




## FIGURE 6

**Recovery and impurity removal results.**

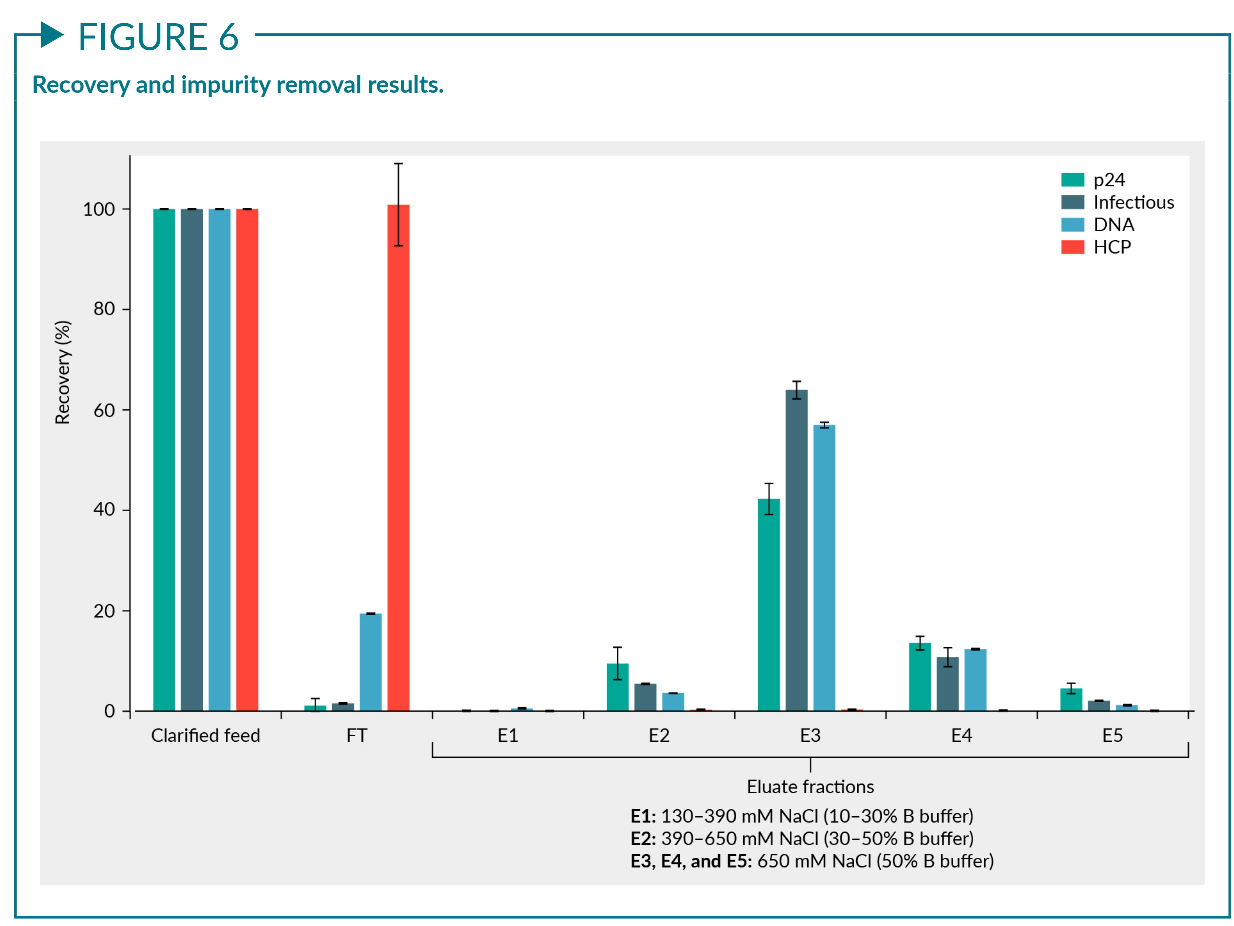
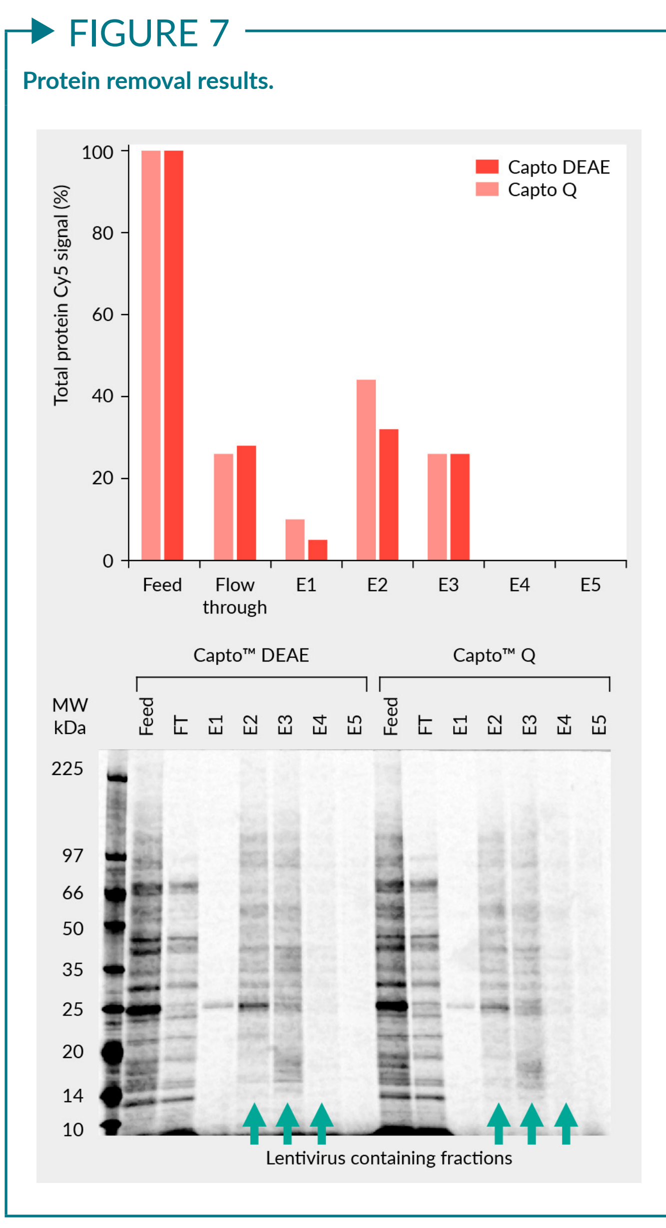


FIGURE 7

Protein removal results.




DQ respectively, were produced by partially reacting the DEAE ligand with propylene oxide.

Table 1 and Figure 4 detail the AIEX capture method and AIEX chromatogram respectively.

Figure 5 shows the virus recovery results, with the green bars representing p24 and the dark blue bars representing the infectious recovery. Results indicated that weak AIEX with lower quaternization degree significantly improved virus recovery, while strong AIEX showed lower recoveries. The two prototype resins also followed this pattern, suggesting that the bind/elute event is milder for the LV using a weak anion exchanger. Figure 5 also shows the DNA removal data in light blue. There was some co-elution with the DNA in anion exchange, but there was no difference in DNA co-elution between the different quaternization degrees from the weak to the strong.

The weak anion exchanger, Capto DEAE, was chosen for further investigation into the recovery and impurity removal for the different fractions. This resin also has dextran surface extenders adding flexibility that may be advantageous for large viruses. As shown in Figure 6, the host cell protein (HCP) comes in the flow-through, and no detectable HCP co-elutes with the LV as determined using the Micro BCA assay. With the eluate fractions (E1–E5) the infectious recovery is highest in E3, but there is also some co-elution of DNA. Some of that DNA comes in the flow-through, but some elutes together with the LV.

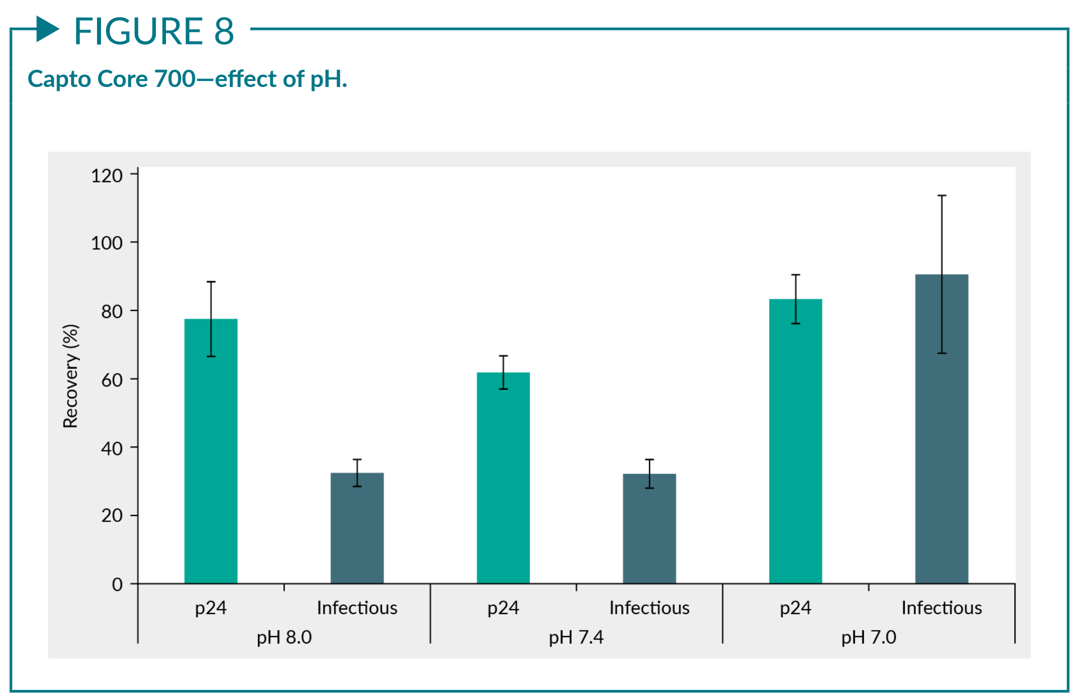
HCP removal was compared between Capto DEAE and Capto Q using a more sensitive protein analysis method compared to the Micro BCA (limit of detection approximately 0.5 µg/mL). Figure 7 shows the results from analysis of each of the different fractions with SDS-PAGE gel using a fluorescent Cy™5 pre-label (limit of detection approximately 50 pg/sample) that has similar sensitivity as silver staining. On the SDS-PAGE image on the right, the LV-containing fractions are marked with arrows. To the left, an image analysis from that SDS-PAGE gel compares the total protein Cy5 signals within the whole lane relative to the load material for the different fractions from Capto DEAE and Capto Q chromatography runs. A minimal difference between the two was observed, even when compared with a more sensitive method.

## POLISHING WITH CAPTO CORE 700

Polishing with Capto Core 700 was employed to remove DNA co-eluting with Capto DEAE. The Capto Core 700 resin, operating in flow-through mode, has high load capacity

FIGURE 8

Capto Core 700—effect of pH.




(up to approximately 30 CV) compared to traditional size exclusion chromatography (0.1–0.3 CV).

This resin has an unfunctionalized outer shell with a pore size (approx. 700 kDa cut-off) that allows impurities to enter and bind to octylamine ligands inside the bead. Meanwhile, large particles such as LVs flow through the resin. It is important that the DNA is degraded by nuclease treatment in order to enter Capto Core 700 beads and be removed. This is a scalable polishing method with low shear and does not require changing the buffer conditions, which is an advantage for retaining stability of the LV.

We wanted to study whether altering the pH could affect the infectious recovery for Capto Core 700 resin polishing. For this experiment, the ÄKTA pure 25 system was employed together with Capto Core 700 HiTrap 1 mL columns.

The Capto DEAE eluates were diluted in buffers with three different pH values before application to Capto Core 700 resin that was equilibrated at the corresponding pH. The buffers were 50 mM Tris with either pH 7.0, pH 7.4 or pH 8.0, all containing 130 mM NaCl and 4% sucrose. The recovery of physical particles (p24 ELISA) was similar, but the infectious recovery was dramatically improved using pH 7.0 compared to pH 7.4 and pH 8.0 (Figure 8). Our hypothesis is that the virus envelope integrity, which is critical to infectivity, is better maintained at pH 7.0 in contrast to higher pH values. Higher pH values could shift dissociation equilibriums toward destabilizing the envelope, and potential integral biomolecules could be scavenged by Capto Core 700 resin, thereby reducing virus infectivity.

Flow rates of 0.3, 0.9, 1.4, and 1.9 mL/min were tested. The flowthrough was collected at approximately 25 CV, or a at the loading volume +1 CV.

The data in Figure 8 is from a run with 0.3 mL/min. Using pH 7.0, higher flow rates were attempted. However, a low effect of flow rates on recovery with pH 7.0 was observed. A 0.7 min residence time could still be run with good infectious recovery. Impurity levels were below the limit of detection using Micro BCA and PicoGreen for all pH values and flow rates (data not shown).

## CONCLUSION

Optimizing LV production and processing involves carefully addressing challenges

associated with virus stability and processing steps. Weak AIEX capture and Capto Core 700 polishing at pH 7.0 proved to be effective strategies, enhancing both physical and infectious virus recovery while maintaining impurity removal. Residence times down to 0.7 min can be used. Following the polishing step, total DNA and protein impurities were below the level of quantitation for all conditions.

# Q&A

**Åsa Hagner-McWhirter**

## Are you working on affinity capture for LV?

**ÅH:** Yes, affinity capture for the more unstable nature of enveloped viruses, such as LV, is of interest but requires completely different types of affinity ligands compared to more stable non-enveloped viruses. Traditional affinity ligands with low pH elution, effective for monoclonal antibodies and AAV, present challenges for LVs. Developing ligands enabling mild elution conditions at close to neutral pH is critical. AIEX is giving rather good infectious recoveries, especially when considering the weak anion exchanger followed by direct dilution of the eluate to reduce salt concentration.

## How would Capto DEAE work for different types of LV feeds?

**ÅH:** In our R&D, we have used LV expressing GFP with the vesicular stomatitis virus G pseudotype. This article shows an optimized process for that pseudotype. Since AIEX is a general type of capture based on charge, I expect it would work with other types of LVs as well, perhaps with some protocol adjustments.



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**Q** Which clarification filters did you use?

**ÅH:** This study relied on a 0.45 µm filter. Alternative options such as depth filters would have also been efficient.

**Q** How long was the loading time for the Capto DEAE resin?

**ÅH:** At large scales, if you don't do the TFF step before, you will end up with quite large volumes, which can lead to long loading times.

We used a 5 mL HiTrap column and loaded a 100 mL clarified feed at 5 mL/min, which took 20 min. We had a 1 min residence time. If you were to go up to a 10 L feed, loading might take a couple of hours depending on your column's dimensions, but you can also consider the option of adding a TFF step up front.

**Q** How did you analyze the impurities?

**ÅH:** We used total protein Micro BCA and also PicoGreen. We used Cy5 pre-labeling, which is a very sensitive method for HCP. Using those assays, we could not detect any impurities after using Capto Core 700.

**Q** Do you have any ideas about what could have been stabilizing the LV at pH 7.0 for the Capto Core 700 step?

**ÅH:** We do not know what it is, but it was very clear that it was probably something small associating with the LV, and then perhaps coming off the virus at the higher pH. It would have to be small enough to be bound inside the Capto Core 700 beads, thereby stripping the virus from a critical stabilizer needed for infection of cells.

**Q** How do you determine the load capacity for the Capto Core 700 step?

**ÅH:** It will depend on your different feeds and how much impurity you have in those feeds. To determine capacity, you load until you see a breakthrough. That would be the impurity binding capacity for the Capto Core 700 step.

It is also very important to do proper nuclease treatment because if you have longer pieces of DNA still present, they will not be able to be internalized. They will go in the flowthrough along with the virus, and you will not be able to remove them.

## How did you measure infectious titer?

**ÅH:** We used a standard cell-based assay. The gene of interest was GFP. We incubated different volumes or dilutions of the sample with cells and then after a certain time, we detached them. We then ran the flow cytometry and measured the GFP-positive cells.

## How did you produce the resins with different quaternization degree?

**ÅH:** We started with the DEAE resin and then did further quaternization of that resin by using polypropylene oxide. We introduced more and more of those positive charges to make it stronger and stronger.

### BIOGRAPHY

**ÅSA HAGNER-MCWHIRTER** has been with Cytiva, based in Uppsala, Sweden, since 2003 and is a downstream and analytics subject matter expert with a broad and deep understanding of viral vector processing. From her long experience, as well as customer interactions, she has gained insights into common challenges and pitfalls in viral vectors and vaccines, as well as general protein purification and analysis. Åsa earned a PhD in Medical Biochemistry from Uppsala University in 1999 based on research around the biosynthesis of proteoglycans. The studies involved polysaccharide structure analysis, enzyme purification, and cloning, as well as characterizing an enzyme reaction.

### AFFILIATION

**Åsa Hagner-McWhirter**
Principal Scientist,
Cytiva

## AUTHORSHIP & CONFLICT OF INTEREST

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INNOVATOR INSIGHT

# Unleashing potential: tackling CAR-T cell production challenges to support the treatment of blood cancer

Narjes Armanet and Felix A Montero Julian

CAR-T cell therapy is a ground-breaking and highly effective personalized treatment involving T cells from a patient's immune system, which are genetically engineered to target and destroy cancer cells. CAR-T cell therapy was considered as a last-line treatment option for patients who meet specific criteria. However, clinical trials demonstrated that these therapies can be used and recommended as a second line treatment for whose cancer has proven resistant to traditional therapies like chemotherapy or radiation or when a patient's illness recurs. As of 2023, CAR-T cell therapy is approved for use in individuals with some types of non-Hodgkin lymphomas, B-cell acute lymphoblastic leukemia, and multiple myeloma. While CAR-T cell therapy has shown remarkable clinical success in treating specific blood cancers, current manufacturing processes have significant room for improvement.

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## INTRODUCTION

CAR-T cell therapy is a ground-breaking and highly effective personalized treatment involving T cells from a patient's immune system, which are genetically engineered to target and destroy cancer cells [1].

CAR-T cell therapy was considered as a last-line treatment option for patients who meet specific criteria. However, clinical trials demonstrated that these therapies can be used and recommended as a second line treatment for whose cancer has proven resistant to traditional therapies like chemotherapy or radiation or when a patient's illness recurs [2–4].

As of 2023, CAR-T cell therapy is approved for use in individuals with some types of non-Hodgkin lymphomas, B-cell acute lymphoblastic leukemia and multiple myeloma. Eligibility for CAR-T cell therapy

is determined based on the nature of the disease and the patient's health status.

## A CHALLENGING AND UNIQUE PRODUCTION PROCESS

While CAR-T cell therapy has shown remarkable clinical success in treating specific blood cancers, current manufacturing processes have significant room for improvement. The CAR-T cell therapy process follows a multi-stage system that typically spans several weeks. The individual stages of production for these therapies, that are all products in the market autologous therapies, can also be challenging and complex, requiring strict quality checks and short timelines [2,5–7]. The main steps involve:

### Leukapheresis

After drawing blood from the patient, white blood cells are separated from the red blood cells and other components. The white blood cells contain the T cells that will be crucial for later CAR-T cells development. Once white blood cells are collected, the remaining blood is reinfused back to the patient.

### T cell activation, transduction, and expansion

From the patient collected white blood cells, T cells are purified and undergo meticulous genetic modification, which progresses through activation, transduction, and expansion phases. Viral vectors (lentivirus and/or retrovirus) are key in introducing the desired genetic material into T cells, converting them into potent CAR-T warriors.

### Formulation and transport

Activated and expanded CAR-T cells are then formulated in adapted media for injection, transforming into a therapeutic elixir poised to combat blood cancers. The final product is only released after being checking against stringent quality control standards.

Regarding transportation, in the case of centralized manufacturing, there may be logistical concerns with cryopreservation or shipping of the final product. In contrast, decentralized manufacturing models allow to minimize the risk of shipping and stability of the product due to cryopreservation.

### CAR-T cells infusion

The enhanced CAR-T cells return to the clinical team and are administered to the patient via intravenous drip. This transformative infusion marks the culmination of a complex, weeks-long process. Now, the therapy is ready to unleash its potential within the patient's body.

One of the most crucial factors affecting patient outcomes in CAR-T cell therapy is the 'vein-to-vein' time, which refers to the duration between the collection of T cells (leukapheresis) and the infusion of the CAR-T product.

For all US FDA-approved products, it takes three to five weeks for manufacturing and quality assessment to be conducted before the product is ready for use. CAR-T cell products available in the market are manufactured in a specific centralized manufacturing facility, aiming for a turnaround time of 16–33 days. However, this timeframe is susceptible to delays, and failure rates range from 1 to 18% [8].

Prolonged manufacturing times have been correlated with a potential decline in CAR-T cell potency, as indicated in studies such as the one conducted by Saba Ghassemi *et al.* in 2018 [9]. This decline in potency raises concerns about compromising the therapeutic impact of the administered CAR-T cells.

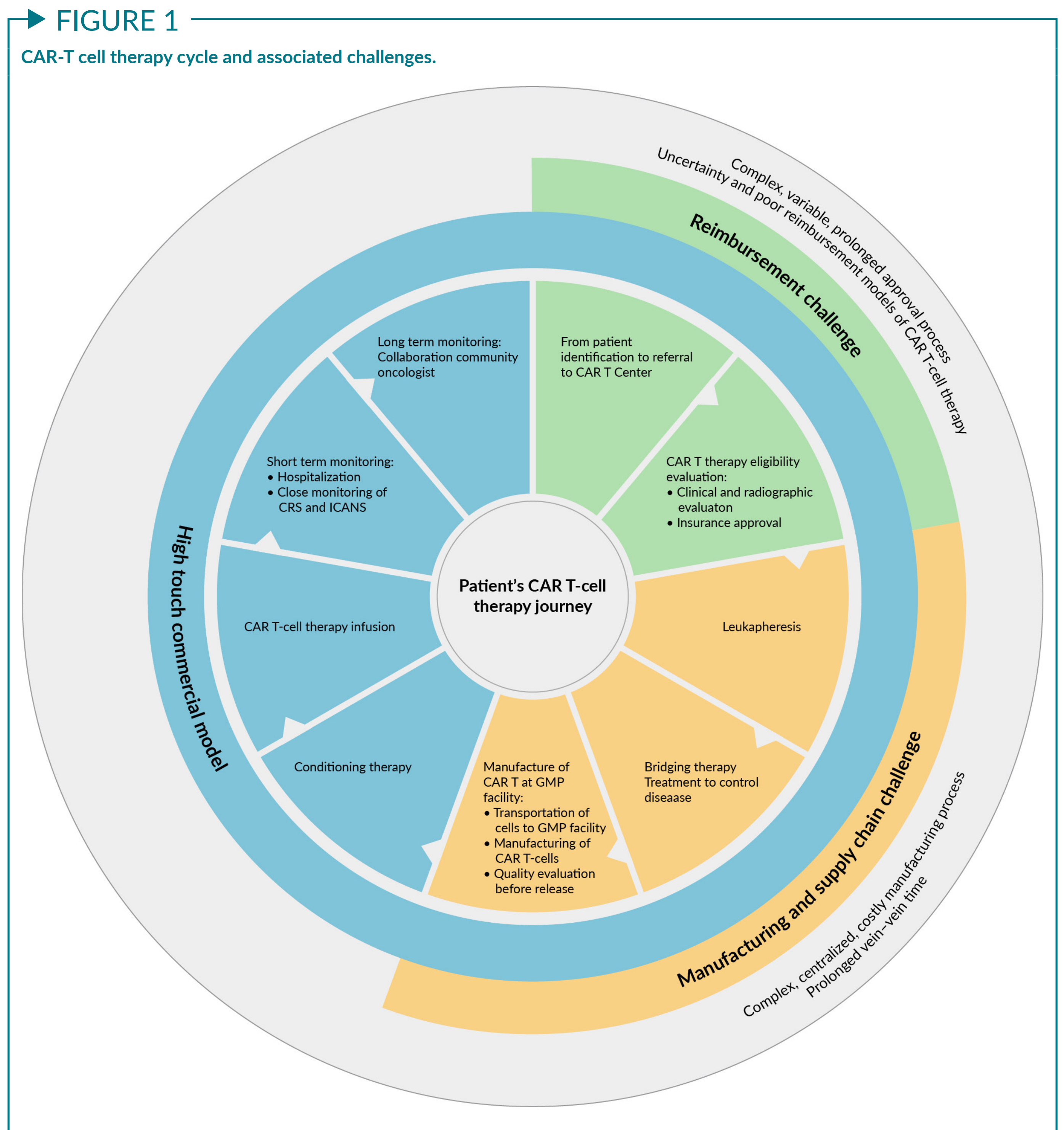
Alternatively, the 'vein-to-vein' time is also influenced by other steps such as shipping to the site of patients, regulatory testing, insurance processes, and broader operational facets, of all whom collectively hold substantial implications for patients with progressive diseases.

The waiting period often imposes patients to undergo additional bridging therapies, which can increase the risk of side effects and complications. Delivering the treatment to the patient as quickly as possible is, therefore, critical because the patient's clinical status could deteriorate very rapidly, and any delay could harm the patient's chances of survival. The current duration for 'vein to vein' time is problematic, particularly for patients in the advanced stages of the disease, as it can impact their eligibility for CAR-T therapy (Figure 1) [10,11].

Because of all the CAR-T products are autologous, another critical parameter is the chain of identity. To maintain the chain of

FIGURE 1

CAR-T cell therapy cycle and associated challenges.



identity, labelling and tracking of material, from leukapheresis collection all the way through CAR-T cells administration, must be extremely well documented to avoid the administration to patient of a wrong batch of CAR-T product.

## THE IMPORTANCE OF SAFETY TESTING IN CAR-T

In the production of CAR-T cell therapies, multiple tests are conducted throughout the process, from the early stages to the final release of the treatment. It is crucial to prioritize safety to minimize the risks associated with these therapies, particularly contamination. These essential tests include mycoplasma testing, sterility testing, and bioburden testing, environmental monitoring, and endotoxin detection [12–15].

### Mycoplasma testing [12]

- Mycoplasma testing is a critical evaluation to detect the presence of mycoplasma, a type of bacteria that can contaminate cell cultures and potentially affect the quality and safety of therapeutic products. Ensuring the absence of mycoplasma is essential to maintain product integrity and patient safety;
- Mycoplasma contamination poses a significant challenge in biologics development and production, potentially compromising cellular products and the safety of biopharmaceuticals. Regulatory agencies worldwide mandate mycoplasma testing during development and manufacturing. Biopharmaceutical companies conducting mycoplasma testing must adhere to Chapter USP <63> Mycoplasma Tests by the United States Pharmacopeia (USP), and European Pharmacopoeia (EP) Chapter 2.6.7 [16,17];
- The traditional method has a turnaround time of 28 days (Figure 2). Making this test critical. A nucleic-acid test-based methods are allowed and give results in one hour to few hours.

### Sterility testing [11]:

- Sterility testing involves assessing the absence of viable microorganisms in the product and verifying that it is free from any potentially harmful bacteria, yeasts, or molds;
- Sterility is paramount in the manufacturing of CAR-T cell therapies. Any microbial contamination can lead to severe adverse patient reactions, making sterility testing critical for product safety;
- Biopharmaceutical companies conducting sterility testing must adhere to Chapter USP <71> Sterility Tests by the United States Pharmacopeia (USP), and European Pharmacopoeia (EP) Chapter 2.6.1, which specify a turnaround time of no less than 14 days [18,19]. However, alternative rapid microbiological methods are allowed to be used to reduce the sterility testing time.

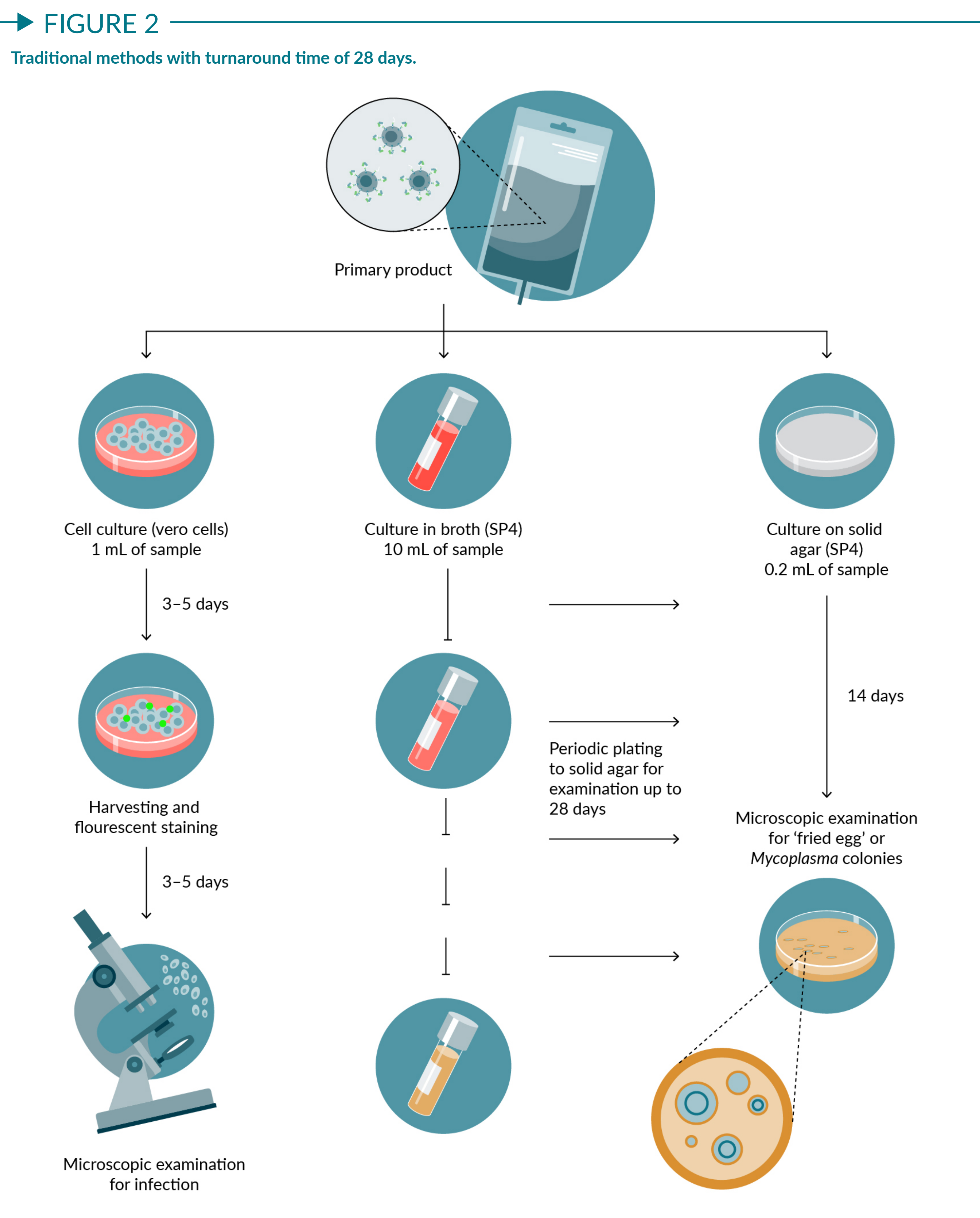
These tests play a vital role in the quality control of CAR-T cell therapy manufacturing, ensuring that the final product is free from harmful contaminants, thereby maintaining the safety and effectiveness of the treatment.

## A DYNAMIC EVOLUTION IN INDUSTRIAL PROCESSES

CAR-T cell therapies are fresh infusion products with a limited shelf life, presenting unique challenges for traditional microbiological testing methods (14-day sterility testing, 28-day mycoplasma testing), which were initially designed for less time-sensitive applications. To meet the urgent demand for expedited therapy delivery to patients, CAR-T cell therapy manufacturers have implemented an evolving industrial process that involves [20]:

FIGURE 2

**Traditional methods with turnaround time of 28 days.**




The mycoplasma analysis for cell and gene therapy products is a traditional testing method that follows specific requirements. The primary product undergoes analysis through three methods: 1) Culture on cells with mycoplasma contamination assessed via fluorescence microscopy. 2) Culture of the primary product in medium followed by subculture on permissive solid agar. 3) Direct culture onto solid agar, incubated for 14 days and examined for distinctive mycoplasma colonies.

- **Increasing digitalization and automation:** this shift toward greater digitalization and automation is crucial to optimize production capacity, reduce costs, and generate valuable data;
- **Reducing turnaround time:** manufacturers are looking to employ rapid methods and modularity to speed up the production process and time for release testing, ensuring that patients receive therapies more quickly;
- **Enhancing instrumentation:** the simplification and improvement of instrumentation aims to reduce complexity in producing CAR-T cell therapies.

Taking a closer look at where CAR-T manufacturing processes could be further adapted, industry pioneers as Kite Pharma recently acknowledged the necessity for rapid quality control methods and emphasized the importance of early QC involvement in the design and development phase. Experts at the company have proposed early monitoring and development of methods with low invalid rates, alongside early investments in rapid methods and innovative lab-in-a-pouch technologies, for use in processes such as mycoplasma testing.

Experts from the Dana-Farber Cancer Institute also highlighted the necessity of mycoplasma testing in product release and the need to address current challenges encountered in ensuring product safety. In response, the institute is exploring testing methods that can be completed in less than 5 min, without advanced laboratory training, where results are available in under one hour and with a reduced risk of contamination.

The changing landscape of CAR-T cell therapy production comes in response to the increasing demand for swift and effective patient care. Driven by the urgency to provide timely access to these transformative treatments, researchers and industry leaders are refining manufacturing processes (e.g., 'Cell Shuttle' of Cellares, platform of OriBiotech) to optimize efficiency and ensure more patients can benefit from CAR-T cell Therapies promptly. With this commitment manufacturers are seeking to transform healthcare delivery, emphasizing scientific rigor and accessibility.

## ADDRESSING THE SKILL GAP IN CELL AND GENE THERAPY

A further challenge faced by manufacturers of these complex therapeutics is the current shortage of skilled workers in key areas that are critical to producing, testing, and delivering these innovative treatments. The shortage extends to manufacturing, analytical development, testing, and quality control roles [21].

One of the most significant barriers to addressing this workforce shortage is the cost of training. While hands-on laboratory training is essential for success in the field, it can also be prohibitively expensive. Both candidates aspiring to enter the industry and educators seeking to provide relevant training face challenges associated with the costs involved. Implementing automated equipment and instruments is one potential solution to bridge this workforce gap. The wider usage of these types of technological advancements could significantly aid the industry in addressing the shortage of skilled personnel and the associated training costs [20].

## WHAT DOES THE FUTURE POTENTIALLY HOLD FOR CAR-T CELL THERAPY?

The future of CAR-T cell therapy is marked by ongoing efforts to streamline and expedite safe, effective, quality treatment delivery to patients **(Figure 3)**. Examples of successful implementations of shorter timelines could be given by University of Pennsylvania's 3-day manufacturing process, Novartis's T-charge, and next-day manufacturing platforms of Gracell Biotechnologies [22].

Manufacturers are actively exploring strategies to minimize the 'vein-to-vein time', but maintaining the efficacy of these product, and employ new solutions to combat other

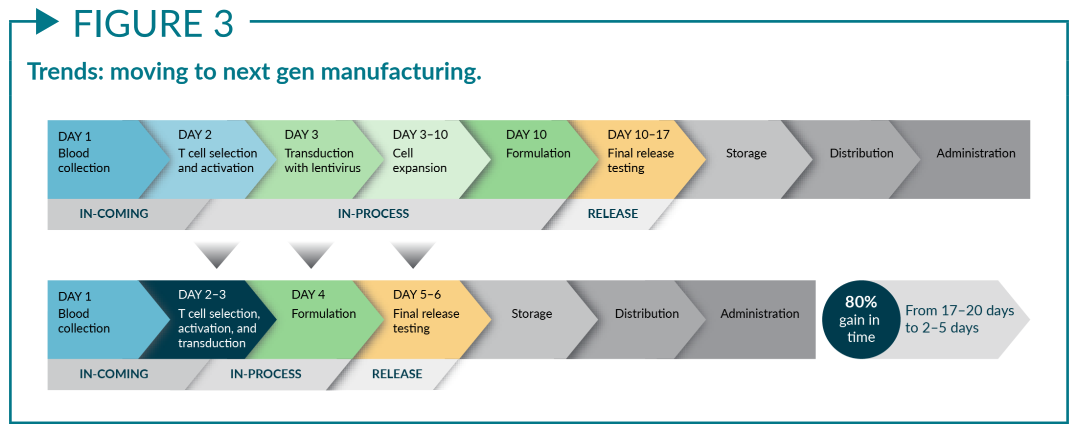



FIGURE 3

Trends: moving to next gen manufacturing.

challenges, such as low production volume, short product shelf life, handling of complex raw materials, tracking and data integrity, scalability and patient demands [14].

Additionally, the expedited nature of shorter manufacturing requires heightened vigilance for:

- Rapid sterility testing. Pharmacopeia are developing new chapters (USP <1071> [Rapid Microbial Tests for Release of Sterile Short-Life Products: A Risk-Based Approach], USP <72> [Respiration-Based Microbiological Methods for the Detection of Contamination in Short-Life Products], and EP 2.6.27 [Microbiological examination of cell-based preparations]) to help release short-shelf life products such as CAR-T cell therapies using modern rapid microbiological methods;
- Potential lentiviral vector persistence, such as replication competent lentivirus testing;
- And finally, control of vector copy number that can potentially alter expression of cellular genes and contribute to tumorigenicity.

A promising solution with the potential to provide cost-effective solutions [23], lies, next-generation CAR-T Therapies in association with CRISPR-Cas9 genetic editing technology and *in vivo* induced CAR-T cells using nanoparticles loaded with mRNA's coding for CAR genes [23]. Though in their early stages, preclinical data suggest that nano-delivery systems for *in vivo* CAR-T cells hold promise for optimizing their efficacy and overall cost [24]. Over the past decade, CAR-T cell therapy has made significant progress, with multiple products available for clinical use.

The industry's focus on digitalization, automation and rapid testing methods reflects its commitment to providing patients with timely access to this life-saving treatment. As the field continues to evolve, CAR-T therapy holds promise as a transformative approach to cancer treatment, offering renewed hope to many patients who have exhausted other options.

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AFFILIATIONS

**Narjes Armanet MD PhD**
Senior Global Medical Advisor,
bioMérieux

**Felix A Montero Julian PhD**
Senior Scientific Director,
bioMérieux

AUTHORSHIP & CONFLICT OF INTEREST

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INNOVATOR INSIGHT

# Strategic raw material selection for cell therapy commercialization

**Kasey Kime, Lili Belcastro, and Kyle Hondorp**

In the ever-evolving global landscape of cell therapy development, selecting quality raw materials is crucial for achieving clinical and commercial milestones. In this Innovator Insight article, Kyle Hondorp asks industry experts, Kasey Kime and Lili Belcastro, to share insights into the key factors influencing raw material selection throughout different phases of development. Kyle Hondorp also discusses the Gibco™ Cell Therapy Systems (CTS™) portfolio of fit-for-purpose media and reagents, cell therapy instrumentation, and viral vector systems, which are GMP manufactured, safety tested, and backed by regulatory documentation to support cell and gene therapy developers along their path to commercialization.

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**Q** KH: What are key factors that organizations should consider when selecting critical raw materials to cater to both early- and late-phase development requirements? How can this help ensure a seamless transition during development?

**KK:** It is important to plan for success, and this involves discussing the regulatory documentation early in the development process. My advice is to review your supplier documentation from both a raw material criticality and a phase-appropriate perspective. This is going to help you identify if you think that there will be a need for any additional information about your raw material.

If you think there might be a need for any additional information, have these discussions early and understand what your supplier can offer you. For example, some suppliers can offer raw material master files. This can be a very efficient regulatory pathway for both the drug developer and the supplier. However, some suppliers do not have master files and some regulatory jurisdictions do not accept them. In those cases, you must have an early discussion with your supplier about how they can support you. They might offer you some kind of regulatory documentation package under a confidentiality agreement.

**LB:** One key factor when selecting critical raw materials is thinking about the 'grade' of the materials. Is it manufactured under GMP conditions? Is it being marketed as a research use-only (RUO) product? Knowing this information makes it easier to move from early-phase into late-phase development. If you start with the highest quality, GMP manufactured materials, you decrease the need for comparability studies in late-phase development. However, non-GMP or RUO materials are usually cheaper, so some companies may want to use them in early development to help save money.

Additionally, as you move from early into late phases, it is important to begin thinking about sourcing. Having a dual source for your raw materials is beneficial, but it is important to introduce the dual sources into your process as early as possible. This will avoid the need to do comparability work later on. When using a sole source for your material, you should ensure that the manufacturer has redundant manufacturing capabilities.

## Q KH: How can organizations strike the right balance when selecting raw materials to optimize their production processes while managing costs?

**LB:** As I mentioned previously, there are options for using cheaper materials, which can help reduce costs in early-phase development. In fact, there are no firm requirements that only GMP materials can be used in commercial manufacturing, but that said, using GMP materials is definitely a best practice to adhere to—if you do not use them, health authorities will ask why you chose a lower-quality material when a higher-quality material was available.

The United States Pharmacopeia (USP) 1043 chapter provides guidelines on materials that are very well characterized and have, for example, a national drug code associated with them, as opposed to materials that are RUO, for example. As you move from a higher quality material to a lower quality material, as per USP 1043, there is going to be an increased amount of testing, documentation, and lot-to-lot comparability assessment of the incoming material. It is therefore up to the user to determine whether starting with GMP materials throughout the process might be more cost-effective than using a lower-quality material and having to perform additional testing.

**KK:** To reiterate, you want to make sure in early-phase development that you have done your due diligence on your raw materials, particularly with regard to safety risks like viral safety or sterility. Doing this exercise early is going to help set you up for filing success later on in the process.

**KH**: Kasey, you mentioned screening raw materials early and assessing whether any additional information is needed. Can you share some examples of additional documentation that might be required?

**KK:** One good example is details on adventitious viral agent testing for human or animal origin materials, or further information around viral inactivation and viral clearance. Often, if a supplier has a master file, these details will be in there, so you would not have to worry about them. But for regions that do not accept master files or for suppliers that may not have master files, these are the instances where you want to have those early discussions about the raw material to make sure that you are set up later for any regulatory questions that you anticipate.

KH: Could you comment on which regions do allow for raw material master files?

**KK:** The USA, Canada and Japan all allow for raw material master files. Sometimes, you can submit directly to a health authority on a sponsor's behalf but this approach is not always accepted. We do know that Europe is undergoing review of their pharmaceutical regulations and there is a push to enable raw material master files in Europe. This would be a very welcome amendment.

**KH:** It sounds like having discussions early on with your suppliers can really help ensure you are aligned on the types of regulatory documentation they are able to support as well as provide awareness of your supplier's continuity plans prior to reaching late-phase clinical trials.

Moving on to the growing demand for rapid drug development in cell therapy, what are the unique challenges that the industry faces in selecting and procuring raw materials, and how can these challenges be addressed effectively?

**KK:** Many cell and gene therapies are eligible for expedited drug development, meaning that they move quickly through the clinical trial phases. It is important to consider whether your supplier can actually scale to meet your commercial demand. For example, at Thermo Fisher, we have seen requests to scale-up five times in as little as 3 years for some cell culture media products.

You should also consider change management. Pre-notification of changes to critical raw materials used in commercial processes is important to manage your post-approval CMC obligations. Occasionally at Thermo Fisher, we observe instances where late-phase customers have not subscribed to change notifications, which can cause them unnecessary challenges such as an unexpected comparability study if a change is made to a product.

**LB:** The landscape for cell and gene therapy drug products has been dramatically changing over the past few years. The same goes for the raw materials that are used in cell and gene therapy manufacturing. Just a few years ago, many of the materials used in the cell therapy manufacturing process, for example, were sole-source materials because there were no other options available on the market.

With the rapid growth in the number of specialist companies providing additional options for different raw materials, it is important to perform technical due diligence for each vendor. It is also important to assess the overall raw material risk before you go about selecting a new material or a new supplier. The challenge here is that there are some vendors who are relatively new to cell and gene therapy drug product manufacturing, so they may not be familiar with the specific raw material requirements in this space. It is the responsibility of the user of these raw materials to ensure that this potential second source provider is not only providing good quality, robust materials, but that they are also a robust supplier in terms of their quality systems and their GMP manufacturing processes.

KH: What are some of the main considerations when evaluating a raw material supplier? What criteria should organizations prioritize when choosing suppliers to meet their specific needs?

**LB:** When you are evaluating a new supplier, you must look at the quality of the material, assess the material in your process, and retest any critical attributes that might be on the certificates of analysis (CoA). Before you get to that point though, you need to evaluate the supplier itself. For example, you must evaluate the supplier's top criteria, the GMP manufacturing processes in place, and the quality management systems that are employed. Does the company have a business continuity or a disaster recovery plan B? Do they have regulatory experience? Have they helped support in the filing of any cell and gene therapy drug products before? Do they have a drug master file or a regulatory support file? What is their general experience in the industry?

**KK:** Again, I would focus on documentation. Having access to adequately detailed and specific CoA is important because these are key documents needed for regulatory filing. Currently, there is no standardization for the way in which raw material quality attributes are reported on the CoA. This can lead to variability amongst suppliers. However, there is some general guidance provided within ISO 20399, and this is an area of focus for some industry groups such as the Standards Coordinating Body.

KH: How could organizations ensure the quality and consistency of raw materials throughout the different phases of development and production? Are there any best practices or standards that can be followed?

**LB:** Materials can be handled in a phase-appropriate manner for cell and gene therapy manufacturing. For example, in early-phase development, verification of the CoA can be an acceptable incoming specification for raw materials. As projects move into late-phase

development and commercialization, you should have some testing specification with critical quality attributes that are important for your final product and your process. At the bare minimum, ensure that the methods are validated, and that identity and safety testing is done.

Luckily, there are a lot of really great best practices and standards that are now available, like the USP 1043 chapter for ancillary materials and the ISO 20399 standard for ancillary materials. In addition, there is a lot of great documentation in BioPhorum, including a raw material risk assessment that can help with determining the best materials and the best practices. There are also some ongoing efforts to standardize raw materials with a standardized CoA.

**KK:** I agree with Lily. The various pharmacopoeias' general chapters on raw materials used in cell and gene therapy manufacturing provide excellent advice on raw material selection and qualification. There are also some product-specific US FDA CMC guidelines. For example, ICH Q8 and Q9 provide details of how to implement QbD and quality risk management into raw material selection.

KH: In an era of growing environmental and ethical concerns, how can raw material selection align with sustainability and ethical sourcing practices? Are there any emerging standards in this regard?

**KK:** This is an increasingly important topic, especially considering the efforts to reduce animal testing in certain raw material testing procedures. A good example of this is USP 88, *in vivo* biological reactivity testing, which is an animal-based test for single-use systems that are used in further manufacturing applications. There is a push from industry to discontinue *in vivo* animal testing when *in vitro* testing is more than adequate for this type of use.

In addition, I know that the Center for Biologics Evaluation and Research at the FDA is planning on publishing draft guidance shortly on the use of human and animal origin components in cell and gene therapy manufacturing. I expect that they will touch further on ethical concerns linked to the use of human-derived components.

**LB:** In general, removing any animal- and human-derived raw materials is key. There are many recombinant sources of both sera and proteins that are available and being used in several approved drug products on the market.

In addition, thinking about sustainability, there is a trend towards using single-use materials and having single-use facilities. While these are great for reducing microbial contamination and cross-contamination, they come with the caveat of increased consumption of single-use plastics. This is a tricky situation because, while an increase in the number of single-use plastics being generated and used is not good for the environment, there is a lot of evidence to suggest that moving from stainless steel to single-use bioreactors, for example, can actually decrease carbon footprint by reducing the amount of energy, water, and cleaning solvents that are used.

> From an end user point of view, you should do your research and look to work with suppliers that have a focus on sustainability.

In addition, single-use systems, manufacturers, and users are also exploring ways in which to recycle these plastics in a safe and compliant manner.

From an end user point of view, you should do your research and look to work with suppliers that have a focus on sustainability.

## Q KH: As the cell therapy field continues to evolve, what future trends or innovations do you foresee in raw material selection and supply chain management? How should organizations prepare for these changes?

**LB:** In addition to the Standards Coordinating Body's ongoing efforts to create a standard CoA for ancillary materials, we are also going to be asking suppliers to have an identity test for all materials. This change will definitely affect suppliers—especially if they supply a complex cell culture medium, for example.

For users of raw materials, I hope that additional clarifications will be made by health authorities. Even with all of the great guidance out there, there are still some grey areas.

Lastly, these guidelines are very focused on the quality of material. They answer questions about what kind of release testing should be done, specifications, and things like that. However, there is not a lot of guidance on the actual manufacturing processes. For example, they do not address if a raw material manufacturing process needs to be validated or not. That is a small gap in information that I hope will be addressed in the future.

**KK:** Building on what Lili just said about standardization, I also think that we will see more standardization of critical raw material attributes and hopefully, better analytical methods to characterize complex raw materials like cell culture media or novel lipids. I think that we will also see a shift towards more defined media formulations and a move away from those high-risk components like human- and animal-derived materials in favor of recombinant or synthetic forms.

**KH:** In summary, it is crucial to conduct a raw material risk assessment and prioritize the critical aspects for your business. Additionally, it is important to ensure that your chosen vendors can meet these prioritized needs. Early communication with suppliers is essential to align the required documentation for regulatory filings and confirm their ability to scale up manufacturing to meet late-phase and commercial demands. Lastly, available and upcoming guidelines and risk assessment tools can be utilized to inform raw material selection and qualification decisions.

Thermo Fisher Scientific can help support raw material selection for cell and gene therapy clinical and commercial manufacturing with the Gibco CTS portfolio of products (Figure 1).

- Gibco CTS media and reagents are GMP manufactured in ISO 13485 facilities and undergo extensive QC testing. They comply with US and EU guidelines for ancillary materials to support the transition from discovery through clinical and commercial manufacturing. Comprehensive documentation packages are available including certificate of analysis, certificate of origin, drug master file, and/or regulatory support files.

- Gibco CTS cell therapy instruments include the Rotea™ Counterflow Centrifugation System, the Xenon™ Electroporation System, and the DynaCellect™ Magnetic Separation

FIGURE 1

The Gibco CTS portfolio of products.

| Media and reagents | Cell therapy instruments | Viral vector systems |
|---|---|---|
| GMP manufactured products designed for cell and gene therapy to support your transition from discovery through clinical and commercial manufacturing | Cell therapy instruments, supporting consumables and software products enable GMP compliant, closed system manufacturing and allow for physical and digital integration | Cost-effective, scalable solutions designed to transition your gene therapy workflow from discovery through clinical and commercial manufacturing |

System. These instruments, as well as their supporting consumables and software products, enable GMP-compliant, closed system manufacturing and allow for physical and digital integration. As with the CTS media and reagents, the instrument consumables undergo extensive QC testing, comply with regulatory guidelines, and include comprehensive documentation packages. Additionally, a global team of Thermo Fisher Scientific cell and gene therapy field application scientists and service engineers provide comprehensive support to maximize uptime.

- Viral vector systems such as the AAV-MAX Helper-Free AAV Production System and the LV-MAX Lentiviral Production System offer cost-effective, scalable solutions to support developers' gene therapy workflows. They were designed to provide high-titers with serum-free reagents and protocols for a scalable, suspension-based platform. The viral vector multi-component systems undergo extensive safety testing, comply with regulatory standards, and include comprehensive documentation packages such as the Cell Line Documentation Package.

To learn more about our comprehensive CTS portfolio and support offering to advance your therapies, please visit www.thermofisher.com/CTS.

## BIOGRAPHIES

**KASEY KIME** is a seasoned professional in global quality and regulatory affairs within the life sciences sector, with over 15 years of experience. She presently holds the role of Director of Regulatory Affairs at Thermo Fisher Scientific, where her focus is centered on technology and tools essential for cell and gene therapy manufacturing. Kasey's specific interests lie in areas such as CMC, advanced manufacturing, and companion diagnostics. Her education comprises a BSc in Medical Laboratory Science, complemented by post-graduate degrees in Medical Microbiology, Quality Systems Management, and Business Administration. Beyond her role at Thermo Fisher Scientific, Kasey remains actively engaged by contributing to

the industry, notably through her roles in the Australia and New Zealand ISCT Regulatory Committee and the Alliance for Regenerative Medicine CMC Advisory Group.

**LILI BELCASTRO** is a Senior Principal Scientist at Bristol-Myers Squibb. She leads the Material Sciences group in cell therapy development. Lili has over 15 years of experience in preclinical and clinical cancer biology, cell and gene therapy product development, and method development, working with a variety of complex biological molecules, small molecule inhibitors, ancillary materials, starting materials, and gene editing materials. Lili holds a PhD in Cancer Biology from a joint program with the University of the Sciences and The Wistar Institute in Philadelphia.

**KYLE HONDORP** is a Senior Manager, Product Management for the Cell and Gene Therapy at Thermo Fisher Scientific, where she is responsible for managing the Gibco™ Cell Therapy Systems (CTS™) immunotherapy and adult stem cell culture media and reagents. Kyle initially joined Thermo Fisher Scientific in 2018 as Product Manager for the Applied Biosystems™ PCR plastics and thermal cycler portfolio, after spending 18 years working for Active Motif, a life sciences company focused on providing solutions for epigenetic research. Kyle earned her BSc in Genetics at the University of California, Davis and MBA at California State University, San Marcos.

## AFFILIATIONS

**Kasey Kime**
Director, Regulatory Affairs,
Cell, Gene, and Advanced Therapies,
Thermo Fisher Scientific

**Lili Belcastro PhD**
Associate Director,
Bristol-Myers Squibb

**Kyle Hondorp**
Senior Manager, Product Management,
Cell and Gene Therapy,
Thermo Fisher Scientific

## AUTHORSHIP & CONFLICT OF INTEREST

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INNOVATOR INSIGHT

# Rapid CAR-T cells: accelerating manufacturing to enable fast transition of CAR-T cell therapies to the clinic

**Tamara Laskowski and Kelly Purpura**

CAR-T cell therapies have revolutionized the oncology landscape, leading to unprecedented successes in the clinic. Despite the remarkable progress and vast growth in the number of CAR-T cell programs transitioning into the clinic since the first approvals in 2017, the complex manufacturing processes associated with these therapies present challenges that impact patient accessibility to these potentially curative treatments. The field has begun to explore rapid CAR-T cell manufacturing approaches that enable the generation of products that possess stronger stem-like properties and exhibit robust potency and persistence when challenged *in vitro* and *in vivo*. In the clinic, first-in-human trials of rapid-CAR-T cells support these observations, reporting notable anti-tumor responses from dose-level administrations lower than those used for products manufactured under longer-term protocols. In this article, the advantages of shorter CAR-T cell manufacturing protocols and the benefits of automation will be explored. Lonza will introduce a rapid manufacturing application that consolidates, within a 72-hour automated workflow, all critical steps required for transforming T cells into potent CAR-T cell therapies. The phenotypic and functional attributes of rapid CAR-T cell products manufactured in this platform will be described, alongside a cryopreservation strategy to support the recovery of stable, viable, and functional CAR-T cells.

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Since the initial approvals of Kymriah® and Yescarta® in 2017, the global pipeline of cell therapy, specifically CAR-T cell therapies, has expanded tremendously, seeing growth in both the number of CAR-T programs in addition to expansion in different modalities

of cell therapies. The field has also seen innovation beyond autologous cell therapies.

However, most of these developments will encounter a major bottleneck in manufacturing due to their complexity. Deploying the right manufacturing strategy to enable therapies to reach patients faster is a critical component of driving a successful cell therapy program. The field has looked at alternative approaches to alleviate some of the burdens associated with delays in manufacturing and the deployment of these therapies into the clinic. These strategies include taking an 'off-the-shelf' approach, with focus on implementing allogeneic programs. This enables manufacturing in large batches in a cyclical fashion enabling cryopreservation of therapies that become available to patients when needed. A second approach is to integrate automation early on in manufacturing decisions. Automation can provide an opportunity to streamline a manufacturing process, reduce complexity, increase robustness and reproducibility, and reduce the overall cost of manufacturing. Lastly, processes may be rethought to shorten manufacturing timelines and enable therapies to reach patients faster.

## AUTOMATION IN CELL THERAPY MANUFACTURING

Whether a therapy is autologous or allogeneic, the elements of the manufacturing process will have many commonalities, and automation can be a solution to alleviate many of the complexities that exist when manufacturing either type of therapy. Automation can also offer significant advantages for enabling scalable solutions in both decentralized and centralized manufacturing models.

Additionally, automation reduces the number of manual operations and end-user interaction with the manufacturing process thus reducing sources of error that can impact the final drug product. This is especially important when deploying a decentralized manufacturing strategy where multiple sites must perform the same manufacturing protocol and ensure robust and reproducible process implementation. By reducing labor complexity and ensuring better process control and reproducibility, costs in manufacturing are reduced, supporting the longevity of a cell therapy program through sustainable and efficient operations.

Logistically and operationally, automation can also add substantial advantages, ensuring better-suited commercially compliant QC and QA measures deployed at clinical manufacturing sites and helping to maintain product chain of custody and chain of identity through electronic batch records and documentation.

## INTRODUCING AN AUTOMATED PLATFORM FOR CELL THERAPY MANUFACTURE

At Lonza, cell therapy manufacturing automation is achieved using the Cocoon® platform. This is an automated scalable platform for cell therapy manufacturing that has been deployed in various clinical programs specifically targeting T cell-based therapies, including CAR-T cells and TCR-T cells.

The elements of this platform include the environmental unit, which houses key components essential for manufacturing cell-based therapies, including the ability to maintain a controlled dual temperature zone and a built-in bidirectional peristaltic pump that supports fluid exchanges throughout the many steps in a given process. The Cocoon environmental unit is also equipped with pH and dissolved oxygen (DO) sensors that allow discrete assessments of the culture conditions throughout the manufacturing process.

The next element is the single-use cassette which is the consumable used for each manufacture. This tool enables functionally closed process steps that are applicable to both suspension and adherent cells within both viral and non-viral processes. It includes an integrated cold chamber for internalizing all process reagents and consumables in one form

factor. When required, media and reagents are pre-warmed prior to entering the proliferation chamber where cell culture takes place.

The final element is the software which monitors and controls process parameters and executes each step as programmed in the manufacturing protocol. The software is enabled by a protocol design component that allows protocol steps to be defined and customized according to the user's specifications. Additionally, pH/DO values can be leveraged to adjust media exchanges, recirculation, and oxygenation of culture, and thus enable process modifications to fit the therapy of choice.

Typically, the key steps involved in manufacturing of most cell therapies start with sample collection at a clinical site and transfer of the sample to the manufacturing center. In the initial phase, an optional sample preparation step may occur, followed by selection of the cell type of interest. Subsequently, an activation step is often involved, and cells are then engineered through transduction or transfection. Once the gene of interest is transferred, the cell therapy product is then expanded to achieve required dose levels. Ultimately, the culmination of the process is final formulation and patient administration.

Several of these steps are commonly carried out by operators who interact with the process throughout each stage. By implementing an automated platform such as the Lonza Cocoon, many of these unit operations can be consolidated into one instrument, facilitating and streamlining the manufacturing process, and reducing the opportunities for errors or batch failures. Moreover, through automation of these various steps, greater reproducibility can be achieved in processes performed by different operators at different locations.

## INNOVATING RAPID MANUFACTURING STRATEGIES

Rapid manufacturing increases the speed at which these potentially curative drugs can reach patients. The Cocoon platform can manufacture CAR-T cells in 72 hours or less. One of the key advantages associated with rapidly manufactured products is the maintenance of stem-like properties in the final drug product. This likely leads to a product endowed with higher potency and persistence, thus reducing the need for high dose levels. By ensuring a product that has greater stemness, potency, and proliferative potential, superior persistence *in vivo* can be achieved, driving improved outcomes in patients. Moreover, by accelerating the manufacturing process, the amount of needed materials and reagents decreases, and labor requirements are reduced, thus lowering the overall cost of manufacturing. Additionally, moving from a 10-day process to a 3-day process allows more batches to be manufactured within the same timeframe, thereby producing more therapies for patients, and doing so faster.

Lonza has created a 3-day T cell manufacturing application suitable for CAR-T cells, shown in **Figure 1**.

In the serum-containing X-VIVO™ medium-based protocol, cells were transduced with Lonza-manufactured lentivirus vector delivering a third-generation CD19-CAR. The product was harvested and analyzed for CQAs including memory phenotype and functional response *in vitro* on day 3. Characteristics were found to be maintained without skewing production or altering the overall composition of the product. Moreover, transduction efficiencies averaging 35% of T cells were achieved in this context. $CD4^+$ and $CD8^+$ T cells showed a similar frequency when compared to the starting material, and observable levels of $CAR^+$ T cells were detected in both cell subsets, demonstrating successful transduction of both T cell subpopulations.

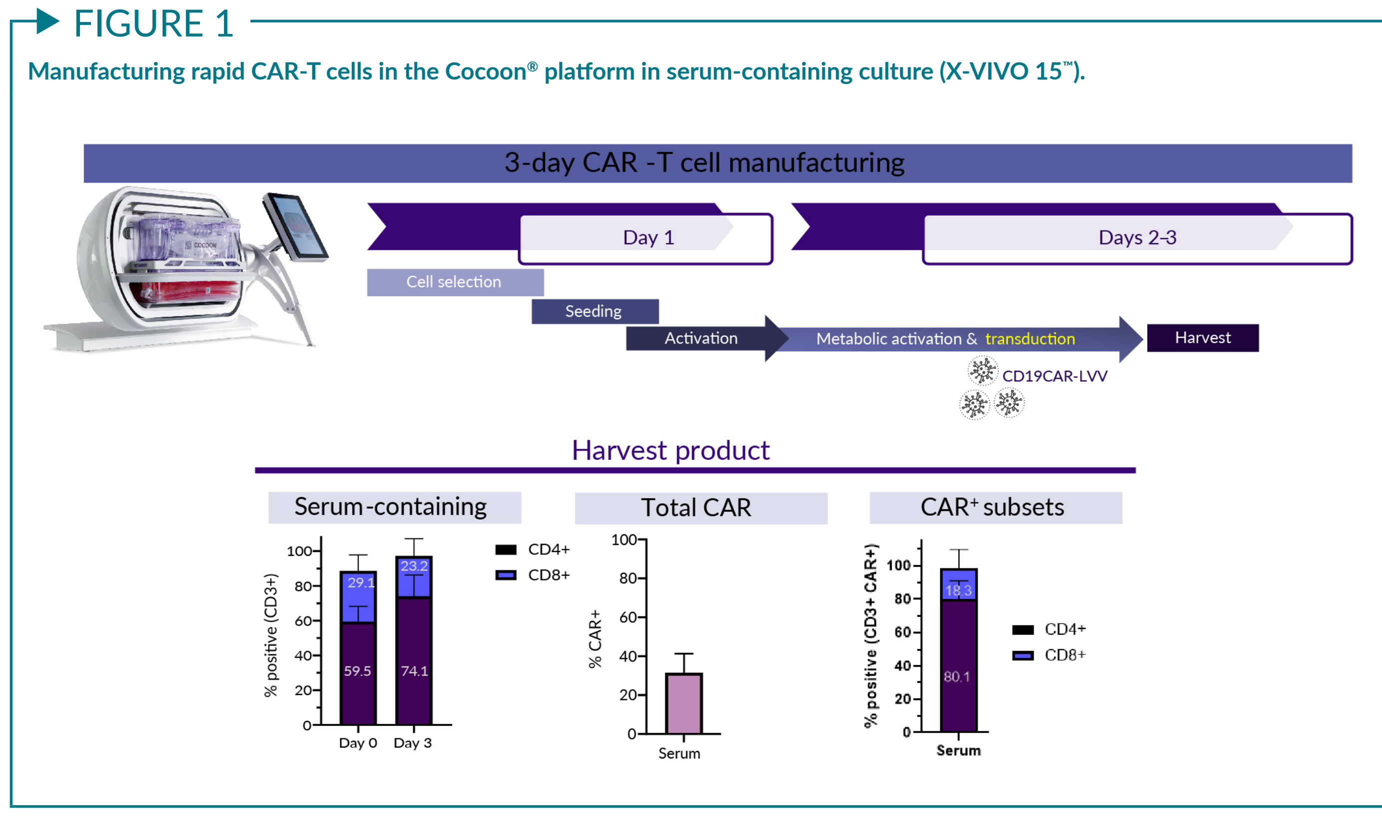
Maintaining the stem-like characteristics of the T cell product is also achievable within short manufacturing. A strategy for interrogating the CAR-T cell products for memory phenotyping was developed. Material from three separate rapid manufactures are profiled in **Figure 2**. Each product is driven into a fate comprising mostly stem memory and central

memory T cells, indicating a more robust phenotype, capable of stronger persistence *in vivo*.

To investigate the potency and persistence of the product further, a persistence anti-tumor cytotoxicity assay was developed, with three stages of tumor challenge. In the first challenge, tumor and CAR-T cells are co-cultured for 3 days. At the end of day 3, the culture is sampled and the performance of the CAR-T cells at eliminating the tumor cells is measured. The second and third challenges involve sequentially introducing further fresh live tumor cells to assess the persistence of

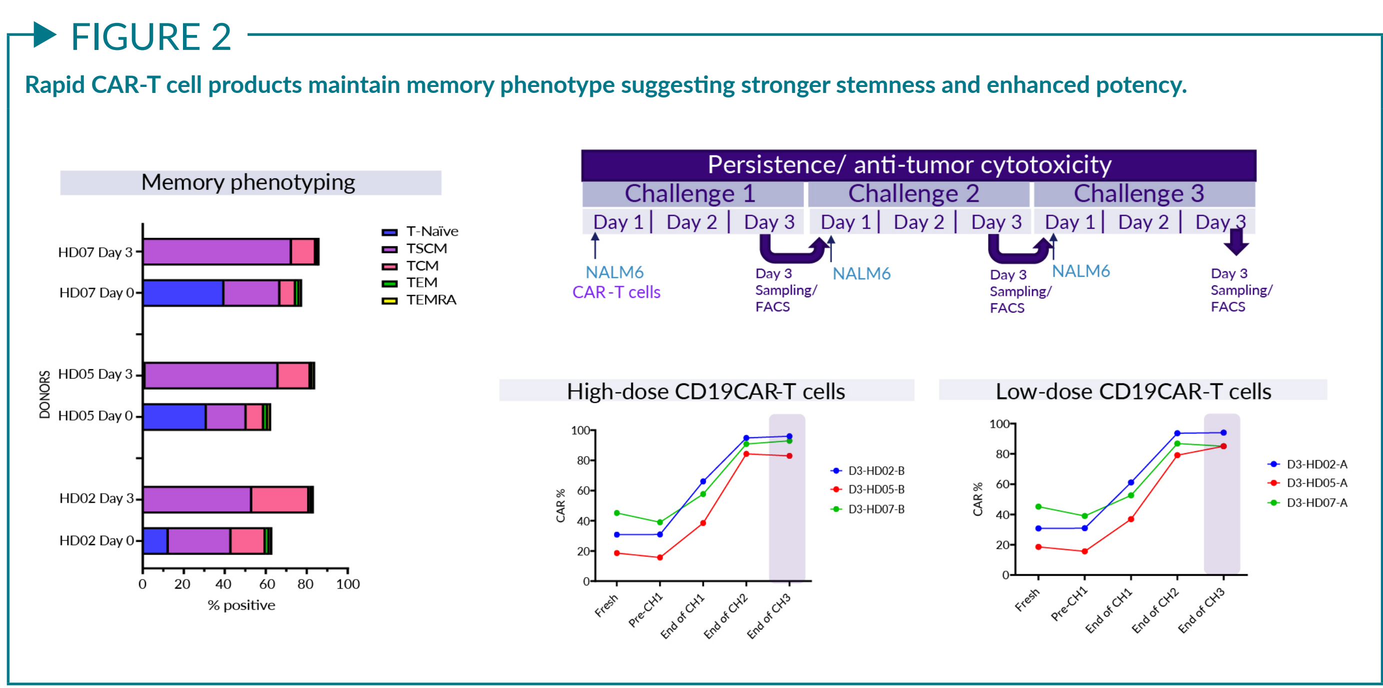
### FIGURE 1

**Manufacturing rapid CAR-T cells in the Cocoon® platform in serum-containing culture (X-VIVO 15™).**




### FIGURE 2

**Rapid CAR-T cell products maintain memory phenotype suggesting stronger stemness and enhanced potency.**



that response. This is performed with both high and low doses of CAR-T cells.

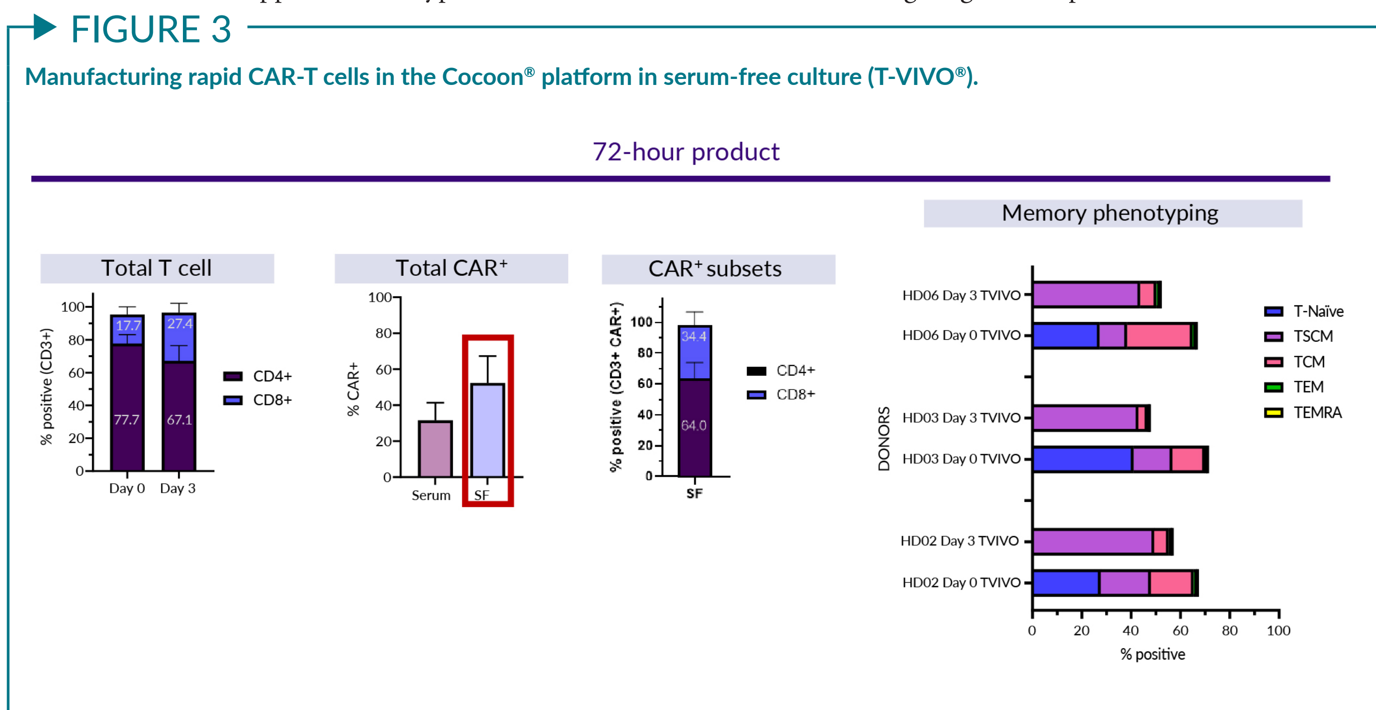
By the end of challenge one, a notable response is seen by the CAR-T cells in addition to an increase in the frequency of CAR-positive T cells. As the CAR-positive T cells increase, a decrease in the tumor cells is seen. All products achieve a high level of tumor clearance at the end of challenges two and three, with the CAR-T cells expanding throughout the sequential challenges and ultimately representing most of the cells remaining. When cells are challenged with an increased tumor burden, they do not experience a decrease in function, instead maintaining the same responsiveness to the tumor. This supports the hypothesis that more robust products lead to robust persistence in this *in vitro* setting.

## DEVELOPING SERUM-FREE CAR-T CELL THERAPY MANUFACTURING

In further modifying and streamlining manufacturing, the possibility of eliminating serum was explored. Serum is a source of variability in a process and presents burdensome responsibilities on the therapy developer to validate serum lots to be utilized in the manufacturing process. The Lonza chemically defined T-VIVO® medium does not necessitate serum supplementation and is highly efficient at supporting the transduction of T cells in serum-free conditions, giving rise to products that

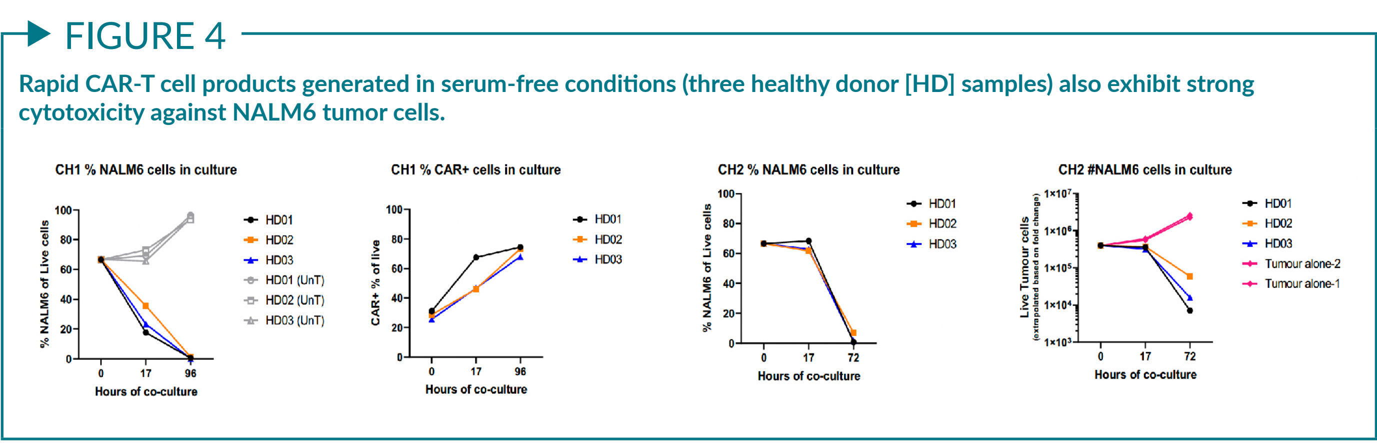
### FIGURE 3

**Manufacturing rapid CAR-T cells in the Cocoon® platform in serum-free culture (T-VIVO®).**




### FIGURE 4

**Rapid CAR-T cell products generated in serum-free conditions (three healthy donor [HD] samples) also exhibit strong cytotoxicity against NALM6 tumor cells.**



possess similar memory phenotypes to those seen in products derived from serum-containing processes (Figure 3).

Manufacturing CAR-T cells in a rapid protocol without serum supplementation was also shown to yield a product with increased persistence and good cytotoxicity through a similar challenge assay as previously described, modified to include two challenges.

The number of live NALM6 tumor cells in the co-cultures decreases over time in both challenges as CAR-T cells in the culture expand in response to the tumor, a finding we observed in three products derived from three distinct donors (Figure 4).

## CRYOPRESERVATION OF RAPID CAR-T CELL PRODUCTS

Next, establishing a robust strategy for the cryopreservation of rapid CAR-T cell products was explored. Various parameters associated with cryopreservation of cells were interrogated and a matrix combination of conditions was performed to refine a strategy for the successful cryopreservation and recovery of products. The parameters investigated included cell density, total volume, cryomedia used (DSMO/DSMO-free), and the type of controlled rate freezing (CRF) platform (liquid nitrogen/electric). To evaluate the various of conditions tested, readouts including total cell recovery post-thaw, recovery of CAR-T cells post-thaw, CAR-T cell expansion in culture, and CAR-T cell potency were measured.

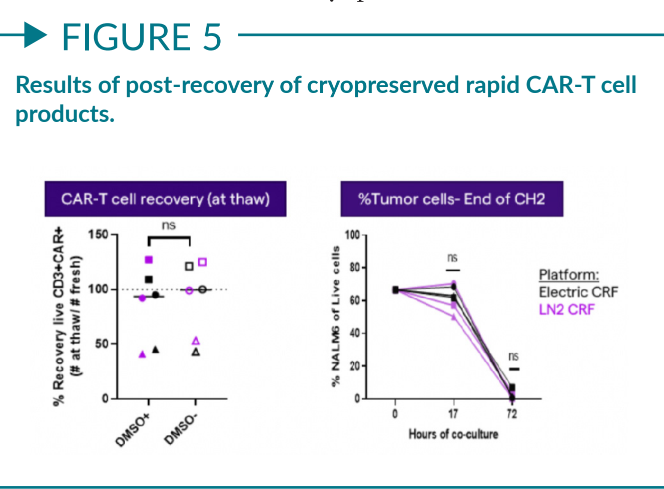
Results for the recovery of CAR-T cells at thaw and CAR-T cell potency are shown in Figure 5. For CAR-T cell recovery, no statistical difference was found, demonstrating that the protocols developed for electric CRF and liquid-nitrogen CRF, when applied in the context of a DMSO-containing or a DMSO-free cryomedia formulation, both lead to robust product cryopreservation and recovery post-thaw. Moreover, as previously shown, all products exhibited strong anti-tumor response, able to eliminate all tumor cells within 72 hours. No statistical difference in the performance of recovered CAR-T cells was identified in this study.

The rapid CAR-T cells were also shown to expand well *in vitro* and maintain robust expression of CAR after thawing, re-activation, and expansion for 6 days in culture supplemented with IL-2 (Figure 6).

Viabilities were high for all products tested. The fold change in the amount of total T cells over 6 days was comparable across the two groups. The CAR-T cells exhibited

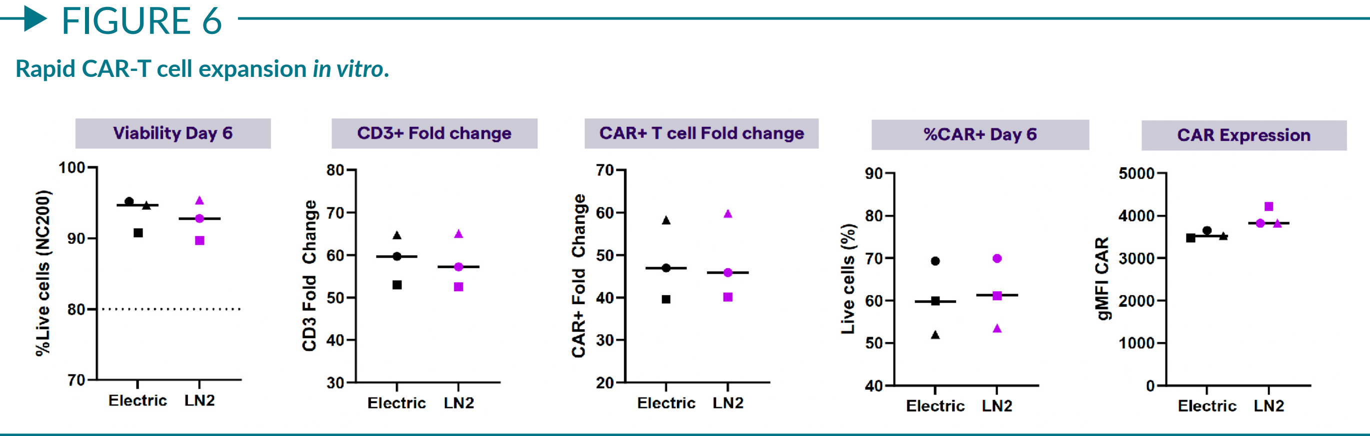
### FIGURE 5

**Results of post-recovery of cryopreserved rapid CAR-T cell products.**




### FIGURE 6

**Rapid CAR-T cell expansion *in vitro*.**



high viability and robust expansion in both systems upon recovery and culture. A high frequency of CAR-T cells was maintained throughout the 6 days in culture with cells showing stable, high expression of CAR on the cell surface.

## SUMMARY

Adopting automation early into manufacturing and building an integrated solution is key to success in the manufacturing of cell therapies. Moreover, shortening the manufacturing of CAR-T cells can provide opportunities for reducing costs and accelerating production. The Lonza Cocoon® Platform enables T cells to be seeded, activated, transduced, and cultured in a streamlined and automated protocol that requires minimal manual touchpoints. Cryopreservation of CAR-T cell products should be robust, reproducible, and enable the recovery of a product that maintains its critical quality attributes associated with function and persistence.

# Q&A

**Tamara Laskowski (left) and Kelly Purpura (right)**

**Q** Is your rapid manufacturing application flexible to use with various media and reagents?

**KP:** Yes—the Cocoon is agnostic to media and reagents. We can use various media including X-VIVO 15 with serum and T-VIVO, in addition to those from other vendors and products. These can be fit into both the rapid process as well as the standard process.

**Q** Have you tested your rapid manufacturing application in the context of different vectors and CAR constructs?

**TL:** Yes, we have tested second-generation CARs and different lentiviral vectors, and have seen equally robust results. We are confident that your vector of choice can work well in this manufacturing process in the context of the Cocoon Platform.

**Q** Are there any specific changes that you recommend when transitioning your CAR-T cell manufacturing process from serum-containing to serum-free culture conditions?

**KP:** We can run similar protocols for both serum and serum-free media. However, we have found that an increase in recirculation for oxygenation in media exchange may be beneficial. This is due to different serum protein levels or how chemically defined media has been shown previously to influence oxygenation. Often, people feed more aggressively for longer processes. However, for a rapid process, aggressive feeding may not be as impactful as we do not typically expand the cells during that short time window. There are minor changes to be made when tailoring to a specific process.

**Q** What are the key differences in the process when transitioning from a standard 9-day to a 3-day CAR-T cell manufacturing?

**TL:** Firstly, a 3-day process is much faster. The expansion phase seen in a longer process will not occur in a 3-day process, so dose levels should be addressed differently. In terms of manufacturing, the critical components, such as transduction and activation, required for transforming T cells into T cell therapeutics will remain the same.

At Lonza Personalized Medicine, our team can help to facilitate adjusting processes and dose levels for those looking to convert a 9-day process to a 3-day process.

**Q** How soon in the development process would you look at manufacturing systems?

**TL:** By designing your process and manufacturing strategy early on, and implementing automation, you can ensure the longevity of the program as it evolves. Today, your need may only be for a small number of patients in the early clinical phase of your program, but the hope is that, as you progress, you will be treating hundreds of patients. Planning a strategy to support the evolution of your program should be considered early on, as these can be critical decisions.

Have you observed any key differences between CAR-T cell products that are generated in serum-free conditions compared to serum-containing conditions?

**KP:** We do often see enhanced transduction in the CAR-T products that are generated in serum-free conditions for lentiviral vectors. There is also strong recovery and maintenance of the T cell expression. Phenotypically, the product is also high in stem and central

> "...some of the same QC assays can be applied to rapid products. Others may have to be modified."

memory expression. That being said, we do not typically see many differences between the products.

## Q Can you outline any common regulatory and technical challenges for rapid CAR-T cells?

**TL:** It is a relatively novel concept for the field, which is still adjusting to what the regulatory requirements for the release of a rapid product will entail. Fortunately, there are some rapid CAR-T cell products already reaching clinical trials, so, in some respects, that pathway has already been defined for us. At Lonza, we have partnered with professionals in the field who have established robust CAR-T cell programs to leverage that understanding.

With respect to product release, some of the same QC assays can be applied to rapid products. Others may have to be modified. We have seen that even at the lower starting cell input in rapid manufacturing, we have sufficient material to assess CAR expression, profile product viability, and perform some of the critical testing that is typically required for the release of these drugs. We await regulatory guidance to further define what the required QC and release assays for rapid CAR-T cell products will consist of.

## Q Do you have *in vivo* data comparing rapid versus standard expansion times for pK and efficacy?

**TL:** Yes, we do. We have infused these rapidly manufactured products in animals with aggressive leukemias and have seen *in vivo* anti-tumor responses. We hope to soon release these data. We have seen encouraging results compared to standard 9-day manufactured products.

## Q In rapid CAR-T manufacturing, the final doses are low. The Cocoon has an optimal seeding of between 50 and 100 million cells. What is the benefit of using the Cocoon here?

**TL:** There are various benefits. The data shown here were generated based on a seeding number of 200 million T cells. We have higher flexibility and can seed more than 200 million in the rapid manufacturing application. We have seen that we essentially recover the number of cells that we put in, or even a few more, as the cells experience subtle proliferation in the 3 days of manufacture. We have made the same observation when

running patient samples through this application. If you seed 500 million, you likely get 500 or 550 million cells in the output. There is a degree of flexibility in the input to achieve the output you wish.

The expectation for a rapid CAR-T cell product is that you can do more with fewer cells. In our *in vivo* models, we have been able to use dose levels as low as 10-fold or even 100-fold lower than a standard dose for a typical 9-day CAR-T cell product.

## Q Did you use CAR-T concentration within clinical levels to limit the adverse events related to CAR-T cell therapies?

**TL:** We performed a cytokine analysis of these products to identify any of the culprits associated with toxicities. We did not see these in the assays that we have run for these products. *In vitro*, we selected doses based on standard settings for *in vitro* assays. Some of those determinations have translated well to *in vivo* studies (in which we did not see evidence of toxicities), but it can be a bigger leap to infer in-human outcomes based on *in vitro* data.

## Q For rapid CAR-T, how do you demonstrate that there is no vector in the final product when levels of free virus quantified by qPCR concern the time of culture? Is there a specific product for washing the cells before harvest?

**TL:** This is something we considered when we first thought about shortening manufacturing and driving cell engineering of any cell type via viral vector in a rapid context. There are a few approaches we are taking to address this. We have looked at how early the expression of CAR can be seen, which in some cases can be as early as day 2. We have also kept side cultures on a small scale and kept the actual rapid manufacture in the Cocoon for the longer term to observe the progression of those T cells. In these experiments, we have carefully monitored the kinetics of the expression of CAR as a result of transduction.

We have also performed vector copy number (VCN) studies on these products. Combined, the data show that day three is a snapshot in CAR expression, and normally by day 5, we have stable expression of CAR. Typically, that number changes ~10 to 20% from day 3–5. We have seen that samples on day 3 can give slightly higher VCN numbers. We are continually designing studies to understand expression kinetics and its correlation with VCN.

## Q Were the target cells irradiated in the challenge experiment?

**TL:** No, they are not irradiated. They are live, fully competent cells in their rapid growth phase. We make the tumor challenge very challenging for the CAR-T cells.

"We have frozen product in both vials and bags and we have not seen substantial differences between these vessels."

## Q What is the maximum manufacturing scale for the Cocoon system?

**TL:** Currently, the 9-day process we have described leads to about 4 billion total cells. We have new iterations of the Cocoon cassette that will enable yields of up to 10 billion cells.

## Q What are some of the common issues expected in the use of the Cocoon system?

**TL:** This will vary depending on the application of choice. As with any other automated manufacturing platform, you need to adjust and design your protocol according to the therapeutic you are developing. In the Cocoon, we can gain insight into how the culture is progressing through pH and DO measurements. This allows us to change process parameters, and optimize key steps in the manufacturing process.

The process development team at Lonza can adjust and refine the Cocoon manufacturing process to meet the needs of your program and prevent problems from happening in the manufacture of your specific product. There is no one-size-fits-all approach, but rather a customizable strategy that allows us to minimize hindrances and obstacles that may impact production.

## Q Is your final T cell product frozen in vials or bags and have you seen a difference between these configurations?

**KP:** We have frozen product in both vials and bags and we have not seen substantial differences between these vessels.

**TL:** The study we have shown here is a snapshot of that. We hope to release an application for cryopreservation in which we delineate the differences between vials and bags, data obtained from those studies, and the protocols we have generated for the CRF-based cryopreservation methods.

## Q What is the data collection interface of the Cocoon to connect to an upper system?

**TL:** At Lonza, we have a central digitization tool that can serve as an upper system. Directly in the Cocoon, the software captures every step in the process in electronic batch

records that can be connected to each specific manufacture. The interface of the Cocoon can be connected to the broader chain of custody and chain of identity tools to enhance data retrieval from sample collection to product infusion.

**Q** Any ideas on applying this methodology for natural killer (NK) cells?

**TL:** This is something that we are looking into. The field of NK cells is continually gaining traction, and we are beginning to see much lower dose levels of NK cells now in clinical trials. This could bring together the idea of memory NK cells with increased potency, reflecting some of the same features that are highly sought after in T cells. We are already working on NK protocols that may be suitable.

**Q** Have you checked the viability and efficacy of the rapidly manufactured CAR-T cells *in vivo*?

**TL:** We have conducted studies in hematological patient-derived xenograft mouse models, and we hope to release the data soon in 2024.

**Q** What has been the highest number of cells that this process has been tried with?

**TL:** We have input 200 million cells to keep the processes manageable and consume less of our viral vector. That input material is flexible–you would require more viral vector particles to transduce a higher number of cells, but the platform can accommodate higher numbers of T cells at the input.

**Q** It has been shown that in rapid manufacturing, high transduction efficiency in the early days may be observed due to pseudotransduction. Have you checked the transduction efficiency of the rapidly manufactured cells after more days in culture?

**TL:** Pseudotransduction has been reported in some cases, and we have methods for identifying if pseudotransduction is occurring or if we have stably transduced T cells. We have kept these cells in culture for 9 days and interrogated the product downstream. We have performed these assessments in products generated in alternative platforms and in the Cocoon. We have completed extensive interrogations on the metabolic profile of these cells and we observed that the product is stably transduced. We have not seen any indication of pseudotransduction.

Is there an option to select CD4/CD8 cells with the Cocoon?

**TL:** The Cocoon has an integrated magnetic selection capability, and in 2024, we hope to release a CD4/CD8 solution applicable to the Cocoon, which will be compatible with rapid or standard T cell therapy manufacturing.

## BIOGRAPHIES

**TAMARA LASKOWSKI** is the Senior Director and Head of Clinical Development at Lonza Personalized Medicine. She supports the transition of novel adoptive cell therapies from pre-clinical stage into clinical manufacturing. Laskowski received a dual doctorate degree in the fields of human molecular genetics and immunology from the University of Texas Health Science Center at Houston, where her work focused on genetic engineering of immune cells and stem cells. As a post-doctoral fellow at MD Anderson, she developed platforms for off-the-shelf production of genetically-modified NK and T cells. Subsequently, Tamara transitioned to a Senior Scientist position at the Immunotherapy Platform led by Dr James Allison where her work focused on characterization of immune response to solid tumors in the clinical setting. For her contributions to the development of innovative analysis platforms, Laskowski was awarded a fellowship to the National Science Foundation Innovation Corps and received a prize for outstanding performance.

**KELLY PURPURA** has a decade of experience in process optimization for stem cell and immunological cell cultures. She has specialized in process translation to the Cocoon® Platform at Lonza for various applications and for immunotherapy. Kelly obtained her MASc and PhD at the University of Toronto through the departments of Chemical Engineering and Applied Chemistry and the Institute of Biomaterials and Biomedical Engineering with work focused on the phenotypic and functional analysis of osteoprogenitor hierarchy and on controlling the emergence of hematopoietic progenitor cells from pluripotent stem cells.

## AFFILIATIONS

**Tamara Laskowski**
Global Head,
Clinical and Process Development,
Lonza Personalized Medicine

**Kelly Purpura**
Process Development Manager,
Clinical Development,
Lonza

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.

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# Reduction of encapsidated hcDNA during AAV production

## Stable and transient vector production without encapsidated chromosomal DNA

**Michelle Hussong**, Head of Molecular Biology and Analytics, Viral Vectors—Genomic Medicine, Cytiva,
and **Helmut Kewes**, Scientist Vector Technology, Viral Vectors—Genomic Medicine, Cytiva

**AAV-based vectors have emerged as the preferred delivery tool for *in vivo* gene therapies. One concern with the use of such vectors are possible process- and product-related impurities. An important example of the latter is encapsidated host cell DNA (hcDNA).**

**This Fast Facts poster presents a method to reduce the amount of this impurity in recombinant AAV (rAAV) preparations to address the steadily increasing quality requirements for AAV-based vectors.**

Residual cell substrate DNA, encapsidated during transient or stable AAV manufacturing processes, holds the risk of causing immuno- or genotoxicity. Safety concerns associated with non-therapeutic nucleic acids are related primarily to possible genotoxicity. This risk has led to the US FDA's requirement of <10 ng residual hcDNA per dose. However, achieving this goal is currently a major challenge, particularly considering the high doses often needed with AAV-driven gene therapy treatments.

To overcome this challenge, Cytiva has developed a process, universally applicable for transient and stable AAV production systems, that decreases the amount of encapsidated hcDNA in AAV preparations. Combining this process with the HEK293-based inducible stable ELEVECTA™ producer cell lines from Cytiva provides a suitable platform for large-scale and high-quality production of AAV vectors.

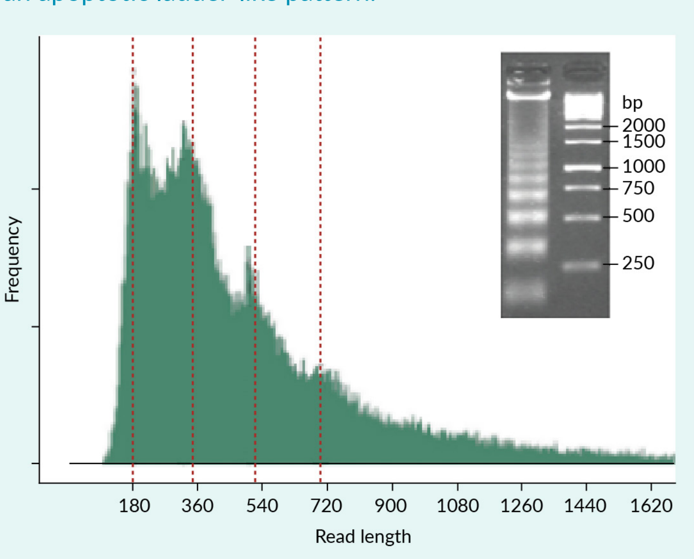
### ENCAPSIDATION VIA APOPTOSIS

Analysis of the read length distribution of encapsidated hcDNA revealed a periodic pattern that corresponded to the apoptotic ladder, which occurs during the induction of apoptosis. (Figure 1). Hereby, chromosomal DNA is fragmented into low molecular weight (LMW) fragments by internucleosomal cleavage.

### REDUCING ENCAPSIDATION

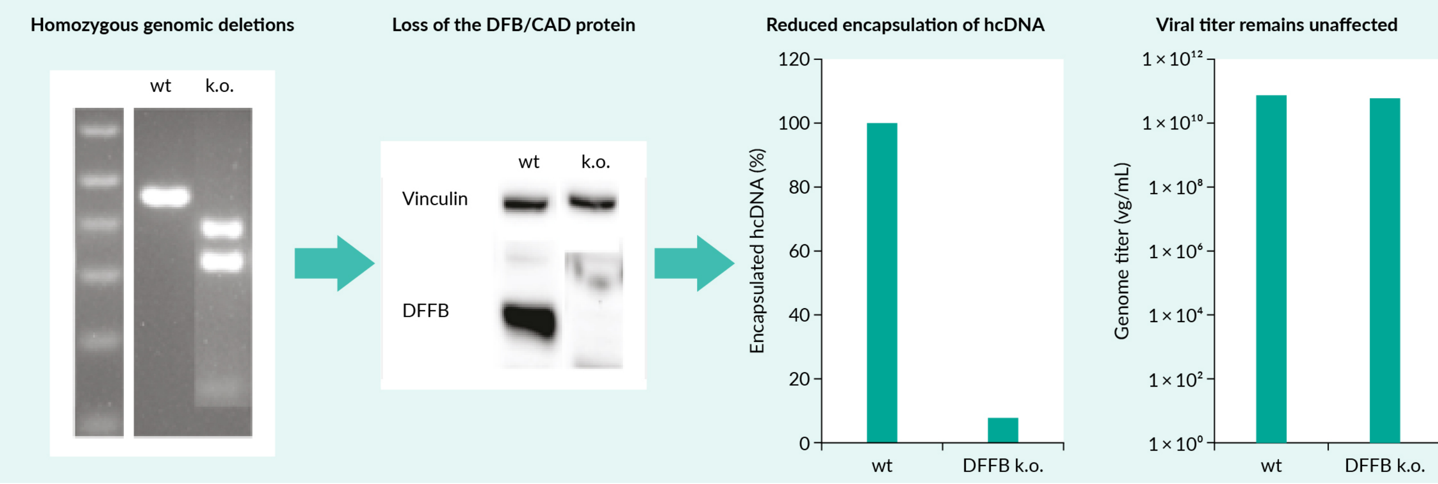
By inhibiting key players in the apoptosis-related caspase cascade (e.g., by knock-down experiments) encapsidation can be significantly reduced. One approach is to efficiently knock-out the DFFB gene/caspase-activated DNase (CAD), which acts as an endonuclease creating dsDNA breaks at internucleosomal linker regions and results in low molecular weight (LMW) DNA.

**Figure 1.** Analysis of size distribution of read length reveals an apoptotic ladder-like pattern.




| Peak | Peak maxima in PacBio seq (bp) | Apoptotic ladder (bp) | Deviation |
|---|---|---|---|
| 1 | 181 | 180 | +1 |
| 2 | 325 | 360 | –35 |
| 3 | 507 | 540 | –33 |
| 4 | 708 | 720 | –12 |

**Figure 2.** New genetically modified HEK293 cell line reduces encapsidated hcDNA without affecting viral titers.




HEK293 cell line was genetically modified with the complete loss of DFFB/CAD. This modification can reduce encapsidation of hcDNA by up to 95% without affecting viral titers (Figure 2) to enhance AAV quality. In this study, E1A and RN7SL oncogenes were undetectable by PCR in AAV particles produced in cells with DFFB deletion (results not showed).

### SUMMARY

As the dosages of AAV-based therapies increase, quality becomes more and more important. A novel technology from Cytiva enables the reduction of encapsidation of host cell DNA by up to 95% in transient as well as stable production systems without affecting cell growth or viral titers.

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FASTFACTS

# Optimizing HEK293-based viral vector production, part 1: cell lines and media

**Sandra Klausing**, Head of Product Development Cell Line and Media, and **David Ede**, Process Technology Manager—Gene Therapies, Sartorius

**In the initial stages of process development, viral vector producers encounter cost and time constraints due to the labor-intensive nature of achieving high titers across various cell lines, serotypes, media, and transfection reagents. This poster will cover the optimization of cell lines and media for HEK293-based viral vector production. This poster is part 1 of a two-part series. See part 2 on process and analytics here.**

## GENERATION OF NEW CLONAL HEK CELL LINES

Clonal cell lines are crucial for efficient viral vector production. The process involves adapting to different media, pool evaluation, and CellCelector device selection for high-performing clones. Ambr® 15 bioreactor stations enable high-throughput clone evaluation, leading to the generation of multiple clones. Process optimization follows, using methods like Ambr®15, with product and process analytics for functional titer and transfection efficiency. Statistical tools, especially DOE (Design of Experiment) software MODDE®, play a crucial role, shortening the 4–6 month process.

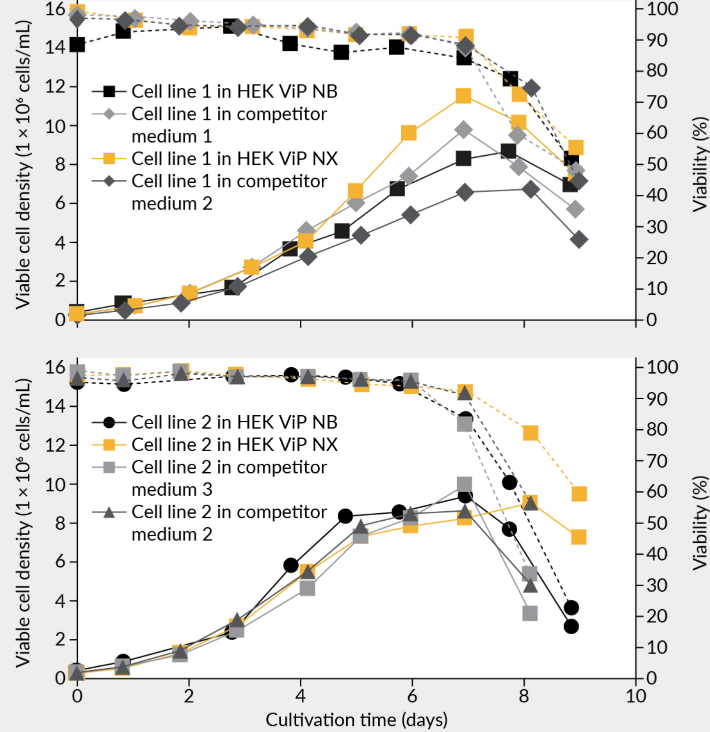



**Figure 1.** Media screening of viable cell density (cells/mL) and viability (%) across four mediums of cell line 1 (HEK ViP NB, Competitor Medium 1, HEK ViP NX, and Competitor Medium 2) and cell line 2 (HEK ViP NB, HEK ViP NX, Competitor Medium 3, Competitor Medium 2) across a cultivation time of 9 days.

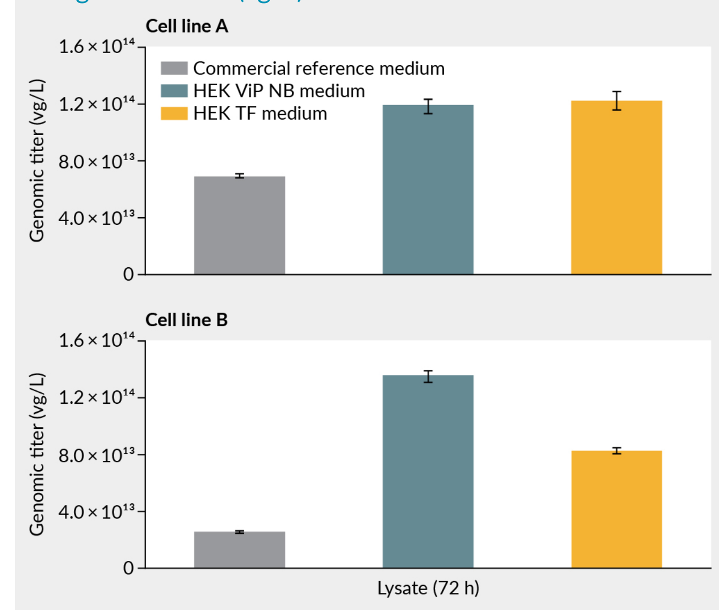
## UNBLOCK EXISTING BOTTLENECKS IN AAV AND LV PRODUCTION

Initiation of the screening process occurs once you have both the media and the cells. The initial focus should be on growth and assessing peak cell densities. We conducted this across four media and two cell lines (commercial HEK293 cell lines), and despite similar performance in the four media, prolonged culture in HEK ViP NX medium for cell line 1 yielded higher peak cell densities, which can be seen in Figure 1.

Viral vector production was then assessed in AAV2 and various cell lines using a reference and HEK ViP NX medium. Some cells show substantial gains by switching the medium; for Cell Line A, there was no major difference in the choice of medium, while Cell Line B exhibited a clear preference for HEK ViP NB medium, which can be seen in Figure 2.

The same principle can be applied to lentiviral vector production while assessing transfection with PEI-MAX-:DNA and harvest after three days. While genomic copies were consistent across all tested media, the functional titer was highest in HEK ViP NX medium which highlights the significance of selecting the right medium for optimal functional titer in both AAV and lentivirus production.

**Figure 2.** Media screening for the production of viral vector AAV2 across two cell lines in three different media (commercial reference, HEK ViP NB, HEK TF) measuring the genomic titre (vg/L).



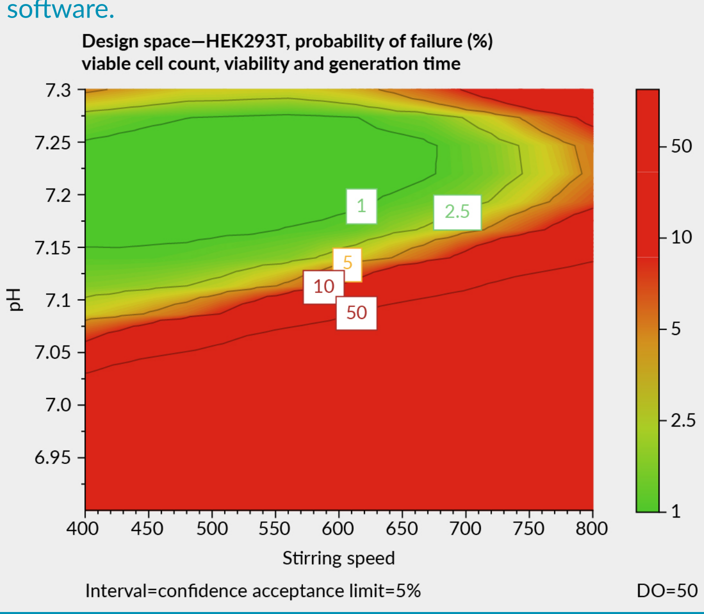

## DEFINING THE DESIGN SPACE OF HEK293T CULTURE PARAMETERS

Application of DOE to optimize cultivation parameters of HEK239T cells in Ambr®15 examined viable cell count and viability as responses in a four-factorial design, assessing all high and low-factor combinations. Cultivating cells for four days with nine different parameter combinations revealed significant differences in cell densities. The critical parameters of the study were identified as stirring speed and pH, with dissolved oxygen having minimal impact. The design space plot highlighted the 'sweet spot' for success with a 7.15–7.25 pH and stirring speed below 650 as shown in Figure 3.

## SUMMARY

Understanding growth dynamics is crucial, and considering the platform process with diverse cell lines is essential to screening and selecting a medium that best suits the entire platform for maximum AAV titer. DOE is fundamental to the optimization of the culture parameters of the cell line.

**Figure 3.** Identifying critical process parameters of HEK293T cell cultivation in Ambr®15 using MODDE® software.

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FASTFACTS

# Optimizing HEK293-based viral vector production, part 2: process and analytics

**Sandra Klausing**, Head of Product Development Cell Line and Media, and **David Ede**, Process Technology Manager—Gene Therapies, Sartorius

**Viral vector producers face challenges in terms of cost and time during early process development, as achieving high titers against multiple cell lines, serotypes, media, and transfection reagents has traditionally been a laborious process. Once an initial screening is complete, other parameters must be optimized to ensure process efficiency. In this poster, transfection and production optimization for viral vectors is performed with a simple DOE screening using MODDE® software.**

## TRANSFECTION OPTIMIZATION

Even with an optimized protocol, only a minor fraction of plasmid DNA (pDNA) is taken up by cells, and even less so by the nucleus. High transfection efficiency does not always correlate with protein expression and viral titers. Thus, optimization of the transfection process is highly relevant and may vary in different cells. To select the best transfection reagent for each HEK293 cell line, transfection efficiency and titer must be considered, rather than cell growth. Cell growth is not an indicator for an optimal reagent, as high transfection often leads to reduced growth due to possible toxicity and cell stress.

In this study, transfection optimization for AAV production was performed using a simple DOE screening. The aim was to optimize DNA:PEI ratio for one difficult, low-producing HEK293 cell line, from an initial 1:4 ratio (and initial PEI concentration of 12 µg/mL). Two different media were tested with a cell density at transfection of 3E6 cells/mL and a pDNA concentration of 3 µg/mL.

The results shown in **Figure 1** demonstrate that transfection efficiency and genomic titer were not at peak with previous conditions. Optimal concentration is 1:3.1 DNA:PEI with both media. This study demonstrated that an optimal set point for DNA:PEI ratio can be chosen with a simple DOE in MODDE®.

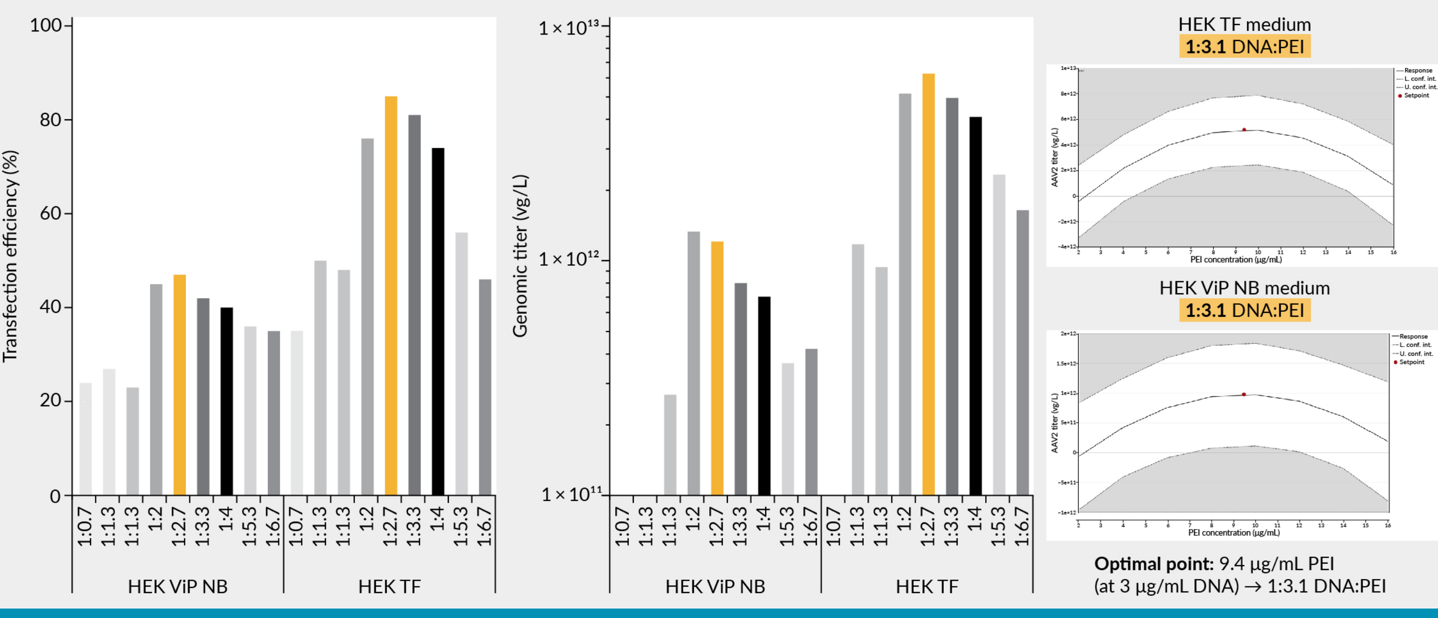


**Figure 1.** Optimization of DNA:PEI ratio for low-producing HEK 293 cell line by simple DOE screening.

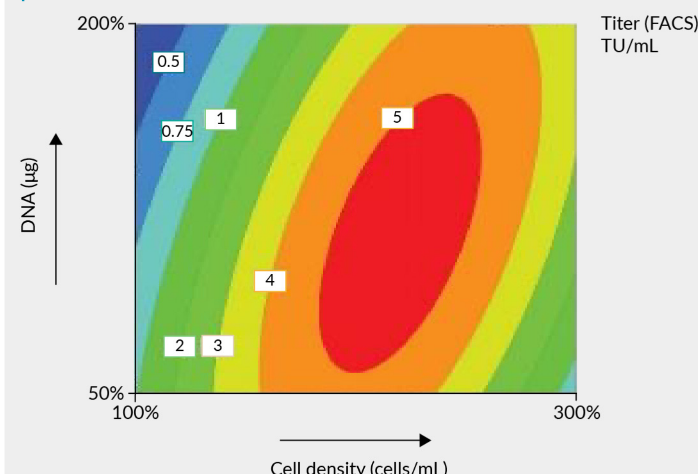


**Figure 2.** Contour plot obtained using MODDE® software showing the optimal seeding density for lentiviral vector production.

## LENTIVIRAL VECTOR PRODUCTION OPTIMIZATION

In this study, Ambr® 15 and MODDE® were used in tandem for the production optimization of lentiviral vector in collaboration with an Oxford-based CDMO–Oxgene. The factors tested were media, cell density, stirring speed, pH, dissolved oxygen, pDNA, transfection reagent, ratios, and feeds. A contour plot example for cell density optimization is shown in **Figure 2**.

With an intuitive approach, many variables would need to be tested after each other, but the DOE approach applied allowed for the optimization of 9 parameters in just two DOE runs. This equated to a 50% reduction in time spent enhancing and validating the viral vector platform. After DOE modeling, a 34-fold increase of overall infectious titer was achieved **(Figure 3)**. This shows how a holistic approach can save time and costs in process optimization.

This poster is part 2 of a two-part series. See part 1 on cell lines and media **here**.

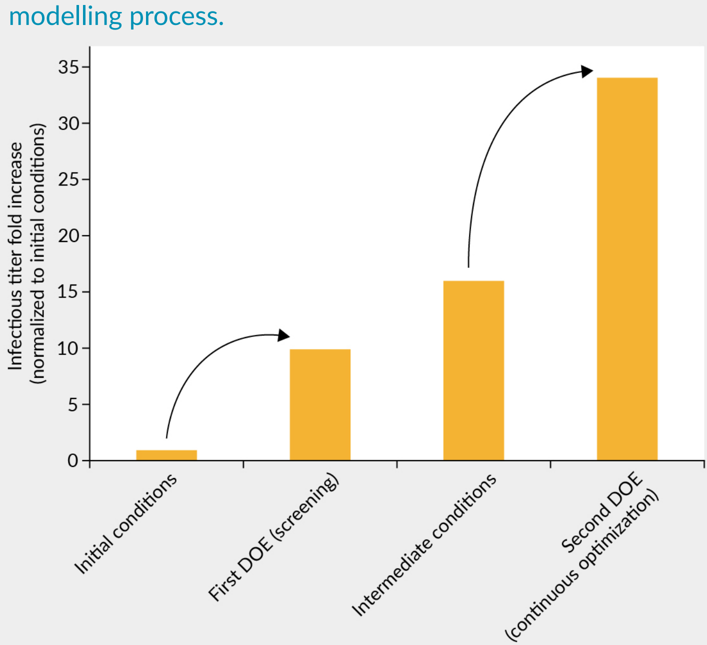


**Figure 3.** Increase in infectious titer throughout the DOE modelling process.

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FASTFACTS

# High-performance analytical solutions for residual DNA testing in cell and gene therapy using real-time PCR or digital PCR technologies

**Christina Bouwens**, Global Market Development Manager for Digital PCR, Thermo Fisher Scientific

**To comply with regulatory impurity analysis standards for cell and gene therapy products, precise quantification of residual DNA concentrations below set thresholds is crucial. The ongoing need for characterizing residual DNA fragments and detecting oncogene presence adds complexity. This poster explores how these challenges can be addressed through a comparative review of PCR technologies and in particular, the use of digital PCR (dPCR) with cell and gene therapies.**

## COMPARATIVE REVIEW OF PCR TECHNOLOGIES

PCR encompasses three main categories: bulk PCR, quantitative PCR (qPCR), and digital PCR (dPCR). Bulk PCR utilizes endpoint data to determine the presence or absence of the target, while real-time qPCR offers real-time measurements, relying on a standard curve for relative quantitative data. In contrast, dPCR achieves absolute quantification of known genetic targets without the need for a standard curve. This absence of a standard curve enhances precision and reproducibility compared to other quantitative methods, making dPCR a valuable tool for accurate and reliable quantification in genetic analysis.

**Figure 1.** Comparative list of quantitative qPCR and dPCR, describing the benefits of each to allow for appropriate selection of technology for an intended purpose.

**qPCR**

- Broad dynamic range (can quantify across a large concentration range)
- Direct readout in mass: no conversion needed
- Standard curve routinely required
- Well accepted by regulatory bodies: simple and well-established path to regulatory approval
- Validated workflow including sample prep ensuring constant LOQ throughout the process
- DNA extraction frequently required to remove PCR inhibitors

**dPCR**

- Emerging technology for resDNA testing
- Highly sensitive, absolute quantification (reduces variability, particularly for low concentration samples)
- Redout in number of target copies per reaction, conversion to mass necessary
- Standard curve not required for quantificaton
- Ease for regulatory approval for residual DNA unknown at this point
- Validated workflow: potentially no sample prep required
- Improved tolerance to some PCR inhibitors

dPCR and qPCR are complementary technologies. qPCR is widely utilized for its broad dynamic range and high-throughput capability. The choice of employing one or the other option depends on workflow preferences and application-specific requirements, showcasing the versatility of both technologies **(Figure 1)**.

## THE APPLIED BIOSYSTEMS™ resDNASEQ™ dPCR KIT: E1A FRAGMENT SIZING

To minimize biological activity in viral preparations, particularly concerning oncogenic sequences, residual fragment length analysis to quantify residual host cell DNA over and under 200 base pairs (bp) is required. The E1A oncogene in HEK293 cells is significant in gene therapy and rAAV production. Removal of HEK293 cells, especially fragments >200bp, is critical. The resDNASEQ dPCR E1A fragment length kit aids in quantitation, showing high linearity and efficiency. Validated for gene therapy matrices, the kit exhibits excellent sensitivity and a broad dynamic range. Measured concentrations of dPCR demonstrate positive results across long, medium, and short amplicon size assays **(Figure 2)**, supporting quantitative results for residual DNA across a broad range of concentrations.

## resDNASEQ™ dPCR: *E. COLI* HOST CELL RESIDUAL DNA QUANTITATION

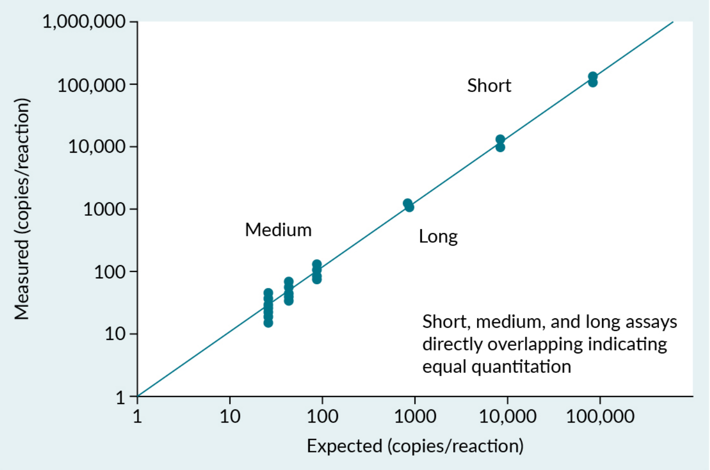
The resDNASEQ dPCR *E. coli* DNA kit provides all necessary reagents and controls, ensuring accurate quantitation of residual *E. coli* DNA. With extremely high specificity and no reactivity to unrelated DNA, the kit demonstrates reliable performance. The included assays exhibit high linearity and efficiency across a broad DNA concentration range, showcasing the kit's quantitative capabilities. Similar to the E1A kit, the *E. coli* residual DNA kit offers a broad dynamic range, illustrated through dPCR data **(Table 1)**. Performance beta testing confirms the kit's reproducibility, highlighting its ability to deliver high-quality data consistent with the specified performance claims.

**Table 1.** Relative accuracy of quantitation of residual *E. coli* DNA (expected, mean, and mass) across a range of DNA concentrations using resDNASEQ dPCR *E. coli* DNA kit.

| Standard | Expected concentration (cp/ul) | Mean of measured concentration (cp/ul) | Concentration in mass (pg/ul) | Relative accuracy (%), measured/expected | CV% |
|---|---|---|---|---|---|
| SD1 | 10,000 | 8887.42 | 86.29 | 89 | 0.96 |
| SD2 | 1000 | 1061.59 | 10.31 | 106 | 0.26 |
| SD3 | 100 | 96.37 | 0.94 | 96 | 2.62 |
| SD4 | 3 | 3.06 | 0.03 | 102 | 13.78 |
| SD5 (LOD) | 1 | 0.87 | 0.008 | 87 | 53.61 |
| NTC | 0 | 0 | 0 | n/a | n/a |

SD: standard dilution; LOD: limit of detection; CV: coefficient of variation.

**Figure 2.** Fragment lengths of E1A oncogene after the removal of HEK293 cells using the resDNASEQ™ dPCR E1A fragment length kit. Data compares the measured and expected copies per reaction of long, medium, and short amplicon sizes.




## SUMMARY

The resDNASEQ dPCR E1A kit focuses on minimizing biological activity in viral preparations, quantifying residual HEK293 DNA with high sensitivity and linearity. The *E. coli* resDNASEQ dPCR DNA kit ensures accurate quantitation with high specificity, linearity, and a broad dynamic range. Both kits offer strong reproducibility, meeting specified performance claims for reliable quantification in genetic analysis and impurity assessment.

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