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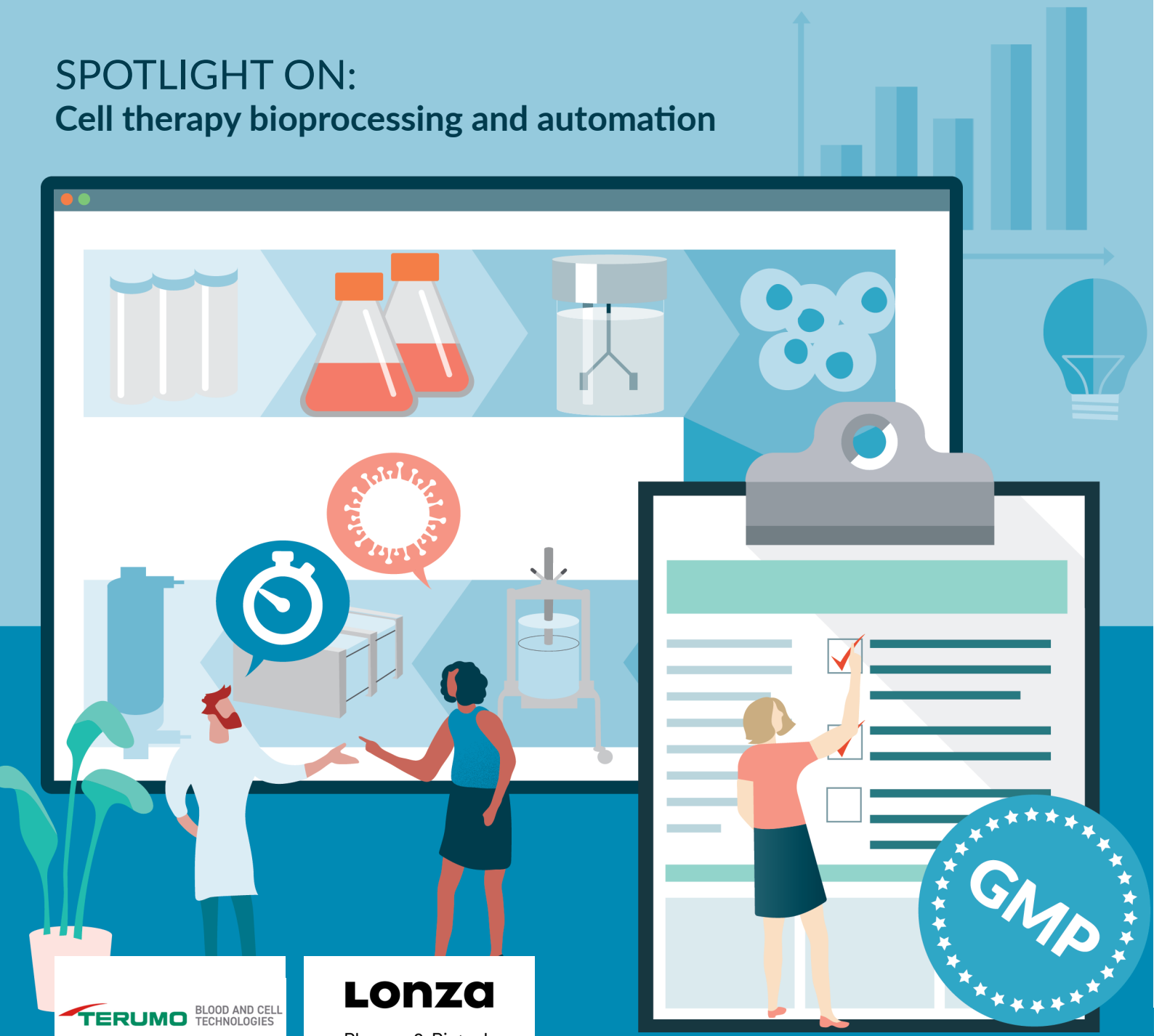
CELL & GENE THERAPY INSIGHTS

SPOTLIGHT ON:
Cell therapy bioprocessing and automation



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Improving allogeneic manufacturing workflows

Evan Zynda & Aditi Singh

Allogeneic T-cell therapies have the potential to improve both the efficacy and accessibility of life-changing cellular therapeutics. However, before this new paradigm can be fully established, there remains a need for improved manufacturing workflows to enable consistent production of highly efficacious allogeneic T-cell therapies. One of the core components of these workflows is the media used for the expansion of healthy donor T cells. In particular, a medium that can rapidly facilitate high levels of T-cell proliferation, maintain the desired central memory T (T_{CM}) phenotype, and increase immune responses by enhancing the production of cytokines such as interferon gamma (INFγ) is a much-needed solution. Gibco™ CTS™ OpTmizer™ Pro Serum-free Medium (SFM), a novel medium formulated for allogeneic workflows, has been developed to meet this need. The following article details the potential of CTS OpTmizer Pro SFM to improve both workflow efficiency and overall therapeutic efficacy.

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INTRODUCTION

The development of cellular therapeutics utilizing T cells has been a major revolution in the treatment of a variety of hematological malignancies. This is particularly true for autologous cell therapies – with over 500 clinical trials currently ongoing worldwide, including a number of therapies which have already achieved both US Federal Drug Administration (FDA) and European Commission (EC) approval [1]. However, as these current treatments are patient-specific, time-consuming

and expensive to produce on a dose-by-dose basis, moving to the development of allogeneic T-cell therapies has the potential to make these cutting-edge therapies more accessible. Through the innovation of a novel medium formulated specifically for allogeneic T-cell therapy manufacturing, the shift toward allogeneic therapies can be accelerated and their efficacy and accessibility can be increased.

Increasing therapeutic efficacy with allogeneic cell therapies

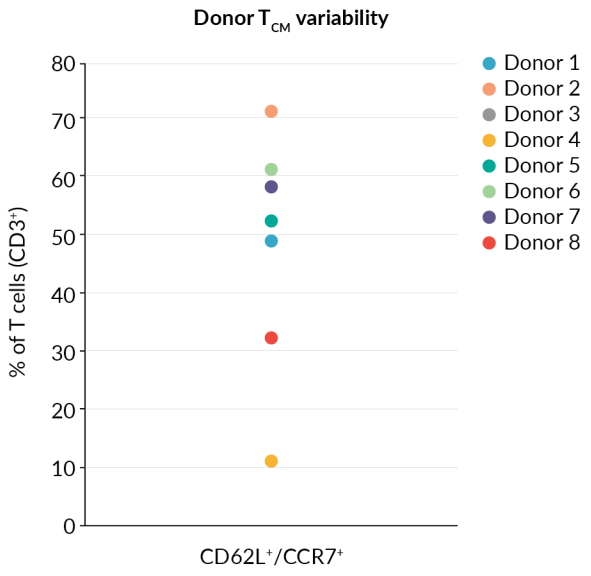
In addition to the well-documented logistical and economic benefits of using allogeneic over autologous T-cell therapies [2], the overall efficacy of the therapeutic intervention is a key factor. This provides an opportunity for allogeneic T-cell therapies, as current autologous treatments often lead to highly variable clinical responses depending on the disease being treated. For example, when looking at clinical trial data, some diseases such as B-cell acute lymphoblastic leukemia have a complete response (CR) – used as a measure of successful treatment – of around 80% whereas others have a CR of below 58% [3].

As a result, a lot of research has been focused on identifying the cause of this variance and it is now widely accepted that this difference is primarily cell based. The results have highlighted the need to use a robust population of young central memory T (T_{CM}) cells, due to the increased engraftment, persistence, and anti-tumor immune response exhibited by these younger T-cell types [4]. This presents an issue for the manufacture of efficacious autologous therapies using cells obtained from diseased patients as they typically present with highly variable T_{CM} populations (Figure 1). This variability usually results from a combination of the patient's disease and any other treatments they may have been exposed to, such as radio- and chemotherapy. To increase efficacy and, crucially, improve clinical outcomes, more suitable donor cells are required.

Healthy donors have been shown to display more consistent, and often higher T_{CM} counts. Therefore, allogeneic workflows could play a key role in improving efficacy through the activation and expansion of cells with more desirable characteristics.

FIGURE 1

Central memory cell variability across donors.



Pre-expansion sampling of T-cell populations in 8 donors for the percentage of cells expressing CD62L and CCR7 phenotypes – characteristic of T_{CM} cells. Variability is between 13% and 72%.

The power of cell expansion media

Much like autologous therapies, manufacturing allogeneic therapies with maximal efficacy cannot be achieved without the use of an optimal T-cell expansion medium. Due to its role in modulating the growth and differentiation of the cells, media can have a profound effect on the overall therapeutic efficacy of the manufactured dose.

One of the biggest challenges when manufacturing T-cell therapies is overcoming the tendency for the naïve T cells to be overstimulated and pushed too far through the differentiation pathway during expansion. If this happens, by the time the cells have been developed into the final therapeutic product, a large proportion of them will be exhausted, reducing the overall therapeutic efficacy. To avoid this situation, the medium used needs

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to be able to drive cells toward early memory phenotypes – such as central memory (T_{CM}) and stem cell memory T cells (T_{SCM}) – and maintain them at this stage to avoid further differentiation and exhaustion.

An additional way that cell expansion media can reduce overstimulation and thus improve efficacy is to eliminate the disparity between workflow lengths for autologous and allogeneic therapies. Using current media, allogeneic workflows require an additional 3 to 4 days compared to autologous therapies to generate enough cells for a therapeutic dose. As each additional day means further differentiation, there is a vital need for a medium that can reduce workflow length by facilitating the production of a greater number of T cells with early memory phenotypes in a shorter period of time.

Gibco CTS OpTmizer Pro SFM

Gibco CTS OpTmizer Pro Serum-Free Media (SFM) is a first-of-its-kind media solution specifically formulated to facilitate the expansion of human T lymphocyte cultures within allogeneic T-cell therapy manufacturing workflows.

By targeting healthy donor cell metabolism, the medium has the potential to enable strong proliferation, as well as robust maintenance of the desired T_{CM} phenotype, ultimately resulting in the production of more efficacious therapeutic products. Crucially, it has the capacity to achieve these results in a shorter time compared to existing T-cell expansion media.

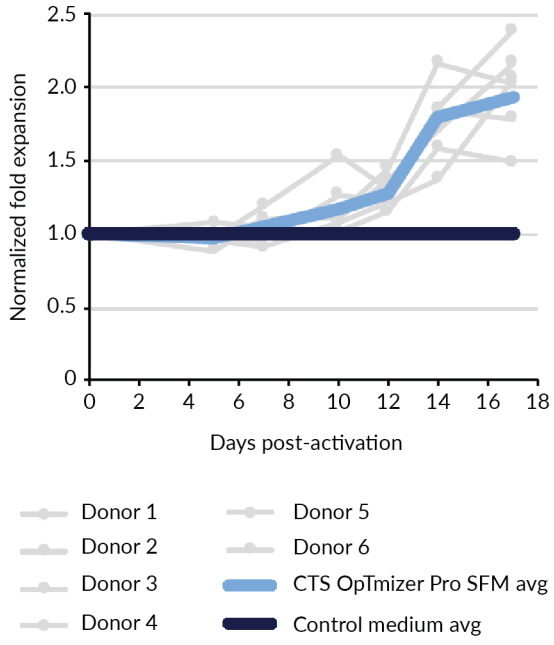
Additionally, the medium has been formulated to enhance overall workflow productivity. Most notably, it eliminates the need for serum, which can improve consistency, lower costs, reduce supply and contamination risks, and ease regulatory concerns. It is also highly versatile – supporting T-cell activation using either Dynabead™ magnetic beads, soluble antibodies, nanomatrices, or stimulatory antibody-presenting cell protocols.

CTS OpTmizer Pro SFM supports T-cell activation using Dynabeads magnetic beads, soluble antibodies, and stimulatory antibody-presenting cell protocol and comes in enhanced design and packaging making it compatible with closed systems.

The capacity of CTS OpTimizer Pro SFM to improve allogeneic manufacturing workflows and enable the production of efficacious therapeutic products is demonstrated in the following experiments.

FIGURE 2

Normalized healthy donor T-cell proliferation in CTS OpTmizer Pro SFM.



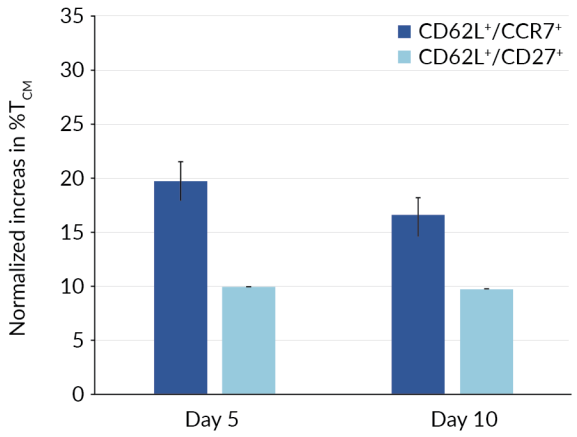
Healthy donors T cells expanded in an 18-day allogeneic-type workflow using CTS OpTmizer Pro SFM demonstrated ~20% higher cell proliferation by day 10, with a ~100% increase by day 17, when compared to a control medium. The average normalized change in cell growth in CTS OpTmizer Pro SFM is represented by the light blue line, with the baseline standard shown in dark blue and individual donors represented by the light gray lines.

PROLIFERATION OF HEALTHY DONOR T CELLS

To assess the suitability of CTS OpTmizer Pro for use in allogeneic T-cell therapy manufacturing workflows, its ability to promote healthy donor T-cell proliferation was measured and compared to an industry standard, serum-free T-cell expansion medium.

FIGURE 3

Healthy donor T-cell phenotype maintenance in CTS OpTmizer Pro SFM.



Six healthy donor cells expanded in an 18-day allogeneic workflow using CTS OpTmizer Pro SFM demonstrated a 10–20% increase in the size of the central memory subset when evaluated on days 5 and 10, following normalization with a control medium. T_{CM} cells express CD62, CCR7, and CD27 as indicated in the legend.

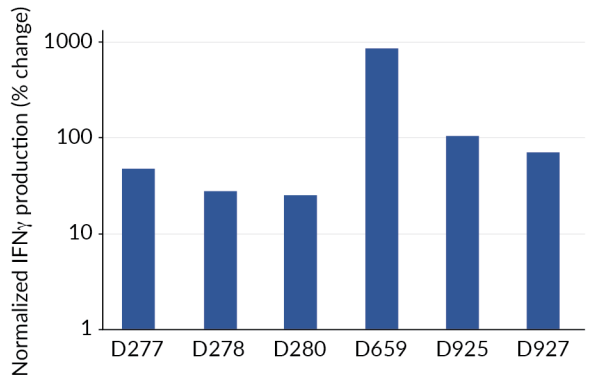
Human primary T cells were first negatively isolated from peripheral blood mononuclear cells (PBMCs) with the Invitrogen™ Dynabeads™ Untouched™ Human T Cells Kit. These cells were then seeded in culture dishes at 1 x 10^6 cells/mL in the indicated medium and activated with Gibco™ Dynabeads™ Human T-Expander CD3/CD28 at a ratio of 3 beads per T cell in the presence of 100 IU/mL of recombinant interleukin-2 (rIL-2). In all experiments using CTS OpTmizer Pro SFM, the complete culture medium was created by supplementing with Gibco L-Glutamine to a final concentration of 2 mM, per the user manual.

Throughout the experiments, T cells were counted every 2–3 days using a Vi-CELL™ Cell Viability Analyzer (Beckman Coulter). Viable cell density was also maintained at 0.25 x 10^6 cells/mL, and rIL-2 was added to the culture to a concentration of 100 IU/mL. In this first experiment, T cells from 6 healthy donors were grown as described above with CTS OpTmizer Pro SFM in an 18-day expansion workflow.

Normalized to the control medium, the average growth of the cells was increased by approximately 20% by day 10 for all donors on average, which rose further to over 100% by day 17 (Figure 2). The normalized results for each individual donor are represented by the gray lines. Moreover, all conditions displayed robust expansion, and no negative effect on viability was observed.

FIGURE 4

Healthy donor cell IFNγ production in CTS OpTmizer Pro SFM.



Normalized to the same cells grown using a control medium, cells from 6 healthy donors grown in CTS OpTmizer Pro SFM demonstrated an average increase of 187% in IFNγ production at day 16.

HEALTHY DONOR T_{CM} PHENOTYPE MAINTENANCE

The T-cell phenotype was also assessed in cells from the same 6 healthy donors by evaluating the expression of central memory markers – CD62L, CCR7, and CD27. This was conducted using the Invitrogen™ Attune™ NxT Flow Cytometer, by staining T cells with Invitrogen™ CD3 Pacific Orange™ dye, CD4 FITC dye, CD8 Pacific Blue™ dye, CD62L APC, and CCR7 PE antibodies.

Normalized to a control medium, healthy donor cells grown in CTS OpTmizer Pro SFM displayed a 10–20% increase in the size of the central memory subset when evaluated on days 5 and 10 (Figure 3). All donors showed normalized increases in the size of the early memory population at days 5 and 10 when expanded in CTS OpTmizer Pro (individual data not shown).

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HEALTHY DONOR T-CELL IFNγ PRODUCTION

Finally, to determine the capacity of CTS OpTmizer Pro SFM to produce T cells capable of stimulating a robust immune response, production of interferon gamma (IFNγ) by the cells was evaluated. IFNγ production is a particularly useful parameter to measure as the cytokine can stimulate both the innate and adaptive immune response and has the potential to boost overall therapeutic efficacy by further stimulating macrophages, neutrophils, and natural killer cells and enhancing host cytokine release [5].

To measure this parameter, a subset of the T cells expanded in CTS OpTmizer Pro SFM were reseeded at 0.5 x 10^6 cells/mL in the indicated medium and restimulated with Dynabeads Human T-Expander CD3/CD28 at a ratio of 1 bead per T cell in the presence of 100 IU/mL of rIL-2. At day 3 following the restimulation, the spent medium was analyzed for IFNγ production on the Invitrogen™ Luminex™ MAGPIX™ system using the Invitrogen™ Cytokine Human Magnetic 35-Plex Panel for the Invitrogen™ Luminex platform (Thermo Fisher Scientific) according to the user manual.

When normalized to the same cells grown in the control medium, healthy donor T cells grown in CTS OpTmizer Pro SFM showed a 187% increase in IFNγ production by day 16 (Figure 4). It is important to note that this observation was not associated with a significant shift in the CD8/CD4 ratio, which was not significantly altered by growth in CTS OpTmizer Pro.

CONCLUSION

These results demonstrate the capacity of CTS OpTmizer Pro SFM to facilitate high levels of T-cell proliferation, robust maintenance of T_{CM} phenotypes, and improved INFγ production using healthy donor T cells – all of which are hallmarks of efficacious T-cell therapies. Furthermore, they indicate that CTS OpTmizer Pro SFM is capable of producing a greater number of T cells with early memory phenotypes in a shorter period of time compared to existing media.

Combined, these results suggest that CTS OpTmizer Pro SFM could have a two-fold effect on the feasibility of allogeneic T-cell therapies. First, it could improve the efficacy of allogeneic treatments by facilitating the production of a large population of desirable T-cell phenotypes and potentially reducing overall workflow time. Second, through this improvement, it could open the possibility of these therapies becoming more prevalent and eventually becoming life-changing, off-the-shelf treatment options for hematological malignancies.

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BIOGRAPHIES

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Dr Zynda has been with Thermo Fisher Scientific for almost 5 years. He serves as a Senior Scientist in R&D for the department of Cell Culture and Cellular Medicine and has been focused Cell therapy process development and product development. He first began studying T-cell biology in 2005 at Roswell Park Cancer Institute, where he received a PhD in Molecular and Cellular Biophysics and Biochemistry. During his academic years, he elucidated mechanisms by which tumor cells to evade the immune system and went on to apply this knowledge in drug development and cell therapy manufacturing.

Dr. Aditi Singh
Global Product Manager, Thermo Fisher Scientific

Aditi Singh has been with Thermo Fisher scientific for over 6 years. She is currently working as Global Product Manager managing products utilized in T Cell Therapy manufacturing workflow utilized in research, process development and clinical settings. Her goal through this role is to improve patient care by making personalized medicine (/cell therapy) more accessible to cancer patients. Aditi has a Ph.D degree in Cell and Molecular Biology from University of Heidelberg, Germany where she worked on infectious disease and diagnosis.

AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

CELL THERAPY BIOPROCESSING & AUTOMATION

SPOTLIGHT

EXPERT INSIGHT

Manufacturing of hematopoietic stem cell gene therapies: opportunities for process automation

Carlotta Peticone & Emma Chan

Genetic medicines are being investigated in the context of a range of different diseases, and in some instances have successfully been commercialized. However, challenges in the manufacturing process have the potential to limit the applications of these approaches to treat an even broader number of diseases. The bioprocessing industry, which historically has been focused on large-scale production of biologics, has recently seen a shift of interest and an increasing number of bespoke solutions emerging for the autologous cell and gene therapy field. Several technologies are now available to automate production, either focusing on individual manufacturing steps or providing a combined platform approach. Here, a review of state-of-the art technologies for the manufacturing of autologous cell and gene therapy products is provided.

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Genetic medicines have potential to treat a range of diseases with numerous investigational and approved therapies at different stages of clinical and commercial manufacturing, with new programs constantly emerging.

However, in many instances, the manufacturing processes for these medicines have been developed years ago in academic settings, on a small scale to treat a limited number of patients. Therefore, they are subject to various

practical and cost limitations and challenges, which could limit their broader commercial application.

With the expansion of the cell and gene therapy field, many novel technologies targeted to this industry that can be used to provide solutions and opportunities for more sophisticated manufacturing processes, are emerging. These include modular or combined equipment designed for either individual unit operations or a full manufacturing process, respectively. Use of these devices offers potential to form automated, closed processes resulting in a more robust and cost-effective manufacturing solution. This provides opportunity for these novel medicines to be used in a more extensive range of indications.

This review discusses some of the available and emerging technologies that could be used for the manufacture of autologous cell and gene therapy products.

HISTORICAL STATE OF MANUFACTURING PROCESS

Manufacture of many cell and gene therapies uses processes that have been established over the last decades, often in academic settings, to support investigative treatment in only a small number of individuals. These historical processes are often highly manual, include multiple open steps, and therefore require highly trained operators and high-grade clean rooms. There are a number of issues and risks associated with translating these early-stage processes to a commercial setting.

The staff requirement is often an issue especially in scale-up/scale-out of the manufacturing process. There are limited numbers of experienced staff in cell and gene therapy good manufacturing practice (GMP) and there is increased demand as the industry grows.

A second issue is that a manual, open manufacturing process has numerous associated risks including contamination risk from many open steps, especially when the final drug product cannot undergo terminal sterilization. Third, manual processes are more prone to human error as they are highly dependent on operator's experience [1]. This determines batch-to-batch variability that is in addition to inherent differences in patient starting material when considering autologous products. Lastly, there is a significant cost for manufacturing per batch that includes the cost of extensive hands-on time of multiple operators and an individual GMP clean room per product to eliminate cross-contamination.

These manual processes, based on readily available technologies, are fit for purpose to allow small numbers of batches to be manufactured and to demonstrate clinical efficacy for new cell and gene therapies. However, for longer-term sustainability, there is a need to optimize and automate manufacturing to allow more extensive production to support demand of supplying drug product to an ever-increasing number of patients globally, across therapeutic areas.

These therapies often have a rapid development pathway, particularly when compared with standard biopharmaceuticals and the product lifecycle does not always allow for easy implementation of significant manufacturing process developments and improvements. Changes to the process often require challenging, time consuming and costly comparability studies, which in some instances require *in vivo* or even clinical comparability. Consequently, the historical processes are often utilized much longer than is ideal, which can lead to challenges and processes that are suboptimal for commercial manufacturing.

TECHNOLOGY DEVELOPMENTS

Cell and gene therapies are still relatively novel medicines and therefore there are limited custom devices to support their manufacture and analytics. Several technologies which have been developed in the past for the manufacturing of biopharmaceuticals are not suitable for cell therapy products, due to significant differences in operating scale (i.e., one

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large scale batch providing multiple doses in biopharmaceuticals as opposed to autologous cell and gene therapy small batch size to provide to a single patient).

However, with the ever-increasing momentum of the cell and gene therapy industry, there are now many companies developing targeted solutions for the problems identified in the field. These technologies are at varying stages of development, from readily available off-the-shelf equipment with supportive regulatory compliance, to those in early prototype stages being developed and tested specifically with cell therapies in mind. Often there are opportunities to test these devices during the development stage to enable customization for specific, real-life manufacturing scenarios.

Two broad methods of manufacturing include the all-inclusive systems designed to perform the full manufacturing process versus modular equipment with each unit operation utilizing a dedicated device.

All-inclusive devices, often referred as 'GMP in a box' technology, are designed to run the process from input starting material to final product in one piece of equipment. A single device means reduced space and training compared with multiple pieces of equipment, and often reduced initial cost output, but could be limited in terms of process flexibility. To ensure that the equipment can accommodate the specific manufacturing process, development with the manufacturer may be required, which adds additional time and cost. However, having a fully closed, self-contained and automated platform that is fit-for-purpose may well be worth the cost. Furthermore, a platform with a reduced footprint and less stringent cleanroom classifications could allow for a decentralized manufacturing model, where production occurs next to or at the hospital site, rather than in dedicated, centralized facilities. The Lonza Cocoon and Miltenyi Prodigy are the most advanced all-inclusive devices, and they provide capabilities for most processing steps, including washing, enrichment, culture and transduction or transfection.

An alternative to a single all-inclusive device is to use several devices as part of a modular system, where each device performs a specific step of the manufacturing process. The initial cost of multiple items may be greater than all-inclusive device and may require a greater footprint in the manufacturing facility. However, it provides more flexibility in the manufacturing process and the opportunity to accommodate for further process evolution, as well as in the facility design and flow. An important consideration with a modular system is the transfer of cells and the compatibility with other devices up or downstream, which may have varying requirements from their respective manufacturers.

Lastly, there are new systems in development that combine both approaches. These systems consist of a fully closed environment where cells are automatically moved from one modular unit to the next in the process, without human intervention and allowing multiple products to be processed in parallel. An example of this type of system is the Cell Shuttle from Cellares.

In the next sections, we will give an overview of typical steps involved in the manufacture of autologous, *ex vivo* hematopoietic stem cell (HSC) gene therapies. In this approach, a patient's own blood stem cells are genetically modified outside of the body and then reinfused back into the patient, with the goal of correcting the underlying cause of disease in a single treatment. The technological challenges for this type of gene therapy manufacturing and requirements or solutions for how these challenges could be overcome are discussed in Table 1.

HSC MANUFACTURING PROCESS

The manufacturing process for HSC-based gene therapies consists of several steps which are summarized in Figure 1, along with collection of the cellular starting material and product administration, and that will be described in the sections below.

TABLE 1

Technological challenges and requirements for autologous hematopoietic stem cell gene therapy manufacturing.

	Technological challenges	Technological requirements
Blood cell collection	Limited control and oversight of procedures at clinical site High level of variability between patients, including volume and cellular content e.g. proportions of red blood cells, granulocytes	Standardization of collection methods at clinical sites Establish specification for incoming starting material
Enrichment	Low frequency of $CD34^+$ cells in starting material Differences in starting material composition amongst patients Isolation of HSC subsets to improve therapeutic efficacy	Standardization of enrichment protocol High throughout GMP sorter with suitable speed, recovery and purity for clinical applications
Viral Transduction	Manual /open processing Variability in transduction efficiency Availability of transduction enhancers for GMP manufacturing and requirement for license High vector requirement due to difficulty in transducing HSCs and the high numbers of cells required to produce DP per patient Cost of vector	Automation GMP-grade transduction enhancers, if used in the process Technologies offering flexible transduction protocols Fewer cells required at transduction if specific HSC subsets selected and transduced (and therefore lower vector requirement)
Expansion	Manual /open processing Sterility Highly skilled operators Requirement of high-grade clean room per batch High dose requirement Prevent HSCs differentiation	Automation Availability of bioreactors at autologous scale GMP-grade reagents for expansion Online monitoring of critical process parameters
Wash	Manual / open processing Sterility QC samples	Automation Compatibility of input/output volumes Compatibility with upstream/downstream equipment
Fill and Finish	Manual / open processing Sterility Highly skilled operators Temperature and time sensitive QC samples Cryopreserved formulation	Automation Flexibility in final packaging DMSO compatibility Controlled temperature Reduce DMSO-contact time DMSO-free formulation Stability and tracking during shipment to clinical site
Administration	Manual / open processing Sterility Highly skilled healthcare personnel Temperature and time sensitive	Standardized thawing Point of care cell washer
Reagents	Sterility	GMP-grade reagents Packaging suitable for closed processing outside of Grade A environment
Process Data Management	High number of complex in-process, QC and characterization data Manual Batch Record	Electronic Batch Record Laboratory Information Management System (LIMS) Automated Data Storage In-Process Data Monitoring Database regulatory compliance and validation

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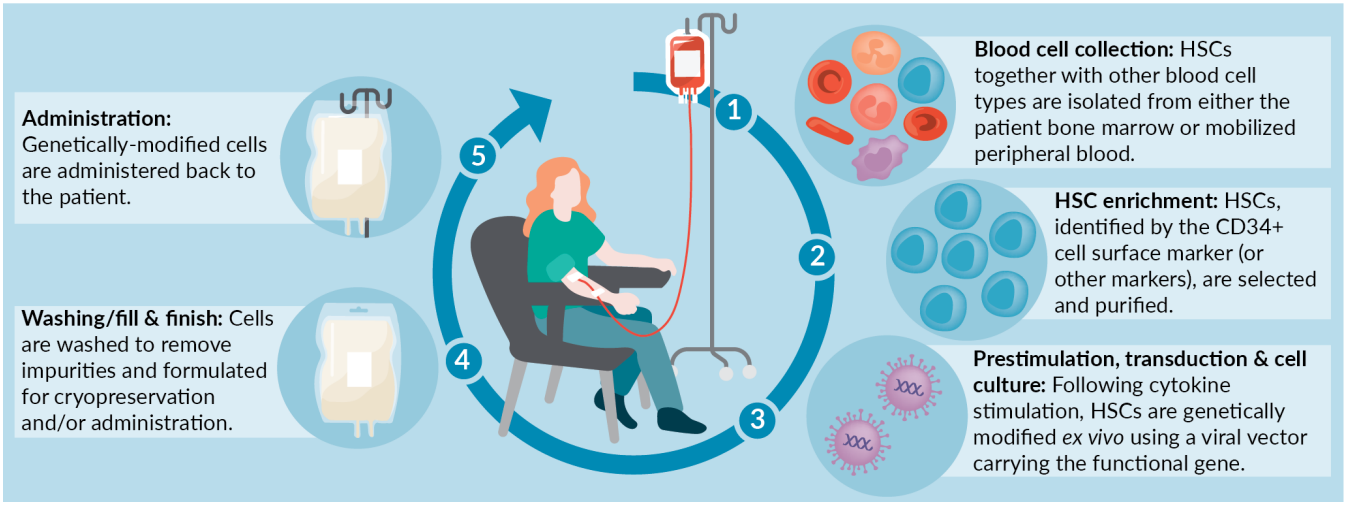
FIGURE 1

Summary of the process to manufacture and deliver autologous haematopoietic stem cell gene therapy from vein-to-vein.



Blood cell collection (starting material generation)

Autologous HSCs are either derived from bone marrow (BM) or mobilized peripheral blood [2]. While the first source requires a surgical procedure to collect the BM from the patient, the second requires mobilization of HSCs from the BM to the peripheral blood. This can be induced by using mobilization agents such as Granulocyte-colony stimulating factor (G-CSF) and Plerixafor [3,4]. Following mobilization, the patient undergoes leukapheresis where white blood cells including the mobilized HSCs are separated from other blood components and collected. Common technologies used for apheresis collection are the Cobe® Spectra and Spectra Optia® (Terumo) MCS+ (Haemonetics), COM.TEC (Fresenius Kabi), and Amicus (Fenwal) [2]. There is high variability of cellular starting material between patients, which can lead to product variation. Therefore, where possible, standardization of the collection procedures is important to aim to reduce this variability. As the apheresis is collected in a clinical setting outside of the GMP facility, and performed by healthcare personnel, this step is not considered part of the manufacturing process.

HSC enrichment

Many, if not most, cell therapy processes require an enrichment step to isolate the cell population of interest from the blood or bone marrow cells harvested from the patient. This is particularly important to remove contaminating cells that could alter process performance during manufacturing steps such as cell activation or transduction [5]. Isolation of HSCs is predominantly achieved via immunomagnetic bead technology targeting $CD34^+$ cells [2]. The transmembrane glycoprotein CD34 is the key marker for long-term stem and progenitor cells. Although its function is not fully defined, its expression decreases with lineage commitment [6,7]. An antibody to CD34 is conjugated to a paramagnetic nanobead that is used to hold the $CD34^+$ cells within a magnetic field. The $CD34^-$ cells are washed away and upon release of the magnetic field, the $CD34^+$ cells can be collected. Purity and recovery in immunomagnetic selection is highly dependent on starting material composition, with high content of cell subtypes such as neutrophils and platelets negatively affecting the selection process [8].

Miltenyi MACS® technology, in either the semi-manual CliniMACS Plus or the more

recent and multifunctional Prodigy system, is used for cell separation and isolation [8]. Miltenyi also produces the GMP-grade $CD34^+$ reagents, making this approach suitable for clinical applications. Similarly, the Cocoon platform also includes a magnetic cell selection module which can be incorporated into the integrated manufacturing process in this system. Novel methods are being developed although not widely implemented or not currently available to GMP-grade. For example, buoyancy-activated cell sorting (X-BACS™, Thermogenesis) uses streptavidin coated microbubbles to select cells previously labelled with biotinylated antibodies by floating them to the surface during centrifugation, while pelleting unwanted cells [9].

Cell surface markers that define more primitive HSCs and potent subpopulations, including $CD133^+$, $CD38^{-/low}$, $CD164^+$, $CD90^+$ and $CD45RA^-$, have recently been identified. These populations have been shown in *in vivo* models to elicit an enhanced therapeutic effect [10,11]. Thus, there is an interest in the field to move beyond $CD34^+$ selection and to be able purify more specific cell subtypes with enhanced engraftment potential [12]. This approach might require selection of both long- and short-term progenitors to support stable hematopoietic reconstitution following HSC transplant [12,13]. While magnetic bead-based selection technologies only enable enrichment or depletion using individual markers, cell sorting could be used to capture cells of a more defined phenotype expressing a complex array of markers. However, the availability of this type of technology in the GMP grade is currently limited. The MACSQuant® Tyto (Miltenyi Biotec) cell sorter for flow sorting using a fully enclosed cartridge designed for GMP use is one of the few platforms available in the space. Cell viability, recovery/purity and speed are critical parameters to consider when switching to a sorting technology and these current limitations need to be optimized to allow widespread implementation in the clinic.

Of note, as $CD34^+$ cells only account for ~1.5% of adult bone marrow cells [14], on one hand, selecting a more defined phenotype might further restrict the starting number of cells available for processing, while on the other might reduce the scale of manufacturing, thus limiting the volume requirement of costly reagents, such as vector [15].

Pre-stimulation & transduction

Viral vector transduction currently is an efficient method for introducing a therapeutic gene of interest into the target cells and both gamma retroviral vectors and lentiviral vectors have been used extensively in the clinic for decades [16]. Despite having been the method of choice in several clinical trials, achieving high levels of transduction of $CD34^+$ hematopoietic stem cells using viral transduction and obtaining consistent efficiency across multiple donors still represents a key challenge in the field. Pre-stimulation of HSCs with an activating cytokine cocktail is required to promote the cells to exit their resting state and to enter cell cycle, facilitating both retro- and lenti-viral transduction [17]. Performing multiple rounds of transduction at high multiplicity of infection (MOI), as well as the use of transduction enhancers (TEs), which are molecules that facilitate viral particle uptake by HSC via different mechanisms, have been used as strategies to optimize transduction efficiency [18,19]. Multiple rounds of transduction require significant amount of vector material, thus significantly impacting the overall manufacturing cost and increases the amount of time the $CD34^+$ are in culture. Using TEs have the potential to reduce vector requirements, however the availability of these reagents at the required GMP standards might limit the options available for clinical manufacturing. Furthermore, the use of some of these reagents might require licensing or might be restricted by intellectual property rights. Not all gene modified HSC products will require the use of TEs but when they are to be implemented, cells must be tested extensively, often *in vivo*, to ensure no negative biological impact on the resulting product.

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From an automation perspective, historically transduction has mainly been performed as a manual step, with addition of viral vectors to HSC growth media following pre-stimulation with an activating cytokine cocktail. However, platforms like the Cocoon and Prodigy enable automated transduction of HSCs, with vector being loaded to the cell culture chamber and the potential to add transduction additives if required, in a controlled and sterile manner. Some reagents might require specific incubation protocols (i.e. surface coating, spinoculation, etc) which may limit the automated options available or require further customization.

As mentioned, although gamma retroviral vectors and lentiviral vectors are the most common method used to deliver genes into HSCs and other cell types in the production of cell therapy products, research into non-viral technologies are also progressing. If these non-viral gene delivery systems become commonplace, alternative processing methods may also need to be employed. To this end, both Cocoon and Prodigy platforms have developed electroporation systems that could be used to deliver relevant mRNAs, allowing more variations in the manufacturing process.

Cell culture & expansion

HSCs are characterized by the dual capacity of self-renewal and multipotency, characteristics that are lost as they differentiate to progenitor cells. Expansion of HSCs *ex vivo*, whilst maintaining these unique features, is one of the key challenges currently faced when developing manufacturing protocols [20]. Optimization of culture media, as well as investigation of supplements to expand HSCs without compromising their multipotency potential still represents a key area of research. In most protocols, cells are cultured for a maximum of 2 to 5 days following transduction, in order to minimize differentiation and to maintain pluripotency [2], as well as to promote vector integration [21].

Historically, cell therapies have been developed in academic labs, with manual culture performed in conventional static vessels like tissue culture flasks or gas-permeable culture bags. Despite several technologies being available for large-scale GMP manufacturing of biologics, most of these bioreactors are not available at a scale suitable for autologous cell therapy and only a limited number of bespoke solutions have now been developed for the cell therapy space and are currently at different stages of development.

The G-Rex from Wilson Wolf is a single-use disposable vessel presenting a gas permeable membrane at the bottom that enhances gas exchange from the incubator atmosphere and that enables the addition of large media volumes without compromising CO_2/O_2 diffusion, limiting the requirement for additional feeds [22,23]. Although the G-Rex has been shown to support efficient cell expansion, it still requires manual handling and use in an incubator, and at present has limited automation possibilities.

There are other options that are available or under development that are designed with more automation and control in place. The methods of culture differ between the devices, and certain cell types may be more suited to different systems. For instance, the Quantum from Terumo is a perfusion bioreactor that can be used to culture adherent and suspension cells and has been demonstrated to be effective for T-cell culture [24]. Rocking bioreactors like the BioStat® RM from Sartorius or the Xuri™ from Cytiva, are also available at a scale compatible with autologous cell therapy manufacturing (i.e. 1–2L bags) and present options for perfusion feeding and culture mixing by wave-induced agitation [25]. A review summarizing technologies used in CAR T-cell manufacturing for clinical trials, identified rocking motion bioreactors as the third most common expansion technology, following T-flask and culture bags [26].

The already mentioned Cocoon and Prodigy platforms both present a culture chamber that can be used to expand cells under controlled conditions (i.e., temperature, %

CO_2), with the option to perform automated media feeds and/or exchange. Dynamic culture conditions can be achieved in both devices (perfusion in the Cocoon and chamber shaking in the Prodigy), as well as closed and aseptic sampling.

Furthermore, high throughput stirred tank reactors like the Ambr® 250 (Sartorius) or the DASbox® (Eppendorf) are particularly suitable for Design of Experiment (DoE) and process development studies due to the reduced culture volume [27], however they are not compatible with GMP manufacturing.

In most of the technologies mentioned above, online monitoring of culture parameters (if available) is mainly still limited to temperature, CO_2 and dissolved oxygen. However, the development of sensors to monitor cell growth, viability, metabolites, and potentially more sophisticated parameters like transduction efficiency or specific cell phenotypes is highly desirable and would significantly reduce the cost and complexity of quality control sampling.

Washing

Cell washing and/or resuspension in different media or buffers are typically required at multiple stages of the process including resuspension of selected cells from selection buffer to culture media, vector removal after transduction and final formulation into cryopreservation buffer. Typically, smaller volumes are required as input/output volumes in autologous manufacturing, compared with standard bioprocessing or even allogeneic products, which cannot be provided in many instances, and therefore, often specialized cell therapy devices are preferable.

As part of their integrated systems, both the Cocoon and Prodigy present washing protocols that can be applied at different stages of manufacturing. Otherwise, available modular systems include Sepax™ C-Pro (Cytiva), Lovo (Fresenius Kabi), Rotea™ (ThermoFisher) and ekko™ (MilliporeSigma). These are closed system devices designed specifically for cell therapy manufacturing, using different technologies for cell washing and presenting single-use disposable kits. As mentioned previously for all modular equipment, of critical importance when incorporating standalone washing equipment in the manufacturing process is the compatibility with other devices up or downstream of the washing step.

Depending on other devices within the manufacturing process, the volumes required, and the cell type used, different equipment may be more or less suitable for each manufacturing process, and options should be evaluated while paying consideration to the cell viability and recovery for the user's specific process.

Fill & finish

At the end of the manufacturing process, the formulation and fill and finish steps, can also be automated using specialized equipment to suit the specific needs of these process steps. Finia® (Terumo), Cue® (ScaleReady) and Signata™ CT5 (Sexton Biotechnologies) are all options that are available and have been custom designed to support cell therapy manufacturing. As part of their all-inclusive manufacturing capabilities, the Cocoon and Prodigy can also be adapted to include steps to wash and formulate the cultured cells at the end of the manufacturing process.

There are overlapping functionalities between filling devices and cell washers. In particular, as fill finish devices typically can wash and re-buffer particularly small volumes, they may be preferable for certain manufacturing steps. In addition, filling devices have mixing capabilities, can aliquot into multiple outputs and have temperature control, which gives them the advantage for the fill and finish process compared with cell washers. They must have flexibility in outputs as final containers may include bags and/or vials, and differing doses between patients and for different indications. It is also beneficial if the system supports filling of smaller aliquots for Quality Control (QC) and retain samples. Also, as the majority of cell

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and gene therapy products are cryopreserved, the device and its tubing sets or consumables must also be DMSO compatible. If the output containers and volumes are compatible with a cell therapy process, using these specialized devices are beneficial as they can reduce variability between operators to provide consistent doses between batches and their integrated temperature control minimizes the negative impact of DMSO on the product.

Cryopreservation of the cell products allows their long-term storage using liquid nitrogen, which provides a long shelf-life and facilitates their global supply and distribution. At the end of manufacturing, as products are cryopreserved, it provides time for the panel of release tests to be performed. The results are assessed to ensure the product has reached the strict acceptance criteria to demonstrate its safety and functionality prior to release.

Administration

Once released, and following patient pre-conditioning, the product is transferred to the treatment site (usually a hospital) in specialized temperature-controlled shippers, where the cells are administered to the patient. As mentioned, both fresh and frozen cell therapy products are currently being developed. In both instances, the logistics to supply the product within a specific timeframe and controlled temperature ranges is very complex. Frozen products require an additional thawing step that is generally performed by the medical team following detailed instructions from the manufacturer. Thawing and administration of the drug product, however, are not considered part of the manufacturing process. Automated dry thawers, such as the Cytiva's ViaThaw™ or the Biolife Solutions' ThawStar® are available to provide controlled and standardized thaw profiles at point of care, limiting variability between different clinical sites and ensuring monitoring of the thawing process via data collection.

Furthermore, while DMSO-based formulations are routinely used in clinical trials [28], there are several side-effects identified with the use of this reagent. While alternative cryoprotectant agents are being developed, automated DMSO removal devices, such as the Exmoor's cPrep, represent a potential alternative for clinical indications where reduction or removal of DMSO toxicity is mandatory [29].

Additional considerations

There are now various data available for all the previous mentioned technologies, however it is worth noting that not all have been tested in the context of HSC culture. Different cell types are likely to respond differently to the varied conditions within each device, so each would need to be tested to determine which is most applicable to a particular manufacturing process.

Implementation of these sophisticated automated devices in the manufacturing process often provides opportunity for integration of electronic batch records, laboratory information management system (LIMS), automated data storage and supply chain software to further streamline and automate manufacturing to support more robust systems and higher throughput.

Ideally these automated manufacturing devices can also be combined with real-time sampling and monitoring and analytics, to allow adaptive control and process-based decisions. This allows flexibility within and between batches and can help to improve the manufacturing success and product quality by minimizing variability at different stages of the process. For this to be performed effectively, it requires a thorough understanding of these complex and relatively novel therapeutics, which is continually advancing as more clinical trials are conducted and newer analytical methods are developed.

If automated technologies are used in clinical and/or commercial GMP manufacturing, there are also regulatory requirements and considerations such as software validation that need to be complied with. As these

technologies are designed with these specific uses in mind, regulatory compliance has to be factored in during the product development, and most manufacturers are adapting to changes in regulatory landscape. Additional considerations and regulations are likely to arise in the future as these newer technologies becomes more commonplace.

Finally, together with technological advancements, full closure of the manufacturing process to reduce the use of Class A clean room use, requires the availability of suitably packaged reagents with aseptic connection such as sterile welding. While an increasing number of suppliers are now providing a range of solutions in this direction, there are still several reagents requiring sterile manipulations in a graded environment.

TRANSLATIONAL INSIGHT

Key features of an optimal manufacturing process include robustness, closed processing to allow production of multiple batches per clean room, reduced cost, time to manufacture and requirement of operators and training. Process automation will assist in all of these areas of process improvements and subsequently aid implementation of manufacturing for a broader range of diseases. Currently many of these technologies are at a relatively early stage of development but are advancing to support the requirement in the cell and gene therapy field.

Implementation of these automation steps will also impact the design and layout of future manufacturing facilities. Closed processing will allow for larger suites designed for parallel processing of multiple batches and reduced or no requirement for isolators. Facility design will also be strongly impacted by whether manufacturers opt for modular versus inclusive equipment. Furthermore, automation will facilitate decentralized or point of care manufacturing rather than the more prevalent model of manufacturing with a centralized or regional facility. This is because a closed, automated process is more reproducible and easier to control. Consistent oversight is more restricted when manufacturing at multiple locations, therefore improving robustness through automation is important to minimize site-to-site variability and maintain product quality. The main source of variability, other than patient cellular starting material, is by using manual, labor intensive operations, which subsequently lead to operator variability between batches. Therefore, implementation of automation to replace this manual processing has the potential to significantly reduce variability and result in more consistent products, even across sites, where the automation equipment can be utilized [30]. Automation will also reduce the burden on the local site in terms of requirements of GMP lab facilities and specialized operators, enabling more sites to perform manufacturing. Potential benefits of decentralized manufacturing could include greater patient accessibility to treatments, less complex shipping logistics and more supply chain security due to manufacturing redundancy between sites.

As process improvements to introduce and improve automation from advanced and emerging technologies become available, the requirement of comparability studies to assess similarity of products manufactured with the new versus existing process need to be considered for those products already in clinical or commercial manufacture. The costs and benefits for introducing changes to an existing process should be evaluated and the comparability studies required will depend on extent of the changes and the stage of product lifecycle.

There are many advances in cell and gene therapy manufacturing that are able to support this vision of fully automated, closed and controlled systems, including the devices described above, with more on the horizon. It is likely that there will need to be different devices to fit varying needs of cells, products and diseases throughout the cell and gene therapy field. These will need to be evaluated by manufacturers to enable them to develop their specific processes depending upon their different requirements to deliver a suitable process for more widespread manufacturing.

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EXPERT ROUNDTABLE

A ddPCR primer: advantages, challenges, and key considerations when adopting Droplet Digital PCR for viral vector manufacture

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Lauren M Drouin leads the Analytical Development group at LogicBio Therapeutics, where she supports the development and implementation of analytical methodologies needed to progress LB-001 and other pipeline products from preclinical development into the clinic and beyond. Current research interests include novel AAV capsid characterization and developing a robust understanding of the factors that influence potency of gene therapy products. Previously, Lauren worked at Voyager Therapeutics where she was responsible for analytical method development and overseeing CMC analytics operations for the Parkinson's Disease clinical program. Lauren received her PhD in Biochemistry and Molecular Biology from the University of Florida where she utilized molecular, biophysical, and structural techniques to characterize the AAV capsid for improved gene delivery.

FABIEN DORANGE
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Fabien Dorange holds a PhD in virology and worked as a postdoctoral researcher in the gene therapy field. Before joining Genethon, Fabien was Head of R&D viral safety in a CRO company (Texcell, France). He joined Genethon in 2016 and currently heads of the CMC Analytical Department. He is responsible for the analytical activities in the characterization and release testing of gene therapy products.

BRYAN SILVEY
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Bryan Silvey has been in the biopharmaceutical industry for over 25 years in both Quality technical roles and Regulatory Affairs CMC leadership positions having responsibilities for both product and facilities lifecycle management. Global experience in regulatory authority requirements and expectations in the CMC space with biologics has proved a grounding platform of quality principles that he has applied to successful development and commercialization of two (2) CAR T-Cell therapies while at Kite (a Gilead Company). As Head of Quality for A2 Biotherapeutics, an early stage cell therapy discovery and development company, this journey in CMC science continues to push forward in an effort to address unmet cancer patient needs.

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Steven Pincus has a PhD in Biochemistry with training in molecular Virology and Immunology. Steven has over 30 years in the biotech industry leading teams in research and development of vaccines, AAV Gene Therapies and monoclonal antibodies in the areas of infectious disease, autoimmune disease and cancer. He has taken projects from discovery through Phase 2 clinical trials. He has developed qualified and validated release assays and assays for measuring clinical and immunological responses. He has led the transfer of assays to partners, developed and managed collaborations with academic and industrial partners, managed CRO's and developed and managed project budgets and goals. During his career Steven has gained experience in Quality control, analytical development, regulatory affairs and manufacturing development for vaccines and Gene Therapies. He is currently Executive Director Analytical Development at Neurogene.

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In this expert roundtable five experts discuss Droplet Digital PCR (ddPCR), exploring its real-world applications, future potential, and their own experiences in effectively managing its use within their organizations.

Q Firstly, could you talk about the specific applications where you each employ ddPCR?

FD: For recombinant adeno-associated viral vector (AAV) the most important assay for vector characterization and batch release is the vector genome (VG) titration. Vector genome is considered the active component of the product, and the genome copy numbers are irrespectively used in preclinical and clinical studies. ddPCR, due to its high accuracy and precision, is a powerful tool for quantifying the vector genome.

LD: In addition to vector genome titration, we also use ddPCR to quantify residual host cell and plasmid DNA in our samples. These product-related impurities are a natural byproduct of our manufacturing process, because the AAV capsid can unintentionally package these non-target DNA species. We strive to minimize the presence of non-target DNA in our process, as it doesn't provide any therapeutic benefit and can lead to an unwanted immune response in patients.

We also use ddPCR as a readout for both vector infectivity and gene expression assays to assess our product potency from an

initial screening either in an *in vitro* or *in vivo* system.

SP: Following on the idea of host cell DNA, one of the key targets can be genes that we know are present in the host cell that may have oncogenic potential.

For example, one of the major cells lines we work with is the HEK293 cell line, which is transformed by adenovirus. One of the gene products, E1A, is a potential oncogene, and we want to make sure we have the lowest level possible of those DNA fragments in our product.

Looking at animal distribution studies, we can use ddPCR to measure how much genome is present in different tissues. We can even look at how much genome transcription is occurring by using some of the new reverse transcription ddPCR (RT-ddPCR) techniques.

> "Looking at animal distribution studies, we can use ddPCR to measure how much genome is present in different tissues."
>
> - Steven Pincus

BS: For a strictly quality control (QC) operation, which we have been involved with at this time, ddPCR applications include identity and vector copy number due to its preciseness and sensitivity, but also for safety tests. Mycoplasma is an ideal candidate due to its selectivity and precision to be able to find low levels of this. So we are also evaluating it in a safety test as well.

MW: This is in line with where Bio-Rad is trying to support the industry – vector copy numbers and vital titers being at the top of the list where most people are starting. As Bryan said, we are now offering a kit for mycoplasma detection. Some of the other areas such as residual DNA, plasmid, and potency are all developing areas that we are watching and hopefully we will be supporting in the future as well.

Q What are some of the key advantages and disadvantages of ddPCR over alternative tools for these applications?

LD: I will start with some of the advantages of ddPCR over traditional quantitative PCR (qPCR) methods.

ddPCR is an absolute quantification method. What this means is that we don't have to rely on a standard curve to determine the concentration of our samples. It offers greater sensitivity for detection of low levels of target DNA, and it's also highly precise and more tolerant to traditional PCR inhibitors.

In addition, we are able to obtain equal reaction efficiency when we test unpurified crude lysate samples or purified final drug products. Because of all of these qualities, we observe less assay variability run to run, between different analysts, and also over time.

The disadvantages to ddPCR are relatively minor, in my opinion. First, the method has a more limited range than qPCR, which means that we may need to perform additional dilutions to ensure we are still within the assay's dynamic range.

In terms of assay throughput, ddPCR can handle a maximum of 96 wells at a time, versus a potential of up to a 384-well format for qPCR techniques. This translates into a

"Clean, accurate, correct primer probes are key. You really have to do your upfront work to make sure you have got the right set for the assay..."

- Bryan Silvey

slightly higher cost per sample run when compared to traditional testing with qPCR.

BS: Lauren has struck on some of the well-known advantages of ddPCR, so I can't add much more there.

In terms of disadvantages it is a volume-based assay, so in terms of QC cost it can be a more expensive assay. You are going to have to weigh out the volume you are going to use it on pretty carefully. Certainly as this becomes more prominent in a clinical setting, and certainly as Mark can attest in the commercial setting, this works out okay in terms of the throughput. But it is something you have to consider.

We'll get to this in a minute, but I would add the upfront investment into ddPCR. By that I mean the assay development itself. Clean, accurate, correct primer probes are key. You really have to do your upfront work to make sure you have got the right set for the assay, and that it is well controlled from that point of view.

SP: The only thing I would add is that ddPCR tends to take a little longer to set up and perform in many cases than qPCR.

This becomes a slight disadvantage, especially in a QC environment. Manufacturing wants to have an answer for how they are doing right away, and we can't promise to deliver that answer in the same timeframe. It changes the schedule of testing a little bit.

But I think the advantages that have been described by my colleagues here do outweigh that, overall.

FD: I am also in line with what has been said. I would add that ddPCR is a very good method with a good inter-laboratory precision. This is very important for when you perform analytical transfer to a CMO – comparing results from different labs is an important thing to help with this.

Q Mark, do the comments you've heard resemble what you typically hear from customers you are working with?

MW: Yes, definitely. What it really comes down to with everyone we work with is the data quality.

While it can be an additional cost to implement ddPCR over some other methods, there is the reliability of getting high-quality data and having tight coefficient of variation – especially regarding what Fabien mentioned about transferring between one lab and another. We know many of our customers now start their process in-house and then they move it out to scale up.

Having that go smoothly and not taking 6–12 months is really important. This is where a lot of companies that have really embraced ddPCR have thought about the entire cost of ownership of the assay. Not just in their group, but as they move through the entire clinical process. That calculation really brings it home regarding why this has become such a gold standard in the gene therapy realm. The accuracy of the viral titer measurement determines what you dose your patient with, so data quality is paramount in that measurement in particular.

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Q Could the panel share some practical tips or practices on how to optimally set up your ddPCR?

BS: I touched on this above: the upfront work with the primary probe set. What we have found is that the work, study and data you need to put into that is pretty substantial. You have to make sure that you ultimately have the critical quality attributes of the assay which are precision, specificity, and accuracy. That takes time. Along with that what you have to really consider is bringing your QC analysts along with you in that journey. Training is very important.

Although this is a highly automated assay, the pre-work necessary in terms of clean lab bench, appropriate set up, and training of the analysts on the different aspects and critical parameters of the assay up front before you put it in the machine, are critical. We have had some painful experiences trying to work through issues that relate to the technical training of the individuals.

Now, the automation and the design of the instrumentation of the assay doesn't require a PhD, but it certainly does require a QC analyst with a four-year degree, and with certain attributes, who of course understands the science behind these things.

Lastly, it is necessary to make sure that you really are focused on what you are going after, how you are going after it, and what you are using it for. That is applicable in terms of not only the method development and method qualification, but in the actual method that analysts use day to day.

Q Steven, does that strike a chord with you in terms of training being one of the critical aspects you think of when looking to implement this successfully?

SP: Absolutely – because of its high sensitivity, one has to be extremely careful that we are not introducing any potential cross contamination. This involves training of the analyst on how to properly segregate the activities in setting up a ddPCR run, having the right stations that segregate those activities, and maintaining the cleanliness.

Another aspect of this training is how to set up your assay in such a way that you have some control over whether or not the run is successful. When you run qPCR we have that standard curve and we can look at how the standard curve behaved, and whether it met certain parameters.

With ddPCR we are not using a standard curve, so we have other parameters of the set-up we can monitor. But having a standard we can run every time and making sure that is meeting our expectations is also very important. As we said earlier with this possible excess pipetting we have to do because we have to dilute, it is confirming that we are doing this correctly every time, and that our pipettes are accurate, because that could add an error in this type of a procedure.

FD: As we know, you have to dilute your vector a lot to be in a good range for ddPCR. Sample preparation is a key step before the ddPCR method in order to limit the sticking of AAV or DNA to plastics, which can occur during DNAse treatment or during the serial dilution steps, for example. Sample preparation is key to a reproductive and robust assay.

LD: I agree with what has been said by the previous speakers. One thing I'd like to add is that during optimization of your ddPCR assay it is also important to screen your primer-probe sets for each gene target you are going to look at.

What we want to see is good separation between the positive and negative droplets. Some practical tips we like to employ during our screening process are to look at temperature gradients and investigate increasing the annealing time to further optimize our ddPCR reactions.

Q Mark, listening to the panel, are these pain points or challenges you are looking to address, or are you working with clients to support them on these?

MW: Yes, these are all the transition things to think about. It is interesting that as we support customers doing the transition, often they have to unlearn some things that they know that are fundamental to qPCR. It is a very different way of measuring.

We are measuring individual molecules in a droplet, so some of the controls that are important are different, as was mentioned by the panel. For example, the source of the primer probe sets – you can have contaminating templates in primer probe sets, so looking at where they are made and doing some quality control on the way in is actually much more important for ddPCR. Bio-Rad has spent a lot of time and energy on making sure that what we offer for ddPCR is clean of any contaminating DNA. Our team has experience with all of that, and we've got multiple white papers, as well as experts on staff who can help with that transition.

It is definitely a short learning curve, and again circling back to what Steven said earlier, the benefits outweigh the drawbacks once you get across that start up and assay development phase and start running. The benefits justify that transition.

Q Next, let's do a deeper dive into the important considerations when transitioning into ddPCR from a different platform. What additional insights can you share?

SP: As has been mentioned, determining the optimal primer probe set is paramount for getting a good ddPCR, and looking at the best separation between positive and negative controls. I have had cases where we have had a qPCR reaction and we tried to use the same primer probe set, and we didn't have a good optimization.

Additionally, have a good reference material that you have properly titered. When you run it in each ddPCR assay, by achieving the same result every time you are running it, you can be confident the assay was performed correctly.

Another thing we found a problem with at one time, and I think you've got to consider, is duplexing. An advantage of these methods is that you can do more than one type of reaction at the same time. By duplexing you could be looking at two different targets in your genome, and trying to get an idea of

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what partials you might have in your material. When we set up one of our duplex reactions we found that we were having a bit of a problem with it, and had to go to a slightly different buffer than normal in order to get that to work properly. These are some of the considerations you have to consider every time you are going to be transferring to this kind of method.

BS: When I think about this question I also think of the analyst element to it. You really have to really hone in on some of the essential and principle GMP compliance aspects of this as you move this through, hopefully into the success of your product and filing.

It is precision; it is accuracy, it is control of reagents – critical reagent control. There is, and should be, an expectation of the stability of the primer probe set, and stability of some of these critical solutions. It is not up to the manufacturer of the primer-probe set, or Bio-Rad, to demonstrate this. When the FDA comes to your shop, it is up to you to have the data for your product.

It is absolutely essential to be thinking not only of the analytical benefits of the assay but also to consider the GMP compliance aspects and make sure you are prepared to put these things in place. The benefits outweigh the effort here, especially as you get into more volume throughput through your laboratory. But it is a key aspect to remember.

MW: Thinking about the compliance, we spent a lot of effort on the Bio-Rad side building in parts to the software to enable our customers to be compliant. Then, like Brian said, it is really up to our customers to validate everything and have that package.

What we are trying to do on the Bio-Rad side is make sure that it is as easy and seamless as possible for our customers, and make sure that we have really high-quality products going in. For some of our newer assays that we have on the market, we are also developing data packages that can help guide our customers on how to put that package together themselves. This means they have a starting point and some material to reference; essentially a step-by-step. They can see the data that Bio-Rad put together and understand what they need to replicate in their lab.

This is another key area, and why droplet digital from Bio-Rad has moved quickly into this space. We are keenly aware of those challenges you all are going through, and trying to build up our support structure to enable that.

BS: I would add that some things don't change; some things are foundational. In terms of assay qualification and assay state of control, the EMA and FDA have plenty of information on their websites on potential pitfalls, or they do surveys of 43s or observations. There is plenty of information out there from those leading agencies, and others as well, on the expectation of assay control.

All of those principles apply to ddPCR. If someone is interested in making this transition – and they should be, as this is where it's going – then there is plenty of information out there to look at in order to make sure you build in not only the scientific efforts but also the compliance efforts.

"For some of our newer assays that we have on the market, we are also developing data packages that can help guide our customers on how to put that package together themselves."

- Mark White

MW: To build on that, one of the things we observe on the Bio-Rad side is that as people are migrating away from the standard curve, it takes a lot of time and energy to maintain an accurate standard curve. When you have to replace it, it is a lot of work to make sure it is the same or recharacterize it. And so, there is a big benefit to not having to do that as much.

But like Steve said, you have to have a standard material that you can use to kind of bridge between lots, and all those types of things. But one of the things qPCR users find out when they move to droplet digital in this area is that if your standard curve is off, every other measurement is going to be off.

Often we have people measure their standard curve with droplet digital and find that the absolute count is quite different to what they think it is. There are lots of different reasons for that, but that's an interesting and really detailed nuance to this whole quality control aspect that is eye-opening for people as they start using digital. I don't know if anyone else has had that experience, but I'd be curious to hear from anybody else on that.

Q We have touched on the need for standardization. It would be great to talk about some of the key issues and requirements related to standardization that are relevant to the various specific applications of ddPCR.

BS: Standardization of an assay in general, in a quality control environment, is something that is critical to a high-throughput clinical, commercial, QC operation.

ddPCR lends itself to that because the methodology for prep and the process is pretty standard. This holds true across different genomes, different pieces of gene you are looking for, whatever your target is – whether it be copy number, identity, or whatever it may be. That is pretty straightforward. It goes again to what Steven pointed out: appropriate laboratory logistics. It's in a clean place. It's well organized. Some of us in the industry do a lot of 5S-ing to make sure everything is organized for this standard flow.

Obviously, with that standardization comes increased assurance of accuracy. At the end of the day that is what we are looking for: a valid, reportable result.

> "...the implementation of standardized reference materials could be very helpful in initial ddPCR assay development to help ensure that our core method is accurate and robust."
>
> - Lauren M Drouin

LD: When I think about standardization, one of the major issues we face is how unique each gene therapy product is both in terms of the capsid as well as the package transgene.

Some organizations such as the USP and NIST are working together to develop various AAV reference materials that could assist in the development of robust analytical methods, including ddPCR. Certainly, the implementation of standardized reference materials could be very helpful in initial ddPCR assay development to help ensure that our core method is accurate and robust.

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However, due to the differences between each gene therapy vector, applying this to both vector genome titration assays and potency assays which are designed to be product-specific could make implementing universal AAV reference materials challenging.

SP: Reference materials would be very helpful, because especially if you are setting up training for a new group to test your material, you would be able to establish that assay is working similarly.

Another thing I have observed is just technique and pipetting. I had an example of working on a project where we were doing ddPCR to support a client, and we weren't able to get the same values with the materials that they sent us. They sent somebody out to us to show us how to do it, so to speak, and it turns out it was a difference in pipetting technique. They were doing some things that we didn't think were proper in a QC environment, and yet that's what led them to get their value consistently.

We tried it their way and it worked the way they said, but not with what we thought was a better technique. So again, that consistency and training is very important. Making sure that everybody is trained on not just how to organize themselves, but on how to perform very simple tasks like proper pipetting.

MW: There is definitely a lot of talk in the field about a standard material that everyone can reference, as a way to test their process.

If you are implementing droplet digital, you can get a material from a NISS-like organization to verify that what you are doing is good. Then when you move to your custom assay, at least you know the basics of droplet digital are working in your lab. So if something isn't working about this new assay, you start focusing on the primer probes, source material, or any inhibitors that may be unique to that material.

That is something that Bio-Rad is supporting and bringing up. What we are seeing on our side from our ITR-2 assay targeting the ITR-2 sequence, is that that was the best sequence that we could find that cuts across as many of the different products that are out there as possible. Everyone getting it from Bio-Rad is at least one step towards everybody running a similar assay as a set point or a start point.

I think it is especially important for the new labs that are moving from qPCR to digital to have one known assay that Bio-Rad knows very well, and the field knows very well. This has been valuable for everyone, so we will continue to make those.

To touch on what Steven was talking about with duplexing, we built a design engine that allows people to take one of these off-the-shelf ITR-2 assays, or a few others that we've developed, and pair it with a completely custom assay. The design engine makes sure those will two work together, so you won't experience what Steven experienced where when you pair them together all of a sudden things go haywire. That's another thing we are doing to try and help advance that aspect of multiplexing in titer measurement.

Q It would be great to explore some of the key additional areas in which ddPCR is beginning to be applied, and the impact so far.

FD: ddPCR's main advantage is to isolate vector particles and enable you to check for genome integrity, and using 2D ddPCR or duplex ddPCR for deeper characterization of the vector genome.

This could also give you key information in the early stages of your project, for example when designing your vector genome, and in the upstream process for comparison of production systems and transfection reagents, in order to improve the quality of your final product.

LD: We can also use ddPCR to measure the potency of our gene therapy products, as I alluded to earlier. Drug candidates can first be screened either *in vitro*, in an animal model, or *in vivo* using cultured cells. Then, we can use ddPCR as a readout to assess various properties of our product potency, such as vector infectivity and integration, as well as target gene expression.

For example, gene therapy products can either knock down existing bad genes, or replace missing good genes. We can assess this by coupling reverse transcription of our mRNA to DNA, and then subsequent ddPCR quantification to measure the expression of our particular target gene. This powerful technique is used in preclinical drug screening, as well as throughout the entire drug development process.

It is particularly critical for the characterization and release of our clinical material to demonstrate both our product strength as well as the batch-to-batch consistency of manufactured drug products. In the last couple of years a lot of focus has been placed on gene therapy potency assays, and I anticipate this trend will continue in the years to come.

BS: Aside from those mentioned so far, one of the interesting areas is coming out of our translational sciences function, due to the unique ability of ddPCR to measure, and its accuracy, precision and specificity in complex backgrounds.

Our translational scientists are considering and evaluating it from a clinical patient point of view. Being able to use it in pharmacokinetic studies, and asking how our therapy is working in patients. How is the CAR T expanding, how are we detecting it?

I find it fascinating that we have now moved from final product or end-process product or drug discovery, and are actually moving to the bedside with patients. I am looking forward to seeing what the future holds there.

SP: My colleagues have defined a lot of exciting things we are working on with ddPCR. Because of the specificity that we can drive it with we can go into patients or animals and look at biodistribution, and be able to distinguish between regions that don't receive a therapy product. Our primers are specific enough to identify endogenous genes that might be present there versus those areas that are expressing and have received our gene product.

This is a very powerful tool for being able to optimize our vectors *in vivo*, which is clearly where we want them to be most potent. And potency is key. We are always looking at how we determine the infectivity of the vector in as accurate a way as possible. Because although the genome titer is what we dose by, we want to know that the ratio of active molecules to just genomes is going to be consistent from lot-to-lot. These are some of the indications that we are continuing to develop and expand this technology for.

MW: I think we are seeing similar things, and it is the obvious next step as people find the value in titer to ask where else we can apply this. I will highlight two areas I have seen that are exciting and up and coming.

One is incumbent plasmid equality. We do a lot of studies at Bio-Rad, and we had plasmids coming in where there was a whole duplication of the entire gene of interest, back-to-back. We could count the molecules of promoter, gene of interest, and poly A, and we saw a doubling of the gene of interest. And it had been concatenated.

When you do a restriction digest it all looks the same. With droplet digital you are counting molecules, so we could tell that this vendor had sent us a concatenated gene

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of interest. This is one area where multiplexing and counting molecules helps with quality.

Another customer of ours did a very elegant study. He took a six well format vector copy number assay and reduced it to a 96-well format with no DNA isolation. They used cell lysate directly, and counted a reference gene copy number to establish the number of cells in the reaction. They also multiplexed an assay for their target vector, and with the reference assay, were able to very accurately count the number of molecules of their target compared to the number of genomes. They were able to scale up dramatically from six-well plates to 96-well plates, and the coefficient of variation came down threefold with the new assay format. Because of ddPCR's tolerance to inhibitors, the 96-well assay format was enabled. I think there will be more creative ways to apply droplet digital going forward now that many people have it and it is becoming more standard.

"By improving the sensitivity of ddPCR and the detection of the target sequence, for example with low cycle, ddPCR may be a good tool for the future of in-process technology."

- Fabien Dorange

Q Finally, given the regulators current priorities and likely future evolution in this regard, what is your expectation for the future application of ddPCR and other such tools? How or where will the technology need to adapt and keep improving?

MW: The one I see is the evolution of the standards of the regulatory bodies. Gene therapies are becoming much more popular, and I think the FDA has been a great partner in helping these get to the market. It is very new and there is a lot of innovation happening, and they are being quite flexible as this is going along.

There is going to be a renewed focus on what the therapeutic piece of DNA is. It's not just the vector backbone; it's the gene of interest itself. And it is making sure it's not just the gene of interest, but the promoter, plus the gene, plus the poly A. That whole cassette is required to deliver the therapeutic molecule, so I could see multiplexing becoming very important. Not just a single number titer, but all the molecules making up that titer, and making sure all of the therapeutic parts are getting into your virus and into your patient.

This goes back to what Steven was talking about. If you just look at one part of your gene of interest, what happens if it is truncated, what happens if the whole thing doesn't get in? As multiplexing is enabled there might be a higher level of scrutiny on those parts of the genome that are getting in.

Looking at identity and integrity of your plasmids, of your vectors after they are packaged, is going to be an interesting area of focus in the next 5 years.

FD: I would add that we need a method for process analytical control. By improving the sensitivity of ddPCR and the detection of the target sequence, for example with low cycle, ddPCR may

be a good tool for the future of in-process technology.

SP: If we take a step back and ask what some of the other issues that we deal with in the gene therapy realm are, one is certainly confirming that the materials we want to take into patients are free of any other adventitious agents. That has been a concern for a lot of people for a long time. Now, there are some newer technologies other than the *in vitro* assays and animal assays, and we do things like next-generation sequencing. I would not be surprised if ddPCR can play a role in that in the future.

Its extreme sensitivity could allow you to identify potential viral genomes that might be present in material at levels where we have problems with some of these other assays. And although DNA itself doesn't mean that you've got a problem, it is something the regulators are always interested in, and how we make sure things are as safe as possible. I think that's an area of future interest.

LD: In the future, we could envision a standardized ddPCR method for titering AAV gene therapy products. This would ensure that companies are dosing their respective drug products at similar levels. So a patient dose of 1E+13 vg/kg would be the same for gene therapy product A as for gene therapy product B. What this would allow us to do is correlate dose and safety data along with other critical quality attributes across the board, from one gene therapy drug product to another. We could then apply these learnings to enhance both the safety and efficacy of future gene therapy products.

BS: I'm going to go back to the promise of it at the bedside, in terms of the clinical patient samples, and what will be utilized in clinical development, clinical studies, and biometrics. How can it advance the knowledge of the performance of the gene or CAR T-cell therapy in the patient itself?

I think in the industry we have become very savvy at characterizing manufacturing processes, certainly in the last few decades. What we haven't become savvy at, and what we're still exploring, is characterizing the effect of these very complex therapeutic molecules, genes, CAR Ts, in the body. I really look forward to seeing how that is further explored.

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CELL & GENE THERAPY INSIGHTS

CELL THERAPY BIOPROCESSING & AUTOMATION

SPOTLIGHT

INNOVATOR INSIGHT

Enabling clinical grade manufacturing of gene-engineered NK cells

Nina Möker, Rizwan Romee & Evelyn Ullrich

In the adaptive cell and gene therapy field, T cells are currently receiving a lot of focus and showing success in making it to the market – but natural killer (NK) cells are beginning to close this gap. In particular, CAR NK cells for cancer cell therapy offer a number of distinct advantages. But what benefits, and what challenges, do CAR NK cells pose? In this article and expert discussion the manufacturing, analytics, and functionality of CAR NK cells will be discussed, along with further engineering strategies to overcome some of the obstacles still facing this modality.

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WHY CAR NK CELLS?

Looking at the adaptive cell and gene therapy industry it is immediately obvious that autologous T cells, engineered either with a CAR construct or T-cell receptor (TCR), are most advanced in terms of making it through the pipeline and towards market. However, natural killer (NK) cells have also been used in a number of clinical trials, and increased numbers of such potential therapies are now heading down the development pipeline, many of which are engineered with a CAR construct.

CAR NK cells for cancer cell therapy offer a number of notable advantages, including:

- Low risk
  - Low risk of graft versus host disease (GvHD)
  - Low risk of cytokine release syndrome (CRS)
  - Limited risk of on-target, off-tumor effect (limited persistence)
- Allogeneic source
  - Beneficial for patients who cannot undergo leukapheresis or face time pressure
  - CAR NK cells can be generated from multiple sources
  - Potential for off-the-shelf use
- Anti-tumor activity
  - CAR-guided anti-tumor action and activity independent of antigen priming
  - Potential prevention of cancer cell antigen escape

If we look more closely at anti-tumor activity, CAR NK cells have a number of helpful features. They can have CAR-guided anti-tumor action, but also innate immunity, so they can be active independent of antigen priming. Therefore they have the potential to prevent cancer cell antigen escape.

One potential limitation is the duration of the response – NK cells and CAR NK cells have limited persistence after adoptive transfer and depend on cytokine support for persistence.

Figure 1 shows a list of ongoing clinical trials which are using engineered NK cells in cancer cell therapy. These include CAR NK cells derived from various sources, including the cell line NK-92, induced pluripotent stem cell (iPSC) -derived, cord blood-derived, and peripheral blood-derived CAR NK cells.

CAR NK CELL MANUFACTURING: TAKING A TRANSLATIONAL APPROACH

At Miltenyi Biotec, we have developed a number of solutions for the manufacturing of gene engineered NK cells, and offer a complete workflow for translational approaches (Figure 2).

FIGURE 1

44 engineered NK cell trials are currently underway as of November 2021.

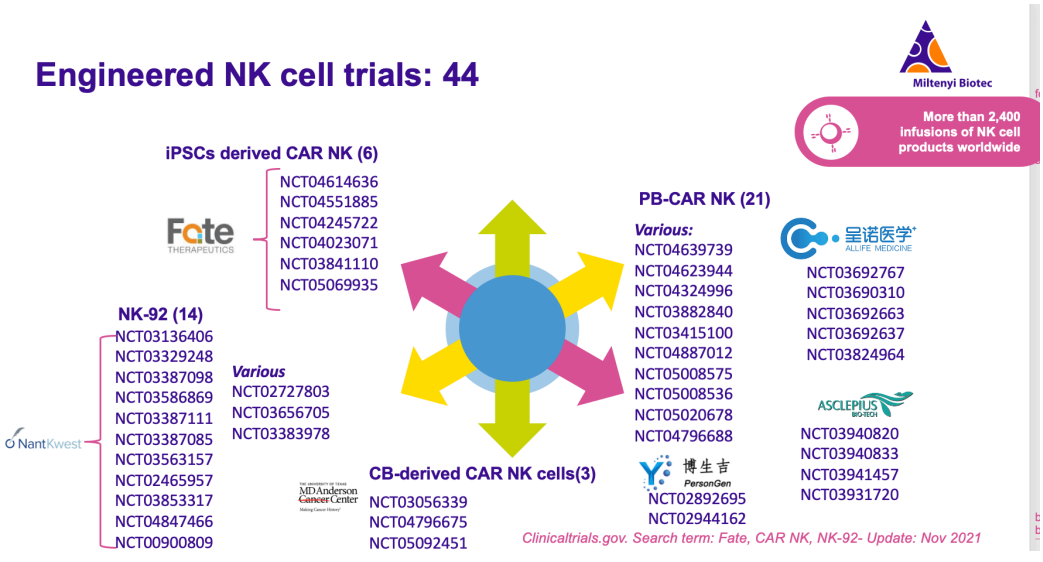

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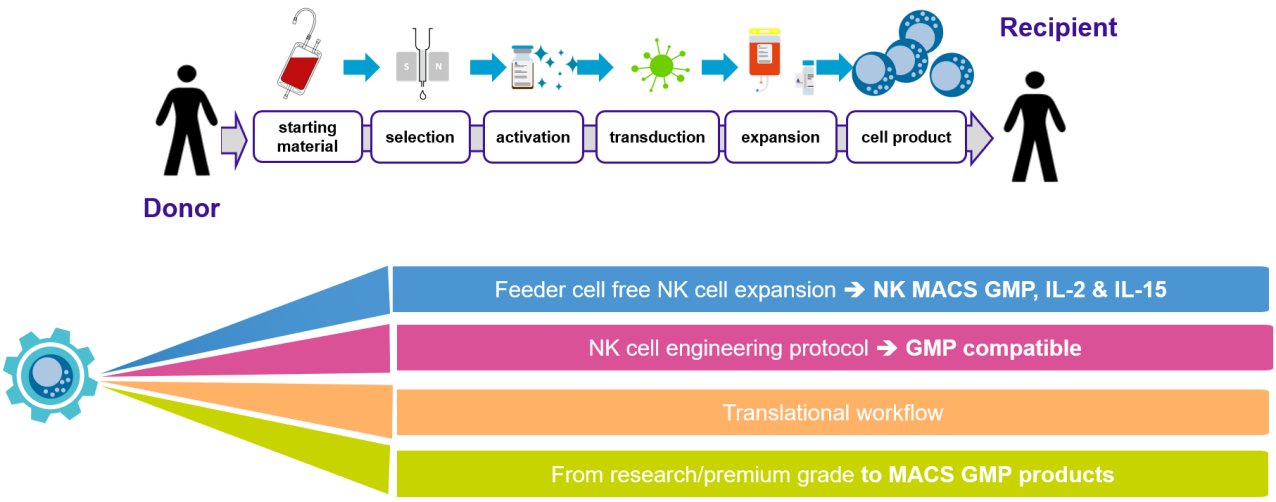
FIGURE 2

CAR NK cell manufacturing workflow.

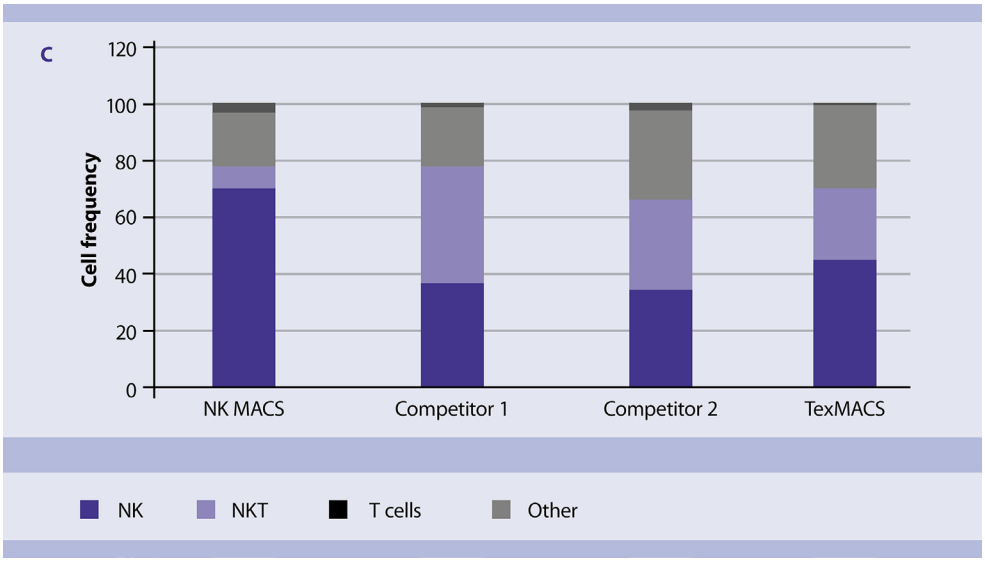


The first step begins with obtaining leukapheresis material from a healthy donor. Next, a selection of highly pure NK cells are obtained using magnetic labeling. This is followed by activation, transduction, expansion, and finally formulation of the cell product for the patient. These workflows include a medium for feeder cell-free NK cell expansion, an NK engineering protocol which is GMP compatible, translation of workflows, and also reagents of GMP standard quality.

Our NK cell expansion protocols enable NK cells to expand without having to use feeder cells in the culture. Another feature of this medium is that it promotes preferential expansion of NKs cells as compared to the expansion of other cells, especially T cells, B cells, and dendritic cells. Figure 3 illustrates

FIGURE 3

Frequency of NK cells following 14 day expansion of PBMCs using NK MACS Medium and other media.



cell composition after 14 days of expansion, using peripheral blood mononuclear cell (PBMC) as a starting material. The major fraction after this expansion is NKs cells. Importantly, the effector functions are preserved after the expansion.

Identifying a suitable pseudotype

Lentiviral vector (LV) -based gene engineering has been widely used for CAR T therapies, and has many known advantages including low genotoxicity and insertional mutagenesis, along with the ability to integrate the genome of non-dividing cells. Unfortunately, for NK cells this vector system has shown low efficiency of transduction when using the same pseudotype as for T cells; i.e. the VSV-G pseudotype. This is due to the lack of LDL receptor, one of the major receptors of the VSV-G protein. After activation, T cells express this at high levels on their surface, whereas NK cells do not. This difference was the basis for selecting the baboon envelope glycoprotein (BaEVgp) pseudotype lentiviral vector system.

The BaEVgp pseudotype uses a different surface expression marker for recognizing the cells it transduces: the ASCT1 and ASCT2 proteins. Shown in Figure 4 is a western blot for ASCT2 expression, and as can be seen, NK cells highly express it after activation. It is also expressed on activated T cells, and on the NK-92 cell line.

The transduction of NK cells with the VSV-G pseudotype lentiviral vector is very low in its efficiency. In contrast, when changing to the BaEVgp pseudotype, transduction of NK cells showed high efficiency, at 73%. IL-2 and IL-15 were also used, along with the addition of an IL-1 family cytokine at the beginning of the culture.

Creating an efficient protocol

Once an LV pseudotype was chosen we created a protocol that gives efficient transduction, so that only small amounts of lentiviral vector are needed. One way to achieve this is to perform the transduction in the presence of Vectofusin-1. In the example shown here, without Vectofusin-1 the multiplicity of infection was around 20%, versus ~80% in the presence of Vectofusin-1 (Figure 5).

A way of further increasing the efficiency of transduction is to add a spinoculation step (Figure 5). This can be done in the CliniMACS Prodigy in a closed system because the cultivation chamber also functions as a centrifuge.

However, the number of CAR expressing NK cells in the culture is not the only important metric – it is also important to consider the vector copy number (VCN). Regulatory authorities require that you stay below a vector copy number of 5. If you increase the MOI of the lentiviral vector, the VCN logically increases, as does the transduction efficiency.

AUTOMATED MANUFACTURING OF CAR NK CELLS

All the data presented above are from a research setting, i.e. in the manual and small scale. Here, data from manufacturing in the CliniMACS Prodigy will be discussed.

FIGURE 4

ASCT2 is expressed on activated T cells and activated NK cells..

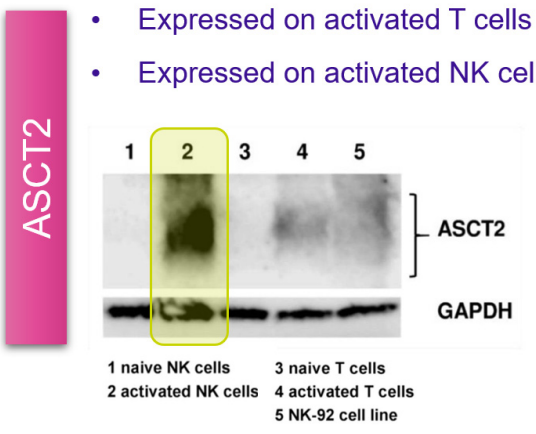


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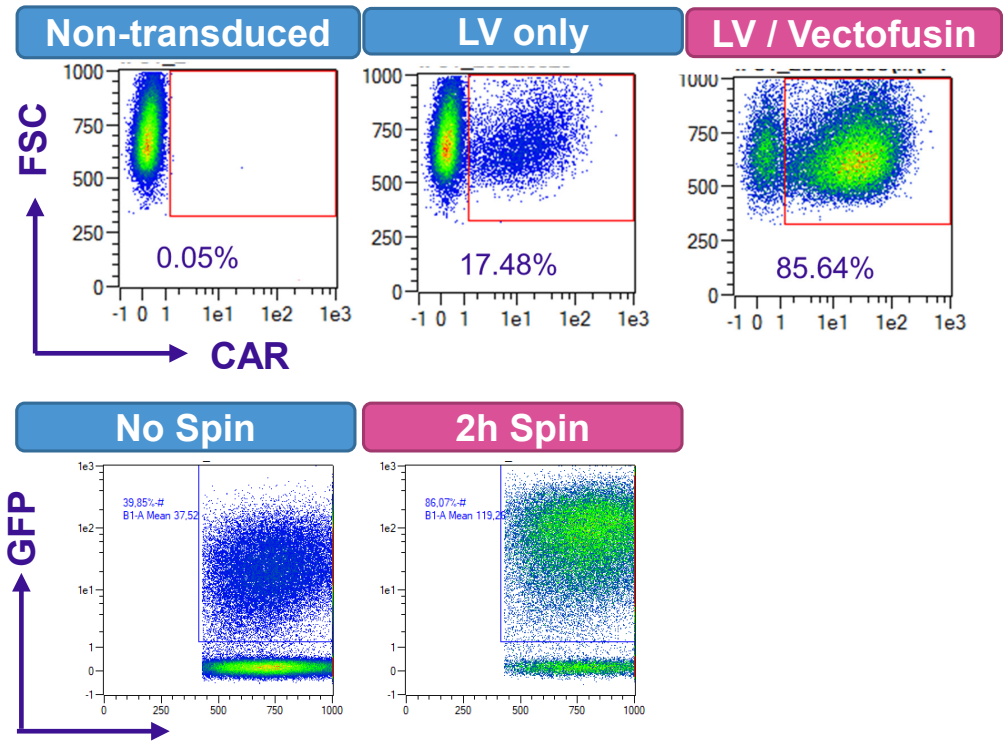
FIGURE 5

Transduction efficiency optimization with Vectofusin-1 and spinoculation..



Selection was performed by actively depleting CD3+ cells, as in an allogeneic setting it is important to have a very low number of T cells left. This is followed by an enrichment step with $CD56^{+}$ cells, to achieve pure and viable NK cells. Following the activation of the NK cells in the presence of the cytokines discussed above, transduction is performed on day 2 in the presence of Vectofusin-1 and with a spinoculation step.

The transduction rate was high and stable (Figure 6) on day 7 and 14. The average is about 50%, and each dot in Figure 7 represents a single leukapheresis; i.e. each represents a different donor.

Figure 7 shows an example of expansion of CAR NK cells in the Prodigy.

The cell product had high purity and viability, with less than 0.5% contaminating cells – which is particularly important for the T cells and the B-cells in the product. NK and CAR NK cell counts in the cell product is shown in Figure 8. The total NK cell numbers were around $2x10^{9}$, with CAR positive NK cells being around $5x10^{8}$. These are the numbers for the current process which is in the standard sized chamber for the Prodigy, and Miltenyi intends to release this in quarter 1 next year. We have also begun the development of a next generation large-scale process for NK cell transduction, aiming at a CAR NK cell yield of 10^{10}.

Considerations for off-the-shelf and large-scale NK cell expansion

The manufacturing of engineered cells is a very complex process, and a lot of different systems have been used and been shown to be suitable. However, a lot of these systems require several manual steps, and this poses a risk for contamination and human error. Our strategy is to automate all of these critical steps by having them in a closed system in the CliniMACS Prodigy. When considering off-the-shelf and large-scale NK cell expansion, there are two options for making use of this automated closed system.

FIGURE 6

Transduction efficiency in the CliniMACS Prodigy.

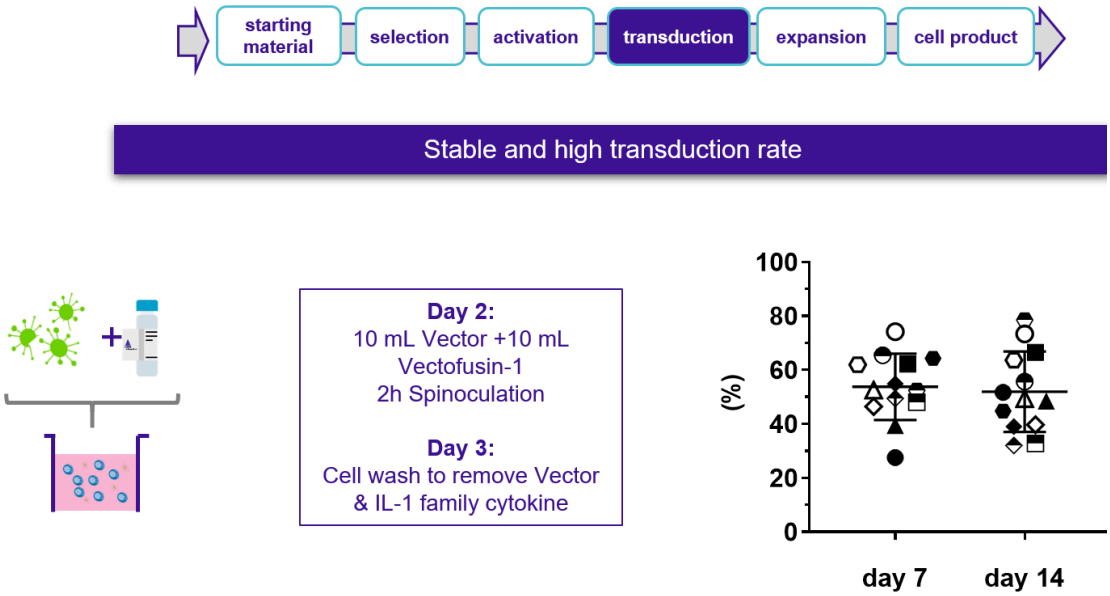
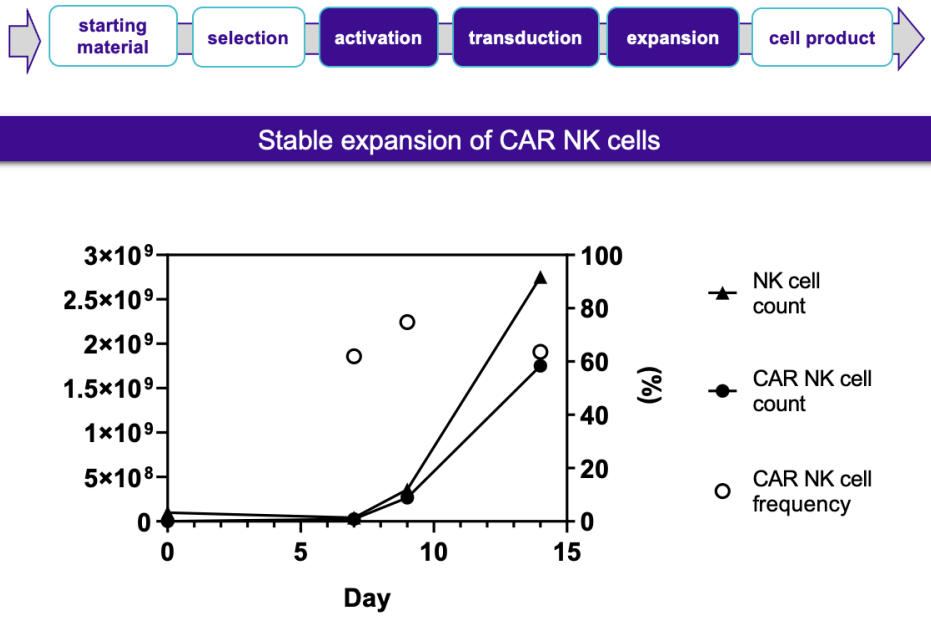


FIGURE 7

CAR NK expansion in the CliniMACS prodigy.



One option to perform all of the separation and engineering steps within the Prodigy, then connect this to a bioreactor system. This can also be connected back to the Prodigy in order to have automated formulation of the cell product. Another option is to generate CAR NK cells in the Prodigy and then freeze the master cell bank, which can then be used as seeding material for a bioreactor.

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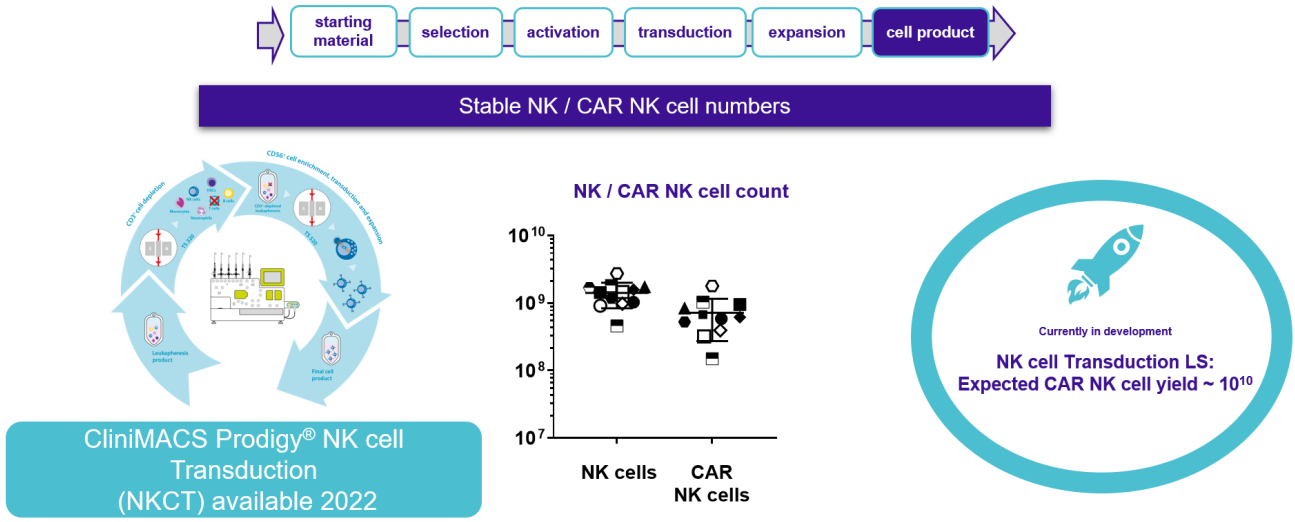
FIGURE 8

Stable NK/CAR NK cells using the CliniMACS Prodigy.



ANALYTIC SOLUTIONS FOR CAR NK CELLS

Setting up analytics for a cell manufacturing process involves as much effort as setting up the manufacturing itself, and it is critical not to underestimate this part of the process.

To facilitate the harmonization of analytics in cell and gene therapy, especially between different centers, we implemented Express Modes in the MACSQuant. Express Modes include automated gating strategies, which were designed as a tool for standardization of data analysis. They are a unique tool that delivers exclusive advantages for in-process control and quality control. The advantages include ease of use, significant time savings, reduction of human error, and simple documentation. The system is quick to integrate into standard operating procedures for robust analysis.

An overview of our analytics strategy is shown in Figure 9. For donor selection antibody panels are available to look at the expression profiles of the NK cells, which are also available with the corresponding Express Modes.

CAR detection reagents

Miltenyi has two types of CAR detection reagent – a biotinylated antigen fusion protein or anti-idiotype monoclonal antibody (mAb). Both CAR detection reagents have a mutated human IgG1 Fc which will not bind to Fc gamma receptors. A number of these are currently available, with more in development.

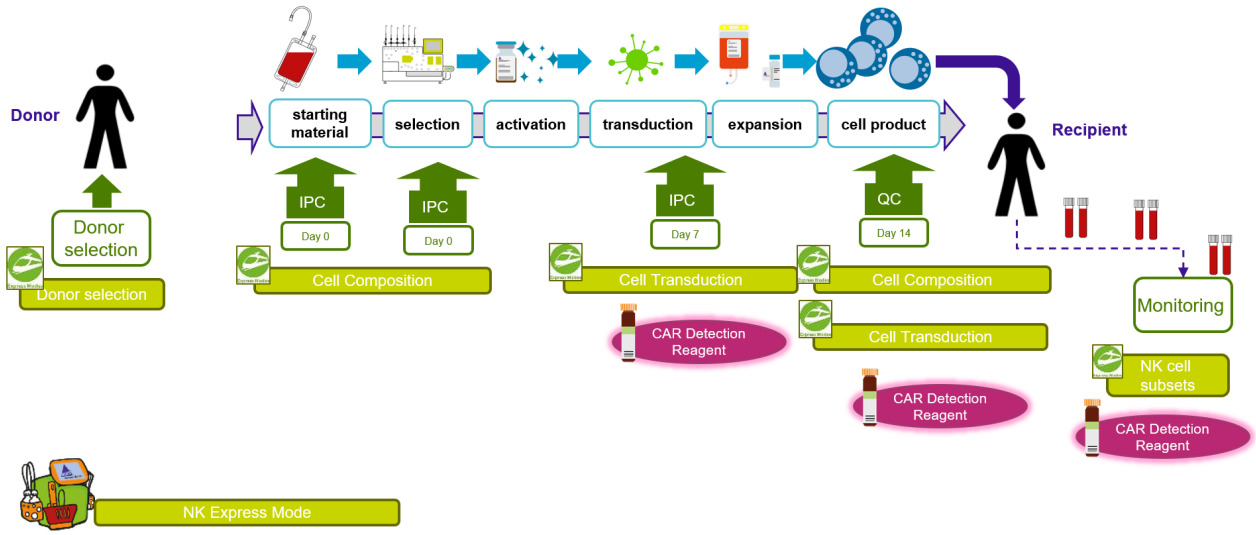
CAR NK CELL FUNCTIONALITY

Functionality data was obtained from CAR NK cells manufactured in the Prodigy as described above. A human acute leukemia cell line that is resistant to NK cell innate immunity was used as a test system. NK cells can kill most tumor cell lines, so a resistant cell line with upregulation of ligands against inhibitory receptors and downregulation of ligands for certain activating receptors was screened for.

When the cell line is transduced with the tumor target, un-transduced NK cells cannot kill it. In contrast, the CAR NK cells very effectively and specifically killed the cells after the tumor target had been introduced into

FIGURE 9

CAR NK cell analytics strategy.



them. This demonstrates two things. One, that the CAR recognition is very specific. Two, that the CAR can overcome the inhibitory effects towards the NK cells.

We also tested the CAR NK cells after manufacturing in the Prodigy in an *in vivo* mouse model with an acute leukemia cell line. We injected IL-2 on day minus 1, and then over a time span of 7 days to look at the persistence of the CAR NK cells. The CAR NK cells showed very efficient anti-tumor activity in the presence of the cytokine. After stopping the cytokine supply, the persistence of the CAR NK cells is limited and the tumor relapsed.

CHALLENGES OF CAR NK CELL THERAPY AND FURTHER ENGINEERING

Manufacturing and functionality aside, one of the major challenges with CAR NK cell therapy is persistence after adoptive transfer. Furthermore, if you use a tumor target that is also expressed on the NK cells, or upregulated during the culture, then you may have a phenomenon known as fratricide where the CAR NK cells kill other NK cells in the culture.

There are various ways to address the listed challenges. To improve the NK cell persistence, one strategy is stimulation with a cytokine cocktail during manufacturing. Another is co-expressing the cytokine within the CAR construct. For an improved NK cell activity, inhibitory immune checkpoints can be knocked out. To be able to further engineer the CAR NK cells we developed an electroporation protocol, and transduction as well as electroporation can be done within one Prodigy run.

To develop the protocol for the electroporation of NK cells we made use of the high flexibility of the Miltenyi electroporator. This enabled us to find settings where we could have a high electroporation efficiency, with more than 80% GFP positive NK cells, while still retaining a high viability. GFP expression after electroporation was also stable over time.

In regards to expansion capacity and effector function of NK cells after electroporation, we were pleased to find that the NK cells were not impaired as a consequence of the electroporation. Expansion capacity, cytokine release after co-culture with tumor cells, and cytotoxic activity were retained. With this

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electroporation protocol as a basis we performed knockout of various markers.

SUMMARY

CAR NK cells offer a range of advantages for treating cancer, including a low risk for GvHD and cytokine release syndrome, effective anti-tumor activity and the ability to generate cell products from multiple sources. However, compared to T cell engineering, many challenges still have to be overcome.

Miltenyi Biotec has developed a number of solutions for the manufacturing of clinical-grade gene engineered NK cells, and offers a complete workflow. We are now exploring further engineering approaches in order to overcome the ongoing challenge of persistence after adoptive transfer.

ASK THE EXPERTS



Charlotte Barker, Editor, BioInsights, **speaks to** (from left to right) **Nina Möker,** R&D Reagents Manager Allogeneic Cell Therapy, Miltenyi Biotec, **Rizwan Romee,** Associate Professor of Medicine, Harvard Medical School, Dana Farber Cancer Institute, and **Evelyn Ullrich,** Professor of Cellular Immunology, Johann Wolfgang Goethe University in Frankfurt

Q What are the main advantages of using NK cells in cell therapy?

RR: This is my favorite question because I get to stress my love of NK cells. We don't see cytokine release syndrome, which is still a big problem despite all the advances in CAR T cell work. Similarly so far neurotoxicity has also not been reported with the adoptive NK cell/NK cell CAR trials.

Furthermore, NK cells maintain both the CD16 as well as other non-CAR mediated killing of the tumor cells, which you can still harness by combining, for example, anti-MICA/MICB antibodies, or you can engage the CD16, and some of the other activating receptors.

This potentially allows tumor targeting by multiple pathways; CAR against a specific antigen expressed by the tumor cells, Fc receptor, when combined with Fc engaging monoclonal antibodies and endogenous activating receptors like NKG2D which can bind to stress ligands expressed.

EU: I completely agree. One issue that I like very much when working with NK cells is all of their intrinsic anti-tumor ability. Even if you think of engineering NK cells for adaptive anti-tumor therapy, NK cells even function in absence of antigen expression and have an intrinsic anti-tumor effect based on this balance of activating and inhibitory receptors, especially in protocols where NK cells are activated with cytokines.

They have a low risk of GvHD and that's really amazing too. We can now engineer NK cells that have a CAR-mediated anti-tumor effect that is very target specific, but in NK cells we also profit from the intrinsic anti-tumor effect.

RR: Evelyn mentioned GvHD, and one of the challenges in immunotherapy in general has been on-target off-tumor toxicity. Applied to NK CAR cells, they have unique ways of differentiating tumor cells from the normal healthy cells even when CAR target is expressed by normal healthy cells, this includes Killer Ig-like receptors (KIRs) binding to class I HLA molecules (primarily HLA-C) and NKG2D binding to the stress ligands like MICA/MICB on tumor cells. This allows effective targeting of the tumor cells with minimal toxicity against normal healthy tissues.

EU: Exactly, that's a well taken point. It may be possible to use NK cells for tumor entities that are difficult to target such as acute myeloid leukemia where you don't have a highly specific tumor target. Most of the potential target molecules are also expressed on healthy, hematopoietic cells.

NM: I would like to add the point of view of manufacturing in the main advantages of using NK cells. We all know that despite the remarkable progress we have seen with CAR T cell therapy in cancer, with FDA approved products currently on the market, its autologous nature still limits the patient's accessibility.

In an attempt to address these limitations, the field is turning towards allogeneic platforms. T cells are not ideal platforms for allogeneic therapies due to their HLA-dependent recognition and potential for inducing GvHD. This is another area in why NK cells are safe and suitable for allogeneic applications, and for off-the-shelf cellular immunotherapy aspects.

Q What are the new trends in engineering NK cells?

RR: I would like to mention key advances in the IPSC technology. Theoretically you could generate any type of immune cells including NK and T cells from the IPSCs , but

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so far it is mostly the NK cells that have been successfully generated in a large scale using this technology. That gives a great head start for the NK cell field. IPSC-derived NK cells are quite attractive as you can generate them in massive numbers thus allowing off-the-shelf manufacturing. Also importantly you can apply CRISPR and other gene editing tools to genetically manipulate IPSCs before differentiating them into the NK cells.

EU: Concerning the engineering of NK cells, innovative technologies have been improved in recent years allowing to engineer primary NK cells from different sources including IPSCs. Nina has already presented these possibilities; both viral transduction and also non-viral procedures for efficient stable transduction of NK cells are very helpful.

In the beginning it was very difficult to overcome limitations of transducing NK cells and the VSV-G pseudotype-based lentiviral transfection protocols worked much better in T cells. But in the last few years, we have learned that NK cells express different entry receptors which are well suited when the corresponding envelope is used. For example pseudotyping with RD114, as we found in our group, or the baboon pseudotype lentivirus work very well with NK cells.

Q What are the challenges of efficient transduction of NK cells?

RR: These are really exciting times in the field because finally we are at a point where gene manipulation of NK cells does not seem to be a major challenge anymore, especially with the baboon and some other unique glycoproteins which we now use to pseudotype the lentivirus for facilitating transduction.

We are finally at the point where transduction is not a barrier any more, which is awesome.

NM: We have focused our efforts on the lentiviral vector-based production because of the known advantages. The clinical safety has been proven, transduction is efficient, and it even transduces non-dividing cells.

As shown, and also as Rizwan has mentioned, changing the pseudotype really helps with transducing NK cells. This goes for every new pseudotype, that new challenges come when changing the pseudotype. If you have a lentiviral vector production system that has been optimized for one pseudotype, things can change. The production protocol may have to be re-optimized. It has to be considered that especially when settings have been optimized for specific pseudotype, for any new one coming, there are production protocols and QC protocols for the LV vector itself that will have to be optimized again.

Once you have defined a lentiviral vector system it is still very important to invest the effort on defining the exact specifications for each new construction that you are introducing. While we don't have to change the general manufacturing protocol anymore, like adding Vectofusin-1 or including a step of spinoculation, we still have to take a close look into new constructs with regards to reproducibility, which MOI to use, the transduction efficiency, the vector copy number, and the stability on the surface expression. And then also *in vitro* and *in vivo* functionality.

If single chain variable fragments for the same target are introduced in the same CAR backbone, but the single chains themselves differ, this can have a different effect *in vitro* and *in vivo*. So there must be a significant amount of research for the best CAR constructs.

Then not to forget, you always need a CAR detection reagent which has to be produced, and it is very important that it is specific and really able to stay in the CAR expression on the surface of the NK cell. Depending on the nature of the target, it can be easy to produce, but this can also be very challenging in itself.

Q We have had an audience request for some practical advice. How do I translate my research workflow into a GMP grade manufacturing process? What are the key considerations and best practices in transitioning from open manual to closed automated bioprocessing? What are the challenges and gaps there?

NM: To translate the research workflow into GMP grade manufacturing process, our vision and strategy is to shift away from an open manual process towards the closed and automated system. We want to reduce the open steps as far as possible, because each manual step poses a risk for contamination as well for human error.

Each manual step is also time intense in the clean room, so one more strategy would be to reduce the required clean room class as far as possible to reduce the cost and effort to maintain the clean room.

In the US for CAR Ts they are manufacturing in the Prodigy, and the recommended clean room class is C. In Germany we are currently working towards approval for clean room class D for engineering in the Prodigy.

Then of course the manufacturing protocol must be highly reproducible and the situation of donor-to-donor variation. It has to be set up in a way that ensures that efficient transduction and extension is obtained from every donor.

Another important point is that all ingredients have to be GMP compatible, so it is desirable to set up the research only processes with reagents that are available at GMP quality.

Another very important challenge is the timing of the manufacturing. Reduce the steps which require people in the clean room as much as possible, because everything takes at least three times as long in the clean room as opposed to the normal lab.

RR: I think the biggest challenge in terms of manufacturing CAR NK cells when translating from preclinical work to clinic is getting access to the GMP grade lentivirus as currently there are only a few commercial vendors or academic centers with the ability to make it. And then if you want to use a baboon-based system it is even more challenging because most of the vendors that make GMP grade virus don't have much experience with it. As a matter of fact, as far as I know, Miltenyi is the only vendor which has experience with a baboon-based lentiviral system.

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The Prodigy system is really attractive. As Nina mentioned, if you can downgrade clean level, that will make everybody's life easier. Otherwise it takes an incredibly long time to manufacture these cells. It is very labor intensive, currently. I think the Prodigy and any similar system that uses closed manufacturing twill be really helpful for both CAR NK cells and the T cell field.

EU: These points are of high importance, first to get excellent GMP grade virus production and then to have the capability for production of a large amount of CAR NK cells. This takes us to the other aspect, we mentioned in the beginning, that new technologies also emphasize on non-viral systems which could also be used in the Prodigy closed system combined with electroporation protocols.

Besides the RNP-based electroporation of NK cells, there are other technologies such as to introduce the cargo via transposons. The *Sleeping Beauty* transposon system is well known for CAR T cell production. These are currently in clinical trials and could also be used for non-viral transduction of NK cells in automated systems. This technology could be less costly, and also has been reported to show a very safe genomic integration profile, which could be beneficial.

However, we will need to find markers and profiles for the quality check of the resulting NK cell products, regardless of whether they are viral or non-viral transduced and engineered cells. It will be of high importance at the end of the procedure to have QC based on flow cytometry, mass cytometry, or PCR, and hopefully to find predictive markers to know the impact and the functional quality of the resulting cell product.

Q Regarding analytical tools, what is the current state of the art, and where do you hope to see further innovation in this regard?

RR: In terms of the current work outflow we have for analyzing our products before they are released, they are manual and slow, and again that is very cumbersome. We use the markers Nina mentioned earlier, like the ones for viability etc., but it is all done manually, making it labor intensive.

If you can build in these analytic tools where you have the capacity in the closed system that give you information on the key elements like transduction efficiency, viability and things like that, and generate a number which meets the threshold for product release, that will make life easy for everyone.

That is in terms of QC analytical tools. But then on the other side, after you put adoptive transfer NK cells and CARs into people, what methods do you use to track them? It is critical that we don't forget about investing into developing detailed analytical tools which will allow us to track these cells and what happens to these cells after adoptive transfer like how long they last, their exhaustion, and if they are able to home in to the tumor site.

Good, qualitative science is very important. NK cells are probably ten years behind T cells, and there is a lot to learn. Having the right analytical tools to be able to do a deep dive after the adoptive transfer is also very important to help advance the field.

EU: I just want to highlight, as Rizwan mentioned, that in the future I hope we will have the chance to further continue learning how to engineer NK cell products to improve functionality, and also migration and persistence of these cells.

NM: By developing the CAR T cell therapies, which Miltenyi and also a lot of other companies and academics have done, we have learned that establishing the analytics of a gene engineered cell product is as least as much effort as establishing the manufacturing process itself. This is something that many people, including ourselves, have been guilty of underestimating.

To help with this, it is important to have automation in the accompanying in-process control and quality control of the cell product. An automated gating strategy to enable harmonization between centers, but also to have defined panels for each cell product. The next step would be to have pre-mixed antibody cocktails or even freeze-dried formulations of the same so this would be even more standardized.

An important aspect of NK cells versus T cells is that in quality control we have the rare cell analysis for CD3. This is one of the risk factors in the allogeneic setting, and also something that can be time consuming for the sign-off QC of the cell product. Rare cell analysis requires you to count quite a volume to be sure that enough cells have been counted to give a really reliable answer on how many T cells are really left there.

It is also very important to have a panel that is very reliable here, and does not give any background. This T cell count is really key in the allogeneic setting.

Q What are the advantages of using NK cells from cord blood versus peripheral blood for cell therapies?

NM: Many sources are available for allogeneic NK cells, including NK cell 92 cell line, PBMC derived, hematopoietic stem cell derived, induced pluripotent stem cell derived, and umbilical cord blood derived. Each source has advantages and limitations. Overall they do share one benefit, which is that they can all be produced for allogeneic cell therapies. This includes the reduced manufacturing time, the increased feasibility for large-scale production, and everything else that comes with that.

When it comes to direct comparison, I would be more interested in what Rizwan or Evelyn would have to say, because our approaches have so far focused on peripheral blood derived NK cells. We are working on other sources, but our main experience is with peripheral blood NK cells.

RR: It is always good to have more options. And it's a good problem to have.

I personally like cord blood because we have hundreds and thousands of untapped cord blood units already stored and HLA typed. If you can tap into that resource, I think that's fabulous.

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Our group primarily uses what we call memory-like NK cells which are generated from peripheral blood. We have shown that these memory-like NK cells are easy to generate and differentiate from peripheral blood, and they can persist for months in an immune compatible environment and also expand. This is a great advantage, and we are now arming them with CARs and doing other gene manipulation to enhance their tumor targeting.

There aren't any direct head-to-head comparison studies comparing NK cells from cord vs peripheral blood or other sources.

EU: We really have to focus on the benefit for the patient. From the CAR T cell field, I believe that CD19 CAR products were really great, but the generation of autologous CAR products could fail in patients who were heavily pre-treated and the immune cells were not expanding as expected. That means, in such cases, a different source could be used to obtain a special NK cell product produced from third-party donors, for example after allogeneic stem cell transplantation or in haploidentical settings. This is a great advantage for the patient.

BIOGRAPHIES

Nina Möker

R&D Reagents Manager Allogeneic Cell Therapy, Miltenyi Biotec

Nina Möker is a R&D Reagents Manager for the development of allogeneic cell therapy for cancer at Miltenyi Biotec in Bergisch Gladbach, Germany. She is leading an innovative team with the focus of developing the comprehensive solution package for the manufacturing and analytics of engineered Natural Killer cells. For the past five years she has been working together with the Miltenyi clinical production and clinical development teams to overcome the challenges of manufacturing NK cells with high functionality in a GMP compliant and feeder cell free system. Prior to joining Miltenyi Biotec and dedicating herself to allogeneic cell therapy, Nina has gained strong experience in big and medium-sized pharma focusing on antibody- and small molecule-based drug discovery.

Rizwan Romee

Associate Professor of Medicine, Harvard Medical School, Dana Farber Cancer Institute

Rizwan Romee is an associate professor of medicine at Harvard Medical School and director of the haploidentical donor transplant program at Dana Farber Cancer Institute in Boston. The research focus of his laboratory is genetic manipulation of human NK cells to enhance their anti-tumor function and modulate the highly immune-suppressive tumor microenvironment. His earlier work at Washington University helped describe human memory-like NK cells with enhanced anti-tumor activity and he led a first-in-human clinical trial of these cells in patients with relapsed and refractory AML.

Evelyn Ullrich

Professor of Cellular Immunology, Johann Wolfgang Goethe University in Frankfurt

Prof. Ullrich, MD, is Professor of Cellular Immunology at the Johann Wolfgang Goethe University in Frankfurt a. M., Germany. She is head of the research unit "Experimental Immunology" and of the "Cancer Survivorship Unit" at the UCT, University Hospital of the J.W. Goethe University Frankfurt am Main, Germany. She obtained her medical degree

from the Albert Ludwigs University in Freiburg and was further trained at the Universities Regensburg, Erlangen and Paris. She is a board-certified specialist in internal medicine and immunology. As junior professor she headed a Max Eder research group sponsored by the German Cancer Aid. Since 2012, she is leading the Experimental Immunology Unit at the Children's Hospital in Frankfurt, supported by the LOEWE Center for Cell and Gene Therapy Frankfurt. She has been involved in the characterization of natural killer and innate lymphoid cell subsets. The current research of her group addresses the understanding of immune regulation following SCT and the development of cellular immunotherapeutics with focus on genetic engineering of immune cells towards translational clinical application. Currently, Evelyn Ullrich is speaker of an NK cell consortium in the Priority Program 'Translational Oncology' supported by the German Cancer Aid. Since 2020, she is co-speaker of the Mildred Scheel Career Center (MSNZ) in Frankfurt, which is an excellence program for promoting young scientists in cancer research.

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AUTHORSHIP & CONFLICT OF INTEREST

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

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CELL & GENE THERAPY INSIGHTS

CELL THERAPY BIOPROCESSING & AUTOMATION

SPOTLIGHT

EXPERT INSIGHT

Considerations for the development of autologous CAR-T cell products

Alexander Davidson, Gerardo Santiago Toledo, Kostadinka Lilova, Michael Merges & Peter Jones

Successful clinical outcomes for cancer patients are leading to significant investment flowing into a wave of novel innovative CAR-T cell products currently under clinical development. Although initial drug approvals have been relatively small to date, the significant pipeline of CAR-T cell therapies currently underway will significantly expand the impact of these treatments, and unleash their genuine, unprecedented potential. These personalized therapies, however, pose unique challenges for manufacturing and commercial scale-up/out. From our perspective, we will address here some of the key challenges to establish an effective process for clinical manufacturing and beyond, namely the patient starting material (leukapheresate), the increasing commercial availability of flexible closed manufacturing platforms for autologous therapies, analytical method development, the suitability of critical raw materials and reagents as well as vector supply.

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INTRODUCTION

Chimeric antigen receptor T cell (CAR-T) therapy involves the genetic modification of autologous patient's or allogenic T cells to specifically target and destroy cancer cells [1,2]. Current CAR-T therapies have shown sustained relapse responses and improved safety profiles that have made these treatments

successful in clinical trials for B cell tumours [2-5], with four CD19-specific autologous products approved by the US Food and Drug Administration (FDA) (Table 1). The manufacturing of autologous CAR-T cell therapy products is a complex process, consisting of

TABLE 1

Overview of commercialized CAR-T cell products.

Brand		Kymriah®	Yescarta®	Tecartus®	Breyanzi®
Name (Code)		Tisagenlecleucel (CTL019)	Axicabtagene ciloleucel (KTE-C19)	Brexucabtagene autoleucel (KTE-X19)	Lisocabtagene maraleucel (JCAR017)
Launch		2017	2017	2020	2021
Company		Novartis	Kite	Kite	Juno/Bristol Myers Squibb
Target		CD19	CD19	CD19	CD19
Starting population		PBMC	PBMC	$CD4^+$ $CD8^+$	$CD4^+$ $CD8^+$ (central memory)
Vector		Lentiviral	Retroviral	Retroviral	Lentiviral
Manufacturing process	Starting material state	Frozen	Fresh	NA	NA
	Enrichment/ Selection	Density gradient enrichment	Ficoll density gradient enrichment	$CD4^+$ $CD8^+$ selection	$CD4^+$ selection $CD8^+$ central memory or $CD8^+$ selection
	Activation	Activation with anti-CD3/anti-CD28 antibody coated magnetic beads	Activation with anti-CD3	Activation with anti-CD3 and anti-CD28 and IL-2	Activation with anti-CD3/anti-CD28 antibody coated magnetic beads
			Wash post-activation	Wash post-activation	
			Incubator culture in serum-free media with IL-2	Incubator culture in serum-free media with IL-2	
	Transduction	Lentiviral Transduction	Retroviral transduction in culture bags	Retroviral transduction in culture bags	Lentiviral Transduction
	Expansion	Expansion in Static culture	Expansion in culture bags in incubator	Expansion in culture bags in incubator	Expansion
		Expansion in rocking motion bioreactor			
	Post-harvest steps, formulation, and cryopreservation	Bead removal	Wash and cell concentration	Wash and cell concentration	NA
		Formulation in Plasma-Lyte A, Dextrose in sodium chloride, Dextran 40 in Dextrose HSA, and Cryoserv® DMSO	Formulation in CryoStor® CS10 and HSA	Formulation in CryoStor® CS10 and HSA	Formulation with 1:1 $CD4^+$ $CD8^+$ ratio in CryoStor® CS10, electrolytes and HSA
		Cryopreservation	Cryopreservation	Cryopreservation	Cryopreservation

NA: Not available.

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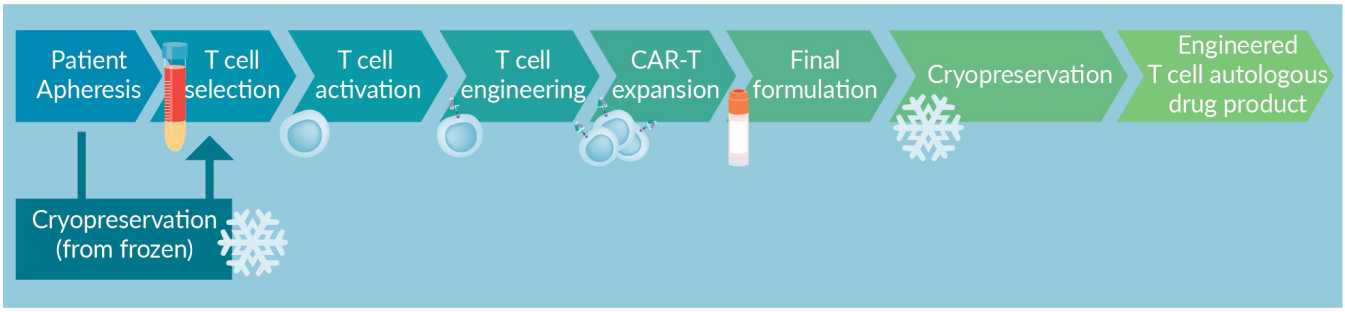
FIGURE 1

Flow diagram outlining the typical process unit operations required for CAR-T cell therapy manufacture. Arrows represent the transfer of product material.



numerous unit operations performed *ex-vivo* (Figure 1). Each one of these steps has an impact on the success of the final drug product (DP) which is designed to be infused back into the same patient, and therefore requires fully trained and skilled operators. To obtain sufficient cells for the manufacturing process, a leukapheresis step is performed to separate the patient's peripheral blood mononuclear cells (PBMCs) from whole blood [6]. Thus, the patient's PBMCs are the starting material for the CAR-T cell therapy manufacturing process, followed by T-cell enrichment and T-cell activation, typically via CD3 and CD28 primary and co-stimulatory signalling without the use of antigen-presenting cells [7]. The activated T cells are then subjected to gene modification through either viral vectors or non-viral based editing techniques, and the subsequent CAR-T cells are expanded to achieve the target dose required. Post-expansion, the cells are formulated or cryopreserved in formulation for infusion once the relevant DP release criteria have been met [8].

Initially, manufacturing processes for autologous CAR-T therapy were developed in academic or research laboratory environments. Subsequently these early processes were transferred to internal or commercial partners to develop efficient cGMP (current Good Manufacturing Practice) manufacturing processes for clinical and commercial use. The drive to develop robust and efficient manufacturing and analytical platforms that can be operated under cGMP in a standardised manner has been critical to the improved product quality, throughput and affordable treatment costs now available [9,10].

In the absence at the time of a flexible fully automated manufacturing platform, Autolus decided to adopt the CliniMACS Prodigy® for its T-cell transduction (TCT) process. The complete manufacturing process occurs in a closed, semi-automated system – from the enrichment of the T cells from starting material to the formulation of the final DP. This approach balances the need for speed at the early clinical development stages with the robustness and reproducibility demands of future commercial manufacturing, thereby avoiding costly manufacturing failures and providing reliable clinical and commercial supply. The use of the CliniMACS Prodigy® in combination with a reliable supply of viral vector and appropriate analytics has benefited development of a consistent and robust process [4,11]. In this insight, we will cover key topics and areas for future focus for the commercialization of autologous CAR-T cell therapies.

UNDERSTANDING & CONTROLLING THE STARTING MATERIAL

The characterization and control around the patient leukapheresate starting material has proven to be critical to develop an efficient and robust T-cell therapy processes that are fit for purpose and deliver doses to ensure the desired quality, safety, and efficacy of the DP. However, the availability of patient material

is subjected to ethical and regulatory constraints and often leads to the use of healthy donor material for most of the initial process development work. This causes a risk of designing a process that will require substantial adjustments during late-stage process development, which may go from the exploration of proven acceptable ranges until the point of failure to the addition of new process step(s). The sooner the starting material is well characterized and understood for a particular pathology in a clinical trial, the more likely it is to develop a process that is suitable to control the patient variability. Additionally, the later that process improvements are implemented to address this variability in starting material, the higher the impact will be on regulatory filing and commercial readiness.

A key consideration is the variability of the starting material from patients as it can greatly affect process consistency and robustness. Therefore, the use of additional processing steps, such as selection or enrichment of target cells can significantly help the manufacturing process to tolerate the variability in the patient cells. The following aspects should be considered as early as possible in the design of the manufacturing process:

- The cell population distributions within in the patient leukapheresate (both target and impurity cells)
- The availability of suitable validated analytical methods to carry out initial T cell characterization
- Cell phenotype (memory, exhaustion, apoptosis) of target cells in the starting material
- Number of target cells that are required for manufacturing the desired DP
- The impact of pre-treatments of the patients on the starting apheresis material and DP critical quality attributes (CQAs). General questions are "could these potentially increase the likelihood of manufacturing failures?" "Should we set assays and extended specifications (e.g. activation and exhaustion phenotype screening) on the starting material prior to manufacturing?".
- The addition of steps that will reduce process variability such as selection or targeting certain cell populations, depletion of cell impurities, and setting specific numbers of T cells for activation. Impact on operations is key to streamline and standardize the scope of resourcing and implementation strategies.
- Fresh or frozen apheresis material; considerations for choosing either of these.

Although it may not be possible to have answers to most of them during early stage product development, leveraging development and clinical manufacturing experiences will no doubt provide insights to refine the plans and strategies for developing better controlled processes for our T-cell therapy products. It is therefore important to align our Research and CMC teams to continuously increase the knowledge and improve our understanding of the product during clinical development.

MANUFACTURING PLATFORMS: ARE WE LIMITED IN OPTIONS?

Autologous CAR-T cell manufacturing relies on a single batch approach for each patient. The initial processes for the manufacture of CAR-T cells were modular and flexible, with different equipment for each unit operation and a combination of open and closed steps. Some of these processes used the CliniMACS Plus® instrument for cell selection and a combination of rocking motion bioreactors, G-Rex® and cell culture bags for activation, transduction, and expansion steps [12]. However, this modularity faced issues with process control and robustness, resulting in

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increased demand for implementing process improvements to overcome scalability issues in commercial stages. The desirability to integrate the process into a single solution led to the emergence of semi-automated platforms such as the CliniMACS Prodigy® system. Although the CliniMACS Prodigy® can accommodate several unit operations such as cell selection, activation, transduction and expansion in a closed and partially automated fashion, this one-size-fits-all approach can lead to process limitations when considering the variability of patient starting material and specific processing requirements (Table 2) [13–16].

In recent years, more novel technologies such as the Cocoon® Platform (Lonza), Facer Cell Culture Platform (Aglaris) and Quantum® Cell Expansion System (Terumo BCT) have emerged for autologous CAR-T cell therapy applications. These systems incorporate trends towards automation, closed processes, integrated process analytical technologies (PATs) and provide more flexible semi-automated process steps (Table 3) [17–20]. Aligning these emerging platforms and technologies into a more modular CAR-T bioprocessing workflow is challenging but can potentially alleviate the limitations associated with a one-size-fits-all approach.

It is important to determine the degree of flexibility with the platform of choice, especially when considering the early-stage process development and characterization requirements that will need to be translated towards future commercial manufacturing. This becomes more critical given the variability of starting material and differences between products. The ability to tailor the process is vital to ensure optimum CAR-T cell quality and quantity for the patient's DP, whilst providing the ability to adapt the system to meet a change in future requirements.

The Aglaris Facer system is a novel manufacturing platform that implements gas-permeable membrane technology, like that of the G-Rex® product line, to culture and process cells in a functionally closed and automated manner. The platform attempts to relieve some of the process limitations highlighted in Table 2, taking the integration of PATs further with in-line sensors for pH, dissolved oxygen, glucose, lactate, and biomass monitoring. The control unit of the system allows for these key cell culture parameters to be monitored, controlled, and recorded remotely off-site, theoretically reducing the hands-on operator requirement in front of the system and associated labor costs. The geometry of the culture area in each cartridge provides the

TABLE 2

Potential manufacturing limitations using mainstream scale-out integrated semiautomated platforms.

Limitation	Rationale
Reduced modular flexibility [13]	High dependence on the platform of choice and the specific supplier for required consumables, risks supply chain disruptions. Supplier monopoly on market costs
Working volume constraints [13]	Programmed steps may require relatively high volumes, not ideal for high-throughput characterization considering companies in early-stage development. Limited standardized scale-down models available
Seeding density constraints [14]	Starting cell numbers demand considering working volumes. Issues present when there is high impact of pre-treatment lines or for specific pathologies that result in low quantity of starting material for manufacture
Limited fold expansion range [15]	Although autologous-scale bioprocessing should focus on cell phenotype quality (memory, persistence etc.) and not only achieving high cell quantities, high-fold increase during culture can drastically decrease the expansion phase duration and associated operational costs needed to achieve a therapeutic dose
Increased capital cost [16]	Large spatial footprint when running multiple systems in parallel for scale-out manufacture (one system per batch)
Limited process analytical technologies (PATs) [13]	General restriction to temperature and gas monitoring/control only. When high cell quality is the desired outcome, increased control of critical culture parameters is necessary. Monitoring of biomass and metabolites could potentially lead to controlling feeding regimes

TABLE 3

Key attributes of available technologies for autologous CAR-T cell manufacture.

Parameter	CliniMACS Prodigy®	G-Rex® 'M' series	Facer Cell Culture	Quantum® Expansion System	Cocoon® Platform
Cell expansion yield	Medium	High	High	High	Medium
End-to-end process (not including formulation)	Yes	No	No	No	Yes
Level of automation	Medium	Low	Medium	Medium	Medium
Complexity to operator	Low	Low	Medium	Low	Low
Flexibility	Low	High	Medium	Low	Medium
In-process control and in-line analytics	Temp, CO_2	Temp, CO_2 (from incubator)	Temp, pH, dissolved oxygen (DO_2), glucose, lactate, biomass	Temp, CO_2	Temp, pH, DO_2 glucose, lactate

advantageous potential for automated passaging steps to occur from a smaller to larger surface area. For programs where the quantity of starting material is low, either because of adverse patient pre-treatment lines or the nature of the pathology, this passaging option enables small cell quantities to be initially expanded at a suitable working volume and scaled-up when required. Given the similarities between the culture geometry in the system to that of a G-Rex® plate, there is the possibility that the G-Rex® product line can be used as a viable scale-down model or control for process development studies, however further investigation would be required to strengthen this hypothesis.

The Quantum® Cell Expansion System (Terumo BCT), a manufacturing platform that implements hollow-fibre bioreactor technology to culture cells, has demonstrated very high T-cell growth kinetics (roughly 500-fold increase over 9 days), whilst maintaining comparable phenotypic cell quality to controls run in parallel [19]. The system cultures suspension cell types by perfusing media through both ends of the hollow-fibre column, thereby maintaining the cells grouped together in the center where cell-to-cell interactions are more prevalent. This mode of culture also alleviates issues surrounding seeding density and working volume constraints and can accommodate much smaller seeding numbers compared to mainstream semiautomated platforms. The Quantum® system is less flexible compared to alternate platforms, with current operation limited to the expansion phase of the CAR-T manufacturing process. However, the high growth kinetics achievable in the system could theoretically enable therapeutic doses to be expanded in a much shorter timeframe.

The question remains whether to proceed with a modular-based closed process or if there is still scope for implementing an open, or partially open, process workflow like those outlined for commercialized CAR-T cell products (Table 1). Although open operations can maintain a high degree of process flexibility, issues surrounding risk of contamination, manual operator variability and the associated operational expenditure can become detrimental to successful manufacture. Technologies such as the G-Rex® 'M' series (Wilson Wolf Manufacturing) have taken commonly used open culture platforms and translated them towards closed processing [21]. Through sterile welding between the G-Rex® 'M' series with the Wilson Wolf GatheRex™ liquid handling system (semiautomated air pump device), both cell concentration and cell harvesting steps can be carried out without cell exposure to the environment. Although this is a partial solution to the issue of product integrity whilst maintaining process flexibility, the lack of PATs and high operator requirement are still limiting factors towards

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achieving a commercially sustainable manufacturing model.

The choice of manufacturing platform, either integrated or modular, should be carefully assessed as early as possible on the journey towards product commercialization, as each pathology and DP will have different processing requirements. The choice of platform will have an impact on process characterization, process validation, cost effectiveness, regulatory affairs, and ultimately in the development of the control strategy for commercial production.

PRODUCT CHARACTERIZATION: FOCUS ON POTENCY ASSAYS

The most challenging assays for the release testing, stability testing, and comparability are potency assays for both vector and DP. These undergo phase appropriate validations and are performed to demonstrate that the vector and the DP meet the released criteria consistently across the process development to commercialization [22,23]. An assay, or combination of assays, can be developed, validated, and used as surrogate measurements of biological activity, to show correlation of biological activity linked to potency. In the context of CAR-T cell immunotherapies, these are designed to be representative of the DP mechanism of action (MoA), in which usually two biological activities are evaluated: the measurement of gene transfer to the autologous T cells and the biological effect of such genetic modification (e.g., specific killing of target tumour cells, cytokine release). The challenge for any developer is the amount of DP information that has been accumulated in the early stages, and the depth of understanding of the DP biological activity itself. Furthermore, is the demonstration of how these measurements are true surrogates of potency and ultimately persistence in the patients. Potency assays are initially designed as cell-based (biological) assays. These must be well-thought out and kept simple wherever possible, with appropriate selection of cell lines, analytical development strategy, and supply chain and forecast of critical reagents. Potency assays present significant challenges in terms of biological variability, availability of reference material and operator training to maintain them under control. Therefore, it is suggested that the strategy to develop these assays should start as early as possible, ideally from the adaptation of pre-clinical testing work. A successfully validated potency assay will decrease substantial CMC matters down the road, with high impact on timelines, regulatory filings, analytical development, and QC readiness as the lifecycle progresses.

SUITABILITY OF RAW MATERIALS

Raw materials used in the manufacturing process are inputs that will have an impact on DP CQAs. The definition can extend from reagents and consumables that are in direct contact with the cells during the manufacturing process. Due to their potential impact on the DP, raw materials should be risk-assessed by criticality using process mapping tools and a material control strategy should be established for each one of them. A process-specific control strategy should therefore first identify which raw material inputs are used at each unit operation, how they are introduced, and what is the criticality of each material in terms of direct DP contact along the whole processing (Table 4).

Once all raw material inputs have been identified, the criticality of each (critical material attributes) should be defined in accordance with relevant DP CQAs. The risk attributed to each raw material should then be evaluated and expected variability determined, then the necessary controls can be implemented to ensure manufacturing robustness and DP safety. Examples of risk types could include those associated with sterility, viral contamination, toxic substances, extractables and leachables (E&L), and supply chain risks. Particularly, leachable studies related to infusion tubing and storage

TABLE 4

Potential ancillary material components that contact the cell product during each unit operation of the CAR-T manufacturing process.

Unit operation	Raw material inputs
Leukapheresis cryopreservation	Apheresis, cryopreservation formulation (with dimethyl sulfoxide), transfer bags
Selection	Selection beads, antibodies & accessory set, buffer, human albumin
Activation	Activation reagents, antibodies buffer, cell culture media, cytokines
Transduction	Viral vector, cell culture media, human AB serum supplement, cytokines
Expansion	Cell culture media, human-derived supplements, cytokines, cell culture platform chamber
Formulation/cryopreservation	Cell wash accessory set, wash buffer, cryopreservation formulation (with dimethyl sulfoxide), transfer bags

devices (i.e., blood bags, transfer bags, single-use kits) made of plasticized PVC must be performed to assess their impact on the formulated DP focusing on patient safety [24]. Regarding clinical manufacture, all raw material components need to be approved for use in humans and focus should be made to ensure cGMP suitability. It is important to consider consumable lot-to-lot variation and ensure acceptance criteria are certified by vendors between batches to ensure DP integrity. Having alternative vendors accessible for key raw materials can avoid supply chain obstructions and reduce manufacturing disruption risk [12].

VECTOR SUPPLY: CONSIDERATIONS DURING VECTOR DEVELOPMENT CYCLE FOR PREPARATION FOR COMMERCIALIZATION

The early success of CAR-T therapies used vector processes mostly developed in academic labs with technologies not designed for industrial scale-up, an issue when the requirements for vector supply are often based on the indication, dosage, and number of patients to be treated. Successful clinical outcomes, an increase in the trial activity and the availability of viral manufacturing technologies based on non-optimal repurposed bioprocessing technologies used for recombinant proteins and monoclonal antibodies (mAb) has not been ideal. Suppliers until recently have lagged in the development of new technologies to meet the dramatic increase in global demand for viral vectors to support this surge of interest in CAR-T immunotherapies. The emphasis on speed to clinic to confirm proof-of-concept and secure further funding rather than the time-consuming process optimization has further exasperated the security of supply issue. It is only later, that the translation from early phase to commercial supply does the scale-up/scale-out issue come into sharper focus.

The vector supply for *ex-vivo* therapies has increasingly focused on lentiviral vectors due to their ability to transduce dividing and non-dividing cells, and recent advances in the development of non-integrating lentiviral vectors have greatly reduced insertional mutagenesis, although gamma-retroviruses (γ-retroviruses) are still used by certain research teams. Shortage of vector can become a major issue when it is not planned strategically from the start. The forecast is important to establish the start of the clinical vector manufacturing, such as choosing an adherent process with transient transfection in which cGMP-grade plasmid quality is critical [23, 25–27], or the generation of master cell banks (MCB) and working cell banks (WCB) of stable cell lines (SCL) under cGMP. Demand for Phase I clinical trials can be satisfied from relatively small productions that will cover from 10 to 30 patients with essential release testing such as infectious viral titer, physicochemical characteristics and compendial safety tests. When addressing the vector late stage and commercial manufacturing strategy, main aspects to consider are the manufacturing site, transient

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transfection or stable cell line, the scalability of the platform and plasmid quality. In the initial phases, in-house platforms, dedicated vector manufacturing suites and limited experience of vector manufacturing can lead to work with experienced contract manufacturing organization (CMO) for some or all T-cell products of the company's pipeline. Some of the challenges working with CMOs are cost – it can be expensive, relatively slower progression of timelines for technology transfer and implementation activities, analytical method development and validation, competing with other companies for manufacturing slots. The positive part of such collaboration is that the CMOs direct experience decreases risk significantly associated to vector supply and its quality and provides better opportunities for early regulatory filings followed by successful product licensing applications. In some instances, the company may want to have better internal control of vector production and decisions are made to retain the vector manufacturing capability in-house. When accumulating more process understanding and product knowledge and moving the manufacturing in-house, a careful vector CMC strategy is essential, considering the development of stable cell lines and their cell banks, or if deciding a transient process path, the plasmid quality (GMP cell banks and raw materials).

Lentiviral vectors have been generated mainly using transient transfection. Initially, calcium phosphate was widely used as the transfection reagent, but polyethylenimine (PEIpro®) and Lipofectamine® are more widely used as a transfection method of choice to achieve reliable viral vector production and high infectious titre yields. Transient transfection is faster than producing a stable cell line but is not ideal for large-scale manufacturing due to variability between batches, the cost of plasmid, and potential plasmid contamination in the final product.

When it comes to manufacturing platforms, the production of lentiviral vectors frequently occurs in cell systems with serum added for rapid scale-up using 2D planar surface technologies such as the layered Cell Factory, CellSTACK® and HYPERStack®. Recent developments such as the iCELLis packed-bed disposable bioreactor by the Pall Corporation has provided a rapid high-density, closed, controlled environment for the expansion of anchorage-dependent cells. The iCELLis Nano provides a surface area ranging from 0.53 to 4.0 m^2, and the iCELLis 500 ranges from 66 to 500 m^2 of surface area and requires less manual manipulation than cell factories [28]. Vector production in a bioreactor allows for more consistent control of process parameters such as pH and dissolved oxygen (DO_2), as compared to an adherent process. Additionally, the use of a bioreactor also allows for scaling up from the iCELLis Nano in process development to the iCELLis 500 in production. This significantly reduces labour as compared to a flask-based process, which must be scaled out rather than scaled up. Regarding shear stress, immobilization in polyester macrocarriers protects the cells from bubble sparging or shear from an impeller while media circulates up through the fixed bed and is replenished with gases as it falls back to the reservoir as a thin film. The high surface area-to-volume ratio of the fixed bed allows for a low inoculation density per surface area with the option to increase media volume with perfusion or recirculation as the cells grow [29]. A variety of viral vectors have now been produced in the iCELLis bioreactor, including gamma-retroviral vectors, adenoviral vectors, adeno-associated viral vectors, and lentiviral vectors and often with little or no optimization.

Serum-free suspension systems for lentiviral vector production are now being increasingly favored over adherent systems and are scalable in single-use STR bioreactors from 50 to 2000 L [30]. In a recent publication, Tangential Flow Depth Filtration (TFDF™) technology developed by Repligen Corporation for the harvest of lentiviral vectors cultured in a serum-free suspension system was described and demonstrated more than a two-fold vector productivity increase compared to the standard batch process

TABLE 5

Characteristics of adherent and suspension culture systems.

Adherent cell culture	Suspension cell culture
Suitable for most cell types	Appropriate for cells adapted to suspension culture and a few other cell lines that can be adapted
Requires periodic subculturing and allows easy visual inspection under inverted microscope. Limited monitoring in cell factories	Easy to subculture. They can require large quantities of media to meet high cell densities
Cells are dissociated enzymatically or mechanically	Cells do not require enzymatic or mechanical dissociation
Growth is limited by surface area, which requires significant amounts of manual operations and consumables	Growth is limited by concentration of cells in the medium, which allows easy scale-up
Static culture and may require surface treating	Suspension culture requires agitation (i.e., shaking or stirring) for adequate heat and mass transfer

harvest [30,31]. Additionally, the authors also demonstrated the potential benefits of TFDF™ to perform multiple vector harvests from a single transient transfection process for increasing overall process yields. A final remark is that process intensification has been greatly benefited from the development of stable cell lines that eliminate the need for transient transfection, thus simplifying the process, reducing manual operations and the number of process-related impurities to monitor.

The scalability of the platform and incorporation of appropriate well-characterized upstream and downstream process steps, either adherent (cell factories) or suspension (stirred-tank reactors), is critical to satisfy the increasing demand during clinical development to commercialization (Table 5). A progressive scalable platform approach will ensure a coherent manufacturing strategy and decrease the amount of substantial regulatory file updates and extensive studies to demonstrate comparability and ultimately the maintenance of vector quality across the development to product lifecycle to ensure patient safety and product efficacy.

TRANSLATIONAL PATHWAY INSIGHTS

The manufacture of a CAR-T cell therapy is a highly complex endeavour requiring both a gene-therapy manufacturing process (often including viral vectors) and a cell process - from collecting T cells, genetically modifying, and expanding them to formulating the DP for delivery to the patient. These complex and multistep technologies and logistics are rife with risk. Developers must keep a very clear vision and oversight during this journey to set appropriate strategies that will meet regulatory expectations, current good manufacturing practice (cGMP) and commercial readiness. The challenges and opportunities in CAR-T cell development, manufacturing and testing should address more critically ways to broaden the abilities, precision, and persistence of these adoptive cell therapies to treat not just approved hematologic blood cancers, but also for those patients in late-stage treatments who are unable to provide sufficient starting materials to make high-quality immunotherapies. Further, as developers we need to come up with scalable systems that are much more flexible in handling the variability of patient starting materials.

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4D-Nucleofector® LV Unit: Efficient, non-viral large-scale transfection for cell and gene therapy applications

Timo Gleissner & Andrea Toell, Lonza Bioscience Solutions

The 4D-Nucleofector® LV Unit allows efficient, versatile, and scalable transfection of cells. This poster highlights some of the key features of the unit and best practices for its operation.

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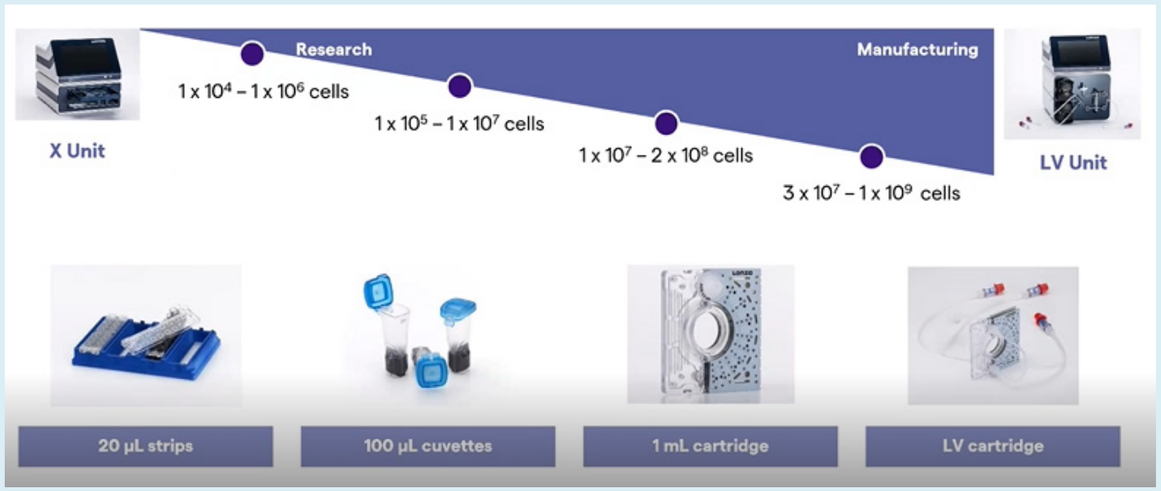
INTRODUCING NUCLEOFECTOR TECHNOLOGY

The state-of-the-art non-viral Nucleofector Technology enables highly efficient transfection of primary cells combined with high cell viability. The technology has been cited in over 10,000 publications and is used in renowned labs in the cell and gene therapy space, including the Yamanaka lab (for iPSC generation), and the Charpentier and Doudna labs (for CRISPR).

The technology can be easily adapted to different substrate types, be it plasmid DNA, mRNA, or ribonucleoproteins (RNPs). Since it allows for efficient co-transfection of these substrates, it is the ideal platform for genome editing, using transposons, ZFNs, TALENs, or CRISPR.

Figure 1. Condition transfer from research to manufacturing.



The platform supports single reaction formats to high throughput formats such as CRISPR screening, and scalable volumes per reaction from as low as 10,000 cells up to 1 billion cells.

In the cell and gene therapy environment, the technology has been widely used for basic research but has now also evolved into manufacturing processes exploring non-viral *ex-vivo* modifications, since it is easily scalable to higher cells numbers in the range that is of interest for autologous cell therapies. The application can be established in the smaller scale formats to save cells and substrate and then scaled up to the larger-scale formats serving 10 million or up to 1 billion cells.

4D-NUCLEOFECTOR LV UNIT

The 4D-Nucleofector LV Unit comprises two parts: a controller (core unit) and a large-volume functional unit (LV unit). The LV unit can handle two different Nucleofection Vessels **(Figure 1)**. First, the 1 ml cartridge, which is suited for transfecting a fixed volume of 1 ml and can be used for up to 200 million cells. Second, the LV cartridge – a flow-through version that can transfect up to 1 billion cells in volumes up to 20 mL, with transfections done in successive fractions of 1 mL. An optional second input line is included for those working with mRNA, which keeps mRNA and cell suspension separate until shortly before the transfection step and thus avoids degradation. The LV cartridge can be operated as a closed system via manual connection or sterile welding to upstream and downstream equipment.

FINDING SUCCESS WITH THE NUCLEOFECTOR TECHNOLOGY

The Nucleofector Technology and LV Unit support five key success factors for setting up cell and gene therapy:

1. **EFFICIENCY AND ROBUSTNESS:** It achieves robust and high transfection efficiencies in cell types relevant for cell and gene therapy.
2. **VERSATILITY:** It has been proven for use with DNA, mRNA, and protein and thus supports applications like CRISPR and CAR-T cell generation. It is suited for many primary blood and somatic cells.
3. **SCALABILITY:** You can smoothly transition from research scale to manufacturing scale by using the same conditions at any scale.
4. **SAFETY:** It is a non-viral genetic modification. Consumables are fit for purpose – available for research and GMP use.
5. **DOCUMENTATION AND SUPPORT:** Lonza can provide technical and application support by its highly skilled scientific support team. Lonza offers installation qualification/operational qualification (IQOQ) service for the device. The system can be operated under 21 CFR part 11 compliant software and regulatory support packages with more detailed product information are available.

CELL & GENE THERAPY INSIGHTS

In partnership with:

Lonza
Cell & Gene

Making billions (of cells) with adherent cell cultures

Catherine Siler PhD, Field Application Scientist, Corning Life Sciences

Adherent cell culture scale-up plays a key role across multiple disciplines of regenerative medicine. This poster will explore some of the challenges and opportunities of adherent cell culture scale up.

Whether the goal is to use cells as the final product or as a vehicle to produce the final product, scaling up an adherent cell culture process is a crucial step in on the path to a therapy reaching patients.

WHAT SCALE-UP PLATFORM BEST SUITS YOUR NEEDS?

Multiple factors play a role in choosing a scale-up platform (Figure 1). If process development time is limited, the Corning® CellSTACK® chamber is a good option, as it is similar to a flask but gives more surface area, saving time and offering consistency. If more surface area or a more ready-to-use closed system is required, the Corning HYPERStack® vessel offers much greater cell growth surface area in a footprint comparable to a CellSTACK chamber.

Corning CellCube® modules are an ideal option for those seeking significantly more surface area as well as control. CellCube modules are combined with a bioreactor to give the benefit of 2D culture along with process monitoring and control. The Corning Ascent® FBR system offers a compact 2D cell culture environment in a fixed bed bioreactor with active monitoring and control, all within the same platform.

In addition, using a 3D system, such as microcarriers, can significantly scale up the process. Here, cells are grown on beads suspended in media, providing an adherent cell culture system in a suspension environment.

SEED TRAIN: HOW CAN YOU BEST ACHIEVE SCALE?

Seed train refers to the process of increasing cells from enough to fill a cryovial up to billions. Many factors must be taken into consideration such as the goal of the process, the amount of product needed, and the number of steps or manipulations needed in the process. For example, depending on the behavior of the cells, it may be possible to seed sparsely and do fewer harvests or seed densely to shorten the number of days it takes to get to the next step. The amount of surface area required, ratio of media to surface area, and the type of cell culture surface treatment desired, also need to be considered. With these parameters in mind, it may then be helpful to decide on your ultimate cell number and work backwards through the steps.

Users often mix products, such as CellSTACK and HYPERStack vessels, depending on the amount of surface area required, and may switch to more advanced systems, such as CellCube modules or the Ascent fixed bed reactor, for the final step. To ensure the desired platform is a good fit for your facility and resources, researchers should consider the required incubator space and available work force. Plus, appropriate gas supplies will be needed if scaling up to the CellCube system, Ascent FBR system or a bioreactor.

Figure 1. Choosing a scale-up platform.

PROCESS CHARACTERIZATION: WHAT IS CRITICAL TO YOUR SUCCESS?

Process characterization involves identifying the parameters that play a key role during a given stage and can be adjusted for an optimal result. For the cell expansion phase, these parameters include pH, temperature, dissolved oxygen, and concentrations of metabolites in the media. For harvesting, key parameters include removal enzyme and physical manipulation of the vessel to ensure even distribution of enzyme.

PROCESS CONTROL AND AUTOMATION

Process control allows you to maintain or manipulate your parameters in a controlled and replicable manner. During growth, a bioreactor can control pH, oxygenation, and temperature. For harvest, process control can be automated. For CellSTACK and HYPERStack vessels, the Corning Automated Manipulator Platform helps ensure consistent liquid handling and manipulation and allows control of vessel motion on all three axes. The Ascent FBR system doesn't require motion of the vessel, but instead controls the motion of liquid within the system, allowing automated liquid handling during media removal and harvest.

SUMMARY

There are a variety of concerns that factor into the choice of a scale-up platform. These concerns will look different depending on your application and timeline. Regardless of the platform you choose, there are methods to help you control the critical aspects of your process, including automation options.

CELL & GENE THERAPY INSIGHTS

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CELL & GENE THERAPY INSIGHTS

CELL THERAPY BIOPROCESSING & AUTOMATION

SPOTLIGHT

INTERVIEW

Critical success factors for tomorrow's cellular immunotherapies

BERND SCHMIDT serves as Vice President, Product Delivery at Quell Therapeutics and has 20 years of industry experience in the pharmaceutical sector, covering a broad range of innovative medicines at different stages in development and post launch. He joined Quell from GSK where he served as MPD Leader with overall accountability for the CMC development, governance and end to end supply chain of a portfolio of medicines (small molecules and cell & gene therapies) covering pre-clinical to late-phase development as well as commercial supply, incl. interactions with regulatory bodies. Prior to that he was the Technical Director and a member of the site leadership team of one of GSK's main manufacturing facilities in the UK. Before re-joining GSK in 2012 he held roles of increasing responsibility in process and product development at AstraZeneca.

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Q What are you working on right now?

BS: Quell Therapeutics was founded just two and a half years ago. Our aim is to develop transformational cell and gene therapies to address unmet needs in autoimmune and inflammatory diseases as well as preventing rejection in solid organ transplantation.

The company is still in a preclinical phase. However, we are moving rapidly towards our first phase 1/2 clinical trial, which will be targeting the prevention of graft rejection post liver transplant. We are also looking at expanding our pipeline, with additional assets in research and preclinical development. I head up Product Delivery at Quell. My role accounts for process development, manufacturing, and supply of our drug products. At the moment, I am heavily engaged in preparations for our first clinical trial.

We have also been working on building our capabilities, infrastructure, and technology platform further, in line with our long-term strategy.

Q Interest in regulatory T cells is growing rapidly – what for you have been the important issues and recent advances in the immunology field that are spurring this increasing activity?

BS: So far, the industry and the investor community have focused mainly on the oncology sector and effector T cell-based technology. However, this field has now become very crowded, and recent advances in understanding regulatory T (Treg) cell biology – largely driven by pioneers such as Professor Giovanna Lombardi and Professor Jeffrey Bluestone, for example – have opened an opportunity to address significant unmet needs regarding immune dysregulations.

Clinical trials in the field of solid organ transplants conducted by Professor Sanchez-Fueyo, using non-engineered polyclonal Tregs, have shown that Treg-derived cell therapies are safe. Todo *et al.* have also shown some clinical efficacy with antigen specific Tregs in transplant patients. These observations, as well as the recent technological advances in the cell and gene therapy field, have signaled the beginning of a second wave of new cell and gene therapies, that have the potential to address unmet needs in indications within organ transplantation, autoimmune as well as inflammatory diseases.

Quell Therapeutics is taking this evolution to the next stage with the development of modular engineered Tregs. The aim is to provide safe, efficacious, and persistent treatments in a number of indications with high unmet need.

Q Can you outline the key bioprocess considerations for Quell's Treg based cell therapies?

"recent technological advances...have signaled the beginning of a second wave of new cell and gene therapies, that have the potential to address unmet needs in indications within organ transplantation, autoimmune as well as inflammatory diseases."

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BS: Treg cells are a T cell sub-population, which represents approximately 5% of all CD4-positive T cells in the human body. Therefore, we have to carefully consider the steps involved to effectively select this specific sub-population of cells, which is a challenge. Importantly, the presence of effector T cells, which have the opposite effect to regulatory T cells, could present a safety risk. We have been developing a very effective cell isolation process that delivers highly purified starting material in order to address this challenge.

We also genetically modify these cells to express the relevant antigen receptor and other proteins of interest, which are key to the performance of the drug product as well as providing immunosuppressive functionality.

In addition to delivering starting material and drug product of the right quality, it is also important to generate a dose which is likely to be efficacious. With the number of Tregs being relatively low compared to the number of effector T cells, this aspect requires a strong focus during process development. Further, a clear strategy is needed in terms of selecting the appropriate manufacturing technology, including method of activation, method of transduction, and having the right platform to drive cell expansion. The latter is a challenge considering the limited number of cells to start from.

Linking process development and the manufacturing model with clinical requirements is also important. Following a liver transplantation, patients usually undergo life-long immunosuppressant treatment regimens. We therefore need to consider a weaning step prior to infusion of our autologous product, meaning we need to take into consideration the time of weaning before the product can be given to the patient. As a consequence, we developed a cryopreservation process which allows us to store the product whilst the patient is getting ready to receive the treatment. Developing this freezing step, which retains the product's viability and functionality, has been important for us – and not just for logistical reasons.

Q Are there any manufacturing-related challenges that you are facing or will face as Quell's product candidates approach and enter the clinic, and what is your approach to addressing them?

BS: Key considerations and challenges in this space relate to having sufficient manufacturing capacity, as well as having trained and capable personnel to conduct the manufacture, QC release testing, and related quality activities. It has been essential to secure manufacturing space early in order to develop our first product into the clinic. As such, we have secured a cleanroom with the right capacity for Phase 1/2 clinical trials. However, in line with our company strategy, I'm already thinking about scaling out our manufacturing capacity. We are currently in the process of securing additional manufacturing space to enable us to deliver our pipeline, and to look ahead at a potential pivotal clinical trial, which we would like to supply using internal capability.

It is important for me to have an integrated corporate strategy, which combines early research with process development and manufacturing, all the way through to the clinic and the planned trial. It is also key to ensure that all the activities with long lead times (e.g.,

setting up the manufacturing space, training staff in manufacturing and QC release techniques, etc.) are considered and mapped out early.

In addition, when it comes to clinical trial manufacture, we aim to avoid manufacturing being a bottleneck. We also try to prevent any delay in the clinical trial timeframe, which can have considerable cost implications for any company, but also means we wouldn't be delivering the value we would like to deliver to the patient. As such, it has been important for us to interact and plan closely with our clinical colleagues in terms of patient recruitment and associated logistics to ensure everything is aligned right from the start.

> "It is important for me to have an integrated corporate strategy, which combines early research with process development and manufacturing, all the way through to the clinic and the planned trial."

Lastly, the general challenge that the whole sector has been dealing with across most indications, is the availability of patient material for autologous product and process development. Most drug development activities are going ahead using healthy donor material, and for good reasons. But we also know that, in a lot of cases, patient material often behaves differently to healthy donor material. And, in our case, we are dealing with patients who have been on strong immuno-suppressants with considerable side effects.

So it is important for us to include patient material in early process development. This has been quite a challenge in the context of Covid-19, as these highly immuno-suppressed patients are in the highest risk category and have been self-isolating. In order to make this happen, we have been planning ahead and working closely with our clinical partners.

Q What are the most impactful ongoing supply chain and manufacturing challenges relating to the COVID-19 pandemic that Quell is facing, and what steps have you been able to take to counter them moving forward?

BS: During the pandemic, it became evident that the demand for raw materials used for the production of vaccines, some of which we and other cell and gene therapy companies use in our processes, would increase significantly. With supply only catching up slowly due to long lead times, as well as the increased demand, this has created a supply shortage in some areas. We have therefore focused on securing key supplies early in order to avoid any immediate shortages and to be prepared ahead of the planned clinical trial.

Q What are the key recent developments in manufacturing technology that are shaping Quell's ongoing bioprocess and analytical development approaches?

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BS: There is quite a lot happening in terms of new technologies that have recently entered the market or are about to do so, which has shaped our thinking regarding our bioprocessing and analytical development approaches.

New technology solutions have been emerging recently, which are very interesting and relevant when talking about closing and automating the manufacturing process. These aspects are key in developing a product that can be successfully commercialized later on. For example, Ori Biotech and Aglaris have developed very promising solutions that close and automate key process steps from cell culture all the way to harvest.

Other processing steps, such as cell isolation and end-product formulation, are often not covered by these technologies and require separate solutions. This results in open transfer steps, which then require tailor-made solutions – a limitation of some of these new technologies. There are other companies such as Cellares, though, who have recognized these limitations and have developed a fully closed and automated end-to-end processing solution, thus providing an almost fully automated environment. This is an exciting new development which we are following closely.

Most technology platforms are built around a more centralized manufacturing model. However, there are also other companies emerging who are taking the opposite approach and starting to look at in-hospital treatments, meaning that patients would be treated on the same day as their blood is harvested and further processed. So the product is produced *in situ* and infused at the same time and in the same session. I believe this is a very interesting paradigm shift, which I would like to look into further, requiring new thinking regarding the regulatory and quality compliance framework. I would also be keen to understand more about the associated cost of goods model – a key element for any company operating in the autologous space, of course.

Q Can you comment on how tools are streamlining and/or accelerating cell therapy bioprocess and analytical development?

BS: When talking about accelerating development, we mustn't forget about the comparability challenges this brings with it, especially if a base process has already been developed. This is something that we have thought about early and are addressing as we speak. Other companies need to bear this in mind at an early stage as well. It is key to ensure that any process changes are not leading to a very different product, which might require additional clinical testing leading to increased costs and longer timelines.

Regarding the acceleration of cell therapy bioprocess development, there are very interesting solutions in the automation space linked to Design of Experiments (DoE).

From the analytical point of view, I'm really impressed by recent advances especially in the area of PAT (process analytical technology) – the increasing availability of in-line probes, for example, and other techniques that would enable us to better understand our biological processes. By their very nature, these biological processes are quite variable and need close monitoring to improve not only our understanding of them, but also our ability to control them,

thus increasing process robustness and reproducibility. This is another area that is shaping our thinking in terms of next steps and the development of future platform technologies.

When talking about evaluation, selection, and implementation of any new technology, it is important to maintain a strategic view overall. Although these technologies are interesting, what we ultimately want is for our process to be reproducible, and to deliver safe and efficacious products of the right quality and right cost of goods. Of course, the latter enables (or should enable) a pricing structure which payers are willing and able to afford. I think anything we do in this space needs to be measured and seen in this context, but it is often something that is not examined early enough in the development process.

Q What is your vision for the future commercial manufacturing and supply model for cell therapy products such as Quell's – and what steps can be taken from an early stage to begin preparations?

BS: In the case of autologous products, I think we will continue to see regional manufacturing and supply models playing a big role, with cryopreservation of starting material and drug product enabling and streamlining a flexible processing and logistics model and approach.

My vision here is to have a highly automated workflow, which enables the manufacturing of reproducible, high-quality drug products at a low cost of goods. Affordability is key, but so is accessing a wide range of indications to better treat patients.

It's important to have a mechanism to test relevant new technologies early, in a targeted way, and to ensure their benefits are maximized. And as I mentioned earlier, having a comparability strategy in place right from the start is also important to avoid meeting a dead-end during process development.

Autologous products have an inherently high cost of goods. This will present challenges in terms of access to a number of indications and consequently, the wider market. In order to compete with biologics, for example, a step change would be needed. Allogeneic products can drive this paradigm shift. I believe that for any company developing cell therapies, it is important to think about this strategically and plan ahead to develop an allogeneic technology approach, which ultimately removes dependence on patient material and allows for a more flexible and lower cost manufacturing and supply model. This is the future goal. Obviously, there are a lot of technical and scientific challenges,

"what we ultimately want is for our process to be reproducible, and to deliver safe and efficacious products of the right quality and right cost of goods. Of course, the latter enables (or should enable) a pricing structure which payers are willing and able to afford."

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but I do believe that with investment and scientific attention given to this field, it will be addressed over the coming years.

Q Finally, what are the chief goals and priorities, both for yourself in your role and for Quell as a whole, over the coming 12-24 months?

BS: Quell is about to start its first clinical trial for its lead asset. It will also continue to grow its pipeline, moving from a single preclinical asset to several programs in preclinical and clinical development.

My immediate focus will be on manufacturing and supplying our lead product to patients in our first clinical trial – this is essential. Our longer-term strategy is to support the organization's ambition and growth in terms of developing the pipeline and to produce clinical and commercial products, which will ultimately be used in patients. Key aspects of that work are the scaling and building out of our manufacturing capacity, and the further development of our manufacturing process platform, including the assessment and implementation of new technologies. We will also continue to recruit over the coming months and years, and there will be numerous opportunities for key talent to join Quell Therapeutics moving forward.

AFFILIATION

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AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

CELL THERAPY BIOPROCESSING & AUTOMATION

SPOTLIGHT

INTERVIEW

Lessons for advanced therapy manufacturing flexibility and productivity

David McCall, Editor, *Cell and Gene Therapy Insights*, **speaks to James Miskin,** Chief Technical Officer, Oxford Biomedica

JAMES MISKIN joined Oxford Biomedica in 2000. He has more than 17 years experience in the GxP environment. In his current role, he has overall responsibility for Oxford Biomedica's Quality systems, analytical testing, lentiviral based bioprocessing development and client programmes. He is also a named inventor on several patents in the field. He holds a Bachelor of Science degree and a PhD in Molecular Biology from the University of Leeds and subsequently conducted post-doctoral research at The Pirbright Institute for a number of years. He is an active member of the UK BioIndustry Association Manufacturing Advisory Committee and is the Advanced Therapies work stream lead for The Medicines Manufacturing Industry Partnership (MMIP).

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Q What are you working on right now?

JM: Oxford Biomedica is working on a number of projects at the moment. We have a significant involvement in the COVID-19 vaccine response in that we are one of the key manufacturing partners for AstraZeneca's AZD-1222 vaccine. We've been involved in that

program ever since we started talking to the team at the Oxford University Jenner Institute very early last year.

We are also continuing to support partners in relation to their cell and gene therapy programs – primarily our core area of lentiviral vectors, but now we are also expanding our capabilities and actively working on other viral vector systems. That's building upon the commercial manufacturing experience and insight we gained firstly with Kymriah®, which also stood us in good stead when it came to incredibly rapid delivery and roll-out of the COVID-19 vaccine program.

And personally, as a member of Oxford Biomedia's senior executive team, I have overall responsibility for several different areas of the business, including quality, analytics, and process development, as well as our client program and alliance management functions.

Q Can you give us some more background on the role Oxford Biomedica has played in the COVID-19 pandemic response to date and how it came about?

JM: We have established ourselves over the years as a significant viral vector-focused, technically expert CDMO, primarily around our lentiviral vector platform. But during that time, we got to know a lot of people in the broader sector, including the team at the Jenner Institute in Oxford University.

In February last year, the team at the Jenner Institute encouraged us to get involved in a consortium they were planning of primarily (but not exclusively) UK-based organizations aimed at trying to roll-out their ChAdOx platform technology into a vaccine. The ChAdOx platform is based on an adenovirus from chimpanzees and in essence, they wanted a partner that could manufacture a viral vector at scale. Obviously, that's exactly what we do and by coincidence, we had just finished the build of Oxbox, our brand-new manufacturing facility in Oxford. This facility gives us four fully independent viral vector manufacturing suites and it's very large at just under 8,000 sq. m. The four suites were designed for use with our own viral vector platform and partner projects.

As a result of COVID-19 there were lots of conflicting forces for us to deal with. Most importantly, there was the safety of our staff – figuring out how we were going to manage our way through the COVID-19 pandemic by ensuring that our staff felt safe, but that we were also able to prioritize our ongoing programs. We focused on making sure that our staff could do their jobs safely and effectively, either onsite or remotely, and that we maintained as much momentum as possible on all our client-facing programs, as a matter of priority. That did mean we had to make some difficult decisions, and we did slow down some internal projects to release as much capacity and manpower to handle the client-facing activities as possible.

Those activities included the vaccine work, which by that stage had grown into a development program with Oxford University. AstraZeneca joined shortly afterwards. We worked with them on further process development to enable what was a small-scale process to be scaled-up to an initial 200L, and ultimately, to 1,000L. That went very well and by late-summer/early-autumn 2020, the process had moved into GMP manufacturing. We had

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managed to get the MHRA license on the new facility for two of the four suites (GMP5 and GMP6), but we had elected to accelerate the roll-out on the other two suites as well.

To mitigate the capital expenditure restrictions we put in place, we were able to leverage some support from a UK government organization called VMIC (Vaccines Manufacturing Innovation Centre.) They provided us with equipment and in return, we entered a five-year agreement to provide VMIC which is a new organization, with a lot of training, documentation, the essence of quality management system, and consultancy, to try to accelerate their progress towards operational readiness. We then managed to get those two additional suites (GMP7 and GMP8) operational and from early autumn 2020 up to today, we've been manufacturing as many batches as we can possibly manage at 1,000L scale in three separate manufacturing suites. To operate at that level, we have obviously had to change our ways of working. For example, we've had to recruit an enormous number of new people and train them, which has been quite a challenge in itself against the backdrop of COVID-19.

Q The importance of establishing and maintaining a degree of flexibility in biomanufacturing is a common theme across the advanced therapies field at present – can you comment on any high-level learnings you've derived from the highly dynamic past 18 months?

JM: First and foremost, flexibility comes from common approaches. Our facilities and ways of working within Oxford Biomedica are all designed for the combined management of both containment for working with viral vectors and compliance in relation to GMP. Additionally, although the viral vector systems and their individual processes are different, there are some significant commonalities across manufacturing platforms. For example, they all involve human cell manipulation and expansion, they are all serum-free suspension processes, and there is lots of liquid handling.

There is also our long-standing general philosophy of utilizing a fully single-use manufacturing platform, which allows for quick turnarounds between processes. The dependence on single use components has been put under enormous stress over the past year because of so many organizations trying to manufacture so much product so quickly, but overall, our experience proves that you can apply good practice and relevant commercial manufacturing approaches to similar technologies quite readily.

"Our facilities and ways of working within Oxford Biomedica are all designed for the combined management of both containment for working with viral vectors and compliance in relation to GMP."

Q More specifically, what are the key advances and trends in manufacturing technology innovation that will give the biomanufacturing facilities of tomorrow the various capabilities they will require – firstly, in this area of flexibility?

> "I think single-use technology is probably the single biggest provider of flexibility. Despite the supply chain challenges in using single-use technology that we face right now, practically speaking it's still a very sensible way to be able to achieve different things."

JM: I think single-use technology is probably the single biggest provider of flexibility. Despite the supply chain challenges in using single-use technology that we face right now, practically speaking it's still a very sensible way to be able to achieve different things. For instance, if you design your facilities with independent and segregated air handling in mind, it helps you to do quick turnarounds between products and utilize all of the facility more effectively. It reduces the requirement for cleaning validation and the like, which could have hampered quite a lot of the activities that we are doing right now.

The other thing from a technology standpoint is having the capability to handle different cell lines and different types of process – transient transfection versus infection, for example.

Q ...and productivity?

JM: This is where we as a company are spending a lot of time. We have always retained a strong emphasis on innovation within Oxford Biomedica. It's one of our core company values. The Chief Scientific Officer and I each have significant teams working on fundamental platform, analytical, and process innovation. We also work on our own therapeutic product innovation, and I think that working both as a CDMO with partners and on our own R&D programs has given us a unique insight into some of the technical and productivity challenges that are out there. It's one thing making viral vector for a CAR-T program where it's a relatively defined amount of vector that needs to be made, but it's quite another to make a lentiviral vector for treating a lung disorder such as cystic fibrosis, which is one of the partner-led projects we're working on with Boehringer Ingelheim and the UK CF consortium. These types of challenges push us to innovate on process and platform technologies, and we have developed a number of different technologies that allow us to enhance productivity and product quality. Some of that is proprietary technology that we are not divulging at this point, but broadly speaking, it's additives and also new molecular biology approaches that allow us to increase productivity during a phase of the upstream process. We also developed a Translational Repression in Production (TRiP) System™, which we have demonstrated in a number

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of proof-of-concept studies with a range of vector systems, including Lenti, AAV and Adeno. Its benefit is realized most where the transgene of a particular product is actually detrimental to the productivity of the vector we are trying to make. In these cases, the TRiP System™ allows us to neatly reduce the level of expression by translational (not transcriptional) repression and allows us to rescue productivity for products where titers are significantly hampered by that challenge, whilst at the same time retaining the use of powerful promoters for maximal gene expression in target cells. We continue to seek to innovate in this way. We have a number of current programs looking at new additives in the process and at the process control strategy, to achieve better consistency and reproducibility.

All of these help us boost productivity and, in some cases, these technologies have been proven in multiple platforms: not just Lentiviral vectors, but other vector types such as adeno-associated virus (AAV) or adenovirus. However, further innovation will be necessary if we are to continue to address the productivity challenges this sector faces. The past 18 months has taught us that viral vectors have broader applications beyond Gene and Cell therapy including immunology and vaccinology.

Q How about quality?

JM: I see quality in two different ways. First, there is quality from a compliance perspective, which I touched upon previously – it's absolutely vital that you have robust quality management systems and ways of working, and that you conduct really rigorous and robust investigations into unexpected in-process incidents. Maintaining compliance for an organization in this sector is obviously hugely important and we take that aspect extremely seriously.

Secondly, there is quality in relation to the product, such as critical quality attributes. This is where process and analytical innovation can really be of benefit -, for example, reducing process and product impurities. On the process side, if you increase yield, typically you can show that the relative impurity content per unit of vector reduces, which is very helpful. On the analytics side, getting a strong, technically detailed understanding of impurities and what causes them gives you insights to help try to remove them or avoid having them in the first place.

Another key consideration is how you do the testing. Many people in cell and gene therapy think about manufacturing facilities in a simplistic way, based around clean room square meterage. But practically speaking, it's also about how you physically test those products - how you ensure you have got analytical methods that are sufficiently robust, reproducible, accurate, precise, and at high enough throughput to be able to cope with increased demand from the manufacturing suite. We are spending a lot of time and energy on innovation related to analytical automation. We have a lot of robotics projects ongoing for some of the critical cell-based and molecular biology assays, and we've also got some interesting projects looking at how to leverage the very rich datasets from development. We are applying artificial intelligence and machine learning to get a better handle on what those impurities are, why they are there, and what you might be able to do to manipulate and improve the quality of the product.

Q ...and lastly, cost efficiency?

JM: Much of what I've described above will have a positive impact on cost simply because as you increase productivity, the net cost of goods typically reduces. This is important because it allows you to tackle other more significant challenges. For example, lung and liver disease targets are big - you need a lot of vector – and it is just not cost-effective to pursue these targets unless you can make enough at scale. This pushes us in the direction of innovation to try to tackle these technical challenges.

It isn't simply a matter of scale-up, though. For some vector processing approaches, scale-up is technically very challenging - transient transfection, for instance. I've often said to my development team that scaling-up the transient process beyond 200L is pretty tricky! We've actually developed some new tech that would enable us to do it but perhaps more relevantly, after some two decades of hard work, we've recently made a lot of progress on stable cell lines for vector production. Today, we have a number of candidate cell lines not for marker vectors such as GFP, but for genuine therapeutic products. This opens up a whole new avenue for future capacity increases and scale-up.

We've also been working on all sorts of approaches to the upstream process – fed-batch process, perfusion, those sort of things - that allow you to improve productivity and yield using the same size of equipment.

Q A further topic of growing relevance to the field is the need to establish a sufficient workforce to allow the sector to continue developing and thriving – what is your view of this issue, and how it could or should be approached?

JM: This is indeed a vital challenge that we must meet. We and any other organization are only as good as the people who work for us. I think there are lots of different approaches to it that are important.

"Many people in cell and gene therapy think about manufacturing facilities in a simplistic way, based around clean room square meterage. But practically speaking, it's also about how you physically test those products - how you ensure you have got analytical methods that are sufficiently robust, reproducible, accurate, precise, and at high enough throughput to be able to cope with increased demand from the manufacturing suite"

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For instance, for us culture plays a huge role. Maintaining and developing our culture is a top priority. We nurture our staff to ensure they feel part of the culture and that it's one they really want to stay in. It's also vital that our people continue learning and have a career pathway laid out that gives them opportunities in the future. Retaining our staff means making sure that we have a competitive offering in what is a very competitive space - we are in the southeast of the UK where there are quite a lot of other organizations in our sector and in adjacent technology sectors, and they know that Oxford Biomedica staff are very well trained, highly skilled, and therefore are attractive. It's our job to try to make sure we are just as attractive as an employer, and the culture is something I personally hold very firmly front and center of that effort. Everyone wants to do a job that they enjoy - they don't mind doing a lot of hard work, but they want to enjoy what they're doing. I think that underlies everything.

It's also about getting involved in schemes that we feel are important for the sector. One such important scheme in the UK is the Advanced Therapy Apprenticeship Community (ATAC). This is an action that came out of a government taskforce (Advanced Therapies Manufacturing Taskforce) that I sat on several years ago, which illustrated the importance of skills to grow the cell and gene therapy industry in the UK. We as an organization are one of if not the biggest adopters of that scheme. We have apprentices at many different levels, allowing new people to come in and learn their trade, but it is also a retention tool that allows some of our key talent to learn new skills and new approaches.

On top of this, we are hopeful that we can increase the amount of doctoral level training that we as a company do. We have always had occasional PhD, DPhil, and EngD studentships in the business but now that we are a much larger organization, we have the opportunity to increase those numbers. To that end we have recently been announced by the UK funding organization BBSRC as a recipient of funding for an OXB-led collaborative training partnership (CTP). We have joined forces with two leading partner academic organizations, the University of Oxford and University College London (UCL), and together we will train 24 doctoral students across three years of intake, starting in October 2022 [1]. Moving forward, it's all about making sure that the sector as a whole has enough talent, and a key part of that is to try to attract school-age people. We have an early careers focus within the business that seeks to do more outreach within the local community, to local schools. In fact, a lot of our people do volunteer within schools, giving talks about science and technology and why it's a good career to follow.

Q Finally, can you summarize the chief goals and priorities, both for yourself in your role and for Oxford Biomedica as a whole, over the coming 12–24 months?

JM: Oxford Biomedica is at a stage in its development where we have never been larger as a company. We've never been more highly valued as a company, nor have we previously had revenues as high as we have currently, so we are in a very strong position to build from.

We are primarily UK-based as an organization, but we are not UK-centric: we are a global organization, as are nearly all our partners. Ensuring we maintain that global perspective and competitiveness is vital for our future and that's something we obviously have to think about moving forward. From my own perspective, Brexit hasn't been positive for our sector, which is very unfortunate. It has necessitated us taking a number of steps to ensure we are well placed to serve all our partners globally, including those in Europe.

I think COVID has shown us that there is a future for our kind of innovative high-tech organization – one that has cut its teeth and has proven institutional competence.

Through Novartis, we are manufacturing product that's marketed in about 36 different countries today, which obviously means that we are scrutinized by numerous regulatory agencies from across the globe. This really gives you an insight into the challenges of commercial manufacturing. It isn't easy: maintaining your ability to manufacture in compliance with those international regulations is critical, and we will continue to invest in that.

Fundamentally, we remain both a product and a platform company - we were founded as a product company, and we continue to develop our own therapeutics. And we as a company are now more able to support innovation not only on the platform technologies - the manufacturing, and the analytics I discussed earlier - but also on the product side. We were the first organization to administer a lentiviral vector directly into patients, quite a few years ago now. I hope for us to continue in that vein with some really interesting product candidates, developing them either on our own or with partners.

We have a board, an investment community, and shareholders that believe in our strategy. I never really understood why having this sort of mixed-mode activity, which makes perfect sense for big pharma, wasn't considered as acceptable for biotech. Certainly, I believe a lot of the insights we have gained over the years and a lot of our strengths have stemmed from us having activity as both a CDMO and a therapeutic developer in our own right. It's not an easy option – you have to think about how to prioritize each activity appropriately - but if you do it well, these are mutually beneficial activities.

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CELL & GENE THERAPY INSIGHTS

CELL THERAPY BIOPROCESSING & AUTOMATION

SPOTLIGHT

VIEWPOINT

Translation and automation hurdles for cell therapies

JENNY A PRANGE As co-founder and CSO of MUVON, Jenny is coordinating all research activities aiming towards the improvement of cell production and the therapy in general and manages the GMP production. She initially joined the team early in 2017 for the setup of the GMP-compliant production of muscle precursor as part of the MUSIC project. Jenny obtained her PhD in Integrative Molecular Medicine from the University of Zurich and gained research experience during her studies at Roche in Switzerland, Novartis in the US, and the Federal Research Institute for Animal Health in Germany. Jenny holds a Diplôme d'Ingénieure in Biotechnology from the Ecole supérieure de Biotechnologie de Strasbourg as well as a Master's degree in Biotechnology and Therapeutic Innovation from the University of Strasbourg.

BEATRIX RAUCH-SCHMID – Innovation Manager - She finished her biotechnology studies 2006 with a diploma thesis in tissue engineering on the regeneration of cartilage at the fzmb in Germany and broadened her R&D experience at the Australian ANU and many years at Roche. She finalized her MAS-MTEC Studies (ETH) at the Chair of Innovation. Beatrix joined MUVON Therapeutics in the beginning of 2021 and is responsible for the coordination of innovation projects such as automating the production process.

VIEWPOINT

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A short experience summary on how to transfer an autologous cell therapy from the bench to the patient's bedside for a phase I clinical trial and its automation challenges for future development.

As a young startup company developing a groundbreaking treatment for stress urinary incontinence (SUI) in women, affecting 150 million women worldwide, we want to illustrate in this article how we set up the foundation for GMP production with limited resources and then ran our first clinical study during a pandemic. From here, automation is the next logical step on the way to develop a safe, robust, and economically viable production of autologous adherent cells that may be able to help millions of patients suffering from this non-lethal, yet debilitating disease. Based on solid process knowledge, we want to achieve great quality of an automated process by design and will provide in the following our thoughts on this important topic.

TRANSLATING FROM BENCH TO BEDSIDE

One of the very first tasks in the preparation of a clinical trial is the translation of the preclinical material and protocols to a cleanroom setting and its associated quality management system. This task of setting up the documentation basis, although not too mentally demanding but clearly requiring a deep knowledge of the whole process, sets the basis for all material specifications and working protocols used for the clinical production and needs to be performed meticulously with great emphasis on the quality evaluation of each individual substance and process step. It is very likely, when translating from the preclinic to the clinics, that material which is research grade quality needs replacement if their use cannot be reasonably argued in a solid risk analysis. The earlier every material complies to certain standards (e.g., Pharmacopeias) the better for the general development of the process. At later stage clinical trials, comparability studies will be significantly more time and resource demanding. The challenging part here is to find the right balance in replacing research grade materials at the right time, without drowning in an endless process of material exchange, before the first clinical results are even in.

Obviously, the goal of every single clinical trial should be to eventually provide some sort of improved treatment or analytical procedure for the patients, but especially in the very early stages of clinical research the chances for a successful trial may not seem too convincing. Although preclinical research provides the basis for any clinical trial, limited availability of starting material (which does not only have to be correlated with a low patient number, but also the degree of investment of the medical personnel) and the search for an ideal pre-clinical *in vivo* model may dictate to which extent such research can be performed and is representing the medical condition in question. This means, diseases will have to be simulated if there is no equivalent model at hand. This may add another bias when trying to interpret obtained research data.

It remains undisputed that all obtained data must be documented in the most accurate and straightforward way possible which highly depends on the procedure in the respective laboratory – but based on our humble experience with university labs, the method of choice is still handwritten laboratory notebooks. This way of documentation does not only trigger readability questions, but also often lacks the required clarity and data accessibility or continuity to be easily referred to during the submission process to the various authorities. So, in the transformation process from R&D to GMP manufacturing, companies must start to introduce electronic batch records rather than handwritten, error prone documents, ideally before a pivotal, registration-directed trial begins, or at the very latest prior to commercial launch of a cell product.

Another point which usually only gets attention when a study is about to begin are QC tests based on product specifications.

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Release criteria need to be defined and associated assays validated. Analytical tests in R&D are not always suitable to be implemented in GMP, because their analysis is either taking too long (so results would only be available when the product is already administered) or the type of method cannot be verified according to commonly used standards (e.g. ICH harmonized 'Validation of analytical procedures Q2(R1)'). In case that the same analytical methods can be taken over for the clinical study, the acceptance criteria need to be well decided. Rigid controls and parameters are required, but based on solid process and product understanding, it should be taken into consideration when it would make sense to widen the respective range a bit further in order not to jeopardize one's own trial. Obviously, any changes in the analytical methods will require re-validation and comparability to the previous assays.

All previously mentioned points should be considered when drafting the actual working instructions for the GMP team and running process validation: You have to include all set acceptance criteria, decide which process steps are critical, and still keep in mind that the operator may need to decide (e.g., in the case of adherent cells) the grade of confluence based on his/her expertise with cell culture. AI-supported systems might be valuable in these and other process-related decisions, especially considering variability in autologous cell manufacturing.

All in all, there is almost no overestimating the amount of time this translation into a GMP-compliant process will take – so be generous in your planning.

MANUFACTURING CHALLENGES

Once a clinical trial is set up, the real work begins with batch manufacturing, deviation handling, change management, and so on. A well-structured quality management system (QMS) is indispensable, but the big question here is: Will you use a paper-based or electronic system? And if you should decide on electronic, a strategy for document signatures is required such as password-controlled initials. But here, it depends on the various providers of eQMS systems as to what is possible. We believe it's worth taking a minute (or two) to evaluate what is really needed. The same is true for a manufacturing execution system (MES). These software-based systems are used to document, control, and manage a manufacturing process. It manages and monitors machines, personnel and support services in real time and can be integrated with other applications used in purchasing, shipping-receiving, inventory control, scheduling and maintenance. It therefore supports not only manufacturing members in their daily work in a regulated environment to adhere to the set standards and rules, but also members of adjoining processes.

A major hurdle that all live cell therapy products face is associated with release: it is often the case that one or other of the analysis results is not yet available (most likely, the sterility test) when the product needs to be released for administration. One solution is the 'conditional release' relying on a risk-based approach with a very stringent release assay definition. Conditional release is typically based on a set of in-process control samples, taken as close to the last production day as possible, so that some degree of quality/sterility assurance can be obtained for use on (conditional) certification day. Because the analytical results of the final product become only available after product administration, it should be considered that conditional release is acceptable for R&D patient trials but is not acceptable for commercial cell products. Establishing a suitable freezing protocol, which shows evidence that critical product specifications remain unaffected, could be one solution to tackle this issue.

Furthermore, sterility, mycoplasma and endotoxin testing also need to be set up with the correct limits and considering sample composition/quality. In the case of endotoxins every material used in the process is

contributing to a potential endotoxin burden. Hence, a detailed calculation of each material is a good point to start one's risk assessment for endotoxin load. Service providers have realized that more and more compendial-alternative methods are required, and also invest in their qualification/validation. Finding the right method is still quite a challenge. For example, an ATP-based, rapid sterility method for microbial contamination detection in cell products needs to be carefully validated for matrix interference to be able to omit false positive results from still active cells in the sample. As an alternative, remote sensors (possibly AI-supported) that monitor oxygen or glucose levels, turbidity or similar, might be an option if the reactor of choice is able to accommodate or implement such.

Another important factor to consider when running a first clinical trial is the manufacturing location. A clinic or startup might not have the financial means to build and maintain its own GMP facility. Hence, the most probable scenario is renting a cleanroom box in a facility that is already qualified. This involves weighing the pros of delegated regular cleaning schedules, equipment maintenance, an established QM system or monitoring visits, with the con of needing to adapt to the facility's QMS, no matter if it is paper-based or electronic.

Therefore, one does not only need to plan clinical workflow and GMP productions, but also personnel. Every GMP operator needs to re-qualify every 6 months, and the same applies for the process. This is without any doubt a necessary requirement from the authorities, even across country borders, and needs to be planned well in advance and reflected in the availability of qualified members to avoid losing time later in clinical production.

To streamline the general approach for manufacturing and conducting clinical trials, global standardizations are desperately needed. The Clinical Trials Regulation EU No 536/2014 entered into force already in 2014, but its application was postponed to 2022 due to technical difficulties implementing the corresponding IT system [1]. Another well-meant attempt has been done with the ICH guidelines [2] but as the name implies, these are "only" guidelines. Another example is the elusive definition of a deviation in any of the guidelines. It is things like these that force every entity to define themselves in their QMS what they consider an event worth pursuing. This rather basic example makes it already very hard to follow up on such events as a third party. Having clear, globally applicable rules would be advantageous and would certainly help a comparison between studies performed under different authorities. Also, we believe that the fast-developing sector of cell and gene therapies will support the various legal entities on their way to a broader understanding of the specific requirements, such as the acceptance of a broader range of alternative microbial assays, for example.

RISK MANAGEMENT & WHY AUTOMATE

Risk mitigation is a fundamental part of not only the manufacturing process but the clinical trial in general: Deciding which material to take (research grade or compendial grade; what risk to accept and what to mitigate); what in-process (IPC) and release controls to implement (depending not only on the material quality but also product specifications); or the frequency for IPCs to ensure a compliant production process. All these assessments have one common goal: Risk reduction. In order to be prepared for deviations, it is worth spending some time breaking down the process into smaller units, assessing what could go wrong and why using a thorough process risk analysis, and defining mitigation strategies.

Risk-associated challenges range from critical materials and basic consumables running out at the supplier's site, to their shipment being delayed without having a new delivery date. Recent events have resulted in shortages of a wide range of products to an extent

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none of us would have anticipated before. It is advisable to mitigate this risk by having a second supplier (for every material and consumable) and in an ideal world, one big manufacturer and/or supplier per continent. Having quality agreements with your supply chain in place is required to ensure that your team is notified when anything changes. This way, quality documents can be adjusted before the actual change happens at the manufacturer's site, and no emergency meetings need to take place to decide how to release a material when its specification does not match the updated certificate of analysis (CoA) anymore.

A constantly present risk to consider, in manual as well as automated processes, are contaminations. Even if well-trained and constantly compliant with the rigid requirements of GMP procedures, operators can introduce impurities themselves, same as with a rushed material lock-in or a shortened cleaning procedure.

Additionally, the robustness of the process can carry risks, especially with autologous therapies: Every patient has slightly different cells in terms of growth or metabolite consumption, resulting in the need to adapt the process without jeopardizing the critical quality attributes (CQAs).

Many of these challenges can be addressed by a well-designed automated and closed manufacturing system that seamlessly fits the process. Closing a process will in general reduce cleanroom costs (with a lower cleanroom grade, lower chance of batch failure, etc.) while automation will reduce reliance on operator skills and increase process control. We hope our approach will result in a robust, scalable, and economically viable production of autologous adherent cells. We defined scalability for our autologous process in being able to process several batches in parallel without having to dramatically increase resources. This is planned to ensure reimbursement of therapies for millions of patients suffering from non-lethal yet debilitating diseases.

AUTOMATION STRATEGIES

When approaching the automation of a manufacturing process, various decisions have to be made based on a thorough process analysis. A solid understanding of the process itself and its key parameters, such as CQAs, the critical process parameters (CPPs), and key process parameters (KPPs) derived from these CQAs, is essential. On the path to defining the system´s architecture to provide the required quality, a few main questions must be answered to find the most suitable design for the process:

- The degree of automation: Does it make economic sense to fully automate? How much flexibility is possible to allow interference by a process expert in case of technical failure to ensure the defined CQAs or prevent manipulation? Does it allow enough flexibility in the future? How are samples for in-process control taken and analyzed? Considerations need to include the COGs of the manual process in comparison to the investments needed to automat that specific step. In some cases, it might make sense to only / first automate the steps that benefit most from this transition, for example, expansion.

- The degree of mechanization: Is human interference desired? Is it enough to control the devices or is actual manual handling required, or at least desired, in case of a failure?

- Connectivity: Does the process need to be fully connected? Is it desirable to have one platform that combines everything in a single box? Or are there components that are either only used for a few hours, or for days up to and including the entire production process duration? Maybe, single individual units are more favorable? Something to consider in that regard could be that upstream and downstream modules usually take less time than expansion.

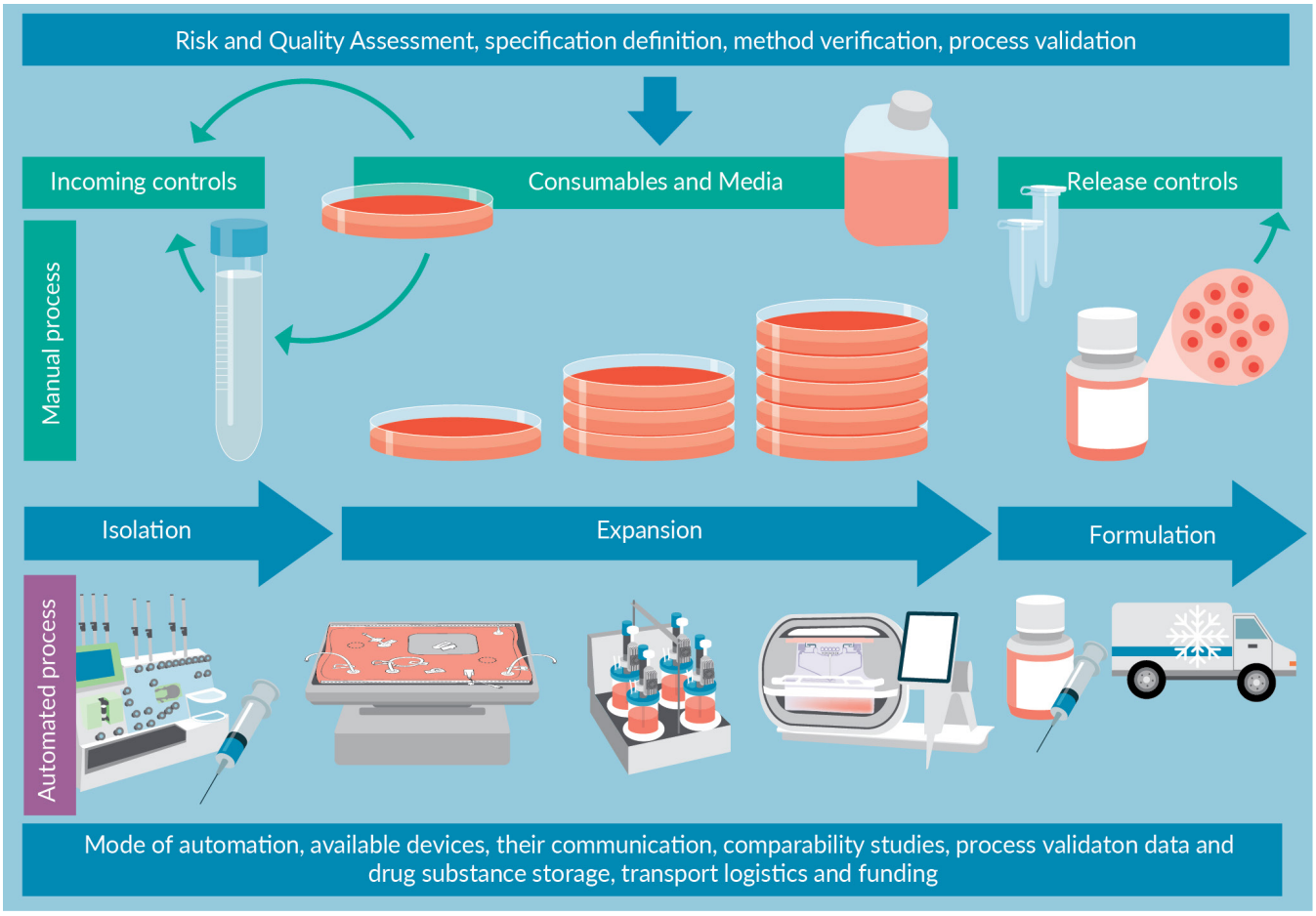
- Degree of closure: This aspect has far-reaching consequences. If a process is fully closed, the cleanroom requirements and with it the cleanroom associated costs can be reduced because cleanrooms are very expensive – not just in acquisition, but also in operation. Maintenance of the GMP status requires a lot of checks, monitoring, cleaning, and quality control. All staff needs to be very well trained and qualified – not just those working regularly inside the cleanrooms, but also the personnel for cleaning and equipment services.

A gap analysis might reveal to which extent the production process steps can be replaced with off-the-shelf solutions (Figure 1). This may reduce the risk and effort of developing required devices oneself. As a startup, this is quite often a question of available resources. In such cases, it would be advisable to work with a two-option scenario: having a fallback strategy available besides the best-case scenario. Then, even in the case that one development path fails (which could sometimes mean keeping manual process steps) automation can be pursued on a second, parallel lane.

If, for example, the ideal solution is a device that is not yet on the market – one that still needs certification or is even still in the R&D stage; a collaboration with that developer might be a valid approach for goal-oriented results. However, it would be key to start this collaboration well in advance of your pivotal studies because the security of a timely development is not assured, especially in the R&D stage. If no device is available in any state of development, though, one could approach an experienced partner to develop a custom-made solution. Of note, this also bears

FIGURE 1

A schematic overview about the modular automation for a manual cell production process.



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potentially high risks depending on which process step and bottleneck needs alleviation. Required time, effort, and funding should be carefully considered in this case. Additionally (and regardless of the specific option) IP considerations must be thought through prior to starting developmental projects.

Despite automation being the next evolutionary step, current technologies are mostly individual units of operation and whilst they can perform certain processing steps in an efficient manner, combining these technologies to develop a closed, standardized manufacturing process remains elusive. As an example, we want to briefly mention the choice of bioreactors for the expansion process. Manufacturers started with models for suspension cells, but to date more options are also developed for adherent cells. For the different reactor types, the initial cell number required to start the expansion step seems the most important one, besides having suitable conditions for the respective cells inside. Although many leading device providers work on their own line of modules for process automation (ranging from tissue homogenization over automated QC analysis to special volume reduction centrifugation techniques), the separate units carry the risk of missing interconnectivity with other devices (likely if the different modules are not all from the same supplier), or missing data integrity throughout the process. Once all these questions have been answered and the process is automated, the final step is to validate the entire manufacturing process to ensure GMP compliance, and proving it provides comparable products to the manual process.

Of course, all these plans and ideas require a substantial amount of funding, which may be collected from dilutive or non-dilutive sources. In the latter case, it is helpful to keep an eye on political developments in case bilateral contracts or framework agreements suddenly cease to exist, meaning one is rendered ineligible to even apply for certain funding opportunities anymore.

CONCLUSION

The number of regenerative, cell-based therapies currently available in clinical practice is still highly limited despite the revolutionizing expectations of global healthcare systems. Major factors for this include high costs and complexity of bioprocess and logistics scale-up to commercial levels, especially in the case of autologous therapies. As such, there is a high unmet need to automate production processes.

To get there, we think that when handling cell therapies at whichever stage, timely planning is of the essence, paired with smart resource management (financial and personnel) to find and tackle the critical questions early on. Events with global impact, whether they be a pandemic or a blocked naval route, will always present unpredictable risks, but recent history shows us that some time should be invested in covering these scenarios in your own risk assessment and mitigation strategies.

Nevertheless, one also needs to have access to the required expertise in a wide range of areas involved in the development of an economically viable, effective, and approved therapy to be able to pinpoint problems early and address them appropriately. This may also be applied to the regulatory space - it is wise to consider from the start which countries are of interest, and what specific requirements their respective regulatory bodies may have, so that potential CMC strategies can still be finetuned in this direction. Don't forget to also consider those countries in IP strategy.

Starting as early as possible to get a detailed and solid process understanding is key to being able to tackle open questions for automation, and consequently having the luxury to evaluate various adaptation options.

In conclusion, we can report that from the perspective of a young startup, it often feels that we are faced with the classic 'chicken and egg' problem. Everything is interconnected and has effects on so many levels that the 'mountain' to be conquered seems steep, and the price to pay if one falls is high. But despite all these hurdles, the pure potential that cell

(and gene) therapies can offer in the personalized medical sector is impressive and without a doubt, worth investing on many levels! Let's keep an eye out together for the latest developments in this rapidly evolving area and learn from one another to bring the best therapies through to the patients who need them the most.

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CELL & GENE THERAPY INSIGHTS

CELL THERAPY BIOPROCESSING & AUTOMATION

SPOTLIGHT

VIEWPOINT

Driving CARs on the biomanufacturing road to clinical success

CHELSIA QIUXIA WANG, PhD, is currently an Associate Staff Scientist, Immune Cell Manufacturing at Bioprocessing Technology Institute (BTI), Agency for Science, Technology and Research, Singapore (A*STAR). Her research is focused on improving immunotherapy using chimeric antigen receptor (CAR) immune cells, with the goal of translating her work to clinical use. Dr Wang received her Ph.D. in Biological Sciences from National University of Singapore (NUS) and completed her postdoctoral training in Institute of Molecular and Cellular Biology (IMCB), A*STAR and Cancer Science Institute (CSI), NUS, where she worked on elucidating the roles of RUNX family genes in leukemogenesis.

ANDY HEE-MENG TAN, PhD, is Senior Scientist and Group Leader of Immune Cell Manufacturing at Bioprocessing Technology Institute (BTI), Agency for Science, Technology and Research, Singapore (A*STAR). One major part of his research encompasses chimeric antigen receptor (CAR) engineering of T and NK cells for tumor immunotherapy. He is the lead investigator in a joint laboratory with a contract development and manufacturing organization (CDMO) to enable process optimization for large scale production of CAR-T cells. Andy also co-directs the Centre of Innovation for Sustainable Banking and Production of Cultivated Meats (CRISP Meats), a multi-disciplinary and multi-institutional research programme which aims to develop innovative technologies for manufacturing of cultivated meat. As Consultant Analytical Scientist with Health Sciences Authority, he supervises the building of capabilities in nucleic acid- and immune-based analytics for characterization of prophylactics. Andy received his BSc and MSc in Physics from National University of Singapore (NUS) and PhD in Immunology from NUS Graduate School for Integrative Sciences and Engineering. He completed postdoctoral training in Immunogenomics at John Curtin School of Medical Research, Australian National University before joining BTI as a Research Scientist.

VIEWPOINT

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Innovations in manufacturing of cellular immunotherapies arising from strategic partnerships between public sector research institutions and CDMOs are crucial to attain substantial reduction in cost of goods. We envision a near future when patients suffering from refractory or relapsing tumors will not have to wait an unconscionable period of time before receiving their dose of 'off-the-shelf' CAR-NK and γδ CAR-T cells.

CAR-NK & T CELL IMMUNOTHERAPY: THE STORY SO FAR

Novel therapeutic modalities resulting from ingenious ways to engineer NK and T cells to achieve targeted therapy against cancer have proliferated rapidly in the field of immuno-oncology over the past decade. To enhance their intrinsic killing abilities, NK and T cells are modified to express chimeric antigen receptors (CARs) that bind to cognate antigens on tumor cells to mediate tumor death [1]. Bench-to-bedside translation culminated in approval by the US Food and Drug Administration (FDA) of five CAR-T cell therapies for treatment of B cell leukemias/lymphomas and, more recently, multiple myeloma which had been regarded as an incurable hematological malignancy for which existing chemotherapeutic options elicit significant toxic side effects (summarized in Table 1).

Despite substantial progress made in purpose-driven CAR modification of NK and T cells, there remains much to learn particularly about the functional longevity and safety of CAR-NK and T cell therapy from ongoing clinical trials. Real-world insights gleaned from challenges encountered by cancer patients who have undergone CAR-NK and T cell immunotherapy will aid future revisions or refinements in research strategy to construct CARs with an advantageous efficacy and safety profile.

BIOMANUFACTURING OF CAR-NK & T CELLS: WHAT ARE THE GAPS TO BE FILLED?

There are multiple steps and associated challenges involved in the entire biomanufacturing process to generate CAR-NK and T cells as cellular immunotherapy products of high quality. The process encompasses leukapheresis of patient to isolate NK or T cells, CAR transduction of the isolated cells, subsequent CAR-NK or T cell expansion, cell cryopreservation and storage, and transportation from manufacturing facility to clinical site where cells are thawed and infused into the same patient. Factors that must be considered are:

i. Donor/cell source

ii. Cell type

iii. Expansion protocol

iv. Type of modification

v. Method of modification

vi. SOPs for cryopreservation and storage

vii. Quality control/assessment of CAR-NK or T cell product

Currently, all FDA approved CAR-T cell therapies involve engineering of autologous, conventional αβ T cells obtained from patients (Figure 1). Often, autologous T cells lack fitness due to prior exposure of the patient to several rounds of radio- and/or chemotherapy. Patient-derived T cells therefore have poor viability and expansion capacity, compromised CAR transduction efficiency, *in vivo* function, and persistence. The limited numbers of CAR-T cells available for infusion into patients is compounded by the long process to produce these cells and the requirement for multiple infusions. One way to circumvent this is to program CARs in innate NK or innate-like γδ T cells derived from healthy donors to develop allogeneic CAR-NK or γδ CAR-T cells. NK or γδ T cells from a single 'universal' healthy

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TABLE 1

Current FDA-approved CAR-T cell therapies.

Generic name	Trade name	Manufacturer	Approved indications	Year of FDA approval	Cell source and cell type	Type of CAR	Method of modification
Tisagenlecleucel	Kymriah®	Novartis Pharmaceuticals Corporation	Acute B-cell lymphoblastic leukemia	2017	Autologous T cell	CD19 CAR (FMC63-CD8a HTM-BBz)	Lentiviral transduction
Axicabtagene ciloleucel	Yescarta®	Kite Pharma, Incorporated (Gilead)	B-cell lymphoma	2017	Autologous T cell	CD19 CAR (FMC63-CD28 HTM-28z)	Retroviral transduction
Brexucabtagene autoleucel	Tecartus®	Kite Pharma, Incorporated (Gilead)	Mantle cell lymphoma	2020	Autologous T cell	CD19 CAR (FMC63-CD28 HTM-28z)	Retroviral transduction
Lisocabtagene maraleucel	Breyanzi®	Juno Therapeutics, Inc. (Bristol Myers Squibb)	B-cell lymphoma	2021	Autologous T cell	CD19 CAR (FMC63-IgG4 hinge-CD28 TM-BBz)	Lentiviral transduction
Idecabtagene vicleucel	Abecma®	Celgene Corporation (Bristol-Myers Squibb)	Multiple myeloma	2021	Autologous T cell	BCMA02 CAR (BCMA-CD8a HTM-BBz)	Lentiviral transduction

CAR: Chimeric antigen receptor; HTM: Hinge and transmembrane; Ig: Immunoglobulin; TM: Transmembrane.

donor can be CAR-modified, subsequently expanded, and cryopreserved at scale to serve as potential "off-the-shelf" therapies to be delivered to many patients. To similarly do so using αβ T cells is far less straightforward as these cells have to undergo more elaborate genetic modification such as ablation of genes encoding the T cell receptor (TCR) α chain (*TRAC*) and β-2 microglobulin (*B2M*) [2]. Such deletions to respectively disrupt expression of TCR and human leukocyte antigen (HLA) class I expression will mitigate the risk of graft-versus-host disease (GvHD), a potentially life-threatening condition in which infused αβ T cells recognize the recipient's tissues as foreign and attack them. Conversely, the recipient's αβ T cells may recognize and reject the donor's αβ CAR-T cells, thus limiting the latter's therapeutic effect. Besides averting GvHD, an added advantage of using NK and γδ T cells is they, unlike αβ T cells, can recognize and eliminate tumor cells by both CAR- and endogenous cell receptor-dependent mechanisms [3,4].

Notwithstanding the favorable properties of innate or innate-like immune cell types for use in therapies, their *ex vivo* expansion following isolation from a single healthy donor to clinically relevant numbers for effective immunotherapy, either prior to or following CAR modification, remains a formidable challenge. Hence, one of the primary goals in our lab is to develop and optimize protocols that enable efficient expansion of NK and γδ T cells from peripheral or umbilical cord blood (UCB) leukocytes of healthy donors. Other labs are exploring CAR modification of human embryonic stem cells (hESCs) or induced pluripotent stem cells (iPSCs) to generate CAR-expressing hESCs or iPSCs that can then be differentiated into CAR-NK [5,6] or CAR-T cells [7]. We are currently exploring novel ways to engineer feeder cells to optimally support CAR-modified or unmodified NK or γδ T cell production and identify compounds that can efficiently promote the expansion of specific γδ T subsets.

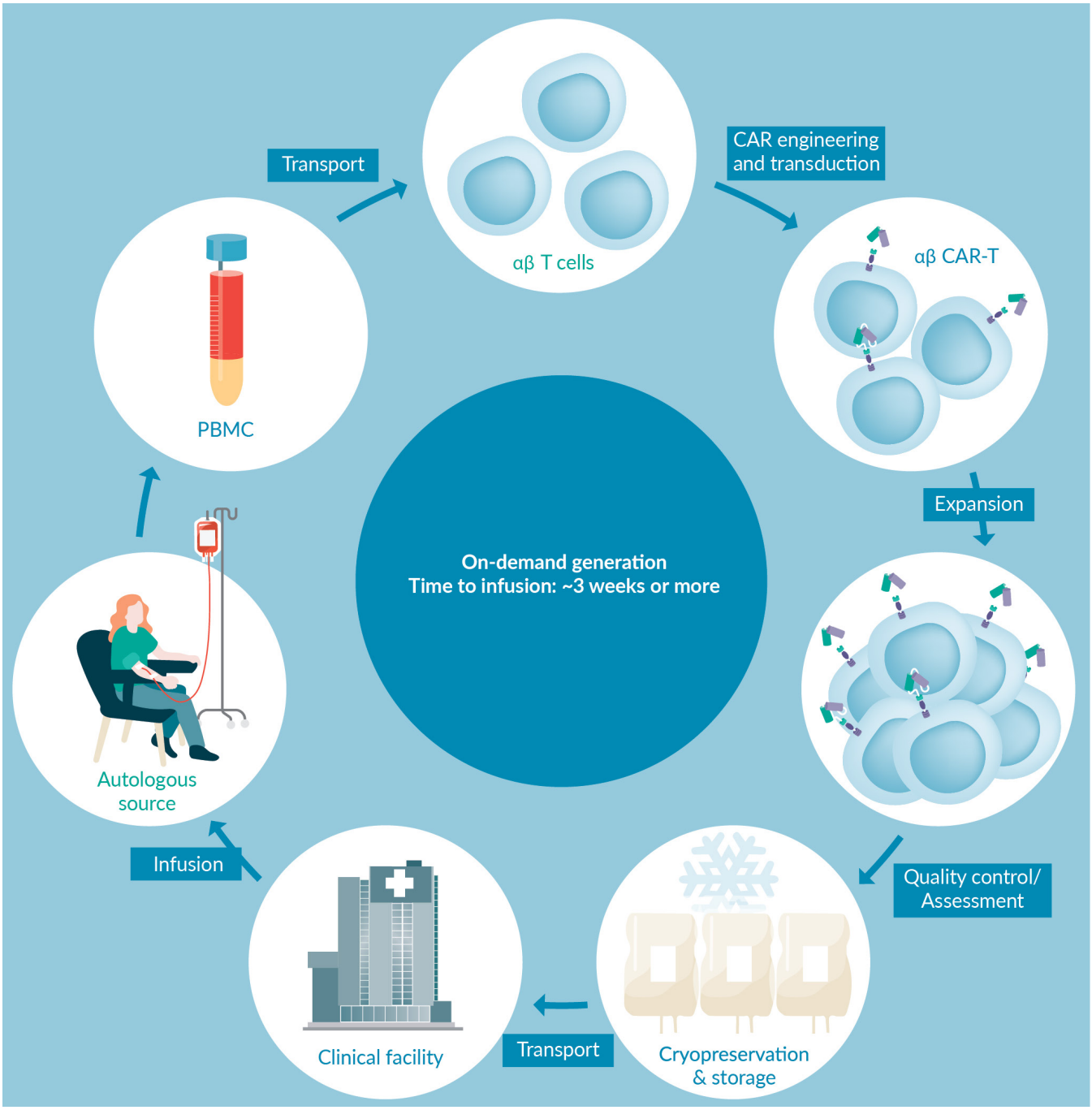
As we explore the therapeutic safety of various immune cell types, we dedicate concurrent effort to investigate how changes in CAR structure influence the efficacy of NK or γδ T cells in killing CAR antigen-positive tumor

cells. The strategy employed in designing a CAR includes selection of [8]:

1. A tumor-specific antigen targeted by the single chain variable fragment (scFv) of a monoclonal antibody (mAb) raised against the antigen which forms part of the extracellular antigen recognition domain of the CAR
2. Hinge and transmembrane domains
3. The identity and number of co-stimulatory endodomains

FIGURE 1

Current biomanufacturing process of CAR-T cells.



CAR: Chimeric antigen receptor; PBMCs: Peripheral blood mononuclear cells.

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The choice of scFv is guided by consideration of 'on-target, off-tumor' toxicity, the manifestation of which may increase in probability with the level of antigen expression in normal tissues. One approach to overcome off-tumor targeting by a CAR is fine-tuning its affinity to the antigen such that binding is significant when the antigen is expressed highly in tumor cells whereas negligible when expressed lowly in normal cells [9]. Another approach is engineering of a bi-specific CAR, such as dual CAR, capable of recognizing two separate antigens, whereby modified NK or T cells are activated only when both antigens presented by tumor cells simultaneously engage the CAR, hence enhancing specificity [10]. We argue the importance of identifying antigens which are highly expressed by cancer stem cells for CAR targeting to arrest the well-known contribution of such cells to tumor relapse [11,12]. In our opinion, this aspect of scFv selection for CAR engineering has been largely overlooked. In deciding which intracellular signaling domains to incorporate into a CAR, we propose the inclusion in a CAR of endodomains of co-stimulatory receptors specifically expressed by an immune cell type for the CAR to be expressed in the same cell type. For example, NK cells engineered to express a CAR harboring endodomains of NK cell-specific receptors will likely exhibit anti-tumor activity surpassing that of counterparts engineered with a CAR endowed with T cell-specific endodomains. Yet αβ T cells programmed with CARs harboring endodomains of B cell-specific receptors have been shown to exhibit potent activity against tumors [13], suggesting more studies are needed to ascertain which combinations of CAR endodomains expressed in specific immune cell types will result in optimal anti-tumor function of the cell types. CAR-T therapy has been spectacularly successful in controlling hematological malignancies but has hitherto not delivered the same promise for suppressing solid tumors. Beyond CAR engineering of NK and T cells *per se*, these CAR-modified cells have to overcome the typically hostile and immunosuppressive tumor microenvironment (TME) presented within solid tumors [14]. Options to achieve this include enhancing the metabolic fitness of CAR-modified cells [15] or conferring resistance to TME-associated immunosuppressive factors by arming cells with dominant negative or switch receptors or ability to release support cytokines [14]. Ongoing work in our lab involves adopting a multi-pronged approach in CAR and other gene-targeted engineering to improve the efficacy and safety features of NK or T-mediated immunotherapy.

CARs are usually introduced into NK or T cells by either virus-based transduction or electroporation-based transfection. Because of viral integration in the genome of these cells, the former usually results in more stable and persistent CAR expression in the cells compared with the latter. Transient CAR expression in cells transfected with CAR mRNA may serve to limit potential CAR-mediated off-tumor toxicity. However, the competing concern is that short-lived CAR expression in NK or T cells could permit evasion of some tumor cells from eradication, resulting in therapy ineffectiveness and tumor relapse. Albeit there has been considerable success in achieving stable transgene expression in T cells using transposon-based system delivered by electroporation [16], lentiviral or retroviral transduction of transgenes is still preferred in most labs, including ours. As the number of viral copies integrated into the genome of T cells is random after viral CAR transduction, CAR expression across T cell clones will be variegated. In contrast, CRISPR/Cas9-mediated knock-in of CAR into the *TRAC* locus leads to CAR expression being under the control of the endogenous TCR promoter and far more consistent across T cell clones [17]. Reduced variation in CAR expression among T cells is correlated with their superior anti-tumor activity and possibly lower toxicity. Challenges pertaining to safety, efficiency and scalability remain when using such gene-editing technologies. These include potential for off-targeting, low recombination frequency of large sized transgenes to be inserted at the cut site and generation of sufficient

gene-manipulated CAR-NK and T cells for infusion into patients since cellular stress imposed by electroporation compromises the capacity of cells to proliferate [18,19]. The CRISPR-Cas9 strategy may be in its infancy, but we foresee that it will gradually supersede traditional transduction methods.

Further downstream in the biomanufacturing process, there are two aspects to consider for virus-mediated generation of GMP-compliant CAR-NK or T cells for eventual clinical use, i.e. manufacturing of:

1. CAR virus-producing adherent cells and
2. suspension NK or T cells transduced with CAR virus.

These involve identification and optimization of critical process parameters (CPPs) that enable manufacturing of virus-producing cells to produce CAR virus of high titer and quality or CAR-NK and T cells of high functional efficacy. The need for robust yet cost-effective manufacturing of CAR-NK and T cell therapies has created opportunities for public sector researchers focused on invention and clinical translation of CAR-NK and T cell therapy assets to license their assets to contract development and manufacturing organizations (CDMOs) in the private sector and collaborate with them to develop closed, modular and semi-automated bioprocessing platforms for scaling up / out production especially of allogeneic CAR-NK and γδ CAR-T assets. Ideally, these therapies should not cost exceedingly more than standard line of care therapies so that they are affordable to and accessible by more cancer patients. In this respect, public-private partnerships have been accelerated to urgently establish and fine-tune CPPs important for various steps of activation and CAR transduction of NK and T cells followed by expansion of CAR-NK and T cells to meet growing clinical demand. Approaches to overcome major cost bottlenecks include engineering of synthetic cytokines or 'synthekines' [20] with heightened bioactivity and culture stability, reduction of the use of activation and transduction reagents to generate CAR-NK and T cells and scaling up / out expansion of these cells from two-dimensional vessels to three-dimensional bioreactor systems. In addition, formulation of serum-free, chemically defined media for culture of CAR-NK and T cells can lessen batch-to-batch variability of manufactured cell products. During expansion of cells in the bioreactor, in-process monitoring of culture conditions such as pH, dissolved oxygen and temperature is conducted to ensure they do not fluctuate beyond set thresholds. Furthermore, the quality of the final cell product in terms of viable cell density, frequency of CAR-expressing cells, secretion of cytotoxic cytokines and molecules, extent of cytotoxicity against selected tumor cells, absence of mycoplasma and endotoxin, etc. is typically measured off-line at the end of product manufacture. In order to efficiently optimize CPPs to manufacture cell products of consistently high quality, product characteristics should not be assessed only at the manufacturing end point but ideally monitored at defined stages throughout the manufacturing process. Such in-line, real-time assessment of critical quality attributes (CQAs) of the cell product can be accomplished using a suite of bioanalytical techniques, the operation of which requires innovation to integrate as automated modules of the manufacturing process. Moreover, defining CQAs that precisely inform product quality at various stages of manufacturing is a matter of priority as the clinical use of CAR-NK and T cells becomes more widespread.

Following manufacture of the cell product, another crucial step to facilitate transportation of product from the manufacturing facility to clinical sites is the careful cryopreservation and storage of the cells such that their viability and functional efficacy are retained after thawing [21]. The use of non-toxic cryopreservative agents to maintain the integrity of the CAR-NK or T cell product is gaining importance as cellular immunotherapy transits gradually from "on-demand" generation of autologous αβ CAR-T cells to *en masse* production of repositories of allogeneic CAR-NK and γδ CAR-T cells. Accordingly, definition

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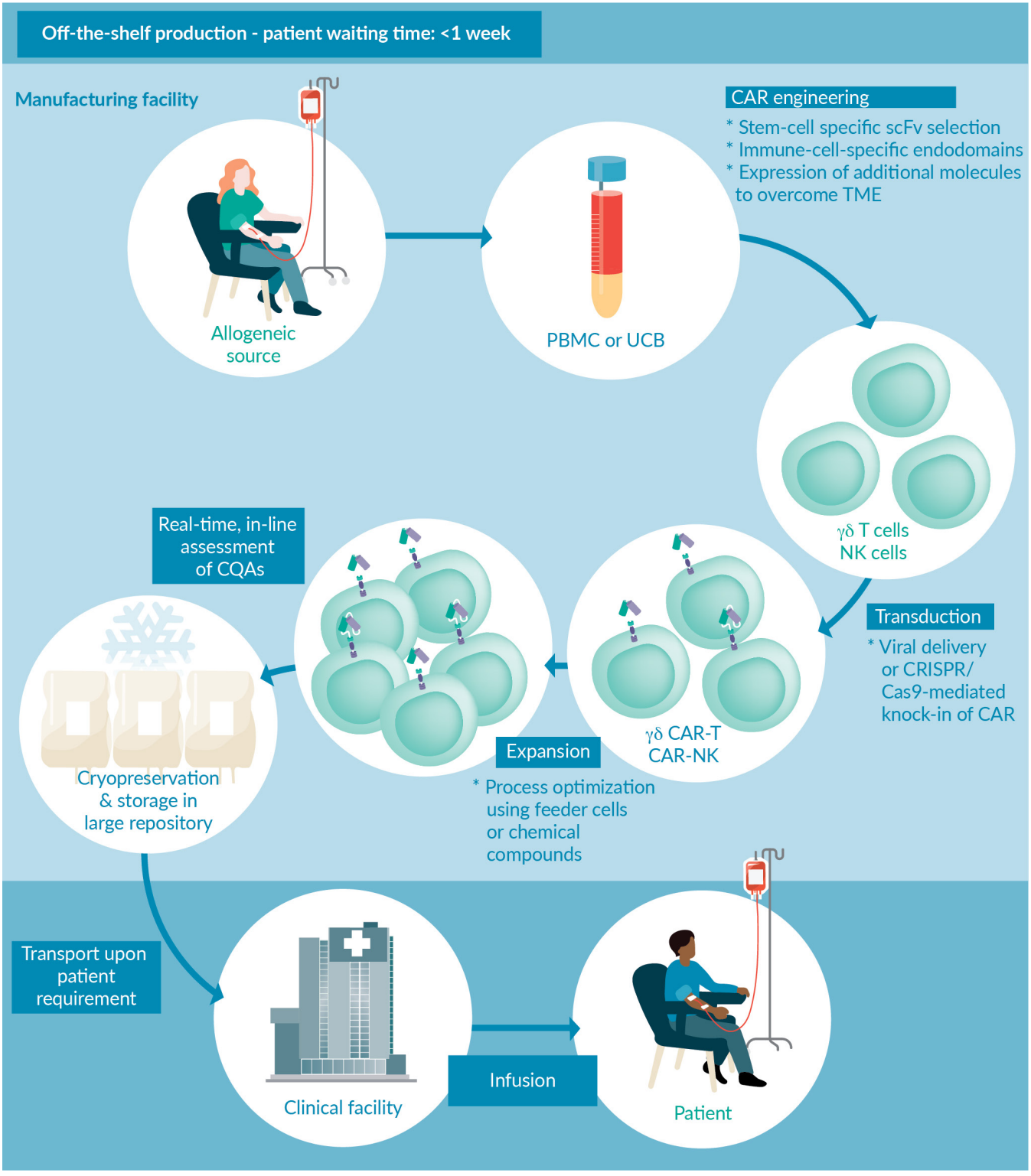
FIGURE 2

The future of CAR-NK and γδ CAR-T cell immunotherapy, highlighting drastically reduced patient waiting time to infusion.



The schematic also depicts (in asterisks) our lab's multi-pronged approach to address some of the challenges associated with the biomanufacturing process.
CAR: Chimeric antigen receptor; CQAs: Critical quality attributes; PBMCs: Peripheral blood mononuclear cells; UCB: Umbilical cord blood.

of product CQAs should be extended to interrogate the quality of the cell product subjected to cryopreservation, storage, and thawing.

THE ROAD AHEAD FOR CAR-NK & T-CELL THERAPY

In summary, despite the proven clinical success of CAR-T cell therapy, many roadblocks remain. One major obstacle is the generation of sufficient numbers of functional CAR-T cells from cancer patients whose endogenous T cells have been incapacitated by multiple rounds of conventional therapy. We propose to move away from the use of autologous αβ CAR-T cells to employing "off-the-shelf" CAR-NK and γδ CAR-T cells for which libraries of NK or γδ T cells expressing CARs targeting different tumor antigens or incorporating different co-stimulatory endodomains can be assembled and stored long before patients present at clinics. We anticipate that it is possible to shorten the waiting time for patients from more than 3 weeks to a few days prior to cell infusion (Figure 2). However, the efficacy of "off-the-shelf" cellular immunotherapies may be limited by host-versus-graft rejection of the infused cells by host NK cells. Progress is being made to overcome such limitations [18].

Multiple challenges in manufacturing CAR-NK and γδ CAR-T cells still lie ahead. These include process optimization to enable efficient and scalable expansion of cells with high anti-tumor efficacy and establishing a "gold standard" for cryopreservation/thawing techniques that maintain cell product CQAs, while bearing in mind that devised solutions ought to be cost-effective. Autologous CAR-T cell therapy currently costs an estimated US$0.5 million per patient. Innovations in manufacturing of cellular immunotherapies arising from strategic partnerships between public sector research institutions (e.g., BTI, A*STAR) and private sector CDMOs are crucial to attain substantial reduction in cost of goods (COGs). We envision a near future when patients suffering from refractory or relapsing tumors will not have to wait an unconscionable period of time before receiving their dose of 'off-the-shelf' CAR-NK and γδ CAR-T cells.

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CELL & GENE THERAPY INSIGHTS

LATEST ARTICLES:

FASTFACTS

How choosing the right transfection reagent can drastically bring down costs of AAV manufacturing

Maxime Dumont, Product Manager-Cell & Gene Therapy, Polyplus-transfection

Choosing the right transfection reagent can significantly lower manufacturing cost and cost per dose, by increasing productivity and reducing the quantity of DNA used. This poster presents the key attributes to consider and describes a case study quantifying the cost savings that can be achieved with the right transfection reagent.

Cell & Gene Therapy Insights 2021; 7(11), 1363 • DOI: 10.18609/cgti.2021.179

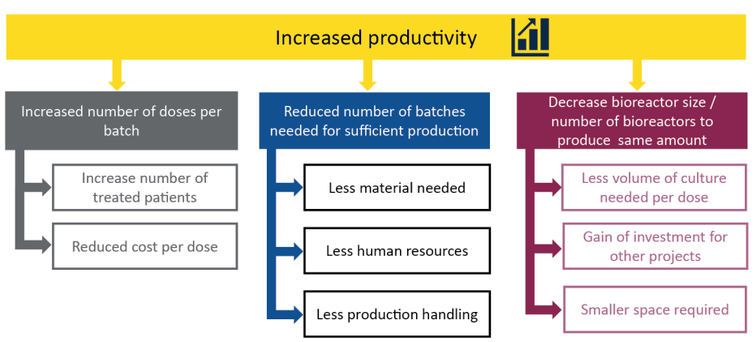
HOW TO CHOOSE THE RIGHT TRANSFECTION REAGENT?

Transfection is a critical step of the upstream process of AAV manufacturing, and transfection performance drives upstream productivity. As such, choosing the right transfection reagent is key to decreasing manufacturing costs.

It is important to choose a transfection reagent that can be used from process development to commercialization and fulfills the following criteria:

- Performance that allowing sufficient functional titers to be reached
- Scalability – provides reproducible results from small- to large-scale bioreactors
- Flexbility – adaptable to different systems
- GMP compliant
- Can be used in combination with a residual test, thus mitigating risk for drug approval
- Supplier support – answering questions related to science, supply chain and regulatory affairs

Figure 1. Impact of transfection performance on manufacturing costs.



IMPACT OF TRANSFECTION PERFORMANCE ON VIRAL VECTOR MANUFACTURING COSTS

Transfection performance can directly impact global manufacturing costs **(Figure 1)**. An increase in productivity will affect the number of doses per batch e.g. If there is a two-fold increase in productivity, then the number of doses produced is also increased two-fold.

IMPACT OF TRANSFECTION PERFORMANCE ON COSTS PER DOSE

A change of transfection reagent can significantly reduce the cost per dose even when using the same bioreactor. If a fixed number of doses is needed and changing the transfection reagent causes a two-fold increase in productivity, fewer batches will be needed to reach the required number of doses.

Figure 2. Cost-modelling case study comparing three different transfection reagents.

Criteria	Polyplus-transfection		Other company
	FectoVIR®-AAV	PEIpro®	PEI
Average dose per patient *	**$8.8x10^{14}$ VG** for 1 dose		
Productivity (VG/mL)**	$4{,}8.10^{11}$	$2{,}45.10^{11}$	$7{,}8.10^{10}$
Productivity of product vs FectoVIR®-AAV (percentage)	100 %	- 48.9 % (≈ -2X)	- 83,8 % (≈ -6X)
Volume of culture needed per dose ***	6,1 L	12,0 L	37,0 L
Number of doses per 2000 L bioreactor (without QCs)	328	168	54
Number of batches to reach 1000 doses using a 2000 L bioreactor	4	6	19

* Theoretical average dose per patient
** In-house transfection experiment in HEK-293 cultivated in suspension using the manufacturer's recommendations
*** Take into consideration a 70% loss for the purification steps (DSP)

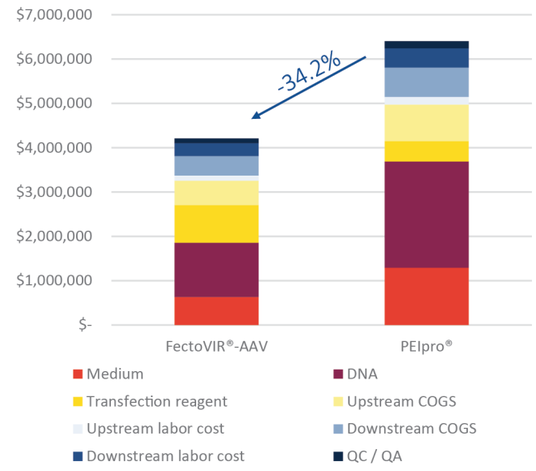
CELL & GENE THERAPY INSIGHTS

In partnership with: Polyplus®

The volume of culture per dose is significantly less for FectoVIR®-AAV in comparison to PEIpro® or PEI **(Figure 2)**. This has a direct impact on the number of doses per bioreactor; in a 2000 L bioreactor, FectoVIR®-AAV produced 328 doses, compared to 168 and 54 doses for PEIpro® and PEI, respectively. Consequently, FectoVIR®-AAV requires four bioreactors to produce 1000 doses compared to six for PEIpro®. This is an example of how the performance of the transfection reagent leads to a reduction in the number of batches and, consequently, the manufacturing cost, by reducing cost of goods, labor, and quality control processes.

As well as driving productivity, choosing optimized transfection reagent for AAV manufacturing can additionally reduce DNA consumption by 25–50%, thereby further decreasing manufacturing costs **(Figure 3)**. In fact, pDNA usually represents 30-40% of the overall AAV manufacturing cost, and being able to decrease the quantity of plasmid DNA is critical.

Figure 3. Manufacturing cost to reach 1000 doses in a 2000 L bioreactor. Using FectoVIR®-AAV provides a saving of $2.1 million compared with PEIpro®. Furthermore, the cost per dose using FectoVIR®-AAV is $3,206 compared to $6,350 with PEIpro®.



Note: the model does not take into consideration the building depreciation

Automate and close your cell therapy process with a flexible system: Sepax™ C-Pro capabilities

Sonia Bulsara, Application Specialist Leader, USCAN, Cytiva

The Sepax™ C-Pro system is a closed and automated system with the flexibility to fit in different steps across your cell therapy workflow. Scientists use it in both clinical and commercial manufacturing.

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THE SEPAX™ C-PRO SYSTEM

The Sepax™ system combines the Sepax™ C-Pro instrument, Sepax™ C-Pro application software, and Sepax™ C-Pro disposable kits **(Figure 1)**. With a single system, you can automate and close multiple steps of your manufacturing workflow. You can configure each application software with open parameters to fit your needs and recreate your current manual process. Once optimized, you can lock and secure the parameters to standardize your process.

LOOKING FOR FLEXIBILITY?

With dedicated application software, Sepax™ C-Pro systems provide an automated and functionally closed solution for more than nine steps of the standard cell therapy workflow **(Figure 2)**. For example, if you're starting with a fresh apheresis unit, you can use PeriCell-Pro to remove plasma, or perform washing steps to remove platelets with PlateletFree C-Pro application software. Then you can use the NeatCell C-Pro application to perform a closed and automated peripheral blood mononuclear cell (PBMC) enrichment with density-gradient medium separation and transduce your activated cells using the dedicated Sepax™ C-Pro application. Finally, after cell expansion, you can concentrate your cells and wash them using CultureWash C-Pro.

The Dilution C-Pro application lets you dilute your cells and split them into different bags to prepare final doses for freezing and shipping.

Other workflows are also possible. For example, if you're starting with a frozen apheresis, you can thaw using our VIA Thaw™ system and use CultureWash C-Pro to perform a functionally closed and automated dilution. Then you can follow with concentration and washing cycles to remove DMSO.

Instead of enriching PBMCs, you might want to perform a magnetic isolation. In this case, you can use the BeadWash C-Pro application to perform your preparation. If required, use an initial dilution, and follow up with a washing step to remove platelets or DMSO. Then use a bead incubation and washing step to remove any unattached beads. After selecting magnetic cells, you can use CultureWash C-Pro to wash your selected cells, remove debris, and perform a media exchange.

Figure 1. The Sepax™ C-Pro system: instrument, software, and kit.

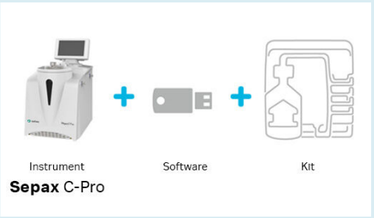
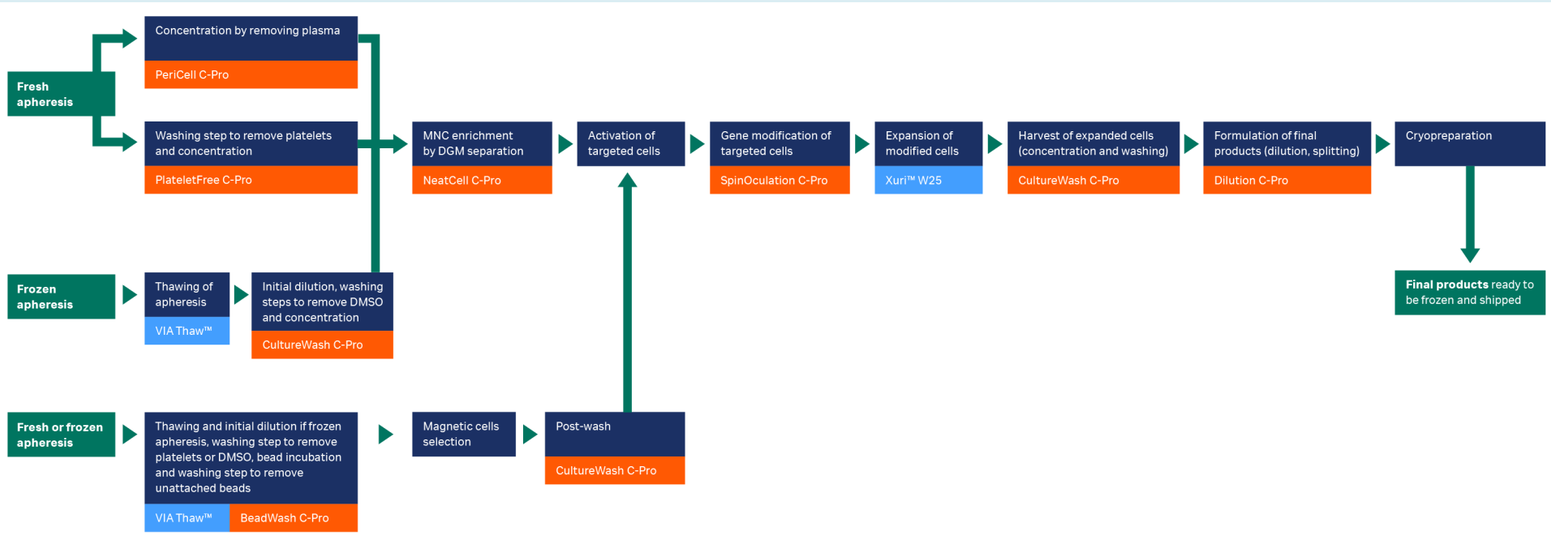


Figure 2. Sepax™ C-Pro system provides a closed and automated platform for multiple steps of the cell therapy workflow.



DESIGNED TO FIT IN YOUR WORKFLOW

Key advantages of the Sepax™ C-Pro system include:

- Automated and functionally closed design to secure and standardize your processes
- Flexibility to support multiple dedicated applications
- Compact design and user-friendly interface for minimal space and resource requirements
- Minimizes hands-on and process touch time while delivering a high-quality final cell product

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INTERVIEW

Finding a happy medium: meeting the rising demand for bioproduction media

MARLIN FRECHETTE has over 30 years of experience in the medical device industry, servicing pharmaceutical and biopharmaceutical customers. As Chief Quality and Compliance Officer at FUJIFILM Irvine Scientific, she holds global responsibility for all FUJIFILM Irvine Scientific sites and affiliates for Compliance, Quality Systems, Global Regulatory, and EHS. She holds a Bachelor's of Science with a major in Business Administration and Personnel Management.

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Recent years have seen shortages of raw materials across the biopharmaceutical industry, including critical components of cell culture media. Here, Marlin Frechette, Chief Quality and Compliance Officer and ISO Management Representative, FUJIFILM Irvine Scientific, shares how the global supplier is adapting to meet the needs of its customers.

Q How is the demand for media for bioproduction evolving worldwide and how are you meeting the needs of this global sector?

MF: With the high rate of growth in the biopharmaceutical market, and pharmaceutical drugs and vaccines moving into clinical trials and commercialization at a fast pace, our customers require full support as their products advance through

"To meet the increasing customer production and reliable, regional supply needs, we are building a world-class cGMP manufacturing facility in Europe."

regulatory approvals. To address these needs, FUJIFILM Irvine Scientific utilizes a global forecasting strategy that incorporates flexibility and consistency while keeping apprised of trends in supply interruptions or bottlenecks to support growth. We also hold Drug Master Files (DMF) for our media products in many parts of the world, stay apprised of global regulations for raw materials and components in each country, and communicate directly with authorities during customer file reviews to answer questions regarding media or raw materials.

To meet the increasing customer production and reliable, regional supply needs, we are building a world-class cGMP manufacturing facility in Europe. The facility encompasses 245,428 square feet of space and increases production capacity by 320,000 kg/year of powder and 470,000 L/year of liquids. The upcoming European site will follow the same certified Quality System as our locations in the US and Japan and includes sustainable initiatives important to FUJIFILM Irvine Scientific and our customers, such as reducing manufacturing water usage, using a membrane bioreactor for wastewater purification, and windmill-powered electricity.

Q What are FUJIFILM Irvine Scientific's procedures for the qualification of raw materials for media?

MF: To guarantee ultimate safety, consistency, and effectiveness of the cell culture media, FUJIFILM Irvine Scientific has a robust and stringent supplier and raw material qualification and maintenance program. A risk-based approach is used during the qualification process to remove and minimize the potential risks associated with materials used. Once a supplier is qualified, the raw material documentation such as Certificate of Analysis, TSE/BSE statements (to confirm components are free from human- or animal-derived materials), and other relevant documentation are evaluated. We then audit the manufacturing site, production history, and the supplier quality system. Once we receive a new raw material, samples are evaluated and tested in three separate manufacturing lots and processed through our Quality System. The decision to qualify a material for use or not takes all of these actions into account.

Q What is your approach to ensuring continuity of supply?

MF: To secure our supply chain, FUJIFILM Irvine Scientific utilizes multiple risk mitigation strategies, continuous improvement, and custom management as fundamental principles of excellence. We strive to ensure continuity of supply through robust

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supply agreements with terms and conditions that protect us, as the customer, should a supplier divest or close their business. These contracts also provide us with the right to procure materials up to a certain point in time – typically nine months to a year – until we find an alternative source.

The upcoming facility in Tilburg, Netherlands, further solidifies continuity of supply for our European manufacturers. Providing customers with our products from a local source enables FUJIFILM Irvine Scientific to reduce time-to-market and helps to lower drug manufacturing costs. The new facility will use the same raw materials, equipment, and certified Quality System as our locations in the US and Japan, with the consistency of finished goods assured by our standardized raw materials program, equipment validation, adherence to SOPs, and environmental monitoring.

AFFILIATION

Marlin Frechette
Chief Quality & Compliance Officer (CQO), FUJIFILM Irvine Scientific

AUTHORSHIP & CONFLICT OF INTEREST

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FASTFACTS

mRNA manufacturing platform using CIMmultus® and CIMac® technology for high recovery of stable mRNA drug substance with rapid HPLC analytics

Rok Sekirnik and Tomas Kostelec, BIA Separations, a Sartorius company

Given the pressure of delivering during a pandemic, vaccine manufacturers have been focused primarily on safety and efficacy of vaccines. Existing purification methods, often adopted from laboratory-scale techniques, allowed rapid implementation and provided adequate product quality. However, future mRNA development will use optimized production and purification processes. Here, we describe a platform for highly productive mRNA purification.

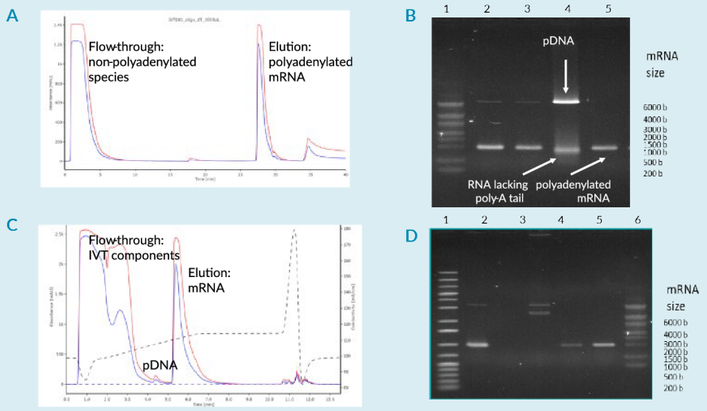
Cell & Gene Therapy Insights 2021; 7(11), 1409; DOI: 10.18609/cgti.2021.201

CHROMATOGRAPHY SOLUTIONS FOR mRNA PURIFICATION

To address the lack of standardization of *in vitro* transcription (IVT) processes and scalability of purification processes, new chromatography solutions were developed specifically for mRNA **(Figure 1)**. Case studies demonstrate the flexibility of monolithic chromatography solutions when they are implemented into next-generation mRNA processes:

- CIMmultus® Oligo dT monolith provides a fast, scalable stationary phase for polyadenylated mRNA capture from IVT with binding capacity of 3–4 mg/mL
- CIMmultus PrimaS® can be used for capture of mRNA without a poly-A tail based on mixed mode ion exchange/hydrogen bonding interaction with binding capacity of 5-6 mg/mL.

Figure 1. Purification of mRNA using orthogonal capture methods. A) capture of polyadenylated mRNA with CIMmultus Oligo dT with corresponding agarose gel (B). C) capture of mRNA using anion-exchange/hydrogen bonding interaction (CIMmultus PrimaS) with corresponding agarose gel (D).



ANALYTICAL SOLUTIONS FOR mRNA PROCESSING

The second key aspect of rapid mRNA process development is analytics.

Production of mRNA by IVT relies on a DNA template, often a linearized plasmid, encoding the gene of interest. Enzymes transcribe the template DNA into the target mRNA from raw materials supplied in the reaction. The reaction takes place at a high rate, typically running to completion in hours, in stark contrast to biologics produced in cell culture. The high productivity of the IVT production process is hampered by the relative low throughput of analytical assays used in process and product characterization.

We developed analytical high-performance liquid chromatography (HPLC) method providing high throughput, automation, and quantification capabilities with low sample consumption. Selecting the appropriate analytical column can also provide the resolution to detect desired contaminants. An example of CIMac PrimaS monitoring of IVT is shown in **Figure 2**, allowing quantification of mRNA, NTP, pDNA and capping reagent with run time of ~5 min.

Combining preparative and analytical concepts can generate new in-process control tools for monitoring product quality at-line that supplements traditionally used agarose gel electrophoresis (AGE). Oligo dT and PrimaS chemistries provide orthogonal purity information (e.g. poly-adenylation and presence of contaminating nucleotides, respectively) which is applied to monitor quality of downstream purification, for example purification of mRNA by CIMmultus Oligo dT **(Figure 3)**. By combining information provided by two orthogonal chemistries it can be concluded that RNA and nucleotides elute in flow-through, as detected by CIMac PrimaS, but the RNA is not polyadenylated (CIMac Oligo dT) and is therefore considered an impurity. The wash step removes additional RNA, which is also not polyadenylated. The elution step contains RNA detected by both CIMac Oligo dT and CIMac PrimaS, which therefore corresponds to polyadenylated mRNA – the desired product.

Figure 2. Monitoring IVT reaction with CIMac PrimaS. A) Chromatogram showing baseline separation of mRNA from NTPs, plasmid DNA (pDNA) and capping reagents (ARCA and CleanCap AG are shown). B) monitoring mRNA productivity as a function of IVT process conditions (A-F).

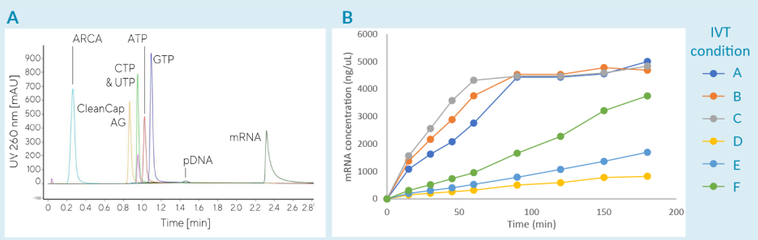
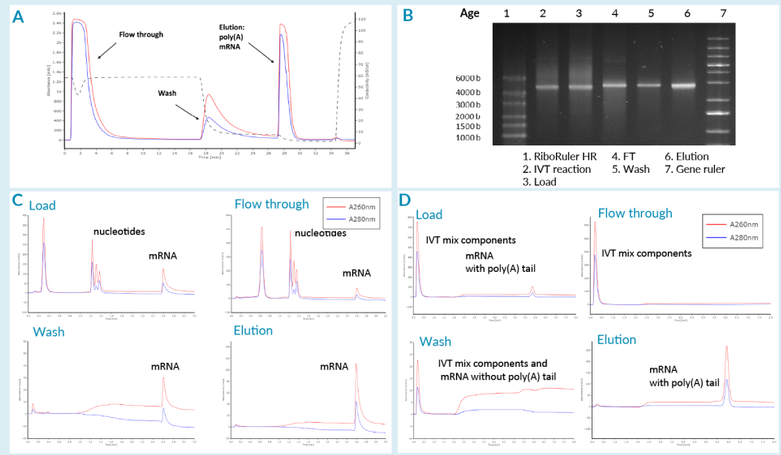


Figure 3. Purification of mRNA with CIMmultus Oligo dT and in-process control using B) AGE, C) CIMac PrimaS and D) CIMac Oligo dT.



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INNOVATOR INSIGHT

Optimizing downstream purification of high-quality plasmid DNA for gene therapy and vaccine production

Alejandro Becerra & Johannes F Buyel

The demand for plasmid DNA (pDNA) has increased in recent years, in part due to its utilization in both cell and gene therapies and mRNA therapeutics. Due to the physical properties of these molecules, plasmid production and purification pose some distinct challenges. A design of experiment (DoE) study was conducted in order to evaluate POROS AEX resins for pDNA capture, with the goals of optimizing process conditions to maximize purity and recovery, determine the dynamic binding capacity (DBC) of POROS AEX resins for pDNA, and confirm optimal operating parameters.

Cell & Gene Therapy Insights 2021; 7(11), 1217–1230

DOI: 10.18609/cgti.2021.162

CONSIDERATIONS & CHALLENGES FOR PLASMID DNA PURIFICATION

Plasmid DNA has multiple uses, ranging from basic cloning in research to therapeutic applications, and in recent years, the demand for pDNA has increased. This is partly due to the growth of the gene and cell therapy industry, as plasmid is one of the key raw materials required for commonly used viral vectors such as adeno-associated virus (AAV) and lentivirus. Plasmids are also one of the key components in the production of mRNA therapeutics, as they are used as a template during *in vitro* transcription.

In the context of plasmid production and purification, there are some important physical properties to consider. Firstly, plasmids are generally much larger than proteins in terms of mass and hydrodynamic radius, which is important for chromatography.

For gene therapy applications, typical sizes of these plasmids are in the range of 5–10 kilobase pairs. More recently, there has been a trend towards larger constructs, for example when two plasmids used for AAV transfection are combined into one, or in the context of mRNA when working on self-amplifying mRNA.

Another key characteristic of these molecules is that they are very highly charged, and maintain a high negative charge over a wide range of pH levels. They are also sensitive to degradation, both by nucleases and shear, which can modify their topology.

pDNA can be found in various forms, including supercoiled, open circular, and linear. Supercoiled plasmid is the most relevant form for therapeutic applications, and in that context, a high purity is generally desired from the purification process.

There are some inherent challenges to the purification of these molecules, including:

- Product and contaminants (gDNA, Endotoxin, RNA, plasmid isoforms) are similar in charge and size
- Shear sensitivity and high viscosity limit operational flow rates
- Plasmid generally represents <1% dry cell mass
- Conventional chromatography resins exhibit low binding capacities for pDNA

A typical downstream process for plasmids normally has multiple steps after fermentation, and anion exchange followed by hydrophobic interaction chromatography are commonly utilized. Thermo Fisher Scientific has developed a variety of resins well-suited for these steps, designed to simplify workflows and increase purity and yield.

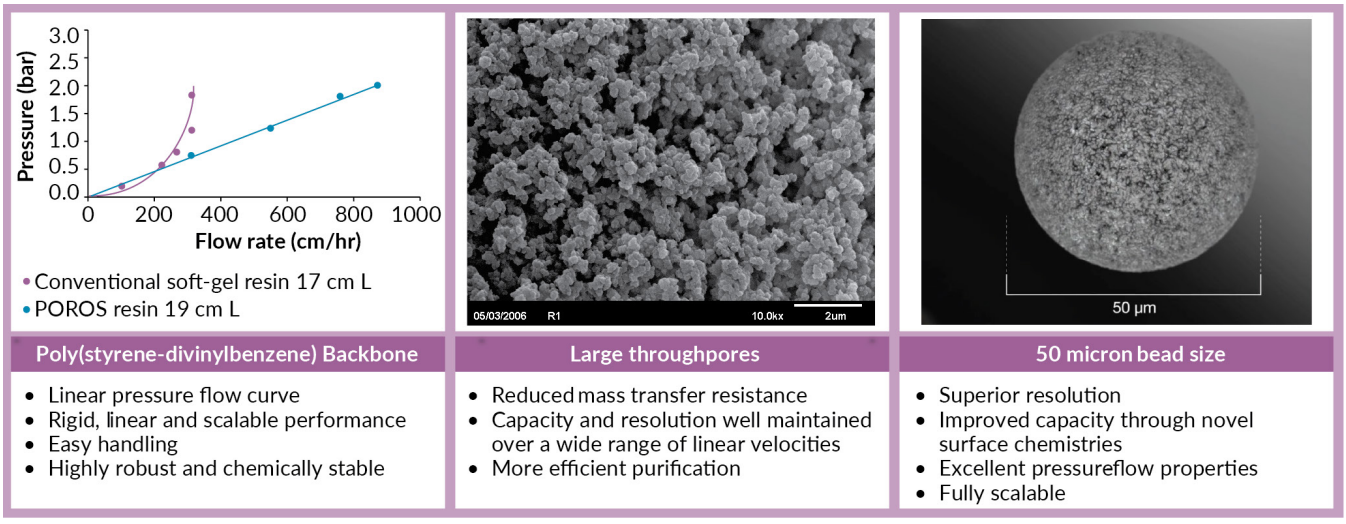
ADVANTAGES OF POROS ANION EXCHANGE RESINS

POROS™ Chromatography Resins from Thermo Fisher Scientific have a number of unique features (Figure 1), and different base beads are available for different resins, allowing for control of pore size, surface area and overall porosity.

Thermo Fisher Scientific offers four different POROS™ AEX resins (Figure 1), and each offers unique surface chemistries, and therefore unique selectivity, as compared to other commercially available AEX resins. This offers a potential solution to unique purification challenges, as a protein of interest or an impurity may bind to a POROS AEX resin differently than it does to other AEX resins.

FIGURE 1

Unique features of POROS resin technology.



DOI: 10.18609/cgti.2021.162

TABLE 1

POROS anion exchange resins.

POROS resin	Type of AEX resin	Surface chemistry	Pore size (angstrom)	BSA binding capacity (mg/mL)	AEX applications
D50	Weak	Dimethylaminopropyl	1100	>100	**Bind/elute:** Protein, virus, plasmid DNA purification **Flow through:** Trace impurity removal by binding impurities (DNA, viruses, HCP, aggregates, endotoxin)
PI	Weak	Polyethyleneimine (mixed amine)	2000	80	
HQ50	Strong	60% quaternized poly-ethyleneimine (mixed amine)	2000	75	
XQ	Strong	Fully quaternized amine	1100	>140	

A full range of weak and strong anion exchange resins with unique surface chemistries, that provide unique selectivity.

In this work, we focused on three out of the four resins in Table 1; POROS™ D50 has a dimethyaminopropyl functional group and is a weak AEX resin, and its chemistry is slightly different than traditional DEAE (Di-EthylAminoEthyl) resins. POROS™ PI (not tested in this study) is also a weak AEX resin with a polyethyleneimine functional group. The functional groups are primary, secondary, and tertiary amines, and are ionizable over a shorter pH range as compared to a strong ion exchanger.

POROS™ HQ is a legacy strong AEX resin. It is unique because it has both weak and strong AEX capabilities. There is a mixture of primary, secondary, tertiary, and quaternary amines on the bead, and about 60% of the tertiary amines are converted to quaternary amines, yielding a strong anion exchanger. This unique PEI-based chemistry and distribution of amines makes POROS HQ50 unlike any other commercially available AEX resin.

The pore size of these resins is also relatively larger compared to other products, which facilitates the diffusion of large molecules such as plasmids.

With this background in mind, the POROS resins were studied for plasmid capture applications in collaboration with the Fraunhofer Institute for Molecular Biology, Germany. The study had two objectives:

- Produce pDNA containing lysate using representative fermentation and primary recovery steps
- Evaluate POROS AEX resins for pDNA capture
  - Optimize process conditions to maximize purity and recovery using a DoE approach
  - Determine DBCof POROS AEX resins for pDNA
  - Confirm optimal operating parameters

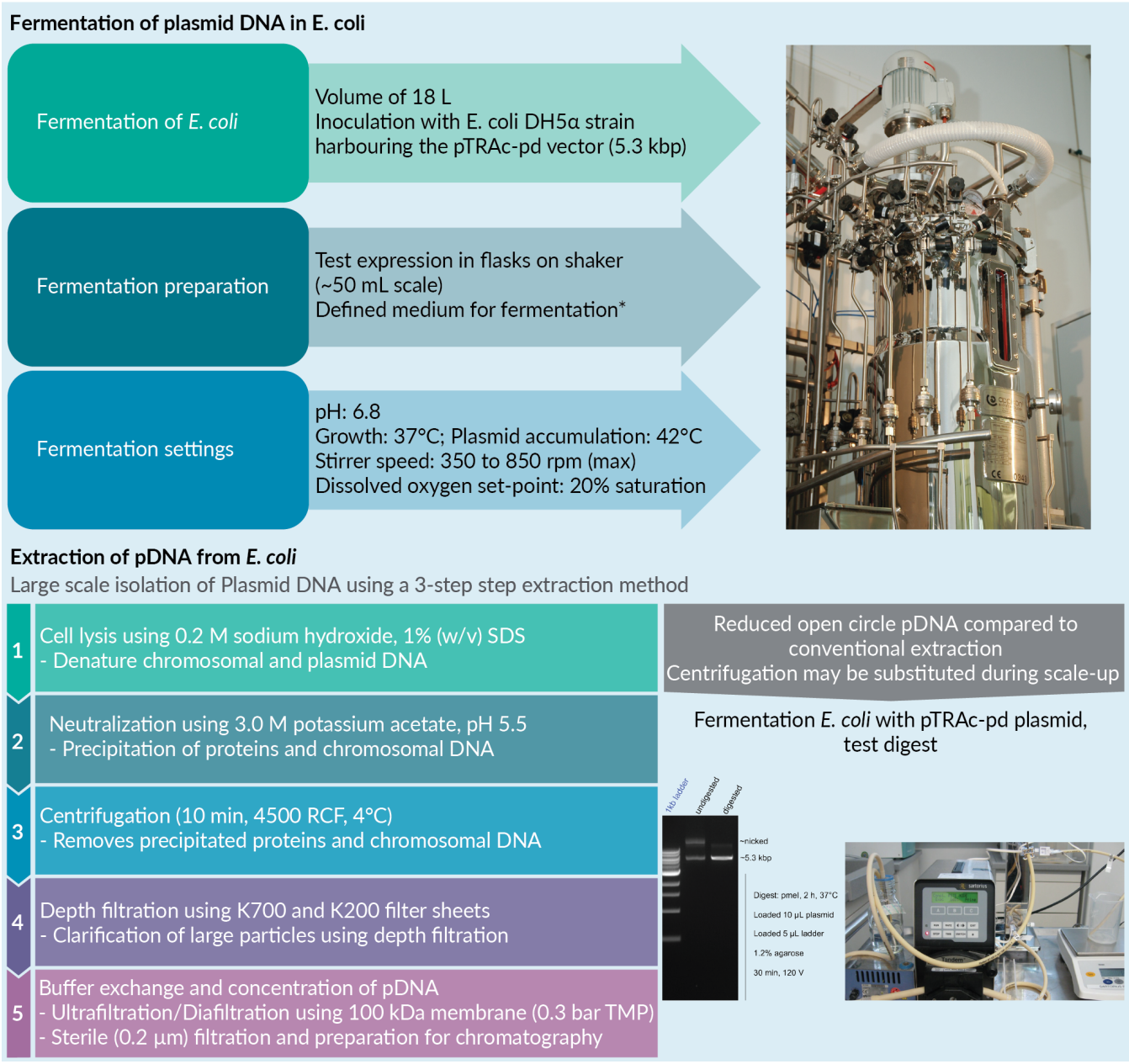
AEX DESIGN OF EXPERIMENT (DOE) OPTIMIZATION

The first step of the study was pre-processing. i.e., generating the materials to be tested for chromatographic separation. *E. coli* was selected as a representative system; the specific fermentation and extraction processes are shown in Figure 2. This preparation procedure provided a starting material with a higher closed circle/supercoiled DNA content than an extraction process that does not use ultrafiltration/diafiltration.

The ion exchange resins discussed above were then investigated, focusing on several parameters: loading buffer pH, loading conductivity, and quantity of plasmid loaded per mL of resin. The design quality was assessed before beginning the experiments, as seen on the right of Figure 3. The flat surface indicates that the model has a good and even predictive power throughout the entire design space.

FIGURE 2

Fermentation and extraction processes used to prepare pDNA for chromatographic separation.



*[1]. TMP: Transmembrane pressure.

pH & purity

Recovery at pH 7.0 was investigated first. Looking at all of the chromatography resins, the initial finding was that overall recovery was fairly high (Figure 4). Notably, for the POROS HQ50 resin, the different parameters had little effect; in this case the load conductivity and load concentration. In contrast, for POROS D50, we found that with an increasing load conductivity the relative recovery of products increased. For POROS™ XQ, the recovery decreased with an increasing load concentration, i.e., with a higher quantity of plasmid loaded per volume of resin.

Using a pH of 6, this initial behavior was amplified (Figure 5). In the case of POROS

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FIGURE 3

AEX Design of Experiments.

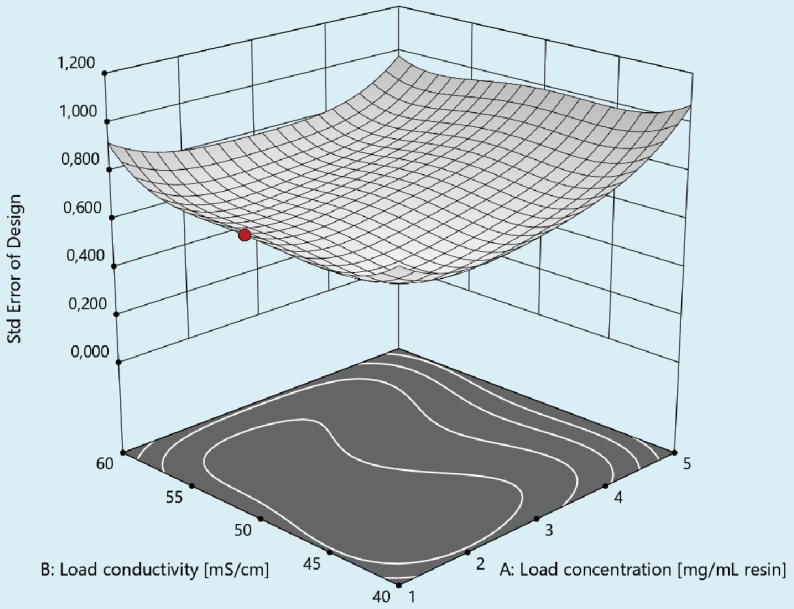
AEX Design of Experiments (DoE)

Design of Experiments Parameters

Parameter	Values tested
Loading buffer	pH 6, 6.5, 7
POROS™ resins	D50, HQ, XQ (RoboColumn® 200 µL)
Load density	1, 3, 5 mg/mL resin
Load conductivity	40, 50, 60 mS/com

RoboColumn® is s registered trademark of ATOLL GmbH

The flat error surface indicates a high-quality design



XQ, the reduced recovery with increasing load concentration was more pronounced. Similarly, for the POROS D50, the effect of load conductivity was more pronounced, and for POROS D50 we also see an effect of the load concentration. In contrast, the POROS HQ50 again showed relatively stable behavior throughout the design space. Interestingly, most pDNA was lost in the elution fractions.

The effect of pH was then compared in more detail for the D50 resin, which showed a dependence on load conductivity and concentration: as can be seen in Figure 6, with an increasing pH from right to left, the recovery increases overall and becomes more robust. In this case, a high pH was favorable to ensure a good recovery throughout the entire design space.

Purity for all three resins was in a good range – between 60 to 75% of total nucleic acid was supercoiled pDNA, and conditions were identified that gave close to 100% recovery for all resins.

Dynamic binding capacity

The DBC of the different resins is an important question to address, as this will ultimately dictate the process economics.

For the XQ resin, based on a UV trace, we calculated a DBC of 5.5 milligrams of pDNA per mL resin (Figure 7). This is in the high range of what is typically reported. Looking at the chromatogram to the top left of Figure 7, a double breakthrough curve can be seen – a steep increase at around 10 mL, and a second increase after 32 mL.

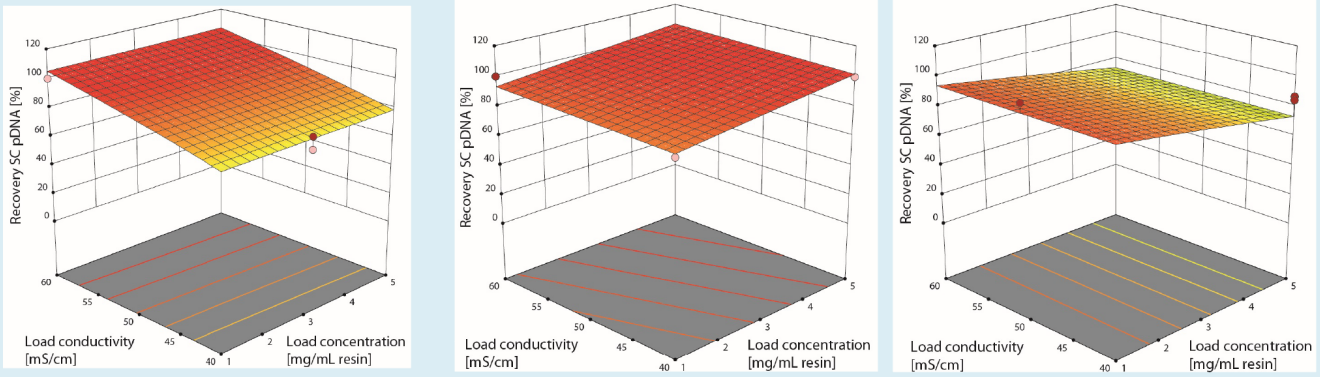
DNA concentration of individual samples was then checked, and it was observed that this second breakthrough is associated with a breakthrough of the relevant plasmid DNA. The initial phase can likely be disregarded as it is likely that other compounds such as proteins are breaking through the column at this point. Based on gel analysis, a substantially higher DBC of approximately 9 milligram per mL resin was achieved.

FIGURE 4

Resin recovery at pH 7.0.

AEX DoE: Recovery (pH 7.0)

- High pH significantly increases recovery for all tested resins
- Load conductivity between 50 and 60 mS/cm increases recoveries for POROS™ D50 resin
- Recovery decreases with increasing load concentration for POROS™ XQ resin



POROS D50 POROS HQ50 POROS XQ

A similar double breakthrough curve was seen for POROS HQ50. However, an inverse behavior was seen, where the DBC based on the UV trace is similar but when looking at the elution fraction and detecting the pDNA concentration, we found that the DBC is lower, at around 3 milligrams per mL.

With the last resin, POROS D50, we found that there is some breakthrough, and also some breakthrough regarding nucleic acid (Figure 8). Looking at the gel, we found that the breakthrough is up to a very late point – around 50 mL – and consisting of small nucleic acids, likely RNA or some fragments of genomic DNA. In this case, we

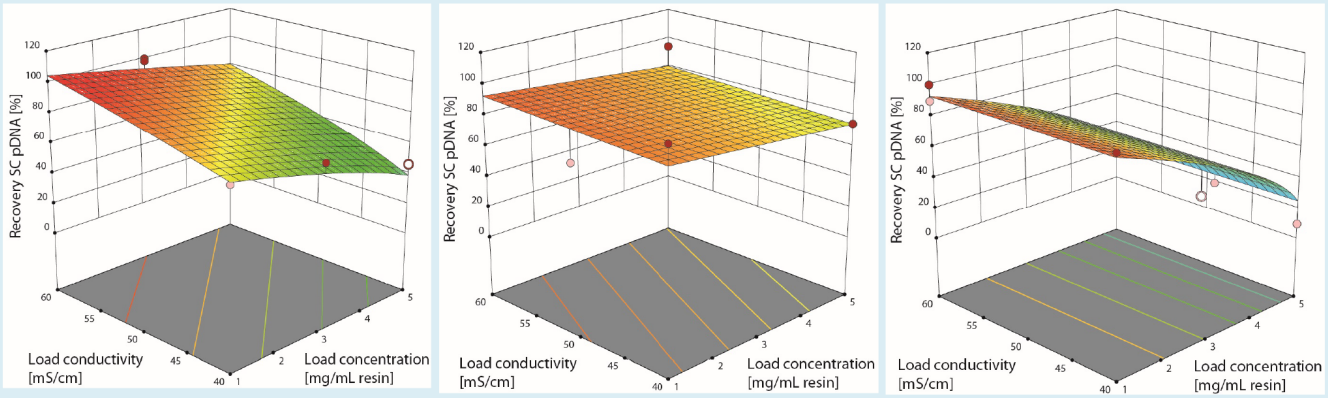
FIGURE 5

Resin recovery at pH 6.0.

AEX DoE: Recovery (pH 6.0)

- Low pH significantly reduces recovery for POROS D50 and XQ resin
- Low pH can reduce recovery for increasing load concentrations
- High pH (7.0) required for pDNA binding at high loading conductivities

Most pDNA was lost in early and late elution fractions with low purities below the load purity



POROS D50 POROS HQ50 POROS XQ

DOI: 10.18609/cgti.2021.162

FIGURE 6

pH effect on POROS D50 recovery.

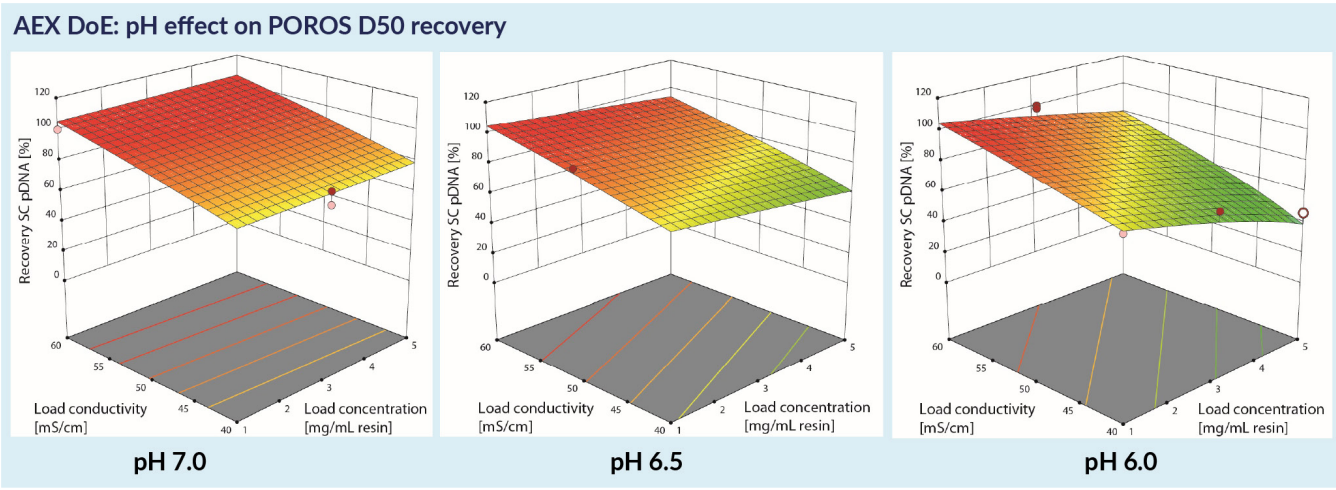
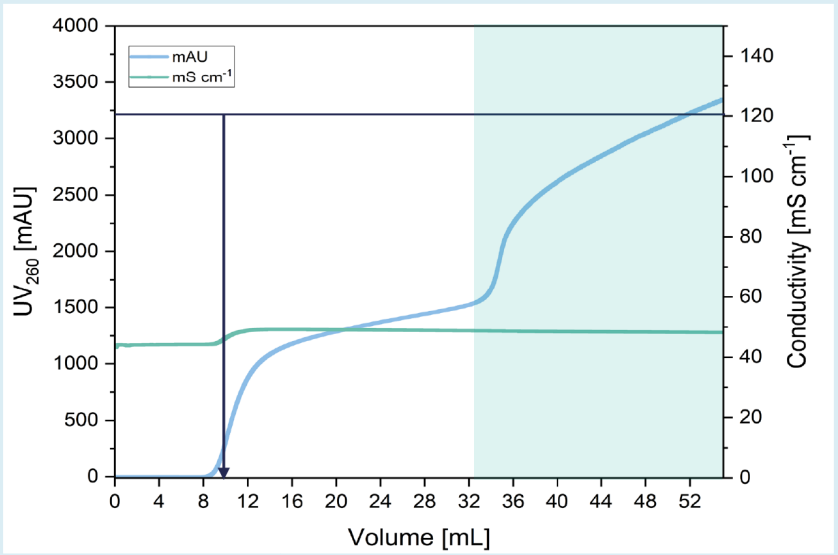


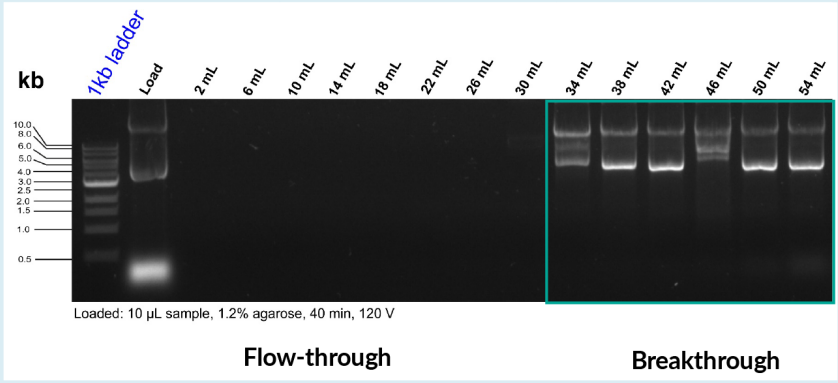
FIGURE 7

POROS XQ dynamic binding capacity.



Parameters for Chromatography	
System	ÄKTA pure
Column Volume	1 mL
Residence Time	2.5 min
Fractionation	2.0 mL
EQ & Load conductivity	~45 mS/cm
Salt Type	Sodium Chloride
pH	6.0

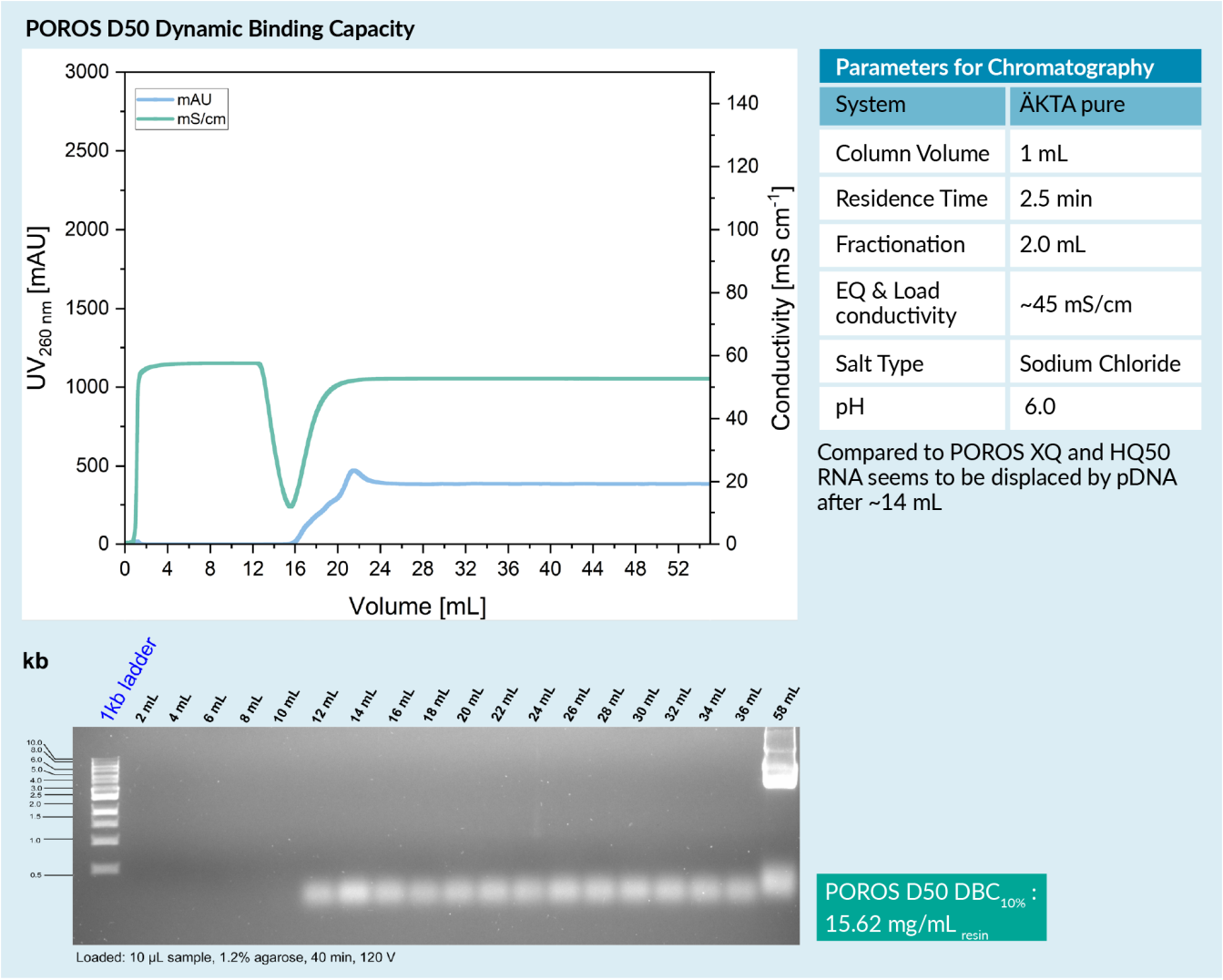
$DBC_{10\%}$ (UV 260): 5.5 mg/mL



$DBC_{10\%}$ (Agarose Gel): 9.0 mg/mL_{resin}

FIGURE 8

POROS D50 dynamic binding capacity.



assume that the supercoiled pDNA is replacing previously-bound RNA or smaller DNA molecules, resulting in an overall DBC of more than 15 milligram per mL.

While the XQ resin has a very high relative surface area, the D50 resin had the highest DBC. Speculatively, this may be because it is not the relative surface area of the bead, but in fact the accessible surface area to a given molecule, that is relevant when it comes to the DBC of the resins.

As the D50 resin provides the highest dynamic binding capacity, it was therefore the best suited to our next step, which was to verify these results using a scaled-up version of the experiment.

POROS D50 scaled up verification

The scaled-up experimental procedure remained essentially the same, but instead of the small-scale 0.2 milliliter columns used initially, a 5 mL column was used for verification.

We verified that the binding capacity was more than 10 milligrams per mL – this loading is less than what was observed previously, but still relatively high (Figure 9). In the gel at the bottom of Figure 9, it can be observed that in addition to the plasmid in the different salt elution steps – which can be seen here as staircase-like bands – there is a fraction of product that is eluting only

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once the cleaning procedure is applied (seen on the right side of the gel, in the lane labeled with CIP). Therefore, it is likely that optimizing the current elution conditions can increase the recovery.

FUTURE WORK

Planned future directions include:

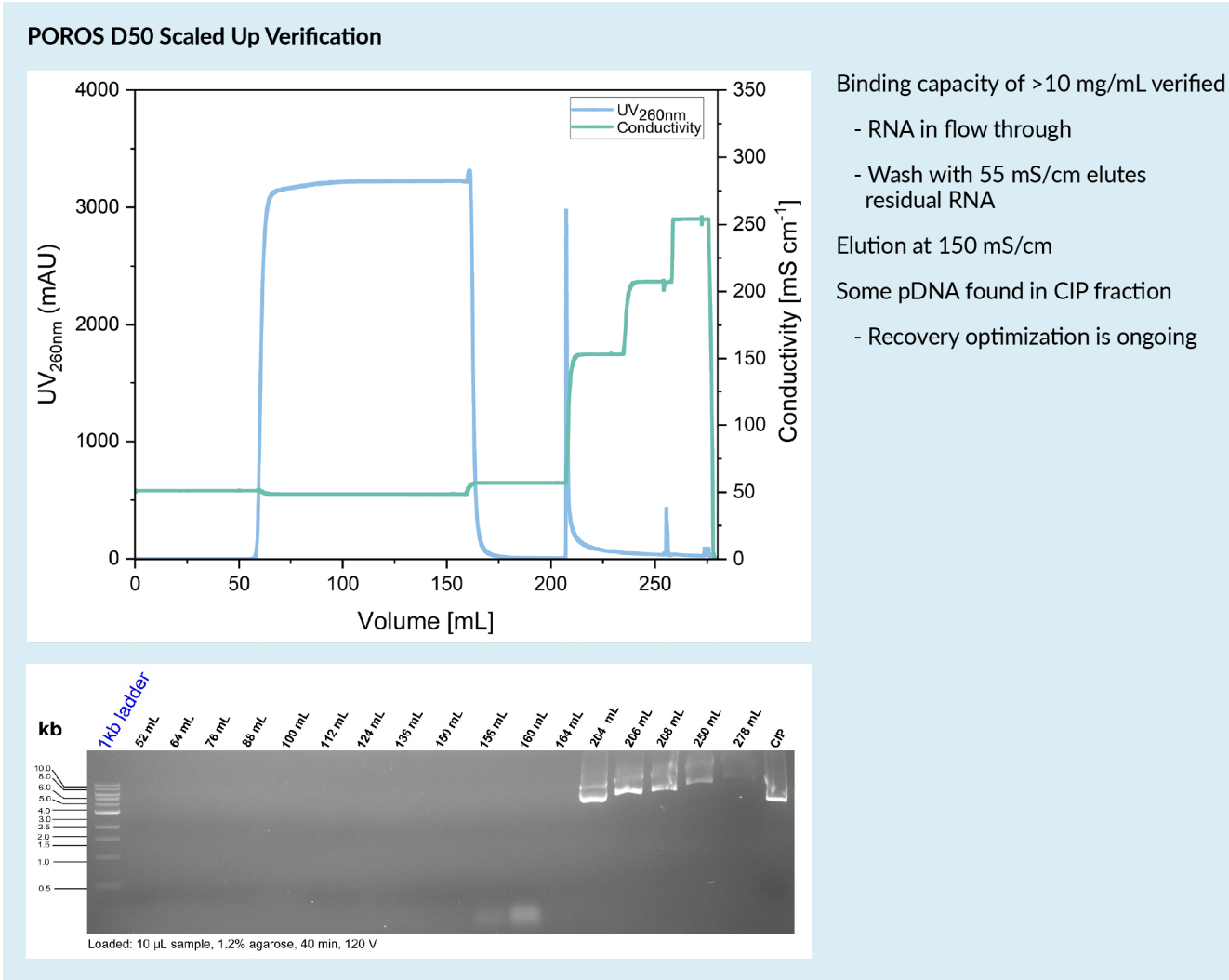
- Further optimization of the capture step with POROS D50
- Separation of pDNA isoforms with POROS hydrophobic interaction chromatography (HIC) resins
  - Preliminary work suggests Benzyl and Benzyl Ultra as potential candidates
- Potential assessment of other chromatography types for isoform separations (AEX)
- Evaluation of larger pDNA constructs

INSIGHT

High binding capacity was obtained for all three resins, with POROS D50 demonstrating the best binding capacity during this work. It is important to note that residence time was at the lower end

FIGURE 9

POROS D50 scaled up verification.



at 2.5 minutes, and increasing this may increase the binding capacity observed. Initial scale-up verification confirmed the high capacity, purity, and recovery for POROS D50, and work is ongoing to optimize the D50 capture step.

These DoE results provide a good guide towards optimal purity and recovery conditions for POROS D50, HQ, and XQ, and demonstrate how optimizing process conditions using a DoE approach can maximize purity and recovery of pDNA.

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ASK THE AUTHORS

Alejandro Becerra
Principal Applications Scientist and Global Purification Technical Lead, Thermo Fisher Scientific

Johannes F Buyel
Head of Bioprocess Engineering Department Fraunhofer IME

Q What is the benefit of using ultrafiltration/diafiltration for preconditioning?

JFB: Even though it is not part of the actual ion exchange step, we chose it for preconditioning because we think it has two benefits. On one hand, it allows us to concentrate the product, so that all the subsequent steps can be operated faster, using smaller equipment. On the other hand, it allows us to bring the plasmid DNA into conditions that are compatible with the ion exchange capture step.

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Q What are the major impurities that remain after anion exchange chromatography?

JFB: This is very relevant in terms of what comes next after this project. At the moment it is mostly nicked plasmid DNA that is not really the target of the production.

We also need to look in more detail at the endotoxin content, and maybe genomic DNA. We will use the samples that we obtained from the scaled-up verification run to analyze them, and build an impurity profile which will then be used to guide the second purification step.

Q Was RNase used in the process, and can this step remove RNA?

JFB: No RNase was used, and the data showed the removal of RNA in the flowthrough by the agarose gel.

Q Which second purification step would you suggest, and why? Which have you tested so far?

JFB: As mentioned earlier, it's most likely going to be HIC as the next purification step as it is an orthogonal method, and that is what typical process development would use as a different mode of interaction to purify.

Multimodal chromatography could also be used, and other ion exchange resins could be an option depending on how the other resins perform. So far, we have done some preliminary testing with HIC, but that is next on the list.

Q How would you design a new resin specifically designed for pDNA purification?

AB: As I mentioned earlier, we are able to control the different characteristics of the beads, as well as the functional group. Based on this work, we could potentially try to further understand how each of those parameters such as pore size, surface area, and ligand density, may influence binding capacity and selectivity. By manipulating those, I think we could further optimize a resin for these applications.

Q How does the binding capacity presented in this work compare to other resins or absorbents?

AB: In the literature there aren't many actual breakthrough curves – at least that I am aware of. Even in the information that is available, those binding capacities are generally in the area of 1–3 milligrams of plasmid per mL of resin. Even for more convective adsorbents,

some of the recommendations for operation are still below 5 mgs per mL. We were pleasantly surprised with the higher binding capacity of these resins, particularly with D50.

Q Would you expect the dynamic binding capacity to be similar with larger plasmids?

AB: We think it will likely be lower. It all depends on the accessible surface area, but generally speaking, the binding capacities tend to be lower with larger molecules.

We are aware that with larger molecules sometimes the recovery suffers more, and that may be related to some potential physical entrapment within that pore network, whether it is a resin or a different adsorbent.

BIOGRAPHIES

Dr Alejandro Becerra
Principal Applications Scientist and Global Purification Technical Lead, Thermo Fisher Scientific

Dr Alejandro Becerra is a Principal Applications Scientist and Global Purification Technical Lead. Alejandro has over 14 years of experience in downstream processing and customer support having worked as Purification Team Manager and other bioprocess engineering roles prior to joining Thermo Fisher Scientific in 2018. Dr Becerra is a subject matter expert in preparative chromatography with expertise in the development, optimization and scale-up of antibody, recombinant protein and viral vector purification processes. Alejandro holds a PhD in Chemical Engineering from Cornell University.

Associate Prof. Dr Johannes F Buyel
Head of Bioprocess Engineering Department Fraunhofer IME

Associate Prof. Dr Johannes Buyel is a Biotechnologist by training and received his Bachelor of Science from the RWTH Aachen University in Aachen (one of Germany's Excellence Universities) in 2006. He continued his master studies in Molecular Biotechnology with stays abroad in Sweden (Lund University, ERASMUS schoolarship) and the USA (Fraunhofer Center for Molecular Biotechnology, Newark, Delaware). Finishing his Masters with honours in 2009 he was awarded the Springoroum Coin. Johannes conducted his PhD at RWTH Aachen University during which he was a visiting scientist at the Rensselaer Polytechnic Institute (Troy, NY, USA) and Karlsruhe Institute of Technology (KIT) (Germany) for several months. He received his PhD with honours and was awarded the Borchers Medal. In 2014 he joined Fraunhofer IME as a group leader and was promoted head of department the year after. In parallel, Johannes started a second PhD in Bioprocess Engineering at the KIT, which he finished in 2017. Between 2018 and 2020 he conducted his habilitation and is an Associate Professor at the RWTH since 2020. Johannes is an active member of the German Biotechnology Association (DECHEMA), and member of the Editorial Boards of Frontiers in Bioengineering and Biotechnology, Frontiers in Plant Science as well as Transgenic Research, where he is Editor-in-Chief since 2021. Since 2021, Johannes is heading the Bioprocess Engineering Department at Fraunhofer IME (~20 employees plus ~10 students, gender balance 1:1). He has 15 years of experience in the development of

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tailor-made upstream production and downstream processing for 50+ recombinant proteins, small molecules and DNA in different expression systems such as *E. coli*, yeast, plant cell cultures and whole plants both under GMP and non-GMP conditions. He is also focussing on integrated processing, i.e. the use of residual biomass for cascading use. Johannes was involved in the two international projects PHARMAPLANT and FUTURE-PHARMA and received a €2.5 million Fraunhofer Attract grant for a project to establish a model-driven high-throughput expression and purification platform for recombinant proteins (FAST-PEP). Johannes is a principal investigator (PI) in the tumor-targeted drug delivery graduate school of the German Research Foundation (DFG) as well as the International Center for Networked Adaptive Production (ICNAP) for which he has so far received funding of €0.9 million and €1.8 million respectively. His work is strongly inter- and cross-disciplinary, combining research in, for example, biotechnology, bioprocess engineering, informatics, mathematics, data science, oncology, cosmetics and nutrition. He has currently more than 50 publications (h-index 19, i10-index 36, 1321 citations as of June 2021).

AUTHORSHIP & CONFLICT OF INTEREST

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

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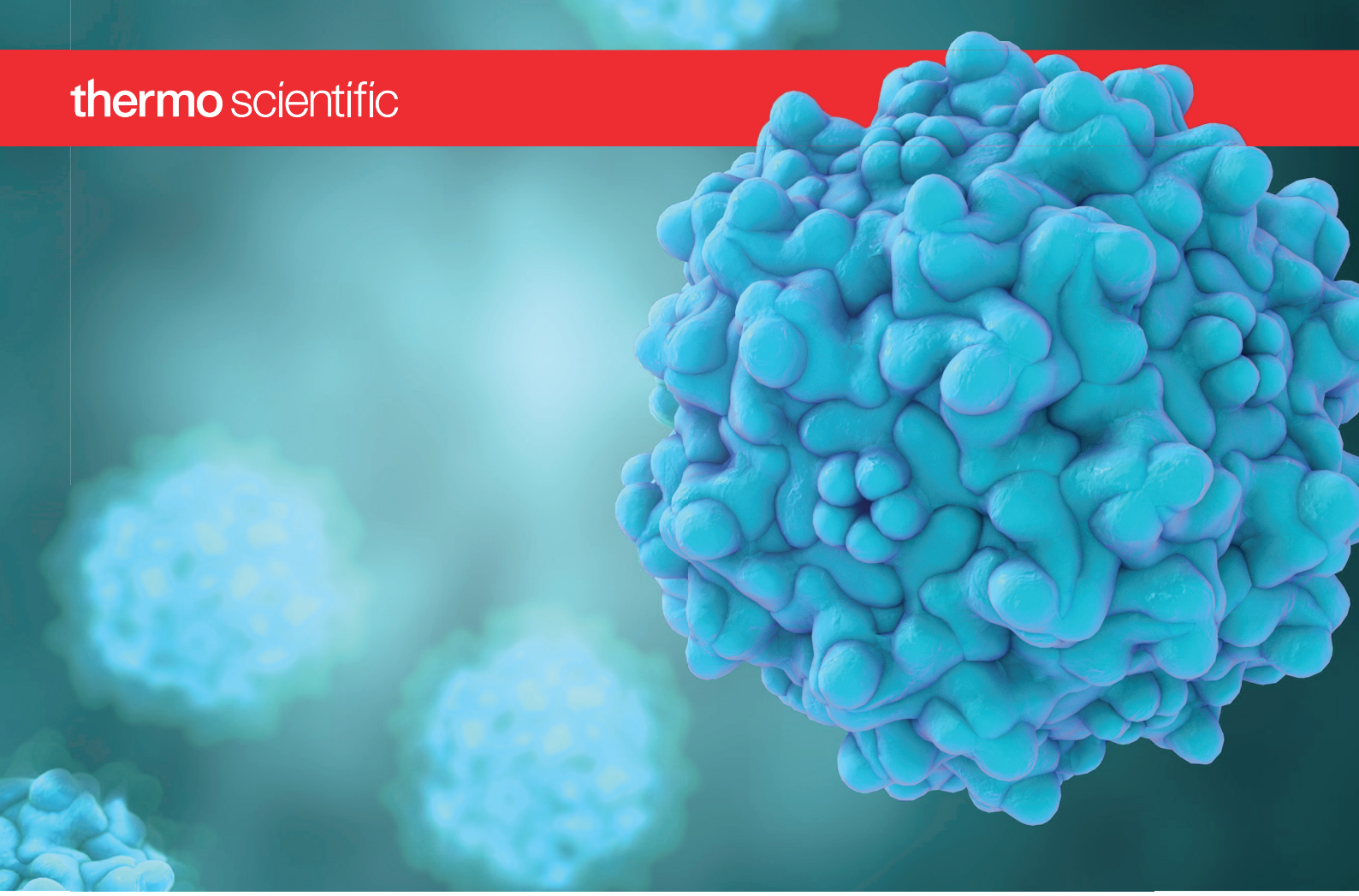

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CELL & GENE THERAPY INSIGHTS

EDITORIAL

Adherent versus suspension-based platforms: what is the near future of viral vector manufacturing?

"Therapeutics developers are searching for a viable viral vector manufacturing platform as the industry is at the inflection point. Paraphrasing Tolstoy, while successful manufacturing platforms are all alike, every unsuccessful manufacturing platform is deficient in its own way."

RAMIN BAGHIRZADE, Global Head for Cell & Gene Therapy, AGC Biologics

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The first sentence of Tolstoy's opus Anna Karenina starts by declaring that "All happy families are alike; each unhappy family is unhappy in its own way." Popularized as 'Anna Karenina principle', and applied in multiple scientific and social disciplines, the concept suggests that successful endeavors all share a common set of main traits, while there are many routes to misery if there is a deficiency in any of the key attributes. Paraphrasing Tolstoy, while successful manufacturing platforms are all alike (e.g. with regards to titers and yield), every unsuccessful manufacturing platform is deficient in its own way.

With over 1,200 clinical trials globally in cell and gene therapy, the field is reaching an inflection point with maturing late-stage pipelines and upcoming wave(s) of commercialization. Over two-thirds of all clinical trials in this area are currently in Phase 2/Phase 3 [1]. Using oft-cited numbers, FDA predicts that by 2025, it will be approving 10–20 cell and gene therapy products per year, with over 200 INDs filled annually [2].

As the field matures, so does the demand for viral vector manufacturing, in particular for lentivirus (LV) and adeno-associated virus (AAV) production, two dominant vectors used for *ex vivo* and *in vivo* gene therapies. While searching for a successful manufacturing platform, the ultimate objective of therapeutics developers is to make the manufacturing process commercially viable. Commercial viability is tied to both quality of manufacturing process acceptable for filing by the regulators and cost of virus per patient justifiable from business point of view as a percent of the overall COGS. The latter aspect is closely tied to process scalability.

When addressing the question of commercial viability, therapeutics developers face a crucial question: do you opt for adherent or suspension based viral vector manufacturing process? The article summarizes the pros and cons of each approach, and concludes that there is place for both.

The case for adherent based platforms:

Dominant approach in the industry, 'good enough' to commercialize at least some products while not letting perfection to be the enemy of progress

In biologics, two techniques of growing cells in culture can be distinguished: adherent and suspension. In adherent cell culture, cells are grown while attached to a substrate as monolayers. In suspension cell culture, cells are free floating in the culture medium. Currently, adherent cells are used in the manufacturing of about 70% of viral vector products [3]. The most common mode of manufacturing AAV and LVV vectors is by using adherent human embryonic HEK293 cells. Typically, human embryonic HEK293 cells – or HEK293-derived 293T cells, are transfected with a vector construct (containing GOI) and helper/packaging plasmids.

Traditionally used adherent culture system units include the likes of roller bottles, flasks, Corning's HYPERStacks® and Thermo Fischer's Nunc™ Cell Factory™ systems. Relying on 2D adherent plasticware platforms as a starting method of choice for upstream manufacturing, is easily understandable from at least three angles [4]:

1. They can be readily procured off the shelf
2. They are relatively easy to cultivate at lab scale
3. They require less expert bioengineering know-how compared to three-dimensional platforms

Moreover, basic adherent culture system units require low upfront CaPex investments and are hence practical starting points for (early) research purposes and beyond. Heavy CaPex investments is hardly a priority – or an option, for, say, an academic player or a fresh university spin-off. Considering that much of the innovation in cell and gene therapy comes from smaller sized biotech companies [5], not uncommonly, cell and gene therapy therapeutics developers inherit the process developed in and/or licensed from academia.

Probably one of the most documented examples of an adherent based process making it to market is that of Luxturna® (voretigene neparvovec), that uses AAV2 to carry a functional copy of the *RPE65* gene into the retinal pigment epithelial (RPE) cells. The product was developed by Spark Therapeutics (now part of Roche), and received an FDA approval in Dec 2017. Luxturna's AAV upstream manufacturing process relies on a roller bottle – basic 2-D cell culture system, using adherent HEK 293 cells process. Classic

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'scale-out' approach applies here – the only way to increase a manufacturing output is to add more roller bottles – rather than increase the volume of the vessel ('scale-up'), which could have been an option if this was a suspension-based process. Diane Blumenthal, at the time (2019) Spark's Head of Technical Operations, argued for the principle of "don't let perfection be the enemy of progress" [6]. What clearly made adherent platform viable enough is a relatively low dosage required (sub-retinal injection) and relatively low number of target patient population.

Another well-documented example of an adherent based process making it to market is that of Zolgensma® (onasemnogene abeparvovec-xioi), AAV9-based gene therapy used to treat spinal muscular atrophy (SMA). The product developed by AveXis (now part of Novartis), received an FDA approval in May 2019. Zolgensma's AAV upstream manufacturing process relies on an iCELLis® fixed bed bioreactor (FBR) adherent platform. iCELLis FBR platform has been cited as the 'most cost-effective option' for adherent cell culture [7], and has been used as a commercially viable solution without the need to switch to a suspension platform. There is extensive data available to demonstrate how one may scale a, say, 48L Cell Factories based process to a 200L iCELLis® FBR without changing critical quality attributes (CQAs) of the product [8].

Apart from commercial launches of Luxturna and Zolgensma, there is documented evidence of some developers making an explicit decision of intending to commercialize part of the pipeline on an adherent platform, and part in suspension, as well as gradual transition to suspension. To note, in its SEC filing back in 2013, Bluebird Bio, one of the gene therapy pioneers, explicitly pointed out that they intend to 'continue manufacturing' its Lenti-D vectors (SKYSONA™ – approved by EMA in July 2021) on an adherent platform, while adapting its Lenti-Globin (ZYNTEGLO™ – conditionally approved by the European Commission in June 2019) vectors in suspension [9]. Interestingly, in its SEC filing in 2020, BMS disclosed that it would assume the contract manufacturing agreements for ide-cel (ABECMA® – a CAR-T product approved by FDA in March 2021) on an adherent platform, while 'over time' the manufacturing will be performed in suspension [10].

The case for suspension based platforms:

Well-established in traditional biologics, still in early stage of maturity in cell and gene therapy industry, though viewed as "must have" for certain types of products/ indications

Adherent manufacturing mode typically implies that to increase the manufacturing output, one has to 'scale out', rather than 'scale up' – well established in traditional biologics and typically, though not exclusively, associated with stirred tank bioreactors (STRs).

Frequently cited limitations of basic 2D upstream manufacturing units include limited options for scale – which can make manufacturing prohibitively expensive, and batch-to-batch consistency, which may pose regulatory challenges. Adherent based manufacturing process also tends to be performed using fetal bovine serum (FBS) – that may pose safety, consistency, and ultimately, regulatory challenges [11]. On the other hand, switching to serum free, suspension platform is not always a viable solution and is far from being a failure free endeavor. While, for example, HEK293 cells have been adapted to grow in suspension [12], and there are alternative suspension-based cell lines, these are not without their challenges – with regards to timelines, costs, quantity and quality of viral vector. Moreover, as 'the product is the process', switching the process may mean that the product is no longer the same, and may require e.g. bridging/comparability studies. A dilemma frequently facing therapeutics developers is whether the existing (adherent) process is 'good enough' for commercialization and how much they are willing or able to wait and invest to try switching to suspension.

Perhaps one of the best-documented cases for the suspension-based process making it to market is that of Glybera, AAV1-based product, launched by UniQure, and widely dubbed as the 'first gene therapy' in the Western world [13]. The drug was approved by EMA in October 2012 to treat hereditary lipoprotein lipase deficiency (LPLD). While adherent HEK293 process was used for the pre-clinical studies and the first clinical trial in 2005, as higher quantity of vector was needed, HEK293 platform was changed to suspension based on baculovirus production system [14]. NIH scientists first demonstrated the suitability of this method by infecting *Spodoptera frugiperda* (Sf9) insect cells with three different baculoviruses – used both as a 'helper' virus and as the vehicle for AAV genetic material [15]. The baculovirus based manufacturing platform is not without its own challenges. For example, in the case of Glybera, while switching the platform helped with generating higher quantities of vector, the impurities profile was viewed as 'unacceptably high' in the assessment report by the EMA [16]. To note, the carryover of the baculovirus DNA was highlighted as a 'major concern', and the therapeutics developer was requested to perform a detailed risk assessment. A comparability study also had to be conducted comparing plasmid based adherent HEK293 process vs. suspension baculovirus based platform [14].

The challenges associated with switching to suspension still seemed to have paid off in the case of Glybera – despite the voluntary market withdrawal of the product in 2017. Depending on the indication/dosage/quantity of vector required, therapeutics developers may feel obliged to opt for suspension, as the only sustainable option. For example, while assessing viral vector needs for muscular myopathies, Salabarria *et al.* (2020), concluded that adherent platforms are 'simply not feasible' for AAV manufacturing for scales exceeding 1–5E+10^{15} vg, and hence would not be suitable for late phase/commercial applications in these indications [17]. For these particular cases, it is suggested that alternative, suspension-based methods are to be used, such as HEK293 adapted in suspension, infection based platforms (e.g. using baculovirus), or a stable producer cell line, with the upstream scale to 500L and beyond. In a similar vein, Pfizer announced ramping up its AAV upstream manufacturing to 2,000L to support its late phase AAV9 trial to treat Duchenne muscular dystrophy – DMD [18].

While, as highlighted earlier in the article, fixed bed bioreactors (iCELLis®) have been assessed as the 'most cost effective' solution on an adherent culture, the same study found suspension based STR manufacturing as the most cost-effective technology "…when a suspension-adapted cell line was available" [7]. The availability of a suspension-adapted cell line, and its characteristics, is a critical qualifier. For example, as has been argued elsewhere [19], producer cell lines in suspension are superior to transient transfection methods – when it comes to cost, reproducibility and scalability, though can be 'cumbersome' and time-consuming to develop, with no guarantee of success.

While there are documented examples of successful manufacturing in suspension, including successful adaptation to suspension from the adherent process [19], in my professional career in the industry, I have also come across multiple cases where a therapeutics developer tried moving to suspension, failed to do so, and focused the efforts on optimizing the adherent process instead. While not uncommon, failures to move to suspension is not something therapeutics developers readily and openly advertise.

CONCLUDING REMARKS: THERE IS NO SILVER BULLET

It is clear that there are challenges associated with either adherent or suspension method of manufacturing. What is also evident is that adherent mode of manufacturing can be a viable solution in certain cases, and not in others. At the same time, suspension-based

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manufacturing, while viewed as a 'must have' in certain cases, might not always be the way to go. There is no silver bullet, and the manufacturing strategy has to be evaluated on a case-to-case basis. As therapeutics developers bring cell and gene therapies to market, they tend to juggle among multiple and at times conflicting dilemmas, including:

- Is the manufacturing process 'good enough' for commercialization in the given disease indication?
- How much process development/ optimization is needed to make the process commercially viable?
- What is the right tradeoff between speed to market and time to develop a manufacturing process?
- At what point (if at any at all) is it wise to switch to suspension: before the market approval or after? (with all associated implications – e.g. comparability/bridging studies).
- If suspension-based process is sub-optimal, should one opt for adherent process instead or continue investing in developing a suspension process?
- What type of suspension-based process should one opt for? (e.g. HEK293 transient transfection, co-infection, stable producer cell line)

While juggling among these and other dilemmas, it is critical for therapeutics developers not to get sidetracked by a mammalian bias – as sometimes, less is more. Depending on the process productivity (total transducing unit (TU) or vg/batch), one might be able to treat more patients from, say, a 48L adherent platform, than from a 200L suspension-based STR.

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BIO

Ramin Baghirzade

Dr Ramin Baghirzade is the Global Head for Cell & Gene Therapy (C&GT) at AGC Biologics. With 15+ years of experience in life science and healthcare, Dr Baghirzade previously held roles of increasing responsibility at Roche and Lonza in global business development, strategic marketing and market intelligence functions. He holds a PhD Degree in Medical Sciences, as well as an MBA.

AGC Biologics provides world-class development and manufacture of mammalian and microbial-based therapeutic proteins, plasmid DNA (pDNA), viral vectors and genetically engineered cells. AGC Biologics' global network spans three continents, with cGMP-compliant facilities in Seattle, Washington; Boulder and Longmont, Colorado; Copenhagen, Denmark; Heidelberg, Germany; Milan, Italy; and Chiba, Japan

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CELL & GENE THERAPY INSIGHTS

INTERVIEW

Combating QC challenges and special needs of cell and gene therapy production

FÉLIX MONTERO JULIAN is the Scientific Director for the Healthcare Business of bioMérieux (Lyon, France). Felix has over 25 years of experience in industrial and clinical diagnostics, and previously served as the Chemunex Ivry-sur-Seine site & R&D Director in bioMérieux. Felix is a member of different scientific organizations, such as the Parenteral Drug Association (MD, USA), and served as an expert in a panel for the Development of Compendial Rapid Sterility Tests for the US Pharmacopeia (MD, USA). Félix has been, and continues to be, extensively involved in the implementation and acceptance of rapid and alternative microbiological methods. He is a prominent speaker at congresses and conferences and a regular contributor to bioMérieux scientific white papers. Félix has extensive technical experience that includes the development of in vitro diagnostic and research use reagents and applications, cell and tissue culture systems, alternative and rapid microbiological methods, sterility testing, Mycoplasma detection, compendia methods, and cell and gene therapy processes.

REY MALI joined Accellix as its Vice President Sales and Marketing on July 2019. She has served as a Product Development and Marketing leader for the prior 14 years in both hi-tech and biotech companies. Rey has a graduate degree in Medical Research and a MBA from The University of California, Berkeley and started her career in pharmaceutical sales at Merck Pharmaceuticals. She then joined a biotech startup that was acquired by Life Technologies (Thermo Fisher) as a Field Applications Scientist (FAS) increasing customer acquisition and retention and later was promoted to a Global Product Manager role at Life Technologies, where she launched and managed products in the molecular biology space. She then transitioned to a Sr. Product Marketing Manager at Illumina, where she launched and managed products in the sequencing space, and later served as the Product Marketing Director at Twist Bioscience leading the global marketing team towards commercial product launches and a successful IPO. Recently, she has been working at the Chan Zuckerberg Initiative (CZI) leading the Product Marketing efforts of free technological tools to advance medical research.

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"Time matters. We need to speed up immunotherapy development and manufacturing. We need to help companies speed up the quality control of these products and make them available to these suffering patients."

Félix Montero Julian sees the clock ticking. As the Scientific Director of Healthcare Business at bioMérieux, a global leader of *in vitro* diagnostics, he understands the urgency of cell and gene therapy delivery as a patient's last line of treatment.

The demand for regenerative medicine is soaring – whether for cancer, sickle cell disease, spinal cord injuries, or bone marrow transplants."It's limitless as far as what [these therapies] can treat," says Rey Mali, VP Sales and Marketing at Accellix, a manufacturer of a cartridge-based, automated flow cytometry platform. "This is the wave of the future for treatments."

With so much at stake, quality control testing of manufacturing processes is crucial. With safety testing and quality cellular attribute testing representing 80% of quality control (QC) needs [1], it's time to ask if QC strategies sufficiently safeguard the accuracy, consistency, and timeliness of cell therapy products.

Fortunately, significant advances are supporting development, manufacturing and control of these therapies for patients urgently awaiting treatment. Félix Montero Julian of bioMérieux and Rey Mali of Accellix teamed up in 2020 with the same end goal: to streamline QC testing – safety and cellular attribute analysis – with a more comprehensive workflow to accelerate production and control of cell and gene therapies. This partnership works with cell and gene therapy manufacturers to:

1. Improve manufacturing processes by providing on-site, automated, and rapid testing to deliver actionable results with reduced hands-on time;
2. Reduce the cost of QC to make these therapies more accessible; and
3. Ensure any cell or gene therapy that is administered to a patient is safe, effective and meets a pre-defined set of quality parameters.

We caught up with Félix and Rey to find out more.

Q How does slow turnaround time affect C&GT production and their patients?

RM: The cell and gene therapy manufacturing process takes anywhere between 2 to 4 weeks, on average. As new research and trials roll out, the time is gradually getting shorter. But the patient is still waiting three weeks to a month, from the point they give their blood to the point that they receive their therapy infused. During that manufacturing process, there are many, many different QC checkpoints that verify: Is the process proceeding as planned? Can we infuse the cells into the patient? Does the batch have the right population of cells? Did the cells get modified correctly?

FMJ: Most microbiological techniques still rely on compendial methods in which samples are placed in culture broth media. By nature, these kinds of cultures take time. Sterility testing requires 14 days, as regulated by pharmacopeia. We cannot afford to wait 14 days to have results on these products because it's wasting time for the patients. Thankfully,

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we have the technologies to detect micro presence in the samples in a few days, as opposed to 14 days. Some technologies deliver results in 5 to 7 days using growth-based methods.

"For cell and gene therapies, it is important to work with the minimum volume of product, and maintain a rapid time to results."

- Félix Montero Julian

Q What is the manufacturing impact of having low production volumes for C&GT?

FMJ: Microbiological testing, including microbial content (bacteria, yeast and molds), endotoxins and mycoplasma, are only one part of the quality control that is done for cell and gene therapy products. Different samples of the final product are needed to perform these three different tests. The other tests are related to the cells themselves – these are what we call quality cellular attributes. Our technologies can tolerate small sample volumes, which is critical because these products are not thousands and thousands of liters. For example, manufacturers of therapeutic monoclonal antibodies are able to produce hundreds of thousands of liters of product, so it is less critical to minimize volume taken out for testing. In contrast, cell and gene therapy final products are only several hundreds of milliliters. In that case, manufacturers need to use the minimum volume of product to do all the testing mentioned before and keep the majority of the product for patient infusion. For cell and gene therapies, it is important to work with the minimum volume of product, and maintain a rapid time to results. This is critical. Our technologies can reduce the sterility testing time by half.

Q How does manpower play into the C&GT QC process?

RM: Every company is kind of writing their own script as to how many QC checkpoints they need because they're all going after something different. Some of them have five, some of them six, some of them have eight during the manufacturing process. And each one of those QC checkpoints monitors environmental sterility or quality cellular attributes, which traditionally takes a lot of time and manpower. Turnaround time there is critical because you have a patient waiting on the other side that can die. With our technologies, lower-level operators can manage the testing easily and accurately, so you don't need to have highly-trained staff and manual processes that add variability.

Q What impact does accessibility have on C&GT patients?

RM: There are boundaries to manpower and a typical 8-hour working day. During clinical trials, you're recruiting patients who come in at all hours of the day, so your lab needs to be accessible 24 hours a day, seven days a week. I'll give you an example. We visited a

GMP facility last week where they're testing bone marrow. They have patients coming in the middle of the night. Do you really want to do manual analysis or manual processes with an operator working at 2 am and then rely on those results to treat a patient? What if they are tired? What if they are exhausted? What happens then? Without QC technologies that run 24/7, the quality of treatment is put at risk.

Q How are C&GT manufacturers maneuvering in this new and fast-moving field?

FMJ: There are many targets that scientists have identified as key in tackling different types of cancers. There is also the possibility of combining cell therapies with other approaches, making them more powerful for cancer treatment. All this, obviously, is making the FDA very cautious and scrutinizing a little bit more. We've heard of filings where the FDA has expressed concerns about the lack of consistent tests and measurements to ensure the quality of the therapy. We can bring a lot of value here because we provide consistent technologies that respond to these FDA requirements.

Q When does custom assay development come into play with customers?

RM: We have a few off-the-shelf assays, but really the long-term plan with most our customers is to develop a custom assay that is specific for the cell therapy that they are developing. Customers typically use our off-the-shelf kits to monitor the incoming quality of cells before processing or to address cellular impurities. After the cells are genetically modified to redirect their specificity, they will use a custom assay. So for each cell therapy, the manufacturer will implement multiple QC checkpoints, and prior to release, this is when the manufacturer monitors and QC's that the sample is not contaminated and that the genetic modification was successful. The custom assay during release testing is a pivotal point in platform adoption because that gets written into their SOPs which they eventually submit to the FDA and determine how the commercial product is manufactured.

Q What do you see for the future of C&GT?

> "The custom assay during release testing is a pivotal point in platform adoption."
>
> - Rey Mali

FMJ: It's so exciting because these kinds of therapies are currently a last line of treatment for the patient. When we look at patient responses to current therapies, it is about 10%. The response rates of some recent CG&T clinical trials were 60%. So going from 10% to 60% is a big win.

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The global outcome is so, so positive because it is only one injection. This can reduce all the pressure on the health economy. Instead of having patients go to the hospitals often and stay for a long period of time, in the case of C&GT, patients goes only one time for the cell infusion, after the product infusion patients stay for a continuous monitoring for a few days due to the fact that current CART therapies have side effects that needs to be identified earlier And yeah, the cost is high. But the goal is to decrease it with new technologies and with new manufacturing approaches.

RM: A cell therapy (for example an autologous CAR-T treatment for hematologic cancer) costs around half a million dollars per treatment. This market is where sequencing was about 20 years ago, where it was so expensive to sequence an entire human genome. Now it's under $1,000 per whole genome, and that's where we're hoping to see cell therapies go and we're part of that solution.

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FASTFACTS

Stable lentiviral vector producer cell line for a scalable manufacture

Roy Liu, Chief Operation Officer, Eureka Bio; email: roy@eurekabio.com

Manufacturing large quantities of lentiviral vector (LVV) for late-phase clinical trials and product commercialization via transient transfection remains a challenge. The use of stable cell lines to produce lentivirus is the best choice for production as it increases the overall quality of the LVV product and addresses issues such as safety and reproducibility, while also reducing production costs.

ADVANTAGES OF THE EULV SYSTEM

EurekaBio has generated inducible stable producer cell lines adapted to high cell-density suspension culture in a chemically defined medium, known as the EuLV system. As shown in **Table 1**, the EuLV lentiviral production system offers a range of advantages, and does not require plasmid transfection. All of the required packaging genes (for example, VSV-G, gag/pol, and rev) as well as the gene of interest (GOI) are stably inserted into the genome of the producer cell lines, and the production of LVV is achieved via chemical induction.

25L-SCALE LVV PRODUCTION PROCESS IN WAVE BIOREACTOR

Figure 1 shows an example LVV production process using EuLV-hPGK-Luciferase-IRES-EGFP producer cells (the insert is ~3.5kb). On day 1, cells are thawed and cultured in a flask. The producer cells are transferred to 1L culture medium on day 7. On day 10 cells are expanded to 5L, and on day 13 to 25L. On day 18, inducer and feed are added, and LVV is harvested after 48 hours. The entire process takes 20 days from cell thawing, and is simple and straightforward. Neither plasmids nor transfection reagents are required.

Figure 2 shows the cell density and viability data when the producer cells were amplified and induced for LVV production in the WAVE bioreactor (Cytiva). On day 10 and 13 when the cell density reached around 5E6/mL, the cells were transferred to the next size of bag, and on day 18, inducer was added for LVV production. Cell viability is controlled by over 80% upon harvest. For the virus titer, we detected 3.8E8 TU/mL in the culture medium, 9.5E12 TU before harvest, and 3.4E12 after purification.

Table 1. Advantages of the EuLV system versus plasmid transfection.

	Plasmid transfection	EuLV system
Production method	Plasmid + transfection reagent	Stable cell line + inducer
Culture method	Adherent or suspension culture	Suspension culture
CDM medium	No, may require serum	YES
Process stability	Variable	Stable
Virus homogeneity	Low	High
Virus specific activity	Around 1x10^5 TU/ng p24 (ELISA)	2x10^6 TU/ng p24 (ELISA)
Titer in culture medium	Low (1-5x10^7 TU/mL)	High (up to 8x10^8 TU/mL, GOI dependent)
Yield after purification	Variable	1x10^{11} TU per liter of culture
Cost of production	High	Signification reduced

SERVICES FROM GOI TO PRODUCER CELL AND BEYOND

Using EurekaBio's service for the EuLV system, it is possible to have monoclonal cells delivered just 4 months after receiving the GOI. EurekaBio also provides optional services such as GOI optimization, small scale production, clone characterization, and process development **(Figure 3)**.

Figure 1. 25L-scale LVV production process in WAVE Bioreactor.

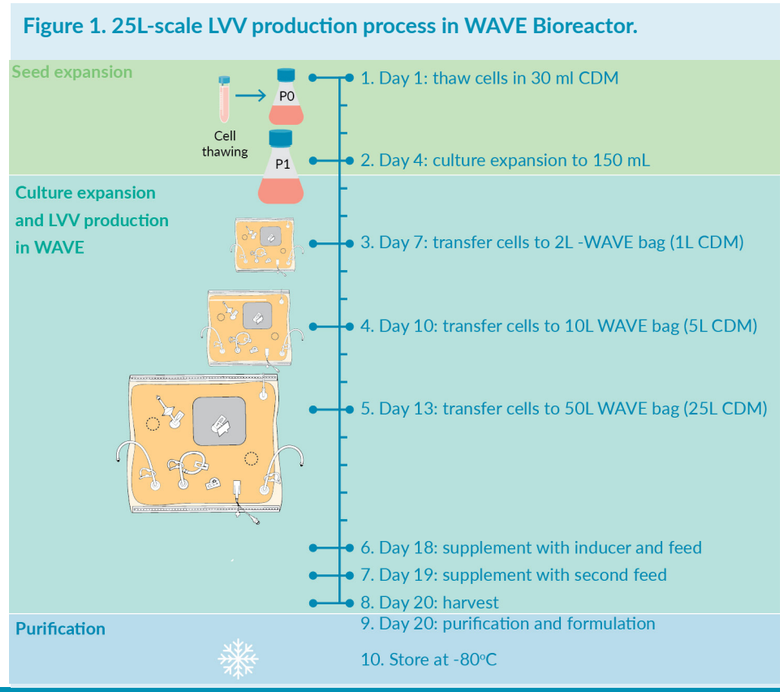


Figure 2. Culture expansion and LVV production in WAVE.

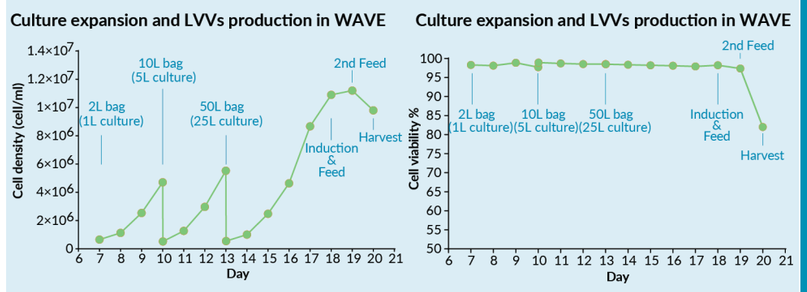
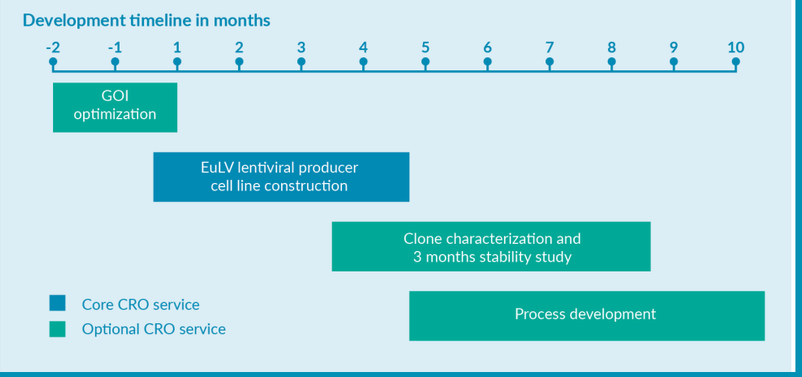


Figure 3. EuLV system development timeline.



CELL & GENE THERAPY INSIGHTS

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CELL & GENE THERAPY INSIGHTS

VECTOR CHARACTERIZATION & ANALYTICS

INNOVATOR INSIGHT

COVID-19 mRNA vaccine approvals: key lessons for cell & gene therapy and mRNA therapeutic development

Joseph Barberio, Christoph Kröner, Venkata Indurthi & Scott Zobbi

In this *Cell and Gene Therapy Insights* Expert Roundtable, our panel of four experts will answer two central questions for novel biotherapeutic developers: what can the cell and gene therapy field learn from the prophylactic vaccine approvals? And how will the vaccine's success help accelerate the progress of mRNA therapeutics?

Cell & Gene Therapy Insights 2021; 7(6), 761–771

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Q & A

Joseph Barberio
Director, mRNA Process Development, Strand Therapeutics

Christoph Kröner
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Venkata Indurthi
Vice President Research and Development, Aldevron

Scott Zobbi
Senior Manager Business Development for Custom POROS Resins, Bioproduction Division, Thermo Fisher Scientific

Q What would you pick out as the key development challenges facing mRNA therapeutics today?

SZ: From my perspective as a vendor, a lot of the challenges come back to the fact that developers like Joseph and Christoph don't have the purpose-built tools they need to get the job done. A lot of the tools that are being deployed in this space are legacy products from mAbs or protein therapeutic production; they work, but they may not be optimized. There's a large development challenge around that, and as the space becomes larger and more invested, you're going to see a lot more purpose-built solutions.

The other thing that I think will be a theme throughout today's discussion is the supply chain. mAbs have been around for 40+ years and have a well-worn supply chain, whereas mRNA therapy has only existed in this iteration in the last year so there are huge gaps within supply chain that are currently getting built out.

VI: I agree with Scott about the supply chain. As the field has exploded over the past year, the demand for raw materials has become very high and there are still only a few companies on the market to ensure supply chain for all processes. Plus, it's not completely clear or defined what quality is needed for which material. There is still a lot of space for development.

JB: With regard to analytics, we need some regulatory guidance to clarify what we're aiming for – the quality of the process as well as what the analytics can tell you and the current state of the analytics.

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There is a lack of experienced CMOs out there, and those that exist are under stress due to demand for mRNA in the biological landscape. The lack of an experienced talent pool for growing biotech companies is also a challenge, making it hard to fill out those positions.

VI: There are challenges in both upstream and downstream. We see certain developmental challenges upstream, right from the enzymes, because most of the enzymes currently are wild types, which have certain disadvantages. The more you can fix upstream, the less pressure you put on downstream.

SZ: Jo, Venkata, Christoph – do you think there is an assumption that we have all the technical challenges solved, when in fact there are a lot of unanswered questions?

VI: Definitely. Just taking the topic of quality level, we are told 'the best available quality level' but that can mean different things.

Even as far as technology goes, there are multiple approaches to get to your end product; there is not just one way to do things. There is no question the field is in its ascendency, but because of the accelerated timeline, there's a lot of information to process and learn in a very short period.

SZ: There has been a lot of pressure in this past year and has accelerated the platform. The level of development, the level of focus, and the amount of funding that has gone into this market are like nothing I have ever seen before.

Everyone's backs were against the wall on this, but I got my mRNA vaccine last week and I wouldn't have put that in my arm if I had any doubts about it. I feel it was produced with the highest level of quality and efficacy available, and I'm glad that technology existed at the right time for this too.

JB: It was interesting how mRNA therapeutics paced the field, whereby the sequence was made known to all different companies at the same time, and all different modalities, and two came out clearly on top.

Q In addition to the influx of funding to the mRNA space, how else has the picture changed since the successful development of COVID-19 vaccines, and how might that alleviate or add to some of the bottlenecks?

VI: The COVID-19 vaccine has accelerated the platform by about 10 years and changed the picture for RNA completely. Now people understand the potential of RNA, more and more people in the space want to promote more and more tools, but that would add to some of the challenges that we've just discussed, such as a shortage of raw materials. Alternatively, I see mRNA being one of the most revolutionary technologies in vaccine and therapeutic spaces.

CK: Now we have approved products, we have a clearer – albeit still developing – picture of the quality level we need to reach for the product.

SZ: I'm looking forward to people paying more attention to mRNA as a therapeutic. You're going to see ideas percolate to the surface – things we've never even thought of before.

JB: One of the biggest gains from the vaccine approvals is establishing trust in this modality from the public and investors. With the efficacy of these two vaccines, there will certainly be more investment in the space – both in the tools and in the biotechs themselves.

Q You all touched on the challenges around the downstream processing side. What are the specific bottlenecks around mRNA downstream processing, and could you point to any recent innovations in this area that you feel are helping to improve or ensure product quality and safety?

CK: We have large molecules with a lot of negative charges so purifying RNA from non-functional RNA or DNA is a real challenge. We have made some large improvements during the purification itself to get the pure product and to achieve upscaling.

BioNTech and Moderna both have large programs focusing on individualized cancer therapeutics, and in both, we had manufactured a lot of mRNA batches for GMP (around 1000 here at BioNTech). That gave us a lot of experience of how to manufacture mRNA in multiple batches quickly and achieve key conditions, which helped a lot.

VI: Downstream purification is a challenge. We've been using tools that were not designed for nucleic acids, leading to lower binding capacity and having to do multiple lots to get to the level of purity needed.

SZ: It's important to take a holistic view of the process. We often see customers focusing on how to solve a downstream problem, but it turns out to be an upstream problem. For example, the titers are very low, the product quality isn't there, or you're trying to remove a reagent or contaminant that wouldn't necessarily be present if you optimized your upstream.

Picking up on Christoph's point, scalability is also a major issue. One of the worries that I have is that a lot of customers will be moving into the space with a very academic or R&D mindset, and they're going to choose solutions that are not scalable and are unsuitable for GMP manufacturing.

JB: I would just reiterate that the binding capacity of resins and the throughput and mass challenges to TFF membranes are low compared to what you see with other modalities.

SZ: As Venkata mentioned, there is no one right way to do this; it's going to be different for different constructs, and different manufacturing scales.

VI: That is a good point, and I'll add a CDMO perspective to that. Often, CDMOs don't control the design of the RNA, and a lot of purification methods are dependent on the

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secondary structure sometimes. That is challenging for us because we see multiple designs with multiple final specification requirements.

Q What more could be done upstream to further alleviate these downstream issues you've mentioned?

JB: The control of process inputs is very important. You must have a deep process understanding and characterization, as well as robust associated analytics to understand how both upstream and downstream iterative process development is affecting the product. You need to ensure high-quality raw materials and starting materials and understand the impacts of those impurities on the profile of the drug product or drug substance.

In my view, that is the most important thing on the upstream side – understanding the inputs you're putting in and how they impact things on the back end.

SZ: Absolutely. Having well-characterized reagents, and the right quality level of reagents (whether GMP or ISO) is of huge importance. Everything has happened so quickly that manufacturers are taking the highest-level quality they can get, but we're now looking to the regulatory agencies to give guidance on what's required in that space.

CK: The most important raw material that goes into the mRNA is the DNA, so as well as the level of quality needed, we need to know the level of sequence correctness that ultimately defines the product.

Q Something we've touched on in this discussion is retrofitting technology and platforms from the mAb space to meet urgent needs in mRNA manufacturing. What enabling technology innovation is needed to help address these bottlenecks we've discussed?

SZ: I'm sure there are a lot of enabling technologies out there just waiting to be discovered. For one thing, I'm convinced we're going to start to see more and better-modified enzymes. I believe that in the mRNA space, we are going to find or modify enzymes to improve yields, transcriptions, and capping that has yet to be discovered or understood.

I also think there is going to be a lot more work focused on polish chromatography. There are different modes of chromatography you can use to purify mRNA, and looking at what the key contaminants are and how to polish those away, whether it be unreacted NCPs or residual enzymes, or double-stranded RNA, will be an important area in the future.

JB: I would add that, to understand what needs to be removed, we need analytics. As a process development person, I would say analytics are almost more important than the process development work itself because if you don't know how to quantify what's happening and understand the effect on the product, that work is useless. The ability to find good functional potency assays or predictive assays, to have predictive models, to minimize

the animal studies are all important. The field does need potency assays to determine efficacy as there is a great deal of difference between *in vitro* and *in vivo* processes when it comes to mRNA.

So it's a priority to work on some high-quality analytics, and have novel approaches to performing functional or potency assays, to minimize the amount of work that needs to be done in the animal studies.

CK: There's a lot of analytical knowledge we can take from diagnostics – but we need to find a way forward to introduce these complex technologies to the pharma world.

Q How could machine learning contribute to the development or production of mRNA therapies?

JB: I would say it is certainly applicable and is currently being implemented at some of the newer startup biotechs. I would expect that it's probably being used in some of the larger mRNA companies as well.

VI: it is a very powerful tool that can be applied in several ways, whether to improve your raw materials, or to understand RNA structure, design, and so on.

CK: I agree machine learning is an important future direction, but the molecule and the reaction itself is so complex and depends on so many parameters that currently there is no straightforward way for us to put the data into the machine and find the perfect mRNA or the perfect process to manufacture it.

JB: You need to understand the entire process. And the entire folding structure of the molecule and how each impurity can affect that, as well as the kinetics of the reaction, to understand exactly what your product needs to be.

There needs to be a better understanding of the important characteristics from sequence all the way to structure, around mRNA as a therapeutic modality, before machine learning can truly be trusted to move forward a platform, as opposed to empirical data and design of experiments.

VI: Initially I think we need to look at applying machine learning in modules, for one particular component in the entire process, rather than holistically.

Q Raw materials came up earlier in our discussion. What specific issues have you encountered and how have you sought to address them?

VI: Extremely long lead time for raw materials is one of the biggest issues in the field at present. There are raw material shortages across the board, and we are starting to see huge enzyme shortages. I do not have a clear answer yet on how we can address that; we are working through it right now.

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SZ: Again, it comes down to the speed the at which field is moving. A year ago, there were no approved mRNA therapies; this year there are two approved mRNA therapies with commitment for billions of doses. The industry is having to build supply chains from scratch for a majority of the reagents, lipids, and raw materials needed. There is a huge investment going on right now to build out that supply chain, but it still takes time.

I find it frustrating when you hear people saying "if BioNTech or Moderna just shared their sequence and their information we could be producing million-dose batches tomorrow." My answer would be, with what? Even if you knew how to make it, there are no reagents, no enzymes, no NTPs available. That's why I think the focus needs to be on the key vendors who already have the infrastructure in place, like BioNTech, Pfizer, Moderna, CureVac.

JB: This would be a supply chain issue for any modality. It's hard to think of a time when the patient population has been, essentially, the entire world. It's not just enzyme shortages, supplies of every kind are stretched, from pipette tips, to bags, to conical tubes. There are queues in CMOs for production, queues in outsourced analytical development organizations.

Q What do you feel are the key lessons that mRNA vaccine and therapeutics makers could learn from each other?

JB: I think we've learned that mRNA-based drugs can be quickly scaled up to make very consistent products. And mRNA is now a proven, safe, and efficacious modality for drug delivery. There are massive datasets that coming out of the vaccine programs, involving hundreds of thousands of doses in all sorts of patients, which will be invaluable to those developing mRNA therapeutics. Once tissue-specific delivery is solved, the sky is the limit for the mRNA space.

SZ: Joe mentioned tissue-specific targeting, and a lot of the work that needs to happen next is not just with the mRNA itself but on the delivery mechanism. Is a liquid nanoparticle really the right way to go? Is it good for certain things but not for others? There are so many novel packaging mechanisms that are being looked at now or have the potential to move forward. There's a lot of excitement in that space.

Q We've had lots of questions from the audience on analytics. What do you see as the biggest challenge in mRNA analytics?

CK: That is a question we are asked more and more often. And it's topical because it is one of the main challenges that we face – mRNA is a large molecule with a complex secondary structure. Having the mRNA as a full-length homogeneous configuration is the aim, but that's not what we get after *in vitro* transcription.

For example, *in vitro* transcription can produce shorter, double-stranded mRNAs. Acquiring knowledge about this completely heterogeneous population of mRNA is very important. In the future, I believe we need to go down to single-molecule analysis of the mRNA.

Q The panel has mentioned that mRNA characterization, particularly folding and forms, is a crucial aspect for downstream processing that needs to be better understood. Could you elaborate on this aspect?

JB: When it comes to the purification and impurity profile, everything matters. Plasmid quality is important, that's your template for the starting material, and different IVT conditions can potentially create different types of impurities, so understanding how those impurities are affecting your downstream purification, or the integrity of the intended full-length product, is important. There are certainly levers that can be pulled that make a higher quality product than others, and you must understand what those are.

The biggest difference between the bench scale and the high-quality commercial manufacturing is the analytics. You don't know you have impurities in the material unless you check for it with high-quality analytics. Bench-scale, silico-purified material looks the same as high-quality multiple chromatographic purified material if you look at it with rudimentary analytics.

Q Do you feel that the BioNTech and Moderna mRNA manufacturing processes and in-process analytics will become the regulatory standard, or will further regulatory scrutiny be in place once the pandemic pressure is removed?

VI: It will be a standard for now, but once the pandemic is over there will be more and more scrutiny. What regulatory agencies are looking for will evolve as the technology evolves, whether from a process impurity standpoint or product impurity standpoint.

JB: I would reply that BioNTech and Moderna have fairly mature processes. They have been working on these technologies for quite some time. I don't think there were shortcuts in the release testing and analytics and qualification of the analytics. So I think there might be a new benchmark in analytics that has been established, but I don't think it will necessarily change the amount of scrutiny on release-testing protocols, although the speed at which everything is reviewed may decrease post pandemic. But I would hesitate to suggest that the release panel wasn't of the highest quality for the approved vaccines.

CK: I think it's a good benchmark, but there are opportunities to improve that. And we will have that opportunity because the situation in the future will be different. I hope we will never again face such high demand in such a short timeframe.

Q What does the future hold for mRNA, and oligonucleotides in general, and how and where will they be deployed next?

CK: We're still at the beginning with mRNA, and there are so many different approaches to use that technology and so many different opportunities.

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JB: In my opinion, it's going to be deployed in almost every setting, unless you need gene addition. We've already seen vaccines for infectious disease, and cancer vaccines will follow. There are companies out there that are using replicating mRNA, and cell-type specific expression using logic circuits. There are the CRISPR tools for base editing and prime editing. We're just scratching the surface with the vaccines. As the supply chains grow and money comes into the space, mRNA will become one of the core modalities for fighting all diseases.

VI: We are already seeing that in the CDMO space, with several different applications, such as protein replacement therapy, coming through. The technology is already accelerating quickly.

SZ: This is an incredibly exciting time and I'm looking forward to seeing the new and novel ways that mRNA is used in the market to cure disease and treat patients. I think everyone here and listening would agree that's why we are all in this business – because we want to help society.

BIOGRAPHIES

Joseph Barberio
Director, mRNA Process Development, Strand Therapeutics

Joseph Barberio is a biochemist and molecular biologist with a proven track record of solving complex problems with innovative solutions. He is the Director of mRNA Process Development at Strand Therapeutics and oversees Strand's manufacturing strategy to support clinical development. With over fifteen years of industry experience, Joe specializes in process and analytical development and has extensive expertise in manufacturing of both viral and RNA based gene therapy medicinal products. The majority of Joseph's career has been focused on building platforms for small biotech organizations. Most recently at bluebird bio, he constructed and led the mRNA process development team, a group designed to enable gene editing programs. Earlier in his career, Joe held process development roles at Moderna, Percivia, and Acceleron Pharma. In addition to his work at Strand, Joseph also serves on the Board of Directors of Sophie's Hope Foundation, a non-profit charity supporting research for glycogen storage disease type 1b (GSD1b).

Christoph Kröner
Director DNA Process Development & Cap Technology, BioNTech SE

Christoph Kröner works as Director DNA Process Development & Cap Technology at BioNTech RNA Pharmaceuticals GmbH with strong focus on developmental work for BioNTech's various clinical projects using mRNA as drug substance. He has more than 10 years of experience working with nucleic acids like mRNAs. His work focused on mRNA-based therapeutics when he joined BioNTech in 2014 as a scientist. Christoph Kröner holds a diploma and PhD in chemistry from the University of Stuttgart, Germany.

Venkata Indurthi
Vice President Research and Development, Aldevron

Venkata SK Indurthi, PhD, is the Vice President of research and development at Aldevron (est. 1998), a biologicals CDMO with sites in Fargo ND and Madison WI. He received his

bachelor's in engineering in biotechnology from SRM university (Chennai, India) and his PhD in Pharmaceutical Sciences from North Dakota State University (Fargo, ND). After completing graduate school, he joined Aldevron as an assay development scientist and was focused developing assay for GMP release for biologics (DNA, RNA and protein). He then transitioned to a role of senior scientist, Product and Process Design (PPD) where he led the development of Aldervon's mRNA process and platform and more recently as the Director of R&D where he established all the R&D efforts for Aldevron. Dr. Indurthi leads efforts that to develop new platforms and innovative manufacturing processes for the company including (not limiting to) the mRNA platform, plasmid DNA, gene synthesis and cell free synthetic DNA platform. His team also focuses on the development of new products for the company such as enzymes with improved attributes (increased yield, stability or activity). Dr. Indurthi's research interests focus on the development of biologics. Particularly, new platform development from idea conception to commercialization. He has developed processes that are currently used for GMP manufacturing. Dr. Indurthi is also the operational head for the Aldevron RNA services.

Scott Zobbi

Senior Manager Business Development for Custom POROS Resins, Bioproduction Division, Thermo Fisher Scientific

Scott Zobbi is a Senior Manager of Business Development for Custom POROS Resins within the Bioproduction Division at Thermo Fisher Scientific. Scott has a B.S. in Biology from University of Connecticut and an MBA from the University of Massachusetts. Scott has worked on chromatography applications in the biotech industry for decades with 22 years spent at Thermo Fisher Scientific. His expertise is finding solutions to complex separation challenges draws on his experience in cGMP manufacturing, process development, customer training, sales and product management. In his current role, Scott is responsible for managing globally the Custom POROS Resin program, including working with customers to identify needs, in-house R&D to develop solutions, and POROS manufacturing to commercialize bioprocess resins for GMP applications.

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CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Supporting development of mRNA-based therapies by addressing large-scale purification challenges

Kelly Flook

The field of mRNA-based therapies is a rapidly emerging area with increasing real-world applications. The potential of these therapies is being demonstrated in various fields. Although the potential of mRNA in therapies is seemingly endless, obtaining the quantities of synthetic mRNA needed for clinical treatment remains a challenging obstacle, and current methods for mRNA purification are creating a bottleneck in large-scale manufacturing. Particularly for vaccine development, obtaining the quantities of synthetic mRNA needed for clinical treatment remains an obstacle. As a result, a robust, scalable and easy-to-use platform to support all mRNA therapies is needed. To support the development of mRNA-based therapies, Thermo Fisher Scientific has developed an affinity resin for the purification and isolation of mRNA from *in vitro* transcription (IVT) manufacturing processes. The following article and case studies will highlight how the Thermo Scientific POROS™ Oligo (dT)25 affinity resin can enable efficient and simplified mRNA purification.

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THE RISE OF mRNA THERAPEUTICS

Whilst mRNA now offers a new therapeutic paradigm, mRNA itself is not a new modality. The first concept proposal and successful study was published over 30 years ago, and

the first clinical trial began nearly 20 years ago – and today, the growing applications of mRNA as a therapeutic have been greatly spurred on by the success of novel mRNA-based vaccines being made available for emergency use against the novel coronavirus.

The rapid growth of mRNA as a therapeutic can also be attributed to the fact that the action of mRNA is relatively simple and well understood, making it a promising candidate for the development of platform technology. Synthetic mRNA has many applications – it can be used to create induced pluripotent stem cells, or induce cell differentiation into desired cell types by introducing proteins that stimulate these processes. It can be used to create secreted proteins such as antibodies, and to express a homing receptor to improve cell migration to specific areas in the body. Additional uses include vaccination of rare and common diseases, and synthetic mRNA can also be used for gene editing using TALENs or CRISPR.

THE PURIFICATION CHALLENGE

For a platform technology to fully succeed, a corresponding purification platform is key. Traditionally, purification of mRNA is achieved by a variety of methods (Table 1), but each option brings disadvantages. Many scientists try to scale up tried and tested methods from the research laboratory – but when moving from micrograms to grams, and potentially even kilograms of mRNA, this may not be the most successful, or optimal approach. Scalability is not the only challenge to tackle – other important considerations include purification efficiency, ease of use, recovery, selectivity, and the option to integrate an affinity resin as a platform solution for various mRNA molecules.

Reverse phase purification

Reversed phase purification is highly effective and achieves high resolution. It offers some selectivity for product related impurities, but

TABLE 1

Methods of RNA purification.

Method	Advantages	Disadvantages
Reversed phase	▶ High resolution ▶ Some selectivity for product impurities	▶ Limited column capacity ▶ Use of expensive/flammable/toxic chemicals ▶ Column fouling impacts resolution
Ion exchange chromatography	▶ Native purification possible ▶ Scalable	▶ Column capacity and recovery (HPLC) ▶ May need toxic chemicals for denaturation ▶ Purified product can contain traces of elution salts
Size exclusion chromatography	▶ Native purification possible	▶ Separation efficiency affected by alternative folding ▶ Flow limited
HIC	▶ Native purification possible ▶ Scalable ▶ Replacement for reversed phase	▶ Non-selective
Affinity chromatography	▶ Native purification possible ▶ Scalable ▶ Platform solution for wide range mRNA molecule sizes – selective to polyA	▶ Requires additional polishing step to remove product-related impurities

Affinity chromatography can be used as a scalable platform solution for mRNA purification.

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when considering this approach from a scale up perspective, there is limited column capacity. An additional challenge is the need for flammable and toxic solvents that pose safety concerns for operators and necessitate intrinsically safe suites which are not commonplace in biotherapeutic manufacturing. These suites are costly to set up, and bring additional cost implications related to disposal of organic solvents. In addition, ion pair reagents add a toxic component that then requires additional purification steps to remove.

Without very stringent cleaning protocols, fouling from smaller proteins and enzymes can impact the selectivity and separation efficiency of the column over time.

Ion exchange chromatography

Ion exchange chromatography is a common approach when working with smaller nucleic acids, and is effective for native purification. When working with increasingly larger constructs, capacity and recovery issues arise – due to the multiple charges on the mRNA, it binds very effectively to ion exchange resins, and in some instances eluting the mRNA molecule from the column with good recovery can prove difficult.

Hydrophobic interaction chromatography

Hydrophobic interaction chromatography (HIC) is a common chromatography technique that is also being used for the purification of mRNA. It allows for native purification, and the resins are scalable. Similar to reversed phase, HIC takes advantage of the difference in hydrophobicity of mRNA and its impurities, and is commonly used by the industry as an orthogonal purification method. It has the potential to replace the traditional reversed phase method as no toxic chemicals are needed. But as with reversed phase, selectivity can be a challenge to remove specific product impurities.

Now that mRNA therapies and vaccines are making their way to the clinic, the need for a robust purification platform becomes apparent – and affinity chromatography can overcome the challenges the field is currently facing. The method allows for native purification, is scalable and highly selective as it uses the poly-A tail to purify the mRNA molecules. Any impurity lacking a poly-A tail will not bind the column and is easily flushed away, allowing all impurities without a poly-A tail to be removed in a single step. Product related impurities containing a poly-A tail such as double stranded RNA can be removed with a second polishing step. Alternatively, it is possible to engineer out the formation of double stranded RNA during upstream synthesis. This approach allows the use of affinity chromatography as a single step purification solution that can be scaled up as manufacturers move through the clinic.

THE POWER OF AFFINITY CHROMATOGRAPHY

Affinity chromatography offers many benefits beyond a selective approach, and is applicable regardless of which modality is being used. It has earned credit in therapeutic antibody development and more recently also in viral vector manufacturing. Depending on the molecule, as well as the process and product related impurities, multiple purification steps may be needed to reach the desired purity. This means that each purification step added to the process will result in lower overall yield.

The graph in Figure 1 demonstrates the number of process steps against product yield. Even with a high step yield, for example 85%, after four process steps the overall product yield is reduced to 50%. Affinity chromatography can address this challenge. Due to high affinity for the target molecule, a higher purity and yield is achieved in the first step alone. This helps to reduce the number of purification steps needed in the overall process, increasing the overall product yield. A simplified purification process

also reduces bioprocessing development time, allowing manufacturers to get to the market faster, and decreasing the overall cost of goods.

THE THERMO SCIENTIFIC POROS™ OLIGO (dT)25 AFFINITY RESIN

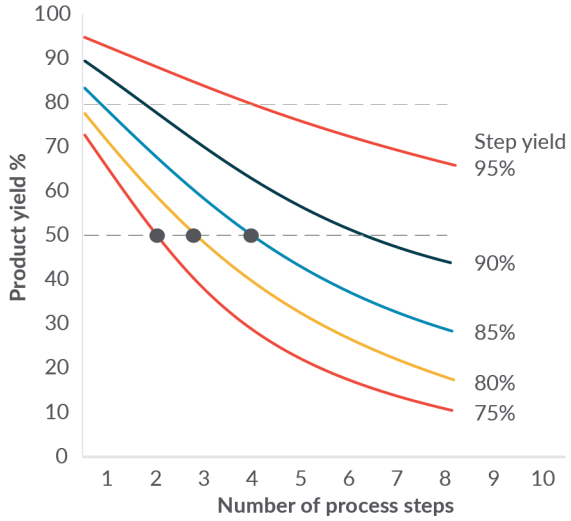
In 2020, Thermo Fisher Scientific launched a new affinity chromatography resin specifically designed for the purification and isolation of mRNA from IVT manufacturing processes in order to address the challenges associated with the purification of mRNA for therapeutic use. Figure 2 shows a schematic of the POROS™ Oligo(dT)25 resin. The resin is comprised of a 50µm porous poly(styrene-co-divinylbenzene) base bead with a polydeoxythymidine (poly-T) 25-mer (dT-25) conjugated to the surface using a proprietary linker.

A poly-T ligand on the surface of the resin allows for simple mRNA capture through AT base pairing. To load the mRNA IVT mixture on the column, salt is added. Once the mRNA is bound to the resin, the column can be flushed to remove process related impurities. To elute the mRNA from the column a low concentration of buffer, or simply water, is used.

The resin has a high binding capacity in comparison to the laboratory-based techniques discussed above, with a dynamic binding capacity of up to 5 mg/mL for 4,000

FIGURE 1

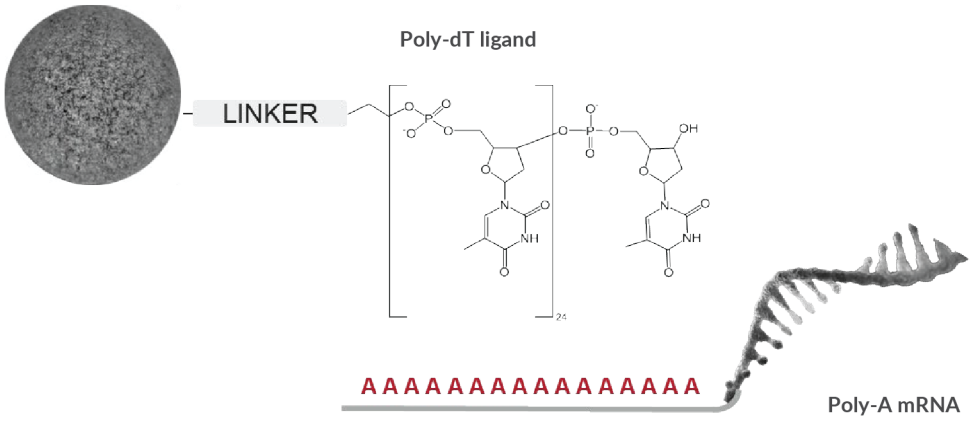
Product yield declines with increasing number of process steps.



Affinity capture can reduce the required number of purification steps, thereby increasing yield.

FIGURE 2

Mechanism of action of the Thermo Scientific POROS™ Oligo (dT)25 affinity resin.



The resin is comprised of a 50µm porous poly(styrene-co-divinylbenzene) base bead with a polydeoxythymidine (poly-T) 25-mer (dT-25) conjugated to the surface using a proprietary linker.

The poly-dT ligand will bind to the poly-A tail of the mRNA through simple AT base pairing.

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nucleotides (nt) RNA. Across a wide range of mRNA construct sizes, the recovery in the first step yield has demonstrated to be greater than 90%, and in most cases, greater than 96–98%.

As the POROS™ Oligo (dT)25 Affinity Resin is a chromatography resin, it is easily scaled, with the ability to pack columns anywhere from a few milliliters or liters, up to hundreds of liters. Like other bioprocess resins offered by Thermo Fisher Scientific, it is a 100% non-animal derived, pharmaceutical-grade reagent, suitable for the manufacturing and purification of clinical therapeutics. The POROS™ Oligo (dT)25 Affinity Resin provides a simple solution to maximize workflow efficiency and reduce the complexity of any subsequent polish steps required.

THE POROS™ BEAD

There are three main attributes that differentiate POROS™ from other chromatography resins (Figure 3).

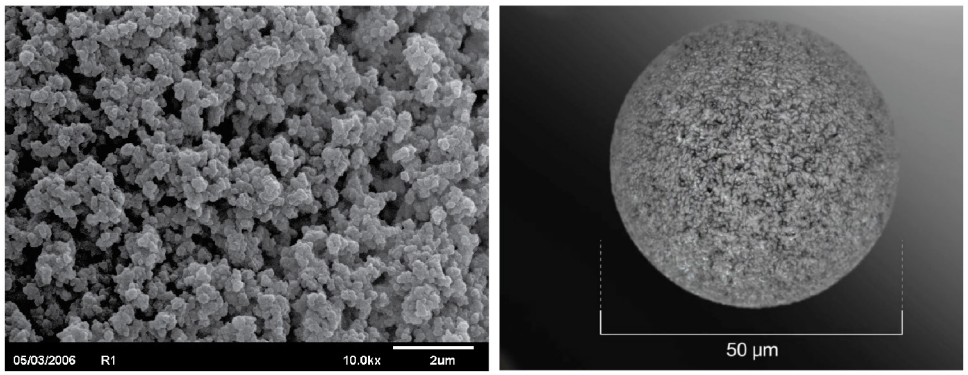
1. **Poly(styrene-co-divinylbenzene) backbone.** The beads are rigid and incompressible compared to agarose type resin. This results in stable column beds as well as linear pressure-flow profile over a wide range of column dimensions, allowing the user to maintain high operational flow rates with a modest pressure drop.

2. **Large pore structure.** The open pore structure of the beads makes POROS™ resins ideal for the purification of larger molecules such as mRNA or viral vectors. The large pores effectively increase the surface area available for interaction between the target molecule and the resin increasing both capacity and resolution. In addition, the larger pores result in reduced mass transfer resistance, which helps to improve process efficiency and productivity.

3. **50-micron bead size.** The average particle size is 50 µm, and this small particle size allows for less band broadening in packed beds, improving the ability to separate proteins and obtain effective impurity removal. Due to the reduced mass transfer resistance mentioned above, this superior resolution is well maintained and independent of linear velocity. In practice, this results in narrower peaks and smaller elution pool volumes which overcomes tank size limitations at large scale.

FIGURE 3

Scanning electron microscope images showing a POROS™ bead (left) and the large through-pores of the bead surface (right).



POSITIONING THE POROS™ OLIGO (dT)25 RESIN IN THE mRNA PURIFICATION WORKFLOW

Ideally, having just one purification step can fully maximize the productivity of the workflow. Purification with the POROS™ Oligo (dT)25 affinity resin will remove process related impurities, such as DNA template, nucleotides, enzymes, and unwanted buffer components. If some product related impurities remain such as double stranded RNA or uncapped mRNA, an additional polishing step can be used.

Affinity purification can also be used in a polish step. Some users may want to retain an initial non-affinity first step, then implement a second affinity polishing step to remove any unwanted components that are left over from the IVT reaction. One advantage of this approach is that it can also be used as a buffer exchange step, as the mRNA can be eluted directly into water.

PROCESS DEVELOPMENT & RESIN PERFORMANCE STUDIES

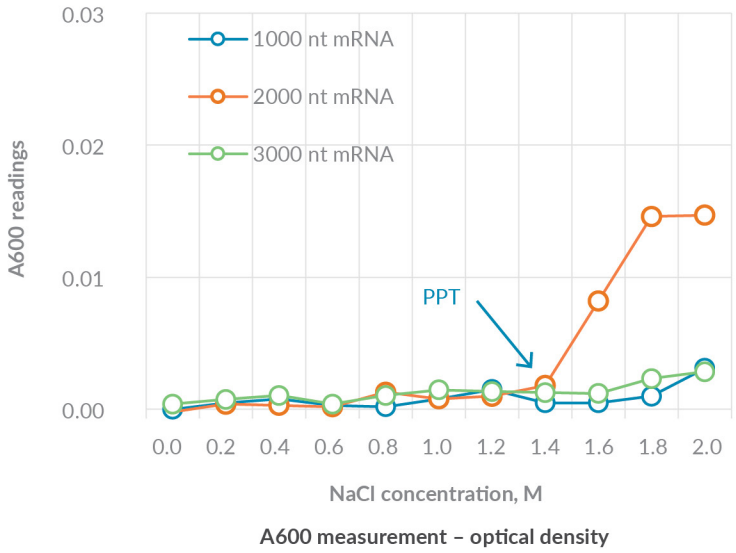
The goal of process development was to first understand how a range of mRNA molecules behaved, in order to more effectively optimize binding capacity without impacting the mRNA. Utilization of a high throughput screening approach allowed rapid optimization over a range of conditions. Once favorable conditions were found, methods were transferred to column format for further optimization.

SALT TYPE & CONCENTRATION EFFECT ON mRNA BINDING

To better understand the stability of the mRNA, and to determine favorable initial loading conditions, various conditions were examined using a 96-well plate design (Figure 4). Three different mRNA construct sizes were studied ranging from 1,000 to 3,000

FIGURE 4

Effect of salt concentration on mRNA stability.



To determine the mRNA precipitation point (PPT) for three sizes of mRNA construct, the optical density (A600) was measured at increasing salt concentrations. Precipitation of 2000 nt mRNAs occurred at lower salt concentrations than 1000 or 3000 nt mRNAs, suggesting that structure, as well as size, plays a role in stability.

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nucleotides using increasing salt concentrations and various salt types. Since the overall structure of these mRNAs is different, different behaviors are expected.

When increasing the sodium chloride concentration up to 1.4 M, precipitation began to occur for the 2,000 nt mRNA. Interestingly, this effect was not seen with the 1,000 or the 3,000 nt mRNAs, which demonstrates that the effect is not related purely to size, but to construct design. When switching from sodium chloride to potassium chloride, the 2,000 nt mRNA was not affected in the same way. Depending on the mRNA sequence being used, it may be necessary to optimize not only the loading salt concentration, but also the salt type used to neutralize the backbone.

Using the information from the 96-well plate precipitation experiment, salt concentration was then studied to determine optimal binding capacity in relation to salt concentration. A decrease of mRNA was seen in the elution pool as salt concentration was increased, demonstrating the promotion of binding – whereas at low salt concentrations, the backbone is not fully neutralized in order to promote annealing. The profile of binding capacity was again different across the three different constructs, indicating that this is another tool that can be used to optimize binding conditions.

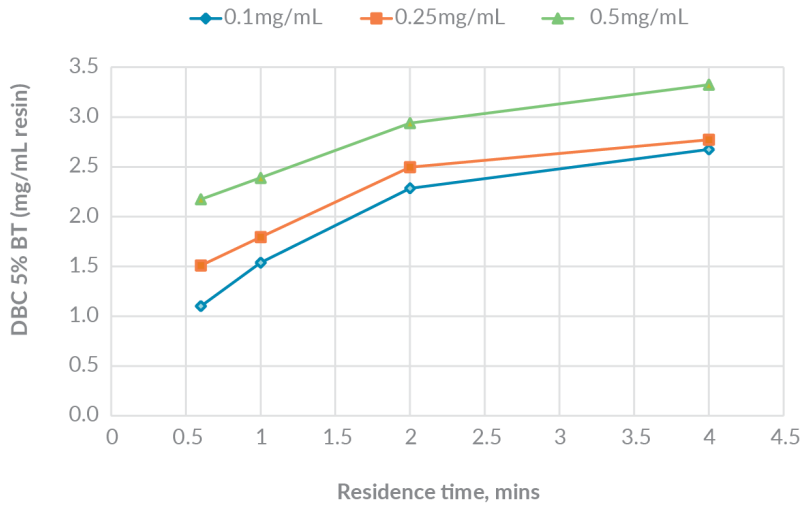
When considering buffer choice, the impact of binding across a range of pH in Tris buffer was studied. Again, optimal binding conditions were not consistent across the range of mRNA sizes used. These differences can be used to further optimize later column experiments, which will in turn assist in optimizing load concentration and flow rate.

DYNAMIC BINDING CAPACITY

The binding capacity of a capture step is an important parameter to determine how much product can be loaded on the column. In a study of binding capacity compared to flow rate, it was observed that increasing residence time resulted in increased binding capacity (Figure 5). This is due to the diffusional effects of the large mRNA molecule, and is common for larger biomolecules. In addition, higher concentrations of mRNA in the load pool better enabled the mRNA to reach the surface of the resin

FIGURE 5

Dynamic binding capacity (DBC) of 3000 nt mRNA at three different feed concentrations.



Residence time for mRNA load (Flow rate)
0.6 min (300cm/hr)
1.0 min (180cm/hr)
2.0 mins (90cm/hr)
4.0 mins (45cm/hr)

DBC increases with higher mRNA concentration and longer residence time.

due to improved binding kinetics at higher concentrations at lower flow rates. However, when considering productivity gains, benefits began to diminish beyond a 2-minute residence time. As a result of this study, a 2-minute residence time was selected for further experiments.

INFLUENCE OF MOLECULE SIZE ON BINDING CAPACITY & RECOVERY

Next, the effect of mRNA size on binding capacity was studied. To study comparative differences this experiment was not optimized for each individual mRNA size – load concentration, flow rate, and column dimensions were all kept constant in order to observe the direct effects of mRNA size. As expected, the size of the mRNA has an impact on the binding capacity and the smaller the mRNA, the higher the binding capacity achieved (Figure 6). As the mRNA constructs gets larger, steric hindrance becomes an issue, and the mRNA lacks the physical room to reach the surface of the resin.

Looking at recovery of the different construct sizes, consistent recovery well above 95% is shown, and is independent of the size of the mRNA.

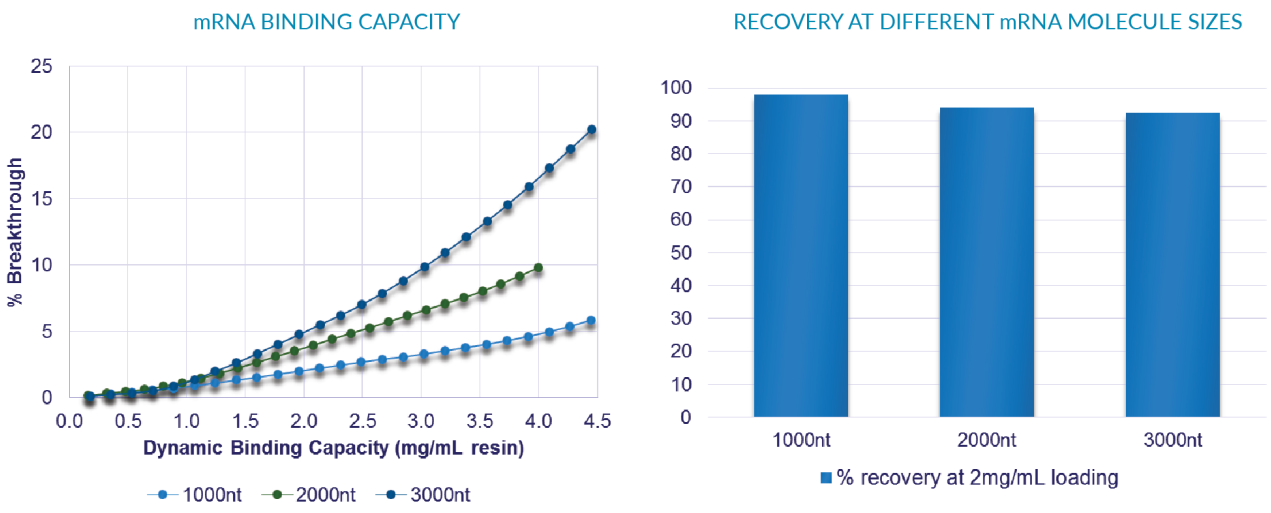
REUSE, CLEANING & STABILITY OF THE OLIGO (dT)25 AFFINITY RESIN

A 2,000 nt mRNA was used to assess the ability to reuse the resin (Figure 7). Multiple purification cycles were performed. The mRNA was bound and eluted over 10 cycles, with a cleaning step at the end of each cycle. Before the first cycle and after the 10th cycle, a blank buffer run was performed to monitor if any mRNA was eluted in the final blank run. The overlays of the blank runs appeared identical, demonstrating no carry over of mRNA from subsequent runs. In addition, this experiment demonstrated that the recovery, measured based on peak area, was consistent over the 10 cycles.

To study the effects of cleaning and sanitization with NaOH, incubation with different concentrations of NaOH was studied.

FIGURE 6

Binding capacity and recovery of three sizes of mRNA construct (1000, 2000, and 3000 nt).



mRNA molecule size impacts binding capacity but not final recovery

Smaller mRNA has a higher binding capacity (left) but size does not impact final recovery (right).

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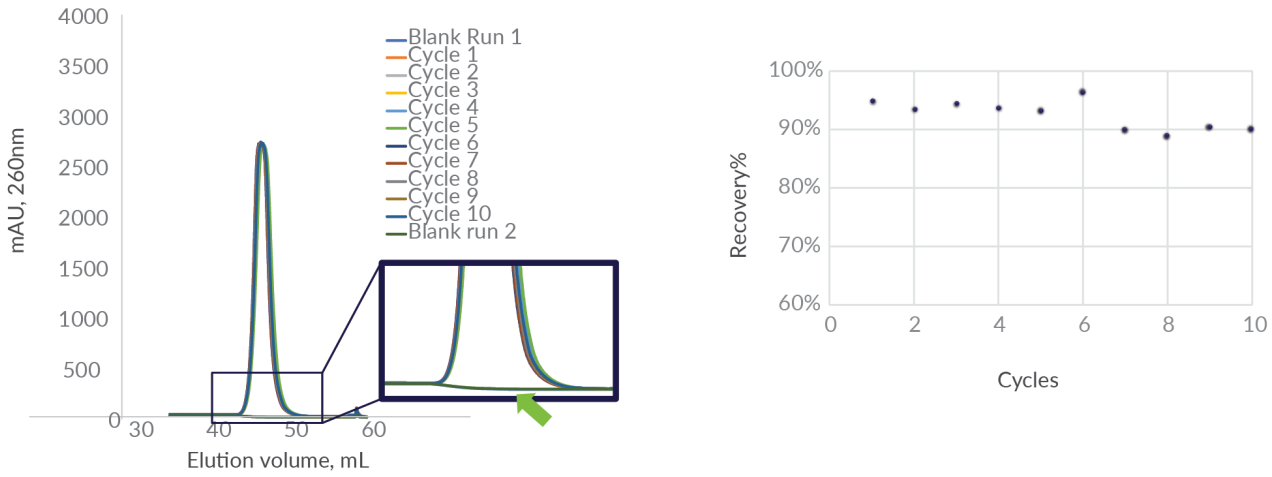
FIGURE 7

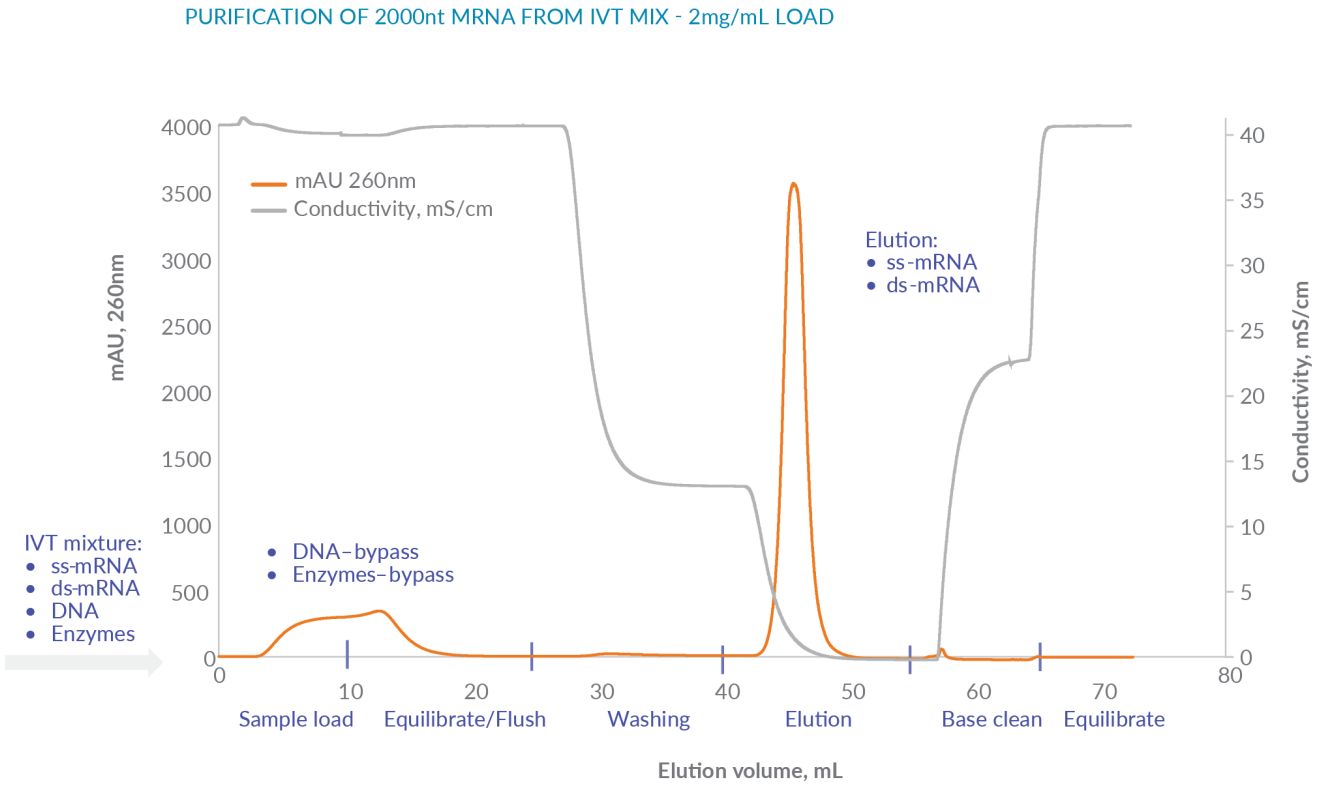
Effect of resin reuse and cleaning on mRNA purification.



Recovery is not impacted by resin reuse and cleaning. Left: Multiple cycles of mRNA (1809 nt + polyA 120 nt) purification from IVT mixture. Chromatograms from blank buffer runs carried out before cycle 1 and after cycle 10 were identical (green arrow), showing that there was no carry over of mRNA. **Right:** Recovery rates for each cycle, showing consistency between cycles.

FIGURE 8

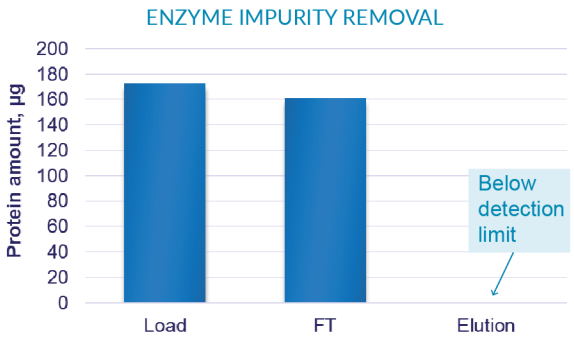
Output of a chromatographic purification run.



Conductivity (representing salt concentration) is shown in gray, while the chromatogram is shown in orange.

FIGURE 9

Enzyme impurity in load, flowthrough fraction, and elution pool.



The amount of enzyme (protein) is high in loading (load) and flowthrough (FT) fraction, but undetectable in the elution pool.

Constant incubation was studied up to a total of 48 hours, which is equivalent, depending on the residence time of the NaOH, to potentially hundreds of cleaning cycles. The experiment demonstrated that the resin can withstand up to 0.5N NaOH, allowing for stringent cleaning and sanitization. In addition, the resin demonstrates good stability over a wide range of pH conditions (1–13).

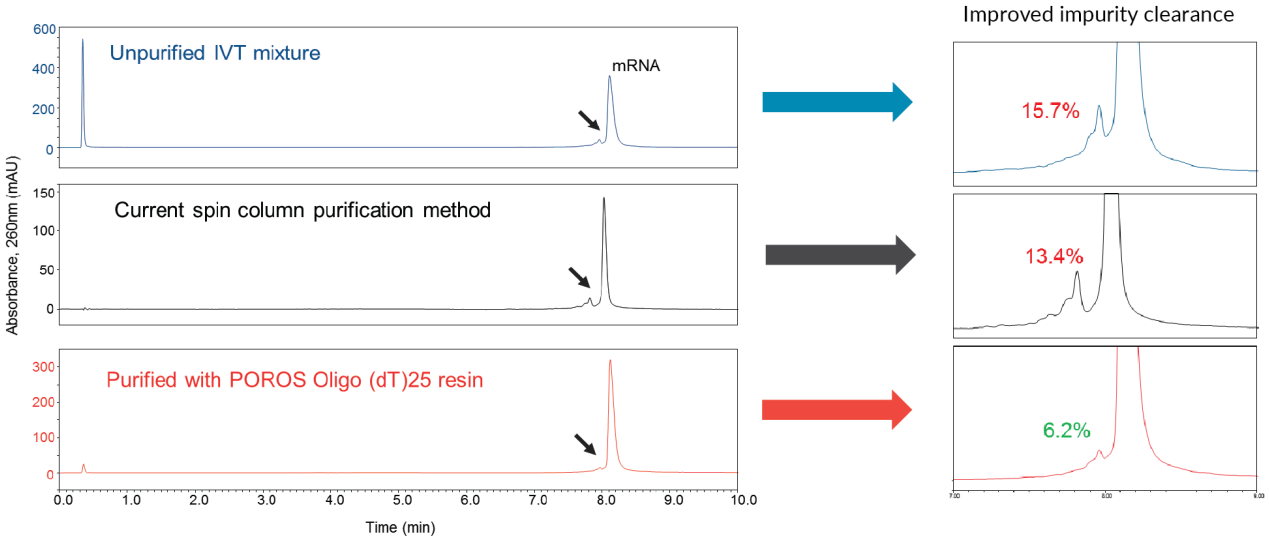
PURIFICATION VERIFICATION

Shown in Figure 8 is the output of a chromatographic purification run. The conductivity trace across the run, salt concentration measurement during the load, a step wash, and then elution and subsequent cleaning is shown in grey. The orange line is the UV 260 nm absorbance measurement and shows the chromatographic profile. At the beginning, an increase in absorbance is seen, which is indicative of DNA and other components flushing through the column. The step elution down to 150 mM NaCl helps to elute smaller truncated poly-A components that bind weakly to the column, as well as components bound to the mRNA itself, and the subsequent transition into water gives a sharp, narrow mRNA elution peak. A small peak is seen in the base cleaning step using NaOH, indicating some residual components were still on the column and are removed by this cleaning step.

The purification run was performed twice – first with already purified mRNA, where excellent recoveries of about 96% were seen. When run again with an unpurified portion

FIGURE 10

HPLC of IVT mixture after no purification (top), spin column purification (middle) and POROS™ Oligo (dT)25 affinity resin purification (bottom).



Purification with POROS Oligo (dT)25 leads to a significant reduction in impurities.

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of the IVT mixture, the same recovery was achieved. This was a key finding, as it demonstrates that the concentration of components present in the IVT mixture does not impact mRNA binding. This is important when considering resin reuse.

IMPURITY REMOVAL

Enzyme impurity removal was also studied using the IVT mixture (Figure 9). A relatively high concentration of protein was initially present in the loading pool, as measured by a BCA assay, and again a large amount of enzyme was present in the flowthrough fraction. When protein was measured in the elution pool, any enzyme present was below the limit of detection.

In addition, a comparison was done between a silica-based spin column method known for efficient removal of IVT components and the POROS™ Oligo (dT)25 resin. The results are shown in Figure 10.

The top trace shows the unpurified IVT mixture, and the peak on the far left represents enzyme, DNA, and smaller components. The impurities eluting the left (before) the main mRNA peak account for almost 16% of the main peak group. As shown in the middle trace, using the current spin column method, smaller enzymes are eliminated, but over 13% of the impurities remain in the main peak.

Applying an affinity resin (bottom trace) significantly decreased the amount of impurity to close to 6%, giving a significant reduction in impurities compared to the spin column method. Further study to identify the remaining components is ongoing, initial data (not shown) suggests the remaining impurities are polyadenylated. Earlier retention also suggests a smaller size than the full-length mRNA.

CONCLUSION/INSIGHT

Affinity chromatography offers a highly efficient and scalable method that has already proven its worth in the development of biologics, and it offers a powerful tool to help address the current bottlenecks in commercial manufacturing of mRNA therapeutics. With high affinity for the target molecule, it can deliver higher yield and purity in the first purification step, helping to reduce the number of purification steps in the overall process, and increasing total product yield. By reducing bioprocess development time, it can result in a decrease in overall cost of goods, and ultimately, a faster time to market for innovative mRNA-based therapeutics.

Q & A

Kelly Flook
Senior Product Manager, Thermo Fisher Scientific

Q Do you need to use heat to elute the RNA?

KF: For purification, we developed this resin so you wouldn't need to use heat. With more traditional, R&D types of mRNA extraction from cells, heat is typically used because the mix in the cell extract is a lot more complex, so it is used to break down a lot of the higher order structures that can bind to those resins and therefore heat aids elution. But in the case of purification, and with this resin, we see a lot of customers using it successfully at room temperature.

Q Does temperature have a negative effect on the stability of mRNA in the chromatography step – and what do you recommend to try and stabilize mRNA?

KF: If there is a stability effect with temperature, it is more related to the construct sequence versus the chromatography. We see people adding EDTA to their buffers in order to help with that stabilization.

Q What sizes of RNA can be purified, and is there a construct size limit?

KF: When we developed this resin, we had relatively small mRNA sizes in mind, typically anywhere from a 1,000 up to about 5,000 nucleotides. We were not really focusing on those larger, self-amplifying RNA up to the 10,000-12,000 range.

What we do see is an impact on binding capacity, as I discussed earlier. With smaller mRNA, you will see a larger binder capacity than you will with something that is significant bigger.

Additionally, the amount of salt you need to neutralize those charges will also be slightly different, because the larger the RNA, the more charges you need to neutralize. You would expect more salt to be needed to achieve that and maximize your binding.

Q How many cycles can you typically get out of the resin?

KF: In this case we looked at cycling just up to 10 cycles. However, we have seen some customers using this resin that are getting 30, 40, 50 cycles, so it is robust. They have a cleaning step in between those cycles as well, this is also a quick sanitization step between cycles.

Q What would you advise for salt concentration to get optimal binding?

KF: We have seen good success starting at about 0.5M sodium chloride in the initial instance. Then either increasing that slightly to increase binding, or simply decreasing that down to the minimum level you need to achieve binding.

Q What is the maximum operating pressure for the resin?

KF: The resin has a robust poly(styrene-*co*-divinylbenzene) core, so the resin itself can withstand pressures over 100 bar. As far as operating and packing for a purification set up, your pressure limitations are really going to be limited by the hardware, and not necessarily the resin.

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Q How can you separate single stranded mRNA from double stranded, and do you have any particular products that fit this goal?

KF: As I mentioned earlier, one of the great things about the dT is that it will bind poly-A well. This also includes double stranded RNA. We recommend our HIC resin range – we have a POROS™ Ethyl, Benzyl and Benzyl Ultra, that can be used to separate the double stranded RNA from single stranded.

BIOGRAPHY

Kelly Flook
Senior Product Manager, Thermo Fisher Scientific

Kelly Flook is Senior Product Manager for Purification products within the Bioproduction Division at Thermo Fisher Scientific. Kelly has a Ph.D in Polymer and Analytical Chemistry from the University of Durham, UK. During her 15 years at Thermo Fisher, Kelly has gained extensive experience in product development across all scales of chromatography and related biological workflows. Kelly has a strong expertise in bead technology and bio-separations. Drawing from a diverse technical background, in her current role Kelly is responsible for new product development and commercialization of solutions across the downstream workflow.

AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

Vector Channel

DOWNSTREAM BIOPROCESSING EDITION

CELL & GENE THERAPY INSIGHTS

VECTOR CHANNEL: Downstream Bioprocessing

November 2021
Volume 7, Issue 11

INTERVIEW

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VIEWPOINT

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INTERVIEW

Stepping foot into a successful partnership to support your viral vector therapy through commercialization

Charlotte Barker, Editor, *Cell and Gene Therapy Insights*, **speaks to Minh Hong,** Head of Commercial, Viral Gene Therapy, Merck, **and Marc Gaal,** Director, Program Management at the Life Sciences Business Sector, Merck

MINH HONG leads the commercial team for Viral Gene Therapy contract manufacturing services within the Life Sciences Business Sector of Merck. He is responsible for account management, business development, and out-licensing activities of innovative viral vector manufacturing tools.

MARC GAAL heads Merck's Commercial Project Management Office and is responsible for contract administration and project management of customer programs throughout the entire life cycle of the program.

Cell & Gene Therapy Insights 2021; 7(11), 1706–1710

DOI: 10.18609/cgti.2021.225

With Merck recently opening a new gene therapy manufacturing facility in Carlsbad, California, we caught up with two of Merck's top experts in viral vector manufacturing to find out more about some of the challenges of viral vector production and how Merck helps customers bring their product safely through to commercialization.

Q What are the key ways in which the quality component of viral vector manufacturing has advanced in recent times?

MG: We've seen a real paradigm shift in recent years from customers saying 'please get me material as fast as possible' to asking us to help them bring their product to commercialization. And having established a proven track record of successful regulatory inspections has afforded us with the first-hand knowledge needed for the evolving landscape that is cGMP.

Q Customers come to you at different stages and with different requirements – how do you ensure that you meet their needs?

MH: I think the industry has a flurry of wonderful activities and great ideas at the research and development stage. However, the challenge contract drug manufacturers face is translating those good ideas into process development and achieving the desired manufacturing outcomes in a robust and reproducible way.

We firmly believe that a robust manufacturing process requires process development insight at every stage of the product lifecycle. For example, to normalize any process that comes into our facility, we have a manufacturing gap assessment service that we provide to our clients. As part of this service, we perform a paper assessment of the customer's process (and potentially a small-scale feasibility run) to build a clear roadmap of what it will take to move the client's process into GMP manufacturing.

We assess the process unit operations, performance, critical quality attributes, analytics, and supply chain considerations through the lens of process development and quality. This results in tailored recommendations for development and manufacturing and helps us to determine the appropriate timelines.

> "...a robust manufacturing process requires process development insight at every stage of the product lifecycle."
>
> - Minh Hong

The manufacturing gap assessment arms us with a comprehensive understanding of our customer's needs and helps the customer to develop a more detailed understanding of how we'll move their process into manufacturing. This service level sets the customer's process to our capabilities, as well as the customer's expectations for the manufacturing journey ahead.

Q Once you understand the customer's needs, how do you support them through the manufacturing process?

MG: Once the manufacturing gap assessment is completed, as Minh already detailed, we'll assign a Project Manager to partner with the customer and the Cross-functional Project Team. The Project Manager then helps to guide the team in refining the target Program Timeline. By employing a combination of traditional Project Management practices and a structured Stage Gate process, we help our customers not only achieve pre-determined definitions of success at each stage of drug development, but also de-risk their manufacturing process. The result is fewer GMP setbacks and decreased release of lot turnaround times.

Q How exactly has your organization chosen to invest in cell and gene therapy manufacturing?

MG: Our new large-scale gene therapy manufacturing facility is comprised of suites that perform buffer media and preparation, cell expansion, midstream, downstream, and fill/finish. All of the manufacturing areas are Grade-C clean rooms, following ISO 7 standards. They are equipped with the latest in adaptable and scalable, state-of-the-art, single-use equipment, providing a safe, closed system for production.

We also have a suite of support laboratories that provides our clients with reliable testing results from process development through manufacturing. These labs perform multiple functions including qualification of raw materials, biochemistry, microbiology, and environmental monitoring.

Q What lead your organization to determine that this was the right time to invest in a new gene therapy manufacturing facility?

> "The new facility ... allows us to use connected, automated, single-use technologies that reduce the need for lengthy changeover practices, thus maximizing batch throughput."
>
> - Marc Gaal

MH: Why was this the right time? I think it's clear that manufacturing capacity is at an all-time high, but it's not only capacity customers are seeking. We've been through the initial 'honeymoon' phase for gene therapies. All our customers remain time-sensitive, but there is definitely a drive to reduce cost-of-goods for manufacturing and increase yield to serve larger patient populations. Upping capacity is great – but building a state-of-the-art facility and implementing enabling technologies to reduce timelines, scale up to decrease costs, and improve product safety and robustness is something that stands out.

Q How do you think the new facility helps to better support your customers?

MH: Our smart facility design, including templated processes and a robust supply chain of our bioreactors and biomanufacturing technologies, can serve many large-volume bioreactor customers with line-of-sight into reducing manufacturing costs and timelines, while increasing yields and quality. That is a huge benefit for our customers.

MG: The new facility allows us to scale up in suspension cultures to produce the three most commonly used viral vectors – lentivirus, adeno-associated virus, and adenovirus. It also allows us to use connected, automated, single-use technologies that reduce the need for lengthy changeover practices, thus maximizing batch throughput.

AFFILIATIONS

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Head of Commercial, Viral Gene Therapy, Merck

Marc Gaal
Director, Program Management at the Life Sciences Business Sector, Merck

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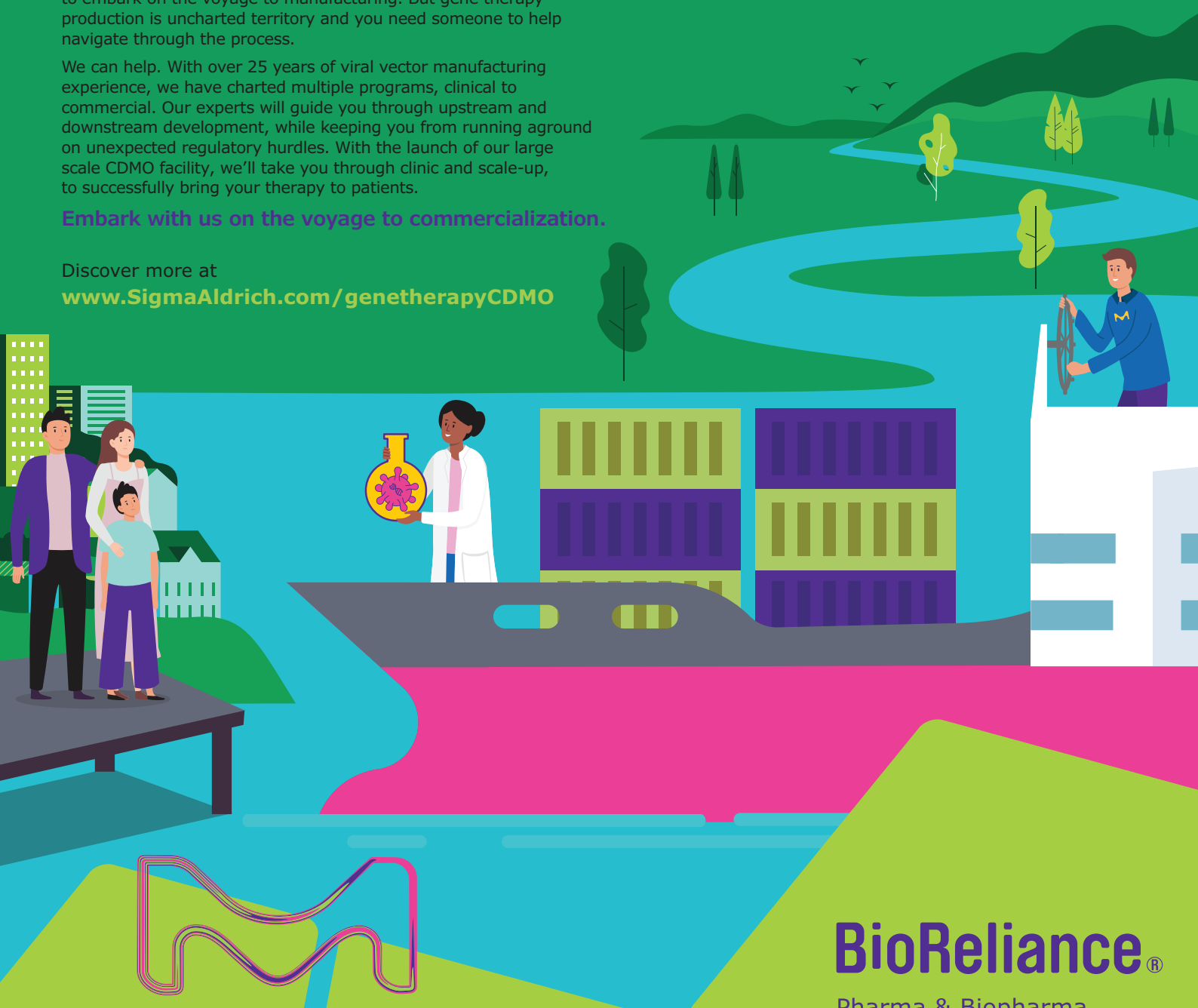

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CELL & GENE THERAPY INSIGHTS

DOWNSTREAM BIOPROCESSING

INNOVATOR INSIGHT

Enrichment of full rAAV capsids in a scalable, reproducible viral vector manufacturing platform

Shawn Tansey, Adam Hejmowski, Rajeshwar Chinnawar, Michelle Olson, Anne MacIntyre, Amanda Rose, Kurt Boenning, Julio Huato, Terese Joseph, Mark Schofield, Aydin Kavara, Nick Marchand, Mike Collins & Todd P Sanderson

Recombinant adeno-associated viruses (rAAV) are the gene transfer vector of choice for many *in vivo* gene therapies. These vectors are synthetic viral particles which can deliver a therapeutic gene to a patient or patients' cells to correct a genetic abnormality. These viral vectors can be produced in single-use bioreactors and purified using scalable single-use technologies. We evaluated the use of scalable, single-use filtration and chromatography technologies for downstream purification of an rAAV5 viral vector. In this testing, vector was produced in the Pall iCELLis® Nano bioreactor by polyethylenimine (PEI) mediated triple-plasmid transfection. The harvest material was clarified using direct flow filtration with a combination of Seitz-P grade depth and 0.2 µm sterilizing grade filters. The product was concentrated using 100 kDa Omega™ Membrane flat-sheet tangential flow-filtration (TFF) before primary purification with affinity chromatography. Affinity purified vector was polished using Mustang® Q membrane chromatography to enrich for full capsids. The rAAV5 product was then concentrated and diafiltered to the final formulation using 100 kDa Omega TFF membrane. The final product was sterile filtered using Pall's Supor® EKV validated sterilizing-grade filters. This manufacturing process was optimized and evaluated for vector yield, low contaminant profile and full capsid enrichment. We established feasibility of a near complete end-to-end manufacturing process using almost all materials available from Pall Corporation. This process resulted in a theoretical whole process yield of ~25% with a low contaminant profile (host cell protein [HCP] and [DNA]) and a ~5-fold enrichment of full capsids to total capsids. The purification process described here shows potential for a scalable, platformable process for rAAV products.

Cell & Gene Therapy Insights 2021; 7(11), 1563–1579

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Recombinantly produced adeno-associated viruses (rAAV) are now the predominant vector for *in vivo* gene therapies. These synthetic viruses can deliver a functional gene to correct a genetic defect and/or inhibit the cell from producing a defective version of the gene to restore normal function [1].

These medicines are a new class of biologics with the US FDA's first approval of an *in vivo* gene therapy in 2017. This viral vector was Spark Therapeutic's Luxturna® which is a treatment for biallelic *RPE65* mutation-associated retinal dystrophy [2]. Subsequently, the US FDA approved Novartis' Zolgensma® for spinal muscle atrophy (SMA) [3]. These treatments are literally lifesaving and can bring sight to the blind [4].

One of the biggest challenges in bringing these life-changing treatments to patients is their production. For products such as Luxturna where the disease indication is rare and the vector amount per dose is low (~1.5 x 10^{11} viral genomes per eye), there is a relatively low manufacturing burden. For more prevalent indications that require systemic administration with a high vector dose (>1 x 10^{14} vg/kg), such as Duchene's muscle dystrophy (DMD), manufacturing becomes a significant bottleneck [1]. Other viral vectors are in development such as Uniqure's AMT-061 (entranacogene dezaparvovec), a gene therapy treatment currently in clinical trials for Hemophilia B. This vector is based on adeno-associated virus serotype 5 (rAAV5) and utilizes a more moderate dose of 2 x 10^{13} vg/kg and has a moderate manufacturing burden [5].

Typical rAAV purification methods used in academic research are generally small scale and utilize ultracentrifugation for purification. This purification method results in very high purity product; however, these methodologies are not scalable [6].

The biopharmaceutical industry has decades of experience producing biologics such as recombinant proteins, predominately monoclonal antibodies, at industrial scale. These molecules are produced in single-use or stainless-steel bioreactors with batch sizes up to 20,000 L. The purification strategies for these moieties rely on technologies with scalable performance including depth and sterile filtration, affinity and ion-exchange chromatography and tangential flow filtration (TFF). These technologies are platformed into a common industrial strategy for the purification of monoclonal antibodies, via the following process steps: clarification by direct flow filtration (combination of depth, bioburden reduction, and sterile), affinity chromatography, ion-exchange chromatography polishing, concentration and diafiltration to final formulation using TFF and finally sterile filtration [7,8].

Here we evaluated the feasibility of applying a similar platform approach using many of the tools applied to mAb purification, for purification of a rAAV5 viral vector. The process was evaluated for product yield, purity and full capsid enrichment. In addition, specific unit operations were evaluated for process robustness.

MATERIALS & METHODS

rAAV5 Production in iCELLis® Fixed-bed Bioreactor

HEK293-T cells (American Type Culture Collection) were recovered from

cryopreservation and maintained in exponential growth using Dulbecco's Modified Eagle's Medium (DMEM, Thermo) supplemented with 10% fetal bovine serum (FBS, Thermo), 4 mM GlutaMax™ (Thermo) and 1X non-essential amino acids (NEAA, Thermo). Seed train biomass was propagated in T-flask and CellSTACK® (Corning) multi-layer trays.

Cell growth in the iCELLis® Nano bioreactor was monitored until the cell density reached ~150,000 cells/cm^2 at which point transient transfection was performed. The culture was continued for 5 days. The spent media was collected and treated with 25 U/mL Benzonase® (EMD Millipore) and 2 mM $MgCl_2$ for 1 hour at 37°C. The cells contained within the fixed bed were lysed with 10 mM tris-HCl, 2 mM $MgCl_2$, 1% Tween 20, and 25 U/mL Benzonase, pH 8.0 at room temperature overnight. After recovery of the lysate, the fixed bed was rinsed with 1 system volume of PBS. All harvest materials were brought up to a 500 mM total NaCl concentration. The final harvest material was a pool of the cell lysate, spent media and PBS rinse.

Clarification

Clarification of the harvest pool was performed using Seitz® P-grade PDK11 depth filters in series with Supor® EKV sterile filters. These are dual-layer cellulose depth filters (2–20 mm retention) and dual-layer polyethersulfone filters (PES, 0.65/0.2 mm), respectively. Depth filter work was undertaken in Supracap™ 50 capsules (22 cm^2) or 5" or 10" Supracap 100 capsules (0.025 and 0.05 m^2). Sterile filter work was undertaken in Mini Kleenpak™ Syringe filters (2.8 cm^2), Mini Kleenpak™ 20 capsules (20 cm^2) or Mini Kleenpak™ capsules (220 cm^2). In all cases capsule size was chosen based on the volumetric loading target. Prior to the clarification step operation, the filters were flushed with 20 L/m^2 of 1X PBS after which, the upstream holdup volume was drained from both filters. Turbidity of the crude harvest and clarified pools were measured by a Hach® 2100Q portable turbidimeter. Flux rates of 100 or 200 L/m^2/hr (LMH) were used on the depth filters, and 200-250 LMH on the sterile filters. Following loading, the filter trains were flushed with 1.5X holdup volumes with 1X PBS buffer.

Concentration

Pall Omega™ 100 kDa single-use TFF cassettes were used to concentrate the clarified pool. T01 (0.01 m^2) and T02 (0.02 m^2) cassettes were stacked in various combinations based on the volumetric loading target. Prior to use, the cassettes were flushed with water and equilibrated with 1X PBS buffer in accordance with their care and use procedures [9,10]. For the flux excursion work, the system was setup in full recycle and loaded with a clarified rAAV5 pool. An initial check showed stable flux over time for set crossflow and transmembrane pressure (TMP) conditions, indicating there was no significant fouling from the feed stream. For each crossflow condition the TMP was ramped up until the permeate flux levelled off. Membranes were depolarized between crossflow conditions by recirculating with the retentate valve open and permeate valve closed for >10 min.

Six concentration studies were performed with filters loaded between 178 and 206 L/m^2. A 10X volumetric concentration factor was targeted for each trial. Following concentration, the membrane was depolarized by recirculating with the retentate valve open and permeate valve closed for >10 min. The system was then drained into the retentate vessel. A 25 mL flush of 1X PBS (equivalent to ~1.5X holdup volumes) was added to the system, recirculated for 10 min with permeate closed, and drained. The recovery flush and concentrated pool were finally combined and filtered through a Supor® EKV sterilizing-grade filter.

Affinity chromatography

Post-TFF, rAAV5 harvest was loaded onto a Thermo Scientific™ POROS™ GoPure™ AAVX

Pre-packed Column, 0.8 x 10 cm, 5 mL. Affinity chromatography was performed on AKTA™ Avant system (Cytiva). To compensate for long loading times and mitigate pool instability from the recommended 3-minute residence time, the post-TFF pool was split into two fractions and loaded onto two columns (~400–600 mL/column). Columns were equilibrated with 50 mM Tris, 0.5M NaCl, pH 7.5. Wash buffer was the same as equilibration buffer. Elution buffer was 50 mM Citric Acid, pH 2.0. 1 mL of 1M Tris pH 8.5 per 5 mL of eluate was added to the collection tube for instant neutralization. The generic Thermo Scientific conditions for elution pH were modified to pH 2 instead of 3.

Membrane chromatography polishing

Post-affinity rAAV5 was diluted to ~1L with 20 mM Bis-tris propane (BTP) buffer at pH 9. Mustang Q chromatography was performed on AKTA™ Avant. After equilibrating the column, the diluted sample was loaded at 50 mL/min (10 MV/min) onto 5 mL Mustang® Q capsules, followed by wash with the equilibration buffer. The sample was eluted using a conductivity step gradient aiming for ~1 mS/cm increase per step, achieved by varying the percent amount of equilibration buffer to elution buffer (Table 1).

Formulation

Pall Omega™ 100 kDa single-use TFF cassettes were used to concentrate and diafilter the purified pool into formulation buffer. Prior to use, the cassettes were flushed with water and equilibrated with buffer in accordance with their care and use procedures [9,10]. The Mustang® Q elution pool was first concentrated to a target volumetric concentration factor of 10X, or until the retentate pool volume dropped to the TFF system holdup volume. The pool was then exchanged with seven diavolumes of 20 mM Tris (pH 8.0), 1 mM $MgCl_2$, 200 mM NaCl, 0.005% Pluronic F68 [11]. A crossflow rate of 7.5 L/m^2/min (LMM) and TMP of 15 psi was used for the concentration and diafiltration. For recovery the membrane was depolarized by recirculating with the retentate valve open and permeate valve closed for >10 min. The system was then drained into the retentate vessel. A 1.5X holdup volume flush of formulation buffer was added to the system, recirculated for 10 min with permeate closed, and drained. The recovery flush and concentrated pool were then combined before final filtration.

Sterile filtration

A Supor® EKV 0.65 / 0.2 µm sterilizing-grade filter was used for final filtration. The filtration was done using Mini Kleenpak™ Syringe capsules (2.8 cm^2) in constant flow mode with a flux target of 500 LMH. Feed and filtrate pools were analyzed for rAAV5 concentration to calculate virus transmission.

Analytical methods

In-process samples were collected and stored at -80 °C.

Viral vector physical titer was measured using droplet digital polymerase chain reaction (ddPCR) assay using the Bio-Rad QX200 AutoDG Droplet Digital PCR System. The PCR primer/probe (IDT) combination targeted an amplicon contained in the gene of interest of the rAAV transfer genome.

rAAV5 Capsid titer was measured using a commercially available AAV5 capsid ELISA (Progen). Host cell protein concentration was measured using a commercially available ELISA kit (Cygnus). dsDNA concentration was measured with Quant-It™ Picogreen™ Assay kit (Thermo).

SDS-PAGE was performed on a 10% Criterion™ XT Bis-Tris gel for 60 minutes at 150V. The gel was then stained using Sypro™ Ruby fluorescent stain and imaged on a Bio-Rad ChemiDoc.

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TABLE 1

The chromatography method used for rAAV5 full capsid enrichment.

Step	Buffers used/details	Membrane volumes used
0 – Priming of membrane	Equilibration (10 mL/min – upflow) Equilibration (10 mL/min – downflow) Conditioning (25 mL/min – downflow) Strip (25 mL/min – downflow) Equilibration (50 mL/min – downflow)	10 per step
1 – Equilibration of membrane	Equilibration buffer	20
2 – Application of sample	rAAV5 sample solution	N/A – Inject all sample using air sensor
3 – Washing of membrane	Equilibration buffer	10
4 – Step gradients	Buffer A – Equilibration buffer Buffer B – Elution buffer Step 1 = 10% Buffer B, 90% Buffer A Step 2 = 15% Buffer B, 85% Buffer A Step 3 = 20% Buffer B, 80% Buffer A Step 4 = 25% Buffer B, 75% Buffer A Step 5 = 30% Buffer B, 70% Buffer A Step 6 = 35% Buffer B, 65% Buffer A Step 7 = 40% Buffer B, 60% Buffer A Step 8 = 45% Buffer B, 55% Buffer A Step 9 = 50% Buffer B, 50% Buffer A Step 10 = 55% Buffer B, 45% Buffer A Step 11 = 60% Buffer B, 40% Buffer A Step 12 = 65% Buffer B, 35% Buffer A Step 13 = 70% Buffer B, 30% Buffer A Step 14 = 75% Buffer B, 25% Buffer A Step 15 = 80% Buffer B, 20% Buffer A Step 16 = 85% Buffer B, 15% Buffer A Step 17 = 90% Buffer B, 10% Buffer A Step 18 = 95% Buffer B, 5% Buffer A Step 19 = 100% Buffer B, 0% Buffer A	10 per step
5 – Strip	20 mM BTP, pH 9, 1M NaCl	10

RESULTS

Clarification

rAAV5 was produced in the iCELLis® Nano bioreactor as described in the methods section. Approximately ~60% of the rAAV5 virus is retained by the cells and ~40% secreted into the production media (data not shown). Functional rAAV vector is reported to be found both retained by cells and in the spend cell culture medium [12]. The crude harvest pool consisted of the fixed-bed lysate, spent media and a phosphate buffered saline (PBS) bioreactor rinse.

Multiple combinations of depth and sterilizing-grade filters were screened to evaluate clarification performance. The screening results showed the combination of Pall's P-grade PDK11 depth filter in series with a Supor® EKV sterilizing-grade filter resulted in the highest performance based on capacity, impurity reduction, and yield (data not shown).

The PDK11/EKV filter train was evaluated for robustness over the course of eight bioreactor harvests. The average titer of the crude harvest material was 7.82 x 10^9 gc/mL +/- 1.17 x 10^9. The turbidity of the crude harvest material ranged from 11 to 129 nephelometric turbidity units (NTU). A summary of the clarification performance is found in Table 2.

The data in Table 2 shows the filter train was able to consistently reduce the crude harvest turbidity down to less than 5 NTU with no significant product loss. Furthermore, we saw strong process robustness against feedstream turbidity with regards to clarified pool turbidity and step yield. A summary of the turbidity reduction is shown in Figure 1. Filter capacity was influenced by crude harvest

TABLE 2

rAAV5 clarification performance summary.

Attribute	Data points	Average	95% confidence interval
Feed turbidity	n=8	57.9 NTU	24.8 NTU
Pool turbidity	n=8	2.9 NTU	1.1 NTU
Yield (ddPCR)	n=8	104%	8.3%

turbidity, however all eight runs showed >250 L/m^2 throughput on the depth filter, and >450 L/m^2 throughput on the sterile filter.

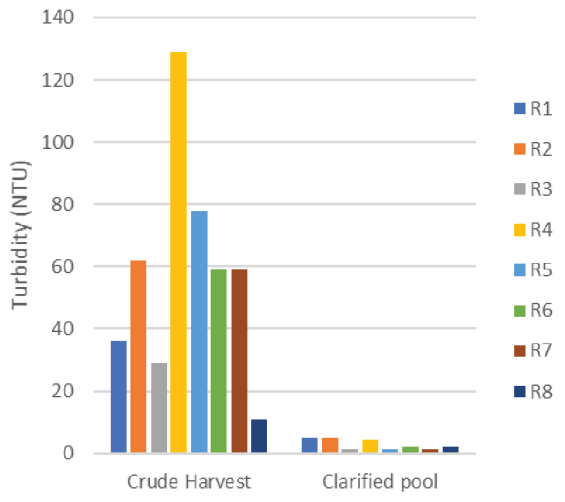
Trial number 4 (R4) showed a significantly higher turbidity at harvest than the other cultures. This culture showed similar cell densities at time of transfection and the resulting titer was comparable to the other harvests used during this testing. A root cause of the high turbidity observed in trial 4 was not found.

Concentration

Due to the ratio of harvest titer to the binding capacity of the affinity sorbent, direct loading onto affinity chromatography would require extended loading times. The clarified material was concentrated by TFF before purification. This reduced the affinity chromatography loading time from >20 hours to ~2 hours. For this process, we evaluated Omega™ 100 kDa PES single-use TFF membrane cassettes.

FIGURE 1

Clarification performance was assessed with eight bioreactor harvests (R1 through R8).



Ultrafiltration of rAAV products with 100 kDa pore size has been previously reported [13]. A flux excursion study was performed to identify the optimal crossflow and TMP process parameters. The results of this study are shown in Figure 2A & B and show a critical TMP at ~10–15 psi with a moderate benefit from increasing crossflow rate, resulting in limiting flux rates between 71 and 97 LMH. Note that after each TMP excursion, rAAV concentration was measured from the recirculating pool and the permeate line. Results from the three crossflow rate trials showed virus retentions of >99.7%. We also observed no significant trend to total gene copies in the recirculating pool, suggesting no significant virus loss due to shear at crossflow rates up to 7.5 L/m^2/min.

Six trials were performed to concentrate the clarified rAAV5 pool to a target volumetric concentration factor (VCF) of 10X. Across the trials, volumetric loading averaged 186 ± 9 L/m^2, virus loading averaged 1.3 x 10^{15} ± 2.6 x 10^{14} gc/m^2, and feed concentration averaged 7.3 x 10^9 ± 1.5 x 10^9 gc/mL. A summary of the filter performance is shown in Table 3 and includes an average vector step yield of 91% ± 8.0%. A representative plot of flux and VCF over time is shown in Figure 2B.

Chromatography purification

Affinity capture chromatography was performed and to speed up loading time, typically a single clarified harvest was purified on two columns simultaneously using two ÄKTA Avant chromatography systems. Across 16 total affinity chromatography purifications rAAV5 recovery was determined to be 68 ± 13% by capsid ELISA and 57 ± 30% by ddPCR method.

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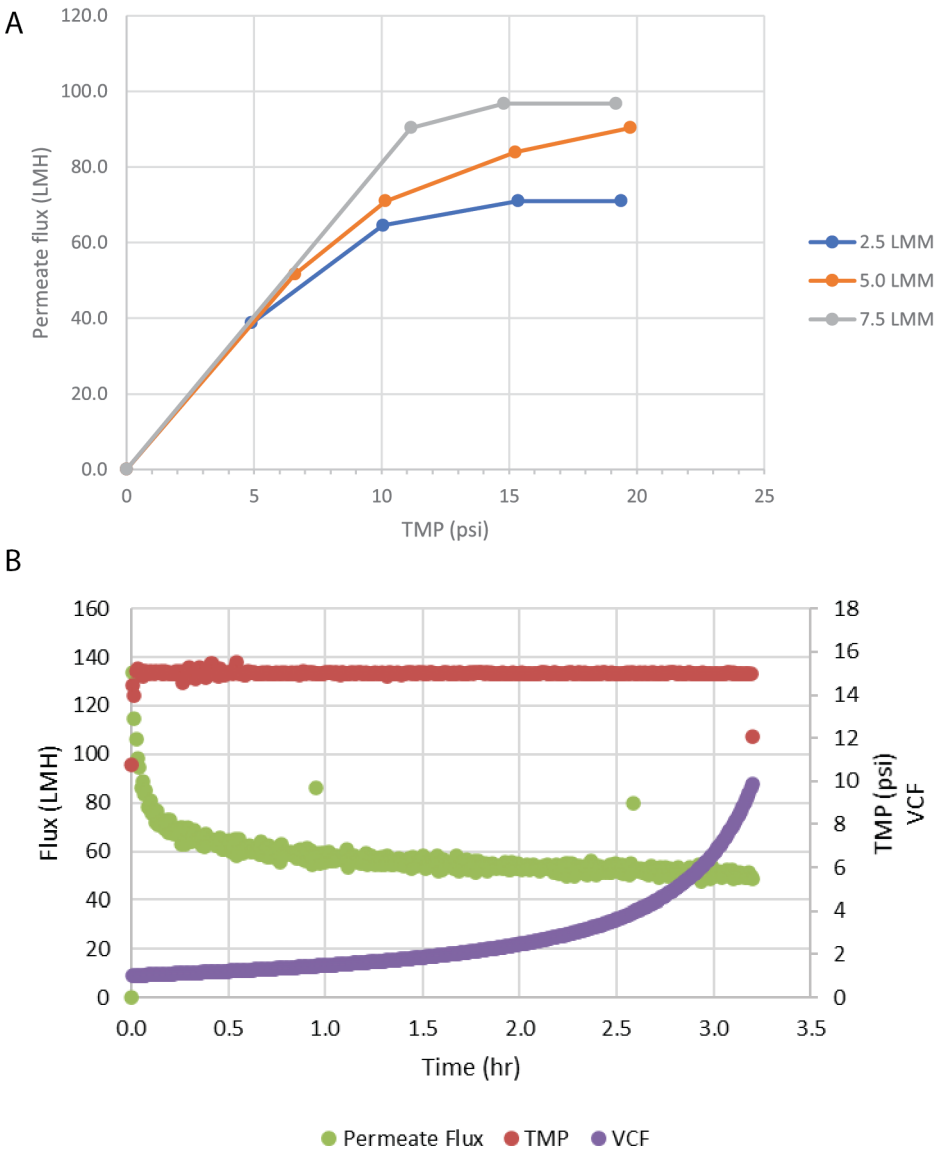
FIGURE 2

(A) Flux excursion evaluation of rAAV5 concentration. (B) Representative flux decay plot of rAAV5 concentration.



The affinity purified vector was polished to enrich for full capsids using Mustang® Q XT Anion Exchange membrane sorbent. The elution pool was diluted into Bis-tris propane (BTP) equilibration buffer to reduce the ionic strength of the material prior to polishing. After column washing, the virus was eluted from the membrane capsule using a ~1 mS conductivity step elution strategy. A representative elution profile is shown in Figure 3.

Elution fractions were analyzed for the gene of interest (the cargo) 'full capsids'

TABLE 3

Summary of rAAV5 concentration by ultrafiltration TFF.

Attribute	Data points	Average	95% confidence interval
Permeate flux	n=6	64 LMH	5.6 LMH
Yield (ddPCR)	n=6	91%	8.0%

using droplet digital polymerase chain reaction assay (ddPCR) and total capsids (AAV5 ELISA). Fractions that showed a higher absorbance at 280 nm than 260 nm correlated to low ratios of genome copies to total capsids, indicating a high percentage of empty capsids. The fractions with similar 280 nm and 260 nm signals showed a much higher ratio of genome copies to total capsids, indicating a higher proportion of full capsids.

When we calculate the mass balance from fraction pooling, we recover and carry forward close to 50% of the full capsids, but only retain 11% of the total viral particles. This results in close to a 5-fold enrichment of full capsids to total capsids.

Full capsid enrichment reproducibility

Mustang® Q polishing for full capsid enrichment was performed on five upstream batches, four of which were further analyzed via ddPCR and capsid ELISA. Figure 4 shows the normalized chromatograms for these five experiments.

Despite variation in the upstream conditions, we found the full capsid enrichment with Mustang® Q to be reproducible. Figure 5 plots the vector genomes (full capsids) for the load and the 5 peaks that were collected for analysis. Figure 6 plots the total number of capsids as determined by capsid ELISA for the load and the same 5 peaks of interest.

The combined peaks 1 and 2 reproducibly contain very little vector genomes (vg) (Figure 5), if any, and thus predominantly contain empty capsids. Peaks 3, 4 and 5 contain most of the genome content while the total number of capsids is low relative to combined peaks 1 and 2, indicating that these peaks are enriched for full capsids. To clearly demonstrate this phenomenon, we have divided the vg/capsid ratio of combined peaks 1 and 2 and combined peaks 3, 4 and 5, respectively, by the vg/capsid ratio of the load and plotted these in the Figure 7. The relative enrichment across processes is generally consistent and is consistent with differences in the UV traces.

Table 4 shows the capsid ELISA yield and vg yield of the empty (1+2) and full (3+4+5) peaks. The total capsid yield in the empty peaks (1+2) is 49 ± 10% while the genome (vg) yield is only 14 ± 8%. The capsid yield in the full peaks (3+4+5) is 19 ± 10% while the genome (vg) yield is 66 ± 13%. This clearly shows that combined peaks 3, 4 and 5 are enriched in full capsid relative the combined peaks 1 and 2.

FIGURE 3

Chromatogram of rAAV5 step elution from Mustang Q Membrane.

Step	LOAD	1	2	3	4	5	6
GC/mL	5.9E+10	1.0E+09	7.9E+10	6.1E+11	3.1E+11	6.8E+10	3.4E+10
Capsid/mL	4.5E+11	3.3E+11	5.0E+12	1.4E+12	4.8E+11	2.9E+11	5.4E+11
GC/Capsid	13%	0%	2%	44%	66%	23%	6%

Blue line represents OD280 absorbance (protein); red line represents OD260 absorbance (DNA); green line is the conductivity (mS/cm). rAAV5 titer based on genome copies (GC/mL) and capsid ELISA (Capsid/mL). The green box highlights elution peaks enriched for full capsids. Viral vector titers of each corresponding elution fraction are shown in the table below the chromatogram. The calculated full percent is also calculated.

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FIGURE 4

Superimposed chromatograms from elution fraction collection of individual Mustang® Q separation experiments.

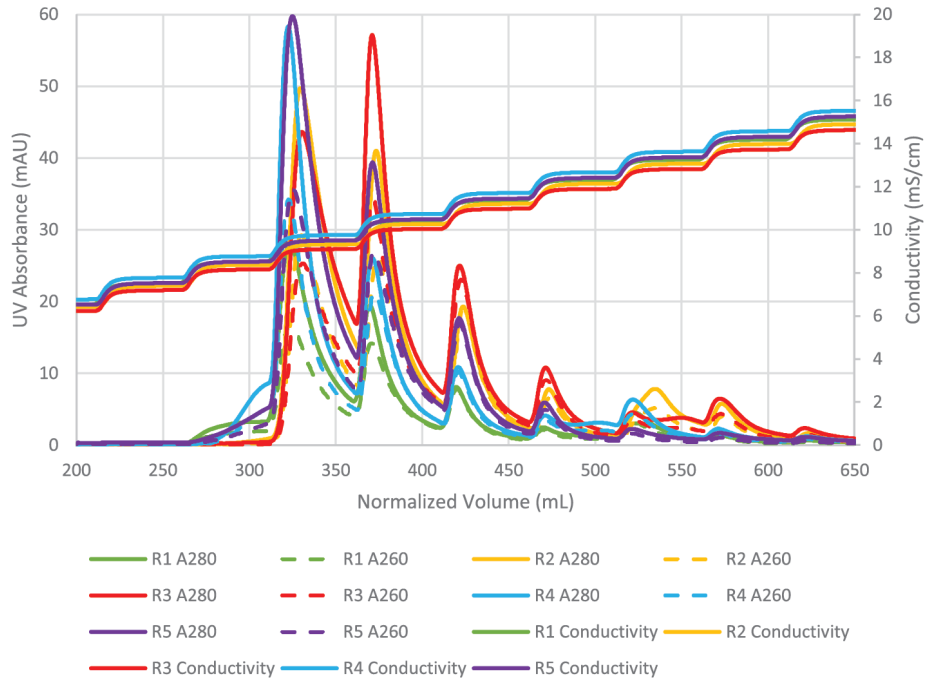
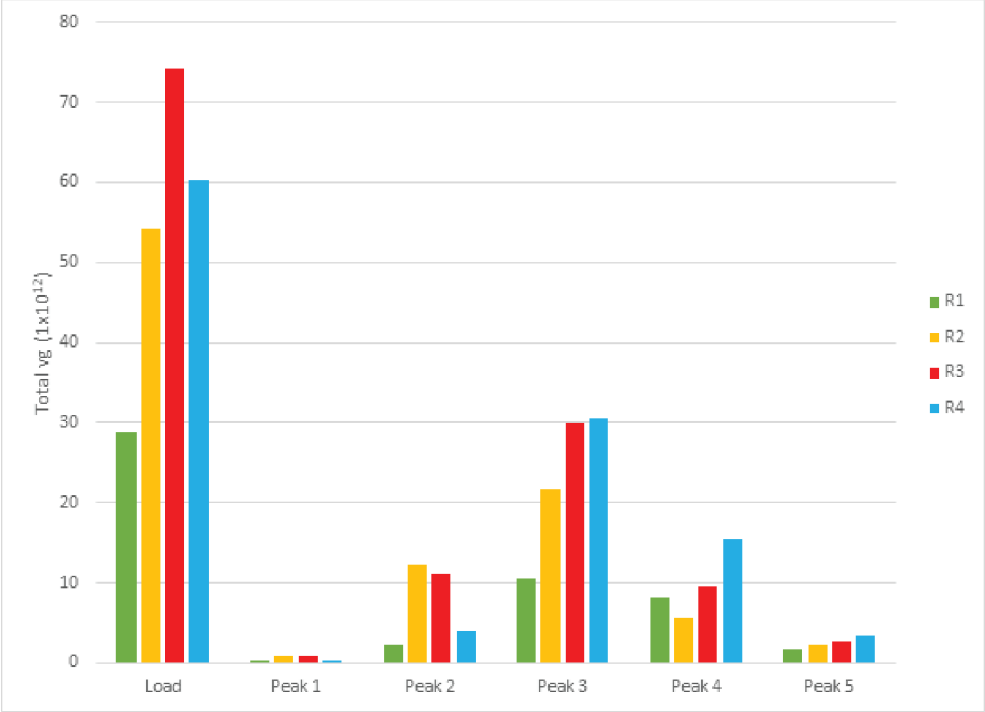


FIGURE 5

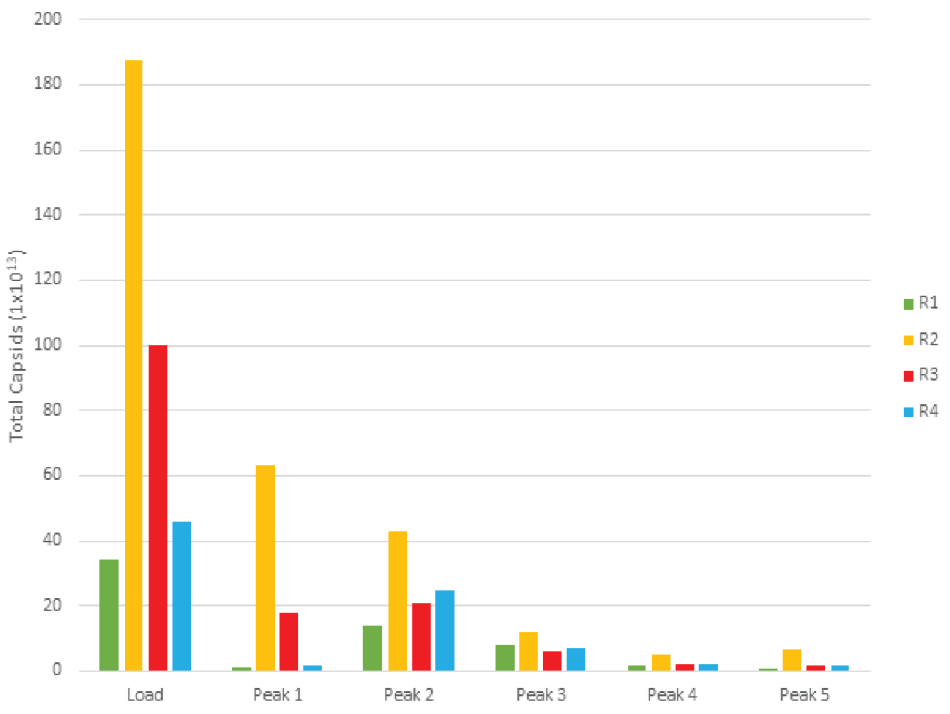
Mass balance of vector genomes during Mustang Q Polishing.



Total viral genome content in the Mustang® Q load material and distribution in each of the five elution peaks. Viral genome concentration determined by droplet digital PCR.

FIGURE 6

Mass balance of viral capsids during Mustang® Q Polishing.



Total viral capsid content in the Mustang Q load material and each of the five elution peaks. Viral capsid concentration determined by capsid ELISA.

Formulation

After Mustang® Q polishing, the next step in the process is to adjust the buffer and vector titer to the final formulation for clinical use. We evaluated the use of Omega 100 kDa single-use TFF filters for this ultrafiltration/diafiltration (UF/DF) step. The purified rAAV5 pool was concentrated to a targeted 10X volumetric concentration factor followed by a 7X diavolume buffer exchange into formulation buffer. The rAAV5 concentration/diafiltration was performed at a crossflow rate of 7.5 L/m^2/min and a TMP of 15 psi. Following diafiltration the filter was depolarized and drained, then flushed with 1.5X holdup volumes of formulation buffer.

Four UF/DF trials were completed using the process described above. Permeate flux measured throughout the concentration and diafiltration remained steady at ~200 LMH (concentration data shown in Figure 8). Virus concentrations were measured in the final permeate pools to measure virus retention. In three of four pools there was no virus detected in the permeate, the fourth pool was calculated at 99.9% virus retention. Virus yields averaged 89% over the four trials but were

TABLE 4

Average Mustang® Q polishing yield across processes.

Capsid yield				Vg yield			
	Empty peaks	Full peaks	Empty + full		Empty peaks	Full peaks	Empty + full
n	4	4	4	n	4	4	4
Average (%)	49	19	68	Average (%)	14	66	80
St dev (%)	10	10	14	St dev (%)	8	13	7

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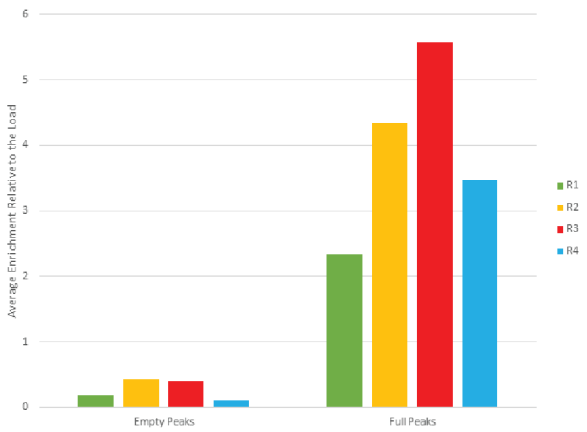
highly variable (95% confidence interval of 69%). We note that the virus and volumetric loading was relatively low due to the material available at these scales, averaging 2.4 x 10^{15} ± 8.9 x 10^{14} gc/m^2 and 11 ± 5 L/m^2 respectively. For a 2–3-hour process we would expect volumetric loading in the range of 150–250 L/m^2, and hypothesize that higher loading would reduce yield variability, but may also reduce permeate flux.

Final sterile filtration

The final step in rAAV manufacturing is to ensure patient safety by sterile filtration through a validated sterilizing-grade filter. We evaluated Supor® EKV for this final sterile filtration step. There was no significant pressure rise observed over the constant flow filtration experiments, though we note the loading was relatively low with the material available (<100 L/m^2). Virus concentration was measured in the feed and filtrate pools to calculate transmission. The results of 4 trials are shown in Figure 9 and Table 5 and show that high virus transmission (averaging 94%), can be achieved at final filtration with an EKV filter.

FIGURE 7

Full capsid enrichment by Mustang® Q Membrane.



Relative enrichment of the pooled elution fractions containing mostly empty capsids (Peaks 1 & 2) and those containing mostly full capsids (Peaks 3, 4, 5) normalized against the starting load material. Data collected from 4 separate bioreactor harvests (R1 – R4).

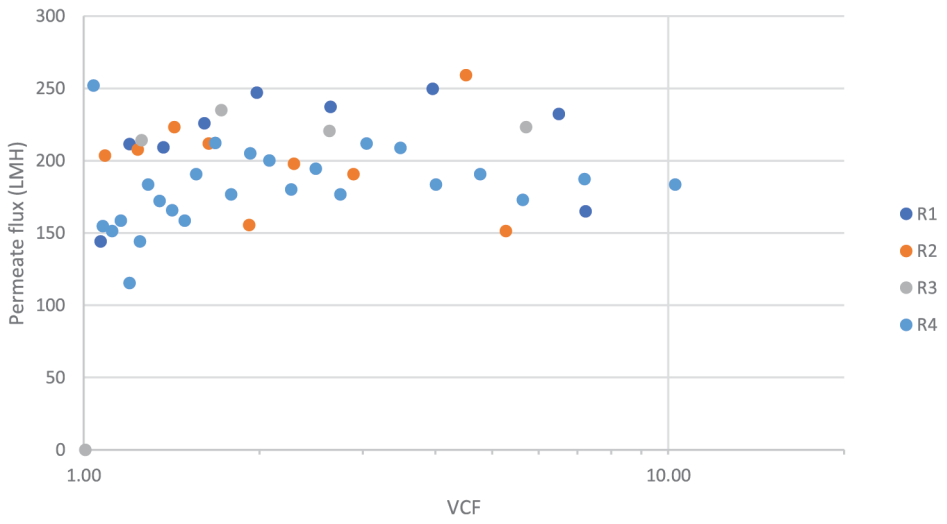
Total process yield & impurity removal

The step and cumulative vector yields from a representative run are shown in Figure 10.

In this run, we observed good vector yield in clarification, concentration, affinity purification and final sterile filtration. The yields observed during Mustang® Q purification

FIGURE 8

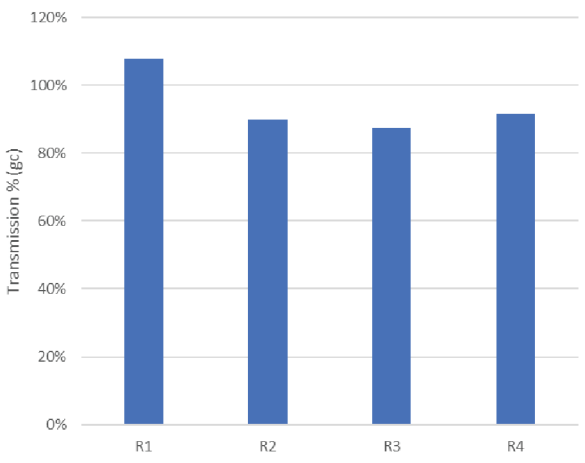
UF/DF of rAAV5, flux compared to VCF.



Correlation between permeate flux and volumetric concentration factor during 4 Ultrafiltration/Diafiltration experiments with rAAV5.

FIGURE 9

Transmission of rAAV5 during EKV sterile filtration.



Calculated transmission of rAAV5 during sterile filtration using EKV membrane filters.

show ~50% vector recovery; however, this loss was also accompanied by an 89% reduction of total capsids resulting in a ~5-fold increase in full capsid percentage.

Final formulation by UF/DF resulted in ~60% vector recovery. This less-than-ideal recovery was likely a result of this process being performed using an atypically low volume to surface area for this application. Based on the process flux measured here, final formulation by TFF could target a volume to surface area ratio of 250 L/m^2 in a 2–3 hour process. In this testing, we were limited by product volume and the resulting surface area to volume ratio was ~15 L/m^2. The pre-purification UF concentration of this vector resulted in an average yield >90% using the same filter, with a more appropriate volume to surface area ratio. We anticipate higher recoveries during scale-up as the relative impact of non-specific loss would be lowered.

The process was evaluated for contaminant removal including host cell proteins (HCP) and host cell DNA. Host cell protein concentration was determined by ELISA (Cygnus). Results are shown in Figure 11. Contaminant DNA was measured by Picogreen™ Assay (Thermo). Results are shown in Figure 12.

The data shown in Figures 11 & 12 show the contaminant HCP and DNA levels are both reduced to below assay limit of quantitation during Mustang® Q polishing.

The protein profile through the purification process was assessed by SDS-PAGE using Sypro™ Ruby fluorescent staining (see Figure 13).

The results from the SDS-PAGE gel show a complex protein mixture through clarification and TFF. As expected, affinity purification shows a substantial reduction in the number and intensity of contaminant proteins. There are only 3 significant bands observed in all samples after Mustang Q polishing. These three bands are the viral capsid VP1, VP2 and VP3 proteins. There are no other significant proteins found in these samples.

The results of the DNA, HCP and SDS-PAGE analysis show this purification scheme results in a very low contaminant profile.

CONCLUSIONS

We evaluated existing filtration and chromatography technologies commonly used in large scale recombinant protein purification, for the purification of a recombinant AAV vector (serotype 5). The purification strategy was based on the general platform process commonly utilized in large-scale industrial monoclonal antibody manufacturing including scalable clarification, affinity purification, ion-exchange polishing, tangential flow filtration and sterile filtration. Each unit operation was evaluated for vector yield based on droplet digital PCR, purity, and robustness.

TABLE 5

Summary of EKV sterile filtration performance for rAAV5.

Attribute	Data points	Average	95% confidence interval
Feed concentration	n=4	3.3 e11 gc/mL	1.5 e11 gc/mL
Transmission (ddPCR)	n=4	94%	9.1%

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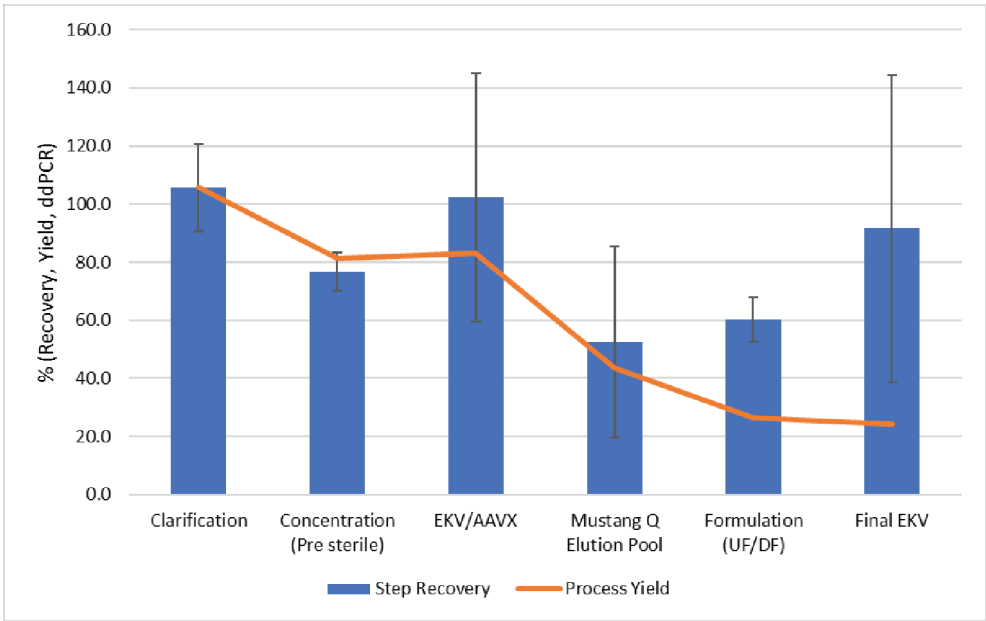
FIGURE 10

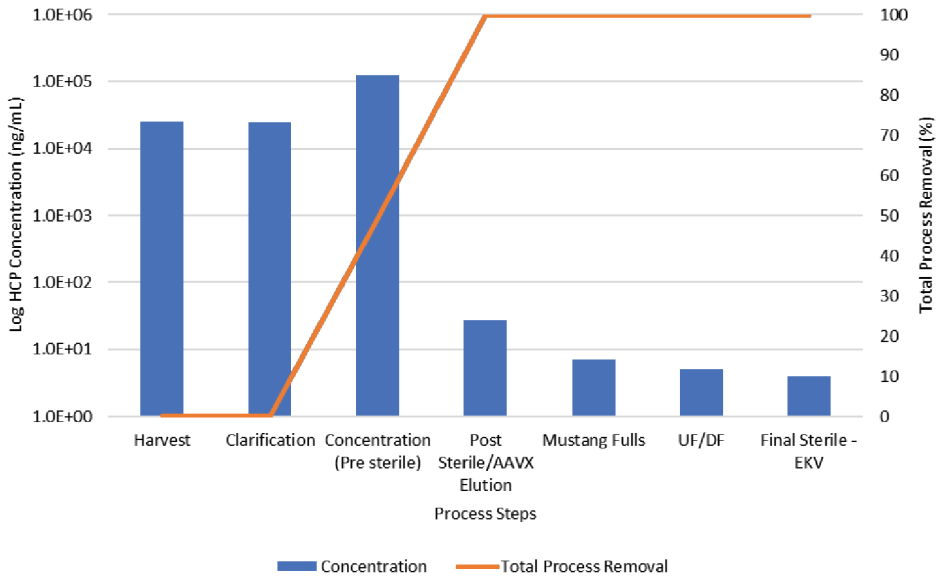
rAAV5 unit operation step recovery and cumulative process yield.



rAAV5 unit operation recovery as determined by ddPCR. Yellow trendline is the theoretical cumulative process yield of rAAV5 through described process scheme. Error bars represent 1 standard deviation.

FIGURE 11

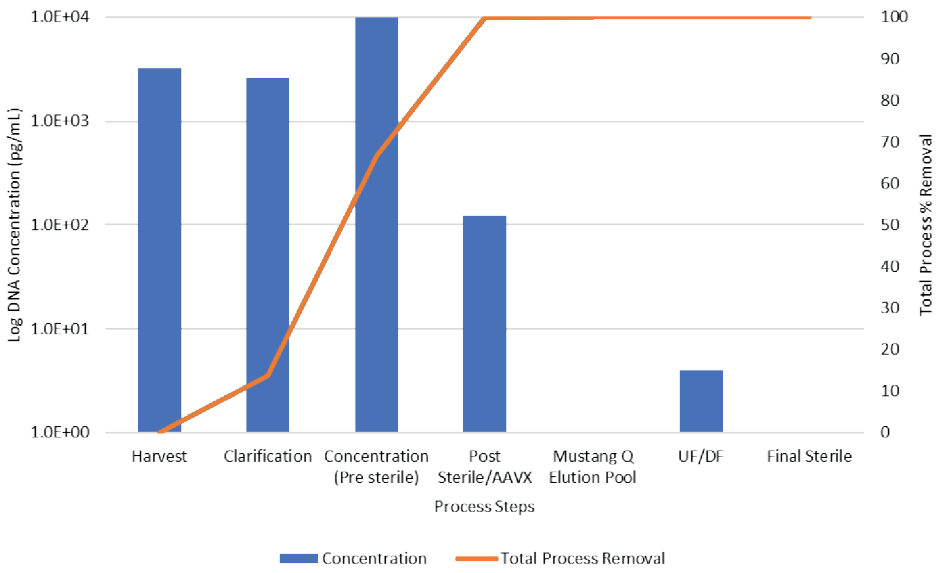
Host–cell protein ELISA results.



Host–cell protein contamination through rAAV5 purification. HCP concentration determined by Cygnus HCP ELISA.

FIGURE 12

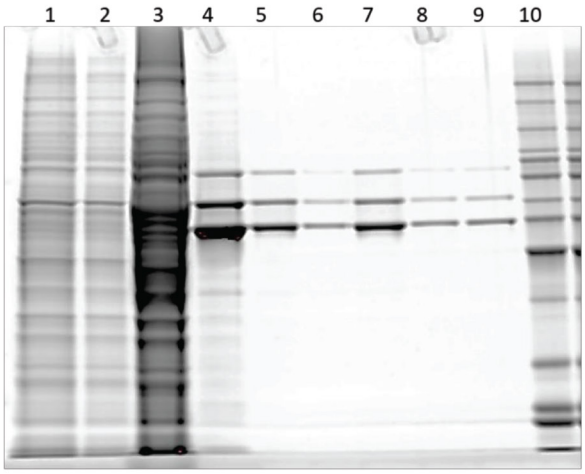
Contaminant DNA concentrations and cumulative removal.



DNA contamination through rAAV5 purification. DNA concentration determined by Picogreen assay

FIGURE 13

SDS-PAGE analysis of rAAV5 downstream purification.



Lane	Sample	VG/mL (ddPCR)
1	In-house AAV5 harvest	6.3E09
2	Post clarification	6.3E09
3	Post TFF	4.8E10
4	Post AAVX (Affinity)	1.0E12
5	Post Mustang Q (Empty Pool)	2.0E10
6	Post Mustang Q (Full Pool)	1.8E11
7	Post TFF/DF	3.2E11
8	Post Sterile (EKV)	3.0E11
9	Post Sterile (NGFF)	2.5E11
10	Ladder	N/A

10% Homogenous gel stained with Sypro Ruby fluorescent protein stain. Estimated viral genome loading calculated from ddPCR titer.

We observed an overall theoretical process yield of 25% full capsids (containing gene of interest) with a full capsid enrichment of ~5-fold compared to the total viral particles (total capsids including empty). This process resulted in rAAV material which had a very low HCP and DNA contaminant profile. Clarification, TFF concentration and final sterile filtration showed robust performance with average vector recovery >90%. The unit operations with the most vector loss were Mustang® Q polishing and UF/DF final formulation. We expect

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the total process yields to improve with further optimization, process understanding and scale-up.

TRANSLATIONAL INSIGHTS

The results presented here demonstrate feasibility of translating proven, scalable purification technologies used in recombinant protein manufacturing to the purification of viral vectors. Almost all the technologies employed are available from Pall Corporation. This enables a near complete end-to-end platform solution for recombinant adeno-associated viral vector manufacturing.

Next steps would likely include scalability of each unit operation, particularly for the final UF/DF and final filtration, where scale-up is required to further challenge the filters. To develop a full Quality by Design (QbD) manufacturing process would also require additional characterization of critical process parameters for each unit operation. A white paper outlining guidance on QbD manufacturing of rAAV products is available from Pall Corporation [14].

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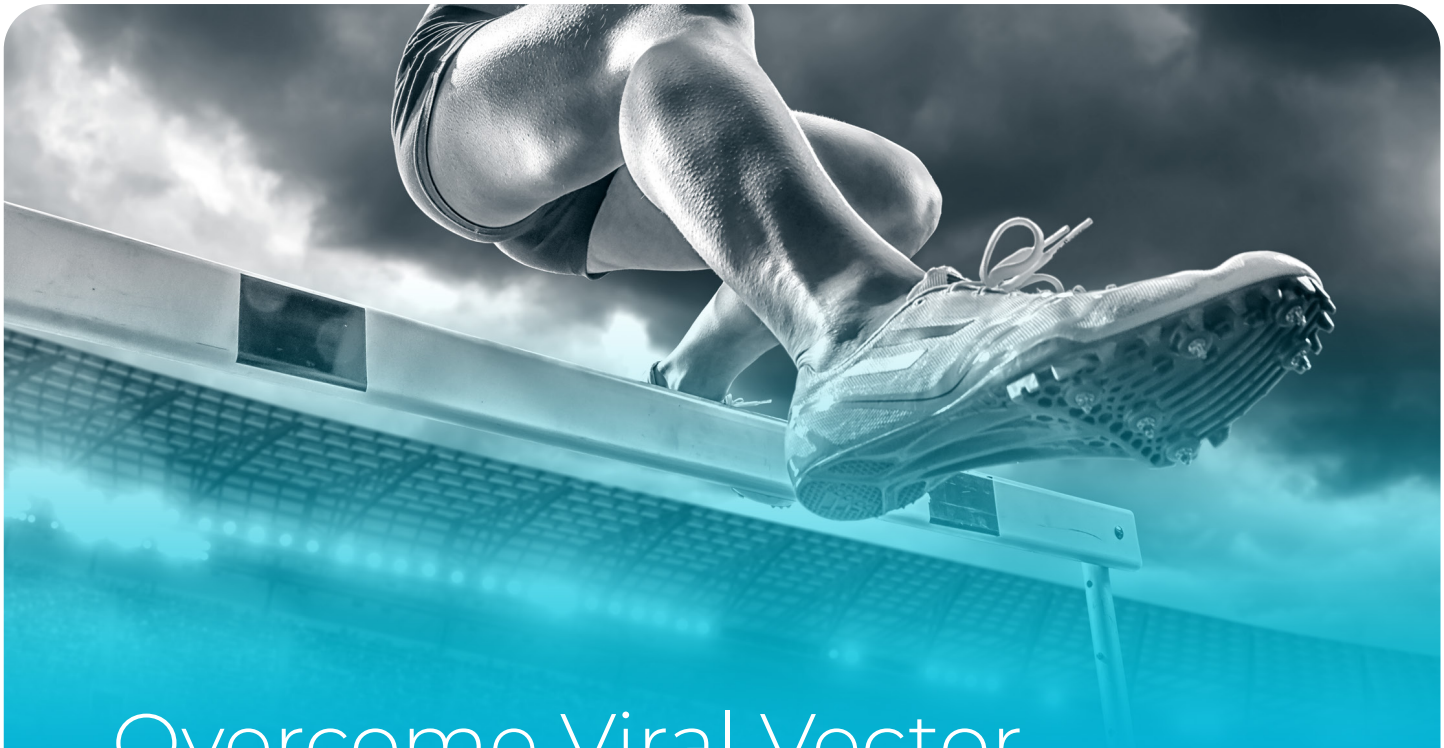

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CELL & GENE THERAPY INSIGHTS

DOWNSTREAM BIOPROCESSING

INTERVIEW

Current trends in AAV downstream bioprocessing and future considerations

MICHAEL MERCALDI is the Senior Director of Downstream Process Development at Homology Medicines. He is responsible for leading the development of Homology's purification and drug product manufacturing processes for their gene therapy and gene editing programs. He has held positions in process development throughout his career at MedImmune/AstraZeneca, Merrimack Pharmaceuticals and Codiak Biosciences before joining Homology. He holds a BS in Chemical Engineering from the Rensselaer Polytechnic Institute and a PhD in Biochemical Engineering from Tufts University.

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Q What are you working on right now?

MM: We are supporting and building up our clinical pipeline. We were able to help the company achieve its goal of having three clinical candidates by the end of the year. We recently had an IND amendment accepted for our lead compound HMI-102, which is a gene therapy already in the clinic for phenylketonuria, and we had two new INDs – one for

"We are thrilled about our first gene editing clinical program with HMI-103. This work is particularly exciting because it will leverage the ability of our AAVHSCs to perform gene editing through homologous recombination, which is one of the cornerstones upon which the company was founded."

our HMI-203 gene therapy for Hunter Syndrome and one for our first gene editing product, HMI-103, for phenylketonuria, accepted as well.

We are thrilled about our first gene editing clinical program with HMI-103. This work is particularly exciting because it will leverage the ability of our AAVHSCs to perform gene editing through homologous recombination, which is one of the cornerstones upon which the company was founded.

Furthermore, we are working on preclinical support for HMI-104, which will be our first gene transfer construct to develop an anti-C5 monoclonal antibody. For this program, we are using the patient's own liver as an antibody factory. It is a novel use of AAV gene therapy, and we are enthusiastic to work on it.

Beyond that, we are also investing significantly in platform development. We have a great production platform already, but we are always considering how we can make it better.

Q What can you tell us about the key downstream bioprocess considerations for Homology Medicines' product candidates?

MM: Our group's goal is to develop highly productive purification and formulation processes that can deliver high purity, high quality, and stable material for patient use.

We ensure our processes are robust and can be easily transferred and executed in manufacturing, which is something we really pride ourselves on. If it can work at the bench but can't work in manufacturing, then it's not going to work, so it's important to always keep manufacturability in mind.

To do this, we have to understand how the construct performs in our process platform. We have invested considerably to build a "Plug and Play" process platform. The creation of this platform was a team effort and result of our fully integrated CMC capability. We were able to get significant support from colleagues in our upstream, analytics, R&D and manufacturing groups to make it a reality. Without the high degree of integration and collaboration we would most likely not have as good of a platform as we do right now.

The power of the platform is that we can take new constructs and serotypes, leverage our platform conditions, and obtain high-quality product with limited development work. This facet of

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the platform gives us a massive speed advantage in that it allows for rapid development and tech transfer.

If we do undertake any development work then it's usually to further refine, optimize or expand our knowledge. This platform provides a significant upside opportunity to accelerate our timeline, allowing us to move quickly towards manufacturing. By using our platform we can streamline the CMC timeline and get promising new candidates into the clinic faster.

Q Give us your assessment of the current technological state of the art in AAV downstream bioprocessing - what have been the valuable recent advances, and where is further innovation needed?

MM: I think the state of the downstream process for AAV has advanced considerably over the past few years. One trend we've been seeing is the alignment of processes within the industry, which consists of harvest affinity chromatography, anion exchange chromatography, and then a final formulation step.

If more companies are using the same basic process, then the field can gain a better understanding of the AAV products and manufacturing. From this alignment, continued improvement in process yield and purity will arise, which is important when developing a better purification process.

We believe this will enable better, more accessible products in the long run, which will be great for the industry, and will ultimately benefit patients.

More specifically, we are seeing the industry moving away from ultracentrifugation and towards anion exchange chromatography for empty capsid removal, which is, I believe, a huge accomplishment for the field. In fact, this is something we have had to do ourselves. Our anion exchange step delivers a product with comparable purity to the ultra-centrifugation process, overcoming a big challenge.

The advantage of moving towards anion exchange chromatography is that downstream processes can be executed in a more manufacturing-friendly and scalable manner, which cannot be achieved easily with ultracentrifugation. Switching to anion exchange chromatography means that larger batch sizes are now within reach. Having a scalable purification process means that bioreactors will also have to be scaled-up. This is something we have already started to do, and we can now go up to a 2000L bioreactor scale. That, coupled with a scalable purification process, means it is possible to make these therapies more accessible to larger patient populations.

Q More specifically, what are your thoughts regarding final formulation and fill-finish?

MM: You hear a lot about the purification and upstream processes but you don't hear a lot about the formulation and the fill-finish process. We really believe that this is an untapped space in the field.

> "We are noticing with our high process productivities that we need to become more sophisticated in our approach towards formulation fill-finish and clinical distribution."

Today, most companies are doing a lot of small volume fills since vector has been limited, which means productivity is a challenge.

We are noticing with our high process productivities that we need to become more sophisticated in our approach towards formulation fill-finish and clinical distribution.

To do this, we're moving towards 2 to 8°C liquid formulation, with our novel high-stability formulation. Most of the field has been using -80°C storage to keep the vector stable. Either they have limited data, a limited amount of material to do the studies, or their product is just not stable at elevated temperatures.

-80°C storage is fine for small patient populations where transport and storage can be kept under better control. However, for large patient populations, this can become expensive and challenging to manage. To alleviate this, we are focusing on developing and executing a 2–8°C clinical distribution supply chain.

Implementing this will give hospitals and pharmacies a significant logistical advantage, as they don't need to worry about procuring, operating, and maintaining -80°C storage systems. We think focusing on cold liquid storage is going to make these therapies a lot more accessible for larger patient populations.

Q Can you comment on the potentially conflicting drivers of increasing efficiency versus increasing sensitivity or robustness of downstream vector processing? And how is this reflected in today's purification toolkit?

MM: This is the purification scientists' major dilemma; how can I balance purity and yield? Is it possible to get both? Of course, that is what everyone wants.

The approach we have taken is to focus on purity first, as that may have an impact on the patient safety profile. By focusing on purity, we believe that we can get trials started and start to see how the drugs are performing in the clinic. This will allow teams to work towards improving yields on a less critical timeline.

However, both need to be pursued in parallel. It's easy to say, 'just focus on purity'. You could develop a process with very high purity but if you have low yield, you may need hundreds of batches to dose one patient, which means you don't really have a good manufacturing process.

We have been able to successfully balance this by ensuring we have high-quality vector that is made using a high-quality platform. This has allowed us to meet clinical demands and will enable us to meet our future commercial demands as well.

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Q How would you frame the key issues and priorities with analytics for downstream AAV processing?

MM: In my opinion, analytics are crucial for developing a high-quality downstream process. They are the eyes and ears of the process. Without good analytics it's difficult to make decisions to create a high quality process.

At Homology Medicines, we have an experienced analytics team who have developed the tools to be able to make effective decisions to define our processes to ensure a high level of product quality. Our early (and continued) focus on product quality has been crucial in allowing us to execute 3 successful INDs, even while the regulatory agencies have been increasing expectations.

The biggest challenge for analytics right now in the field is cell-based potency. This has been emerging as a top priority for all the regulatory agencies as well, which we are acutely aware of. With our current platform, we have two orthogonal potency methods of infectivity and gene expression, which allow us to rapidly assess potency with more accuracy.

We believe the field has a good grasp on biochemical assays such as titer, purity – anything that's not a biological assay. Many of these biochemical analytics were leveraged from the recombinant protein space. This has enabled us to get these assays up and running very quickly. In the future, however, I believe we will start to see analytics developed that are much more specific for assessing whole virus particles.

Examples of emerging virus particle analytics are whole-particle mass spectrometry and analytical ultracentrifugation. There is starting to be emerging reliance on analytical ultracentrifugation to assess capsid packaging profiles in the field. I think that when we start looking at more analytics developed for the virus particle, that's when we are going to increase our understanding, and ultimately make better products.

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

MM: We will continue to deliver and grow the pipeline. Through leveraging of our platform, we will continue to quickly develop and prepare new product candidates for clinical manufacturing.

More specifically, we are going to be working towards building our late-stage and commercial plans for HMI-102, our lead drug to treat phenylketonuria. We are anticipating dose expansion data by the middle of 2022,

"...analytics are crucial for developing a high-quality downstream process. They are the eyes and ears of the process. Without good analytics it's difficult to make decisions to create a high quality process."

which means we will need to start preparing for commercial manufacturing. This means that we will need to support commercialization efforts by working on process characterization and process validation.

In parallel, we will also be supporting the supply for HMI-203, our Hunter syndrome candidate, and HMI-103, our gene editing phenylketonuria candidate, while also supporting the organization through preclinical development of HMI-104, which is our gene transfer anti-C5 monoclonal antibody construct.

Finally, outside of our core business, our group will also continue to improve our platform. We are looking at ways to reduce cost of goods by eliminating, intensifying, and streamlining our current operations.

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Development of a scalable adeno-associated virus purification process for gene therapy

Åsa Hagner McWhirter, Principal Scientist, Bioprocess applications R&D, Cytiva

Adeno-associated virus (AAV) has become the main vector for gene therapy, but scalable, cost-efficient, and robust filtration and chromatography-based processes are required for purification. This poster will describe purification process development of AAV2 and AAV5 serotypes, including harvest by cell lysis, clarification, concentration and buffer exchange, affinity capture, and finally anion exchange polishing to reduce the empty capsid product impurity.

Each step of the purification process for AAV2 and AAV5 was evaluated and optimized, initially at a small scale.

HARVEST

This was carried out directly in the bioreactor with 0.5 % Tween™ 20, 300 mM NaCl, 1 mM $MgCl_2$, and 40 U/mL Denarase™. This mix was incubated in the bioreactor at 37°C for 4 hours, before clarification by normal flow filtration, with a recovery of 74–80%.

CONCENTRATION AND BUFFER EXCHANGE

Tangential flow filtration with hollow fibers was used for concentration, with a 300 kDa cut-off proving most effective. Recovery was approximately 75–80%.

CAPTURE

Capture was done using affinity chromatography with Capto™ AVB. We found that AAV2 and AAV5 have different optimal conditions **(Table 1)**. For AAV5, recovery of virus particles was better with glycine compared to citrate. The presence of salt negatively affected elution, even at reduced pH.

The eluate from the Capto AVB was very pure. For AAV2, the eluate showed a strong virus band but no detected HCPs, whereas the flowthrough fraction showed a lot of HCPs but no virus protein **(Figure 1A)**. Additionally, the TEM image shows the high purity of AAV2 particles **(Figure 1B)**. Similar results were observed for AAV5.

Table 1. Affinity capture protocol for AAV2 and AAV5.

Step	Volume	Buffer/sample
Equilibration	5 CV	20 mM Tris, pH 7.8 + 200 mM NaCl
Sample load	170–215 CV	TFF retentate. Load 1-3 x 10^{14} VP/mL resin
Wash	10 CV	20 mM Tris, pH 7.8 + 200 mM NaCl
Elution AAV2	4 CV	50 mM citrate pH 3.5, 500 mM NaCl, 500 mM arginine
Elution AAV5	5 CV	50 mM glycine pH 2.7

POLISHING

Ion exchange chromatography was used to reduce empty capsids. As full capsids have a slightly lower pI compared with empty capsids (5.9 vs 6.3), the charge difference can be used in anion or cation exchange chromatography with salt elution **(Figure 2)**. In cation exchange, the full capsids are less charged than the empty capsids and therefore elute first. In anion exchange, the less charged empty capsids elute first. We also found that high concentrations (15–20 mM) of $MgCl_2$ are needed to maximize separation and achieve a high percentage of full capsids with high viral genome recovery.

ANALYTICS

The main key considerations when using analytic assays are detection range, assay variation, accuracy, and matrix/sample buffer interference. As different buffers can affect the assay, orthogonal methods should be used for full/empty capsid analysis and throughput, and processes should be automated as much as possible.

We have also developed Biacore™ assays for AAV2 and AAV5 using surface plasmon resonance. This is less hands-on than the ELISA assay and has been shown to be stable and robust.

SUMMARY

It is critical to obtain harvest material with high levels of full capsids. Conditions for affinity capture may differ by serotype. Analytics are critical and the most challenging analytics is the full–empty analytics. We have developed a scalable AAV5 purification process, which is GMP compatible.

Figure 1 (A) Fluorescent multiplex SDS page and Western blot of AAV2 and AAV5. (B) TEM image of AAV2 viral particles in Capto™ AVB eluate.

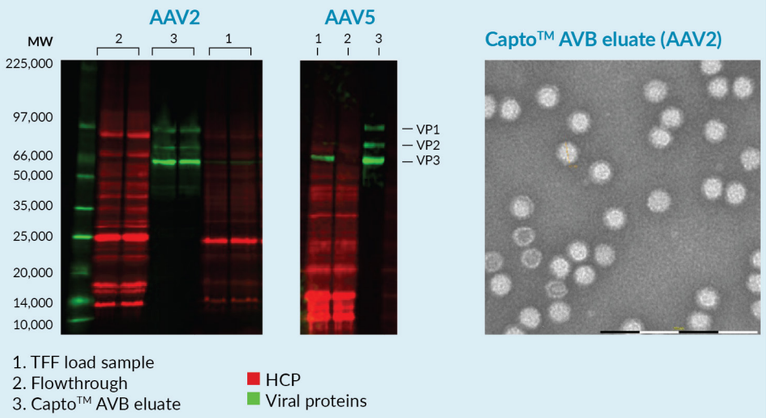
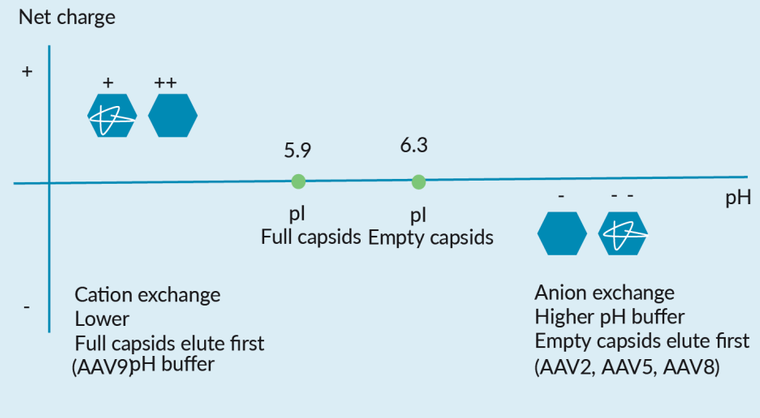


Figure 2. Ion exchange to reduce empty capsids.



CELL & GENE THERAPY INSIGHTS

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DOWNSTREAM BIOPROCESSING

VIEWPOINT

Titration for research grade rAAV: understanding challenges and minimum requirements

JAVIER F ALCUDIA,
Director, Stanford Gene Vector and Virus Core

"...when designing the vector, it is not always clear to the user what is needed from a manufacturing standpoint to make a product that will be suitable for patient use."

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The AAV (adeno-associated viral) vector field has expanded dramatically over the past few years with an increasing number of people using this technology for research purposes. Despite this, many are still using recombinant AAV (rAAV) as a tool, rather than understanding its function as a virus. Furthermore, many demand a high titer without firstly gaining an in-depth understanding of the process it takes to achieve it; the process to obtain a viral preparation in the range of 10^{12} to 10^{13} is very different to achieving titers over 10^{15}. Therefore, it is essential that users become more aware of these differences.

Viral preparations in the laboratory are done on a small scale for high throughput testing and as such, can be produced in a relatively short timeframe. Occasionally, users do introduce extra quality control (QC) steps, mimicking those that are commonly and routinely used in industry manufacture. Although there is nothing wrong with introducing these steps, these extra processes are not particularly required for research-grade vector material: the key requirement in the laboratory is a viral preparation that can be produced rapidly and quickly tested, as well as being able to function in both in vitro and in vivo models. Therefore, it is important for users to keep in mind the minimum criteria required for a viral preparation.

Another area of importance is understanding the range in titration for rAAV, especially between different laboratories. Each laboratory uses its own reagents, protocol, etc., thus creating diversity between viral titers. Inverted terminal repeats (ITR) are used as a main standard sequence to determine titers for rAAV. However, due nature of the sequence (repetitive, high GC content) it is difficult to obtain reproducible and accurate titers when comparing samples from multiple sources. Therefore, we would discourage this and would strongly recommend using an internal probe.

My team at Stanford University has been validating protocols for a number of different users. We have now achieved a protocol based on qPCR and hydrolysis probes that gives accurate viral titers using a set of internal sequences present in most rAAV. Currently, we are in the final steps to validate a digital platform (dPCR) system allowing us to do multiplexing on the same sample tittering with different probes for better accuracy. Additionally, we use in all our assays the reference material from ATCC (American Type Culture Collection) as well as an internal rAAV preparation control that we have been validating extensively over the past few years.

Differences in titration for the same virus between laboratories is a further challenge. Occasionally, the titration numbers can vary significantly between laboratories such that when receiving viruses from other cohorts, validation of the protocol is required. It is critical that accuracy is maintained here, especially when researchers have to use rAAV from different sources to dose their animals for in vivo studies.

One of the complications we face in our laboratory is the fact we are producing multiple rAAVs every week. Therefore, it is necessary to have a platform that can be applied across several different rAAVs produced with multiple serotypes. However, it is difficult to find a probe that has been validated to the extent that it is suitable for all the potential combinations from a user.

It is vital for us to build more awareness and educate users about these challenges, helping them appreciate the complexity of the process. It is also necessary that users are aware, from the start of the process, what the limitations are and the multiple steps that need to be taken to reach the end goal. Importantly, not all processes and steps conducted in the laboratory to achieve a research grade viral vector are transferable to later phases of GMP manufacturing.

As the field is rapidly evolving, there is little time for newcomers to the field to develop an in-depth understanding of the requirements and criteria for an AAV product that will one day be commercially approved and available

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for patients. In fact, when designing the vector, it is not always clear to the user what is needed from a manufacturing standpoint to make a product that will be suitable for patient use.

In an ideal scenario, a platform would be developed that is transferable between systems, allowing AAV vectors to be produced in the laboratory, data to be obtained and validated, and a proof-of-concept study to then be undertaken. Using the same platform, the process can then be moved to GMP. However, currently, the system we are using is either not scalable or not transferable, meaning the entire process must be started again from the beginning.

BIOGRAPHY

Javier F Alcudia has been serving as the director of the Stanford Gene Vector and Virus Core since 2015 producing thousands of custom rAAV preparations for users across the world. As a molecular and cellular biologist, he obtained his PhD in Biomedicine from the University of Barcelona and did his postdoctoral training in the dermatology department at Stanford.

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CELL & GENE THERAPY INSIGHTS

DOWNSTREAM BIOPROCESSING

INTERVIEW

The route to downstream process and analytical innovation for efficient AAV vector manufacture

MATTHEW ROACH Matt leads the AAV Process Development group at Precision BioSciences, which is focused on designing and implementing new strategies for the production and purification of adeno-associated virus. Matt completed his Bachelor's degree in Biological Sciences at North Carolina State University and his Master's degree in Microbiology and Cell Science at the University of Florida. Prior to Precision, Matt spent time at Pfizer working on the purification of AAV and the Biomanufacturing Training and Education Center training industry professionals on downstream bioprocessing operations.

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Q What are you working on right now?

MR: We are continuing development of our AAV production process, with an emphasis on ensuring that it remains a platform. We have a diverse portfolio of products that require various serotypes and transgenes to achieve our desired clinical outcomes, and we

"overall, we are most concerned with purity and safety. We don't just need to produce enough product - we need to produce enough of a safe and efficacious product."

need to be prepared to support that with a robust process and with robust analytics that are fit for purpose to measure the specific quality attributes we are looking at.

More specifically, we are spending a great deal of time optimizing our polish chromatography step for each serotype - running through screening of columns and buffer conditions, and then poring over the analytics required to determine sufficient purity.

Q What would you pick out as the considerations in downstream processing of Precision Biosciences' gene therapy product candidates?

MR: Purity and safety are paramount. I think we have seen this reflected throughout the field over recent times, with the FDA's CTGTAC committee toxicity meeting, for instance, really bringing this issue to the forefront. Additionally, recent clinical trial outcomes at other AAV-based gene therapy companies mean that our focus must be on ensuring we have a safe product, first and foremost.

We are also focused on demonstrating efficacy for our product, of course, and what drives that efficacy. That work centers on determining which quality attributes are measurable and how they correlate with infectivity or potency.

Recovery is also key, but it is dependent on a program and the dosage. For example, if we think about an eye disorder or a liver disorder, we don't need to produce as much vector as we do for a muscular disorder.

So recovery may be more key for some programs than others but again, overall, we are most concerned with purity and safety. We don't just need to produce enough product - we need to produce enough of a safe and efficacious product.

Q Where do you see progress being made in streamlining AAV vector downstream processing, particularly through automation and/or reducing the number of process steps?

MR: I think automation is an interesting one. It is talked about a lot, and it's considered the ideal because it eliminates some of the risk with manual operations. But it also requires a large amount of time and resources to implement.

To date, I have mainly seen companies moving towards automation as a product approaches commercialization - at Phase 3 and beyond - and foregoing it for early-stage

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manufacturing to save time and reduce risk prior to knowing whether a product will be successful in clinic. If you built it in as a platform early, and it is a genuine platform, there is no reason why you couldn't implement it early on and have it in place for those early-stage programs. However, I haven't seen a lot of companies do this - I just don't think the field is far enough down its development path yet.

I do see a lot of trimming of process steps, though, and that's something we have done, too. If we can reduce the number of steps overall, firstly our recovery is probably going to improve significantly just because of the reduction in how much we are manipulating the product. But it also just makes manufacturing easier and makes the product more manufacturable, which increases the likelihood of success.

Q How are the potentially conflicting drivers of increasing the efficiency versus the sensitivity/robustness of downstream vector processing reflected in today's purification toolkit?

MR: These two things definitely conflict quite often for us. We want efficiency and to be able to run at optimal conditions that may be very specific. But we also need the process to be robust so we can actually manufacture this product in a manufacturing setting, where systems might not be able to achieve a given specification.

I really focus on this more in terms of optimizing the robustness that the overall platform can achieve. We want our platform to be able to achieve good quality production and purification of different serotypes, different transgenes - basically, anything we throw at it.

In terms of the specific steps that will get us to that optimal robustness and efficiency, we have explored the whole design space to identify our optimal ranges - a larger design space than what we are currently running with. We have chosen to run with a specific, much smaller window within that box. That is a lot easier said than done, especially with early-stage programs, but it is our goal.

Q Can you frame for us the key issues with vector manufacturing assays/analytical tools currently and promising potential avenues towards addressing them moving forward?

MR: I think the biggest issue is that we are still so early in figuring out what some of these critical quality attributes are, how to measure them, and what they really mean. If you think about what has traditionally been measured for a monoclonal antibody, for instance, there are some things that we assume will also be really impactful for vector manufacture, or really impacted by vector process conditions, that maybe aren't. This then becomes challenging because it's a whole new side of the field.

We have seen a lot of progress already, though – for instance, with PCR: *the* key assay for AAV as it determines dose. I think the transition from qPCR to ddPCR has in my experience generated much better results. That gives me hope as we move towards the next wave of

"I think the biggest issue is that we are still so early in figuring out what some of these critical quality attributes are, how to measure them, and what they really mean."

analytical tools. And as the analytical tool providers share more information around what they are developing, you can already see this new generation manifesting in additional analytics such as viral particle titer. There are a number of solution providers coming out and competing to try to provide tools that require less time or that are high throughput, but that also require low sample volume or low viral genome content to produce a result. So I think the focus is on the right things - it's just going to take a little bit of time for the more advanced techniques to really come through.

Q What for you are the key questions for gene therapy developers and manufacturers in terms of viral clearance and adventitious agent control at the moment? And do you have any related advice or best practices to suggest?

MR: People just entering the viral vector manufacturing space from biopharma are often alarmed by the prospect of having to show they have cleared a virus from what is a viral product. And there was some initial concern around that, but there have been numerous groups now that have shown that it's not only possible, it's the logical thing to do, and perhaps not as scary as it initially sounds. With that in mind, the advice I would suggest is thinking about viral clearance early and often in development.

For one thing, the US FDA requirements differ from EMA requirements in terms of the stage at which you need to have the supporting data. The FDA requires data later on, near Phase 3 trials, whilst the EMA requires it prior to initiation of a clinical trial. So for the EMA, you definitely have to think about it very early and probably really often.

However, I think that's also the right strategy for the US FDA, even if it's not strictly required, because you don't want to have to implement an additional step or change your process significantly later in order to achieve a higher log reduction value of the given model viruses. The easiest time to change the process is at the beginning, obviously, so whether it's introducing a detergent step to knock down enveloped viruses, or something like low pH hold off capture chromatography, it's important to get a hold of it early on, and to plan to meet those regulatory requirements in advance.

Q Can you go deeper on how and where you see the field making progress in reducing the amount of final vector product required for QC and release testing? And how to drive further improvement here?

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MR: As I mentioned earlier, I have seen encouraging improvements in this area lately. The first example that comes to mind is the development of new analytical equipment like the Stunner from Unchained Labs. This instrument uses two microliters of sample to generate viral genome titer, viral particle titer, percent full, and aggregation, amongst other results, and it can get through an entire plate in under 30 minutes. We have used this instrument and found it to be really useful, and it's kind of a model for what we need: we need things to be measured really quickly because we are moving through experiments rapidly.

In general, I think the bottleneck is analytical. That's not in the least a slight to my analytical colleagues - it's an extremely hard task they have been set.

However, I do see that the vendor community is driving improvement and importantly, they are starting to see that there is a unique opportunity for them in the gene therapy market. I think this has changed quite dramatically inside the last 4–5 years: the need for innovative tools is far more evident today, and the teams using the equipment are more eager and willing to try new technology. From what I've seen and heard, that's actually quite a refreshing change for the vendors, certainly compared to the world of traditional biologics.

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

MR: Our overall goal as a company is to progress some of our *in vivo* gene editing products into the clinic. Some of these programs will use AAV while others will use our lipid nanoparticle technology. Overall, our aim is to treat patients with a safe product that is effective and hopefully cures some of the debilitating diseases that we are up against.

More specifically around my own team's work, we want to achieve a successful tech transfer into our internal manufacturing facility in Durham, North Carolina, and to continue tackling problems quickly on the development side as they appear. I think that is something my colleagues in viral vector process development will fully understand. We can't always predict what issues are going to crop up in the next year or two, but we can work on having the right processes and analytical toolkits to hand that will allow us to address them.

"...the need for innovative tools is far more evident today, and the teams using the equipment are more eager and willing to try new technology.."

AFFILIATION

Matthew Roach,
AAV Process Development Team Leader, Precision BioSciences

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INTERVIEW

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EXPERT INSIGHT

No cell left behind: engineering gene therapies for cross correction

Jill M Weimer & Jon J Brudvig

First-generation AAV gene therapies generally transduce a minority of target cells and may thus have limited efficacy. Cross correction strategies offer a solution to this problem by turning transduced cells into therapeutic protein "factories" that deliver transgene product to both themselves and more broadly to their untransduced neighbors. Multiple engineering strategies can enhance cross correction, with several preclinical programs demonstrating marked efficacy improvements in animal models. Cross correction strategies are furthest along in preclinical development for secreted lysosomal hydrolases, which can be delivered by endogenous surface receptors. Cytosolic proteins, transmembrane proteins, and mitochondrial proteins are more challenging targets, but new developments in cell-penetrating peptides, exosomal targeting, and tunneling nanotubes are opening the door for cross correcting a wide-variety of intracellular locales. Thus, while wild type gene therapies will enter the clinic first as important treatments, new cross correction strategies may greatly enhance therapeutic efficacy in subsequent next-generation therapies.

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While dose-limiting toxicity presents what is perhaps the principal safety concern for AAV gene therapies, the closely related issue of incomplete transduction is often the most limiting factor for efficacy. For loss-of-function genetic diseases, in particular, an ideal gene therapy would restore expression of a disease protein within every target cell. This is a far cry from even the most optimistic estimates of transduction; sustained expression

is generally achieved in less than 10–50% of target cells in human patients and nonhuman primates treated with high-dose AAV vectors [1–3], and capsid engineering has thus-far made only incremental improvements. Given this limitation, many first-generation gene therapies will leave a majority of target cells untreated, yielding suboptimal efficacy.

Cross correction strategies are an attractive solution to this problem. Rather than attempting to transduce every target cell, cross correction works within the confines of transduction limits to turn transduced cells into therapeutic protein 'factories' that deliver transgene product to their untransduced neighbors, both locally and distally. This offers the potential to treat a far greater cell population while reducing dose requirements and has thus been the subject of intense interest in recent years. Thankfully, the field is on the cusp of breakthroughs with next-generation cross-correcting gene therapies showing marked efficacy improvements in animal models. At Amicus Therapeutics, we are developing a broad toolkit of cross correction technologies with the goal of eventually addressing a wide range of lysosomal, cytosolic, and transmembrane targets. These tools will be a key part of the technological foundation for Caritas Therapeutics, a next-generation genetic medicine company that Amicus is planning to launch in late 2021/early 2022.

ENGINEERING CROSS CORRECTION FOR LYSOSOMAL HYDROLASES

For some disease proteins, endogenous export and import mechanisms can achieve a limited extent of cross correction without any intentional engineering. Many lysosomal hydrolases, for example, are post-translationally modified in the Golgi apparatus to contain terminal mannose 6-phosphate (M6P) residues. When secreted, these enzymes bind the cation-independent mannose 6-phosphate receptor (CI-MPR) on the surface of neighboring cells, which then mediates uptake and lysosomal delivery [4]. We and others have exploited this pathway in gene therapy programs for Fabry disease, a multisystemic lysosomal storage disorder (LSD) caused by a deficiency in alpha-galactosidase A (GLA) and have demonstrated supraphysiological GLA activity in plasma following systemic treatment with AAV vectors [5–12]. Unfortunately, GLA is unstable outside of the acidic environment of lysosomes, and multisystemic cross correction through the blood is limited by a rapid activity decline in plasma. To circumvent this problem, we introduced artificial disulfide bridges into the natural GLA homodimer interface, which dramatically increases stability in the neutral pH outside of the lysosome (Figure 1A & 1B). When this engineered transgene is utilized for gene therapy, the enhanced stability results in improved efficacy in disease-relevant tissues (i.e., kidney, heart, and dorsal root ganglia; Figure 1C) [9,11]. Similar engineering approaches that enhance cross correction via stability improvements could be broadly applicable to many gene therapy programs.

Unfortunately, not all lysosomal hydrolases have the capacity to naturally cross correct with the same high efficiency. Acid alpha-glucosidase (GAA), a deficiency in which causes the multisystemic LSD Pompe disease, has the potential for cross correction through the M6P-CI-MPR pathway, but wild type GAA is inherently poorly phosphorylated resulting in low affinity for CI-MPR [13–17], which limits its effectiveness for cross correction. Inefficient phosphorylation is intrinsic to the natural cellular machinery and cannot be resolved with higher dosages and is worsened with over-expression of the enzyme. To address this, we and others have developed strategies that express GAA fused to lysosomal targeting tags that function without M6P (Figure 2A) [18–21]. In addition to trafficking lysosomal hydrolases, CI-MPR also scavenges excess insulin-like growth factor 2 (IGF2), shuttling it to the lysosome for degradation [22]. We have leveraged this natural high-affinity ligand for CI-MPR by engineering a fusion protein consisting of GAA fused to the

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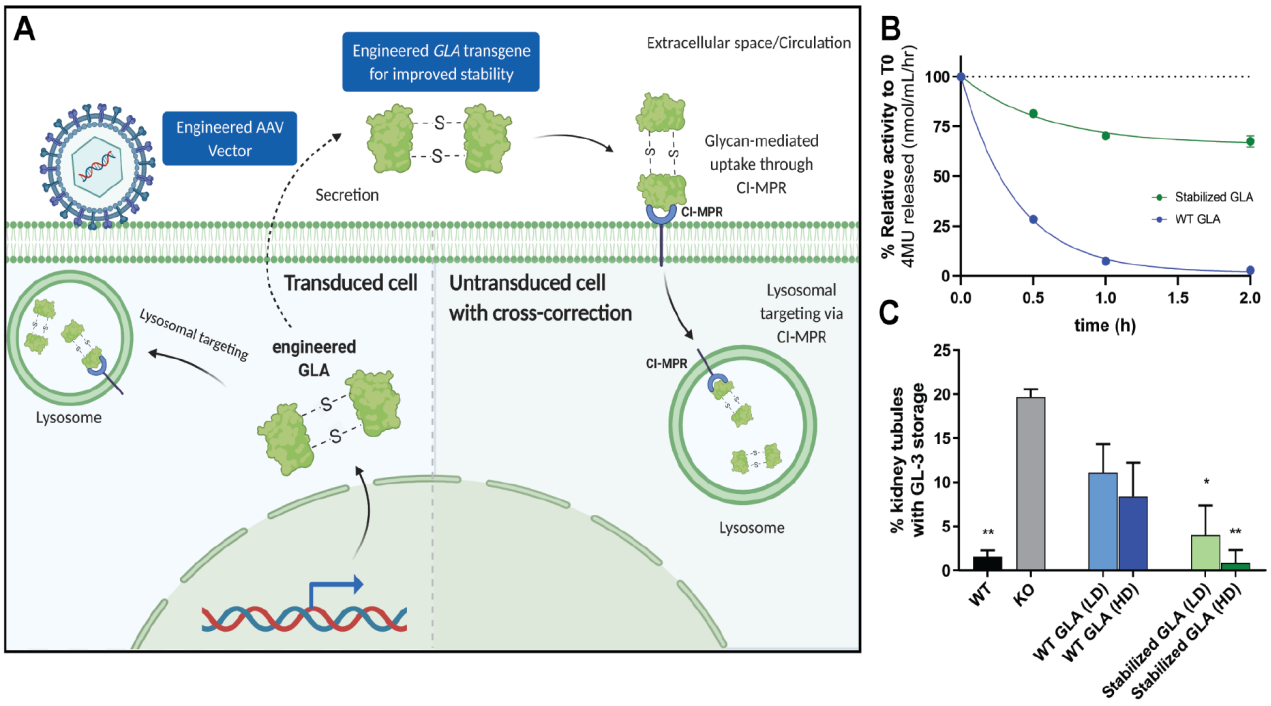
FIGURE 1

Engineered disulfide dimerization in a GLA gene therapy stabilizes the transgene product and improves storage material clearance in a mouse model of Fabry disease.



(A) Depiction of cross correction enhancement with transgene product stabilization. An engineered transgene is delivered to transduction-amenable cells with an AAV vector. These transduced "producer" cells (left) generate the recombinant GLA product, which is stabilized with intermolecular disulfide bridges at the dimer interface. The engineered GLA is secreted and subsequently delivered to local or distal cells, including untransduced cells (right) that are thus cross corrected. **(B)** GLA stability at neutral pH and 37 °C was assessed in plasma. While the Amicus stabilized GLA construct was stable over the course of 2 hours, wild type GLA (WT GLA, agalsidase beta) lost more than 50% of its activity within 30 minutes of incubation. **(C)** Stabilized GLA exhibits improved efficacy *in vivo*. 3.5–4.5 month-old male Fabry (Gla knockout [35]) mice were treated with PBS control (KO group in graph) or a low dose (LD) or high dose (HD) of intravenous AAV9-like vectors expressing wild type GLA or stabilized GLA. Four weeks later, tissues were examined for storage material (globotriaosylceramide, GL-3) accumulation in disease-relevant tissues. Only the stabilized GLA vector reduced GL-3 at statistically significant levels in kidney tubules, with similar results observed in dorsal root ganglia [9,11]. n = 2 replicates for **(B)** and 3–8 mice for (C). *p<0.05, **p<0.01.

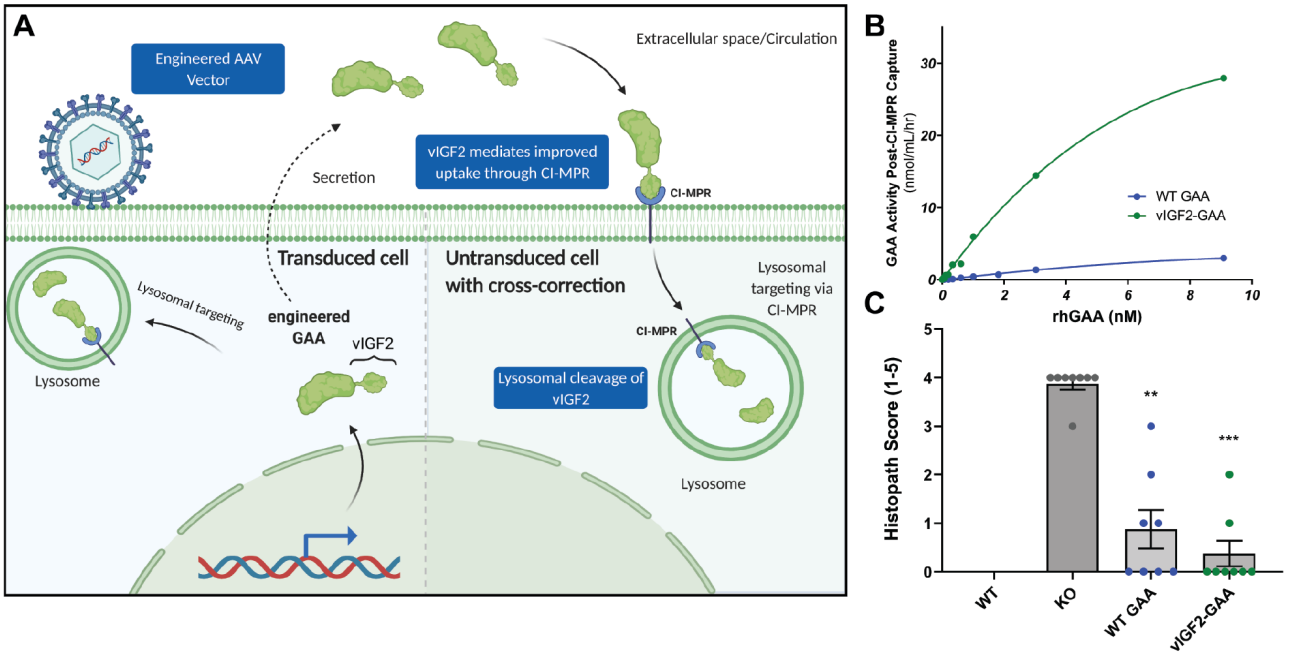
minimal sequence of IGF2 that is necessary for CI-MPR binding (designated as variant IGF2, vIGF2). Off-target interactions driven by wild typeIGF2 (e.g., insulin receptor and insulin-like growth factor 1 receptor binding) have been eliminated with rational design, a key feature that distinguishes our approach. Further, we engineered a proteolytic cleavage sequence between the two elements to ensure that the vIGF2 tag is cleaved and degraded by resident lysosomal proteases, leaving free GAA available for catabolism of glycogen. This vIGF2 –GAA fusion exhibits a profound increase in CI-MPR-mediated uptake efficiency while retaining wild-type activity and elicits greater pathological improvements than a wild type GAA construct when utilized for gene therapy (Figure 2B –2C) [18].

CROSS CORRECTION THROUGH CELL-PENETRATING PEPTIDE MOTIFS

Lysosomal enzymes are relatively amenable to cross correction due to the presence of lysosomal trafficking receptors on the surface of recipient cells, but can engineering also achieve cross correction for non-lysosomal proteins? In producer cells, signal peptides can be engineered to direct a variety of proteins through the secretory pathway, destining

FIGURE 2

An engineered vIGF2-GAA fusion protein improves CI-MPR binding and histopathological rescue in a mouse model of Pompe disease.



(A) Depiction of cross correction enhancement with engineered uptake tags. An engineered transgene is delivered to transduction-amenable cells with an AAV vector. These transduced "producer" cells (left) generate the recombinant GAA product, which is terminally tagged with vIGF2. The engineered vIGF2-GAA is secreted and binds CI-MPR with high affinity, which delivers the enzyme to the lysosomes of cross-corrected cells. **(B)** CI-MPR-coated plates were incubated with varying concentrations of wild type GAA (WT GAA, alglucosidase alfa) or vIGF2-GAA to measure binding efficiency (as reflected by post-capture GAA activity). WT GAA exhibited only low-level binding, reflecting the known low abundance of the M6P glycans that facilitate interactions with CI-MPR. The Amicus vIGF2-GAA exhibits greatly improved binding due to the high-affinity interaction between vIGF2 and CI-MPR. (C) vIGF2-GAA exhibits improved efficacy *in vivo*. 1–1.5 month-old Pompe (Gaa knockout [36]) mice were treated with PBS control (KO group in graph) or intravenous AAV9-like vectors expressing wild type GAA or vIGF2-GAA. Four weeks later, disease-relevant tissues were processed with periodic acid-Schiff (PAS) staining to label glycogen and assessed for histopathology on a 1–5 scale, with 5 reflecting the most severe glycogen storage. While both constructs reduced glycogen storage in quadriceps femoris, the vIGF2-GAA construct outperformed WT GAA. Similar results were observed in the central nervous system. n = 2–5 replicates for **(B)** and 8 mice for **(C)**. **p<0.01, ***p<0.005.

soluble proteins for secretion regardless of their intracellular residence. In recipient cells, cell-penetrating peptides (CPPs) can mediate cell-surface membrane associations and uptake by endocytosis. Finally, a variety of intracellular targeting motifs have been identified for targeting CPP-tagged cargoes to various organelles and intracellular compartments including the nucleus, endoplasmic reticulum, cytosol, and mitochondria [23–25]. In combination, these strategies could offer a powerful approach for developing cross-corrective gene therapies.

CPP-based approaches have been explored for numerous therapeutic applications, including several with utility for gene therapy. CPP fusions have been shown to mediate uptake and targeting of several disease proteins including cyclin-dependent kinase-like 5 (CDKL5, the cytosolic protein lacking in the neurological CDKL5-deficiency disorder) [26], frataxin (FXN, a mitochondrial protein lacking in another neurological disease, Friedreich's ataxia) [27], and myotubularin (MTM1, the cytosolic phosphatase lacking in X-linked myotubular myopathy) [28]. In all cases, these CPP fusion proteins elicit functional improvements in disease phenotypes *in vivo*, demonstrating their therapeutic potential. However, these and other existing studies have utilized CPPs only in protein-replacement therapies, which exhibit

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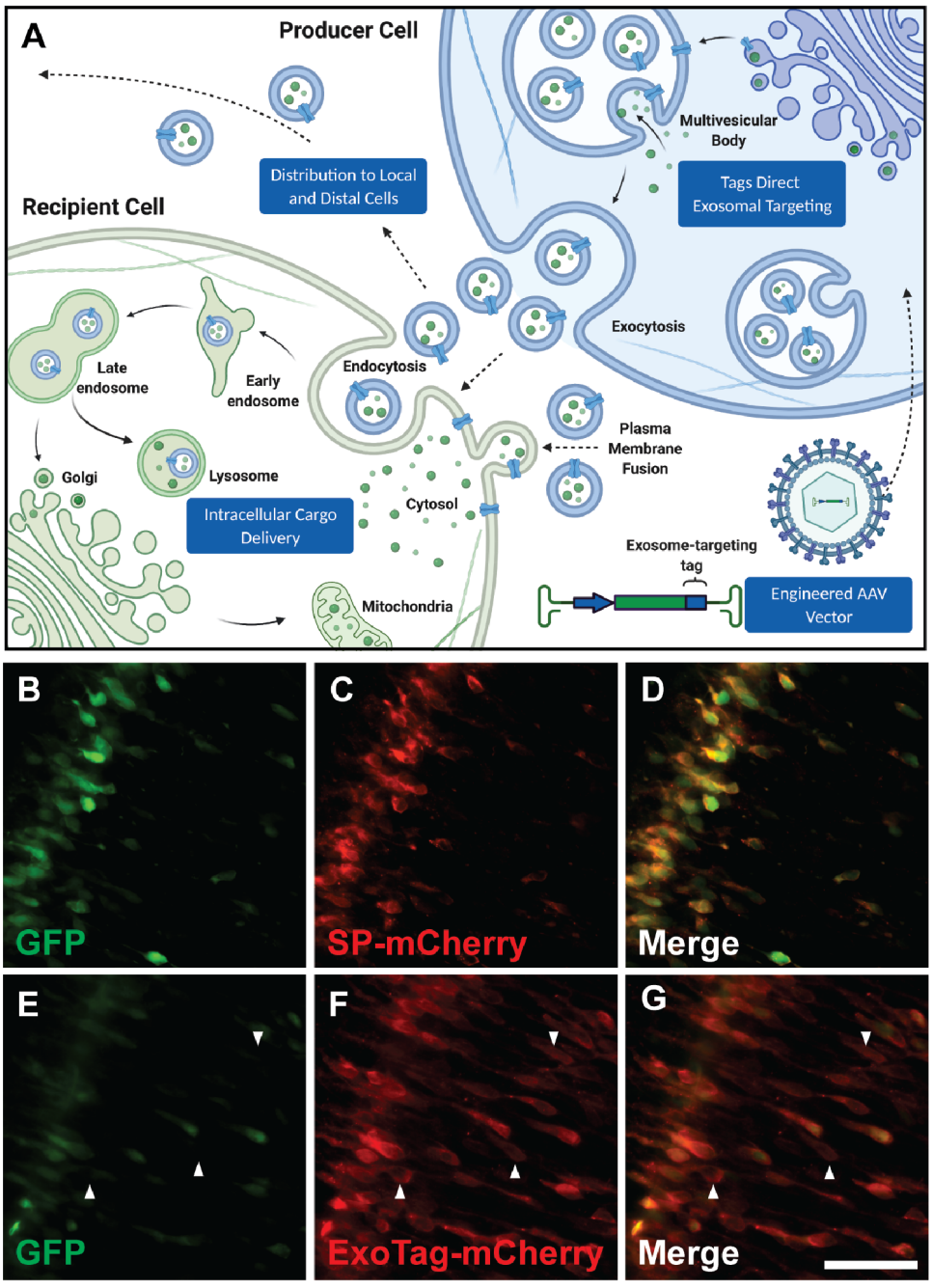
FIGURE 3

Exosomal targeting achieves cross correction of model proteins.



(A) Depiction of cross correction through exosomal targeting. An engineered transgene is delivered to transduction-amenable cells with an AAV vector. These transduced "producer" cells (right) generate the recombinant protein product, which contains exosomal targeting tags. These tags direct packaging of the target protein into exosomes at the multivesicular body (MVB), either at the cytosolic face of the MVB membrane (for soluble cytosolic proteins) or through incorporation of outer (limiting) MVB membranes into exosomes (for transmembrane proteins). Exosomes filled with cargo are then released via exocytosis and distribute to local or distal cells. Recipient cells can receive exosomes through endocytosis or plasma membrane fusion, followed by targeting to a variety of intracellular compartments. **(B–G)** Proof of concept for exosomal cross correction of a model protein. Mouse cortical neurons were transfected in vivo via in utero electroporation at embryonic day 14.5 (E14.5) with plasmids expressing secreted signal-peptide-mCherry (SP-mCherry, **B–D**) or mCherry fused to an exosomal targeting tag (ExoTag-mCherry, **E–G**). Both constructs also expressed cytosolic (non-secreted) green fluorescent protein (GFP) expressed from an internal ribosome entry site (IRES) to label transformed cells. At E18.5, cross-corrected cells (GFP-, mCherry+; white arrow heads) are observed only for the construct with the ExoTag. Scale bar 50 µm.

suboptimal biodistribution and require repeated drug treatments for the lifespan of the patient. Translation of these approaches into gene therapies could reduce treatment burden while enhancing target-cell delivery and uptake.

THE ELEPHANT IN THE ROOM – CROSS CORRECTING TRANSMEMBRANE PROTEINS

Receptor-mediated uptake and CPPs have great potential for the cross correction of soluble or membrane-associated proteins in a variety of cellular compartments, but neither approach will be useful for the plethora of diseases caused by deficiencies in transmembrane proteins (e.g., Niemann-Pick Type C, CLN3 Batten disease, Parkinson disease type 9). Since both single and multi-pass transmembrane proteins are inserted permanently into membranes during translation, cross correction will most likely require the transfer of therapeutic-protein-containing membranes from producer cells to target cells in the form of vesicles or membrane-limited organelles. Endogenous vesicle and organelle transfer mechanisms have only recently been characterized, and cross correction engineering strategies for transmembrane proteins have thus lagged behind. Thankfully, new biological insights are pushing this frontier forward.

Tunneling nanotubes were first described in cultured cells in 2004 as transport structures facilitating the cell-to-cell transfer of vesicles and organelles including lysosomes, but their *in vivo* relevance has remained controversial [29]. More recently, tunneling nanotubes have not only been confirmed to exist *in vivo*, but have also been shown to facilitate the *in vivo* transfer of vesicles, mitochondria, lysosomes, and have even been implicated in the efficacy of some gene-replacement strategies [30-31]. In a mouse model of cystinosis (a multisystemic lysosomal storage disorder caused by a deficiency in the transmembrane lysosomal protein cystinosin, CTNS), transplantation of wild type hematopoietic stem cells results in widespread correction of the disease phenotype, presumably due to the transfer of CTNS-containing lysosomes via tunneling nanotubes [31-32]. Unfortunately, the molecular mechanisms underlying such vesicle transfer are poorly understood, and this knowledge gap limits the ability to engineer transgene products for enhanced nanotube correction. This may quickly change as new insights are revealed.

On the other hand, a wealth of mechanistic knowledge exists for exosomes, another form of cell-to-cell vesicle transfer. Exosomes form through the luminal invagination of endosomal membranes and are released at the cell membrane in bulk upon fusion of multivesicular bodies [33]. Subsequent uptake occurs through multiple mechanisms, including fusion with the cell membrane (releasing soluble luminal contents into the cytoplasm and transmembrane proteins into the plasma membrane) and endocytosis (directing contents to the endolysosomal pathway). Exosomes have been explored by Amicus and others as delivery vehicles for numerous therapeutics but have yet to be exploited for cross correction in gene therapy [34]. We have investigated this approach (Figure 3A) and have developed exosomal targeting sequences that efficiently direct transgene protein products into exosomes. These targeting motifs facilitate cross correction of model proteins *in vivo* (Figure 3B –3G) and could soon function as a new modality for the cross correction of a wide range of therapeutic proteins using gene therapy. In addition to facilitating the transfer of transmembrane cargoes, exosome-mediated delivery could also provide immunological benefits by shielding therapeutic proteins from neutralizing antibodies and could facilitate broad biodistribution with the potential to traverse the blood-brain barrier.

Collectively, a number of distinct engineering strategies are emerging that could enable widespread, efficient cross correction in gene therapy across a range of targets, protein types, and diseases. These strategies have the potential to work within the confines of dose

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and transduction limits to correct untransduced cells, greatly enhancing efficacy. While the greatest progress has been made for lysosomal hydrolases, new technologies are opening doors for cross correction to nearly every cellular locale including those traditionally viewed as inaccessible or refractory to treatment. Thus, while wild type gene therapies will enter the clinic first as important treatments, new engineering strategies will greatly enhance therapeutic efficacy in subsequent next-generation therapies. When combined with other advances in capsid engineering, immune modulation, and manufacturing, these new therapies will bring the field, and more importantly patients, closer to long-sought cures.

TRANSLATION INSIGHT

Multiple engineering strategies could improve cross correction for next-generation gene therapies. These strategies have the potential to treat every affected cell, and thus offer some of the greatest therapeutic potential for patients living with loss-of-function genetic diseases.

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CELL & GENE THERAPY INSIGHTS

Innovation Insights

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INTERVIEW

Engineering approaches to adopt automation in tissue engineered and cell therapy product manufacture

IOANNIS PAPANTONIOU is an Associate Professor head of the Tissue Engineering Lab of the Skeletal Biology and Engineering Research Centre, Department of Development and Regeneration, KU Leuven. He is also part of Prometheus, the translational division of the Leuven R&D, KU Leuven. In addition he is also a visiting PI at the Institute of Chemical Engineering Sciences, Foundation of Research and Technology (ICEHT/FORTH) in Greece. He is the coordinator of the H2020 'Jointpromise' project aiming at automating the biomanufacturing of organoid-based osteochondral implants and is also a participant and Board member of the recently launched H2020 project 'AIDPATH' aiming at the integration of Artificial Intelligence technologies in Cell Therapy Manufacturing. He has also obtained funding from European, regional and national sources and has coached many young researchers into obtaining personal Post-doctoral and PhD grants. He has been invited in several academic and industrial conferences to serve as session and track chair as well as invited panelist. He has provided numerous invited lectures in leading academic institutes across Europe. During his research activities multiple industrial collaborations have been carried out successfully with leading players in the ATMP manufacturing field. His main research focus is develop designed 3D cell-based products with built-in arranged quality attributes through high-precision bioengineering technologies for skeletal regeneration. In addition the integration of automation aspects in organoid based tissue products has been at the forefront of his interest. Recent research breakthroughs resulted in the development of cartilaginous organoids for the bioprinting of bone regenerating living implants. Their subsequent use in various contexts has been evaluated while their production for clinical application is under way at KU Leuven university hospitals.

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"Tissue engineering has become very complex – the products being made are more complex than ever before. However, I think there is not enough investment in this field at the moment."

Q What are your working on right now?

IP: I am involved in two large Horizon 2020 programs, which are consortia composed of industrial and academic partners from the European Union. Both projects have a common theme, which is the development of an automated bio-manufacturing pipeline, but they cover two different applications: organoid-based skeletal tissues and CAR T cell therapies.

I am a coordinator of one of the products (JOINTPROMISE) through KU Leuven, the coordinating partner. This project looks at automating the pipeline from an engineering perspective; how do we design and produce a sequence of units of operation leading to the manufacturing of a tissue engineered product, from the single cell to the microtissue, to meso tissue, and finally, macro-tissue living implant? Here we must account for bioreactors, robotics, and bioprinters, which all need to be integrated into the process flow. And not only do we need to integrate the hardware but also their software to enable seamless operations. We also are employing metabolomics, genomics, and imaging, which all must be integrated within the manufacturing process at-line. Although we are not making a yet product, we are taking regulatory requirements into account early on so that the manufacturing process is GMP-ready and therefore ready to be translated clinically.

In the JOINTPROMISE project, we aim to also conduct studies at the preclinical level, where the efficacy of the tissue product (osteochondral implants) is being tested in mini pigs. As the osteochondral implants are made from two different types of tissue, it is a challenge to design and manufacture them as well as to test them in large animals.

The second Horizon 2020 project I mentioned, is called AIDPATH. This is coordinated by the Fraunhofer Institute for Production Technology, based in Aachen, Germany. For this project, we are focusing on CAR T cell therapy - specifically automation, as well as some point-of-care manufacturing focus. Here, we will be addressing how a small factory can be contained within a hospital and how artificial intelligence can be integrated into the existing hospital IT infrastructure. We are also looking at how quality control can be automated. This requires integration of a bioreactor with a robotics system, as well as robotics for the analytics. How can assays and quality control procedures be done in a standardized way? The two projects are quite similar. The main difference is the end product - one is a tissue and the other is a cell. The whole manufacturing approach is tailored with this in mind. Like all cells, CAR T cells have markers, which means they can be sorted with FACS (Fluorescence-activated Cell

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Sorting) and the product is then analyzed, providing a quantitative result. In a tissue, there are no markers, so you need to analyze something else in order to understand the physiology and phenotype of the cells and the tissue. Tissue also has an extracellular matrix, making it a more complex product.

Q How has the field of tissue engineering evolved over the two years since we last spoke, and what represents the cutting edge at the moment, for you?

IP: Tissue engineering has become very complex - the products being made are more complex than ever before. However, I think there is not enough investment in this field at the moment. If you compare the active clinical trials of tissue engineered products with CAR T cell therapy, the field is lagging by maybe a log scale in terms of investment.

I believe this may be due to the innate complexity of tissue engineered products. In addition, novel surgical procedures might be needed focused on the handling of living products. As such, the chances of success can vary, and may not always be attributed to the product itself. All these factors can compromise the success of your product.

It is difficult to characterize a very complex, three-dimensional ATMP, as I feel the technologies are not yet mature enough to characterize and to capture their complexity. And of course, in order to obtain marketing authorization from regulatory bodies, it is necessary to effectively characterize the product. Over the past few years, technologies that were not active in the realm of life sciences have now found a place within the field, such as Raman spectroscopy and other types of mass spectroscopy-based methods, which have been used to characterize tissues. Today, these technologies are slowly gaining traction in our field and will progressively become inherent ATMP product Quality Controls enabling us to characterize quality attributes with high precision, non-destructively.

In addition, we have multi-omics technologies that are being developed and used more and more frequently. From these tools, libraries and atlases are created that can comprehensively characterize ATMP products. We are also now seeing a transition of the tissue engineering differentiation process used to mimic processes observed in developmental biology. Development is a robust process and hence, provides robust quality metrics that are derived from developing systems such as the embryonic system, post-natal system etc., that will enable us to engineer and industrialize components of human development in future factories.

This gives us a reference that we can use to compare our measurements from *in vitro* or tissue engineering and understand where our product is lying compared to the gold standard. This helps us identify tissue functionality, which is also extremely important for the induced pluripotent stem cell (iPSC) field. You have a paradigm to follow and finetune your process so as to mimic something that is functional. Mimicking the tissues of the body is not a case of trial and error anymore. Rather, it is a case of mimicking the regeneration process which in turn mimics development. Now there are panels of metrics (we could call them innate Critical Quality Attributes) that can help us achieve this. Having this set of metrics and functional attributes is important as it helps us identify in vitro process - critical quality attributes, which in turn, allows

"...we also have a new generation of skilled personnel (e.g. researchers, engineers, technical leads) who have a more holistic knowledge of these advancements, and have a solid understanding of how to apply them."

us to engineer or re-engineer our process to ensure the product complies with this 'criticality'. I think that now, with the help of so many new technologies, we are slowly bridging between the *in vitro* and *in vivo* realms.

Also, this last decade has seen the rise of more and more bio-fabrication technologies that have higher precision, higher capacity, and higher throughput. For example, products can be printed for tissue models that are already differentiated, and that are being made into larger or more complex implants. Alternatively, you can keep differentiating for later timepoints. In either case, you have tools that will help you achieve precision in what you are trying to make.

Different technologies also exist that can synergize, either for a material component or for manipulating living modules. This is something we are thinking about in our JOINTPROMISE project: how can we synergize extrusion bioprinting with laser bioprinting in a single process flow? One has better throughput, while the other has more precision so can we print certain features of the tissue with either method? In other words, can we use higher precision for more refined and smaller components, and then use high throughput for the bulk of the tissue? This will enable the product to be generated in 1 or 2 hours, rather than a day.

However, there are still challenges and bottlenecks we need to tackle. I think we have more technologies coming up that can help with scalable production of tissue modules - high precision bio-fabrication, and biomanufacturing of assembly processes for larger tissues.

These advancements are helping us tackle some of the main challenges in tissue engineering, making processes more efficient, attractive, and translational. I think we may soon see the emergence of three-dimensional complex products - tissue products that will mimic development and regenerate defects, or even heal organs.

We also have modules for *in vitro* screening for diseases that are more predictive. These can also be applied in other ways - for example, to discover new molecules that we might then engineer to improve products. Currently, we are seeing a snowball effect, which will accelerate the development of new 3D-tissue-engineered ATMPs.

Furthermore, we also have a new generation of skilled personnel (e.g. researchers, engineers, technical leads) who have a more holistic knowledge of these advancements, and have a solid understanding of how to apply them.

Q In terms of increasing genuine automation of cell and tissue manufacturing, what is the current situation and what challenges remain left?

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IP: I think there has been a lot of progress in this field lately. I've seen many small companies popping up and trying to help with the way GMP operations are being conducted – by providing services that will digitize the way we collect information about assays, for example.

Right now, processes are still outdated in that they are very rigid; data is collected manually; dossiers submitted to the regulatory bodies are handwritten. This is currently how GMP operations are being conducted officially. It's a bureaucratic system. However, there has been some progress in that a lot of companies are calling for regulatory process to be digitized. We also need to digitize the way orders are made and raw materials tracked; making a log of what is coming in and how that might affect the quality of what is being made.

A lot of manufacturing devices are now digitized, too. All the bioreactors, all the downstream processes (e.g., centrifugation, filtration) have sensors that provide some information. The question is, 'how do we integrate this?' This is what is lacking. In addition, with autologous products, the cells used are a part of someone, which gives rise to issues regarding data security. For example, let's say we have a decentralized manufacturing scenario in one country, and we have a facility producing tissues or cellular product in another country - we cannot simply provide information from one to the other, just like that. We need technologies that can encrypt data, which will enable these operations to happen in a compliant manner. These technologies exist in other sectors, such as banking and investment - maybe we can use them here. I know companies in cell and gene therapy are trying to account for this and take on these initiatives.

In an ideal world, we would have a databank or data library where the data is structured, and by mining this, we could make sense and inform our processes. This library would include information from raw materials as well as who is doing what, what the process conditions were, the result for a particular patient, what the donor-to-donor variability might be, stratification of patients and how this will impact the outcome of your process, etc. Data would be securely uploaded to the cloud and then we could use AI to extract knowledge. Information will need to be grouped in a modular fashion, where each module will address regulation and translation, process optimization, etc., thus making the business more viable and efficient.

In terms of human-based products, there is no consensus yet on how these will be manufactured. However, with autologous products, I think it is likely manufacture will eventually be decentralized. Again, this presents the challenge of covering a larger geographic domain. It is not clear yet whether hospitals will have GMP facilities themselves, so will there be a CMO covering many hospitals? These sorts of aspects have not yet been defined. Regardless, I think we need this type of approach to address logistics challenges, efficiency requirements, and to meet the demands of regulators.

Q Tell us more about the AIDPATH project and how it is addressing point-of-care manufacture: is it GMP-in-a-box or separate unit operations? What does the QC process look like?

IP: I wouldn't say it is GMP-in-a-box; rather, it is GMP-in-a-room, as we are thinking about a bigger infrastructure. GMP-in-a-box would be a single bioreactor system.

"Maybe in several years' time, integrated processes and systems will become smarter and perhaps decentralized manufacturing and the logistical complexity will become more risk averse. It may be that in the future, the process is operated remotely, especially given that everything is becoming digitized."

Here we are talking about entire manufacturing designed to be embedded in hospital environment and process flow.

Maybe in several years' time, integrated processes and systems will become smarter and perhaps decentralized manufacturing and the logistical complexity will become more risk averse. It may be that in the future, the process is operated remotely, especially given that everything is becoming digitized. For example, quality control experts may digitally sign-off on the final product. At the moment, a lot of processes, including sign-off, have to be done in person. This can slow things down and disrupt the process.

Even if a process is described as GMP-in-a box, there are still many hurdles and bottlenecks. As a result of these hurdles in regulation, legislation etc., promising advanced therapeutics may not be viably translated and marketed as successful products. The concept of GMP-in-a-box may be a little risky right now and I think people would rather minimize risk.

My big question is whether the technology is going to be feasible as a business model. Also, will it provide something that will encourage people to invest in it?

CAR T cell therapy is so expensive, but it is potentially addressing a life-threatening disease, which makes it suitable as an area for investment. So there is perhaps more scope to adopt this type of advancement. In my opinion, my project is not about GMP-in-a-box, but it is about point-of-care manufacturing in facilities either close to or within the hospital. This can ultimately help with surgical planning - e.g., the technology can be produced and be ready for implantation, keeping in mind the planned date of surgery for the patient.

In terms of analytics, I don't think we are making significant progress. At the moment, we have metabolomics, but also robotics to standardize the process of sampling and measuring readout. A lot of the quality characterization techniques are quite complex, and we also have to factor in human error in terms of making and utilizing the assays

What we are saying is, what if we used robotics to standardize the way we are developing the assay before it's measured? In other words, automating preparation of samples. Currently, much of this process is manual but by standardizing we can eliminate this variability, reducing human error. This will allow people to approach regulatory bodies with a quantitative readout of their FACS, metabolic readout, etc. For example, you could have a panel readout that might be reflective of potency.

I also think it is necessary to undertake several orthogonal assays, allowing you to measure different parameters with different analytics technologies. This will allow users to characterize their product in different ways, thus minimizing risk. Orthogonal assays can also be used in

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conjunction with metabolomics, mass spectrometry, FACs, etc. This means several assays, all independent and certified, may be used concurrently to prove the product is good quality, safe, efficacious, and will work once implanted.

Q What is your general feeling about the state of advanced therapy innovation and relevant initiatives in Europe?

IP: I would say that within the EU, there is support for the field. There are specific goals covered in the needs of regenerative medicine and cell and gene therapy. I'm actually applying for another grant where we will harness iPSCs to try to make allogeneic CAR T cell therapies.

In the Netherlands and Belgium (Flanders) there is a large collaborative initiative, called REGMEDXB, which is a commitment of long-term, sustainable funding for 'Moonshot-type' projects. This is a public-private partnership where the Netherlands and Belgium (Flanders) have come together to commit additional funds in the region of >€100 million over the next 10 years to manufacturing-related 'Moonshot' projects. This demonstrates the commitment of the Belgian and Dutch Governments to support this domain and become European leaders in it.

I am also aware of major initiatives in the UK. There has also been a lot of growth with many centers of excellence for ATMPs there, not only in London with the Cell and Gene Therapy Catapult, but also in the Midlands and Scotland. There is a lot of support from the UK public domain to allow small companies to come in and take their place in the market. The same is true for France, where there is also a lot of investment.

I am also involved in an alliance which promotes the field of translational regenerative medicine and drives for more funds to be committed. Generally speaking, I would say that throughout Europe there are quite a few initiatives pushing for public sector investment in the field.

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