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SPOTLIGHT ON

Review of 2024 and previewing 2025

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CONTENTS

SPOTLIGHT Review of 2024 and previewing 2025

VECTOR CHANNEL Characterization and validation

LATEST ARTICLES

CELL & GENE THERAPY INSIGHTS

REVIEW OF 2024 AND PREVIEWING 2025

SPOTLIGHT

INNOVATOR INSIGHT

Generation of novel AAV serotypes with enhanced infectivity, specificity, and lower toxicity via AAV capsid engineering platform

Ye Bu, Yue Pan, Yujian Zhong, Huan Chen, Zhiyong Dai, Youwei Zhang, Ying Fan, Junlin Chen, Keqin Tan, Rui Duan, Min Guan, Irene Song, Luyan He, Xin Swanson, and Paul Li

AAV-based gene therapies pose a number of challenges to developers, including ineffective uptake to target tissues, off-target effects and toxicity, and virus neutralization. Therefore, naturally occurring AAV serotypes cannot fulfill all the requirements of targeted gene therapy. Capsid engineering can provide a solution to this issue by enabling successful development of novel AAV serotypes with enhanced specificity, infectivity, and reduced toxicity. In this article, a capsid engineering platform is described, along with study data on promising AAV variants generated using this approach.

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Adeno-associated virus (AAV) is a small DNA virus that has become a prominent tool in gene therapy research due to its targeted gene delivery capabilities [1,2]. The ability of an AAV to infect and transduce specific cell types based on the presence of particular surface receptors or other factors on target cells is referred to as AAV capsid tropism [3–6]. Unfortunately, the naturally occurring AAV serotypes cannot fulfill all the requirements of

targeted gene therapy. *In vivo* gene delivery poses several challenges that can significantly reduce the efficacy of new AAV therapies [7,8], these include ineffective uptake into target tissues or organs, off-target transductions that may cause toxicity or unwanted immune responses, and virus neutralization by pre-existing antibodies (Figure 1).

To enhance the precision and efficacy of gene delivery, AAV capsid engineering is a crucial research approach that involves modifying the AAV capsid [9–13]. While AAV vectors are commonly used because they efficiently transduce various cell types without triggering an immune response, their effectiveness may be limited in certain tissues or cell types. Naturally occurring AAV serotypes are insufficient for targeted gene therapy, and many challenges still exist for *in vivo* gene delivery. AAV capsid engineering can address these challenges and is a promising approach to improve gene therapy outcomes. Capsids can be engineered using rational design and directed evolution to enhance AAV transduction in the targeted tissue or cell types while also evading the immune response [14].

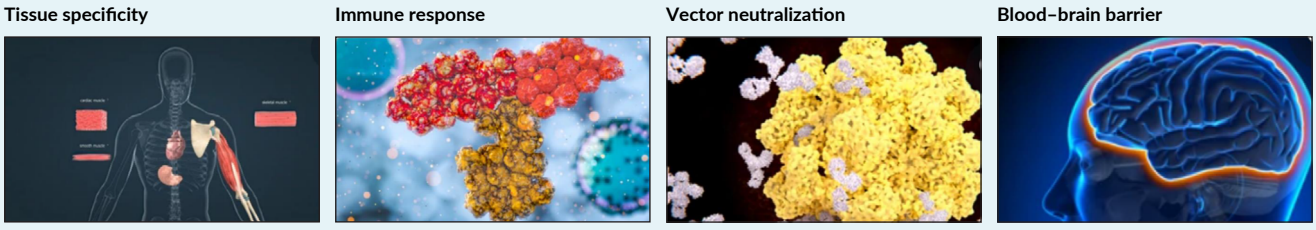
PackGene has developed an advanced AAV capsid engineering and screening platform that provides a reliable solution for overcoming challenges in gene and cell therapy. This platform involves constructing capsid libraries and conducting animal screenings [15], aided by a proprietary algorithm that predicts new sequences based on screening results. These sequences are then incorporated into subsequent screening rounds, increasing the likelihood of identifying optimal AAV capsid variants with enhanced organ targeting, reduced off-target effects, or other customized features. This process is referred to as the π-Icosa capsid engineering system.

The first step is to generate a large capsid gene library by combining deep mutation and rational design techniques. Deep mutation, in this context, involves the use of combinatorial saturation mutagenesis, where a few or up to all 20 possible amino acids are introduced in a combinatorial manner for the selected positions, such as AAVR, or glycan binding motif. This is achieved by inserting degenerate oligonucleotides or precision oligo pools, enabling the exploration of a vast diversity of capsid variants. These deep mutations are strategically designed to uncover novel capsid properties, such as altered receptor binding, improved tissue specificity, or enhanced transduction efficiency.

The next step involves generating a unique pool of AAVs from the capsid gene library, which is directly tested *in vivo* to assess the biodistribution and tissue tropism of each variant. This process begins by administering the capsid library to an animal model, followed by tissue extraction and RNA isolation from the target tissues. The viral RNA is then reverse-transcribed into cDNA using RT-PCR and analyzed through next-generation sequencing (NGS) to quantify the abundance of each capsid variant. The NGS data are normalized against the original input library to determine the relative biodistribution and providing valuable insights into

FIGURE 1

AAV-based gene therapy challenges.



AAV tropism. These findings guide a second round of library design and *in vivo* testing [16], aimed at identifying the most efficient and effective AAV variant (Figure 2).

Experimental data generated from extensive testing demonstrates the utility of PackGene's π-Icosa capsid engineering platform, which has been used to successfully engineer several AAV variants with improved specificity in the central nervous system, muscle, and other tissues. The platform's ability to identify and validate AAV capsid variant sequences with enhanced target tissue transduction, reduced off-target effects, and improvements across many other customizable features makes it a promising approach to improving gene therapy outcomes.

There are currently two major trends in AAV capsid library design:

1. Rational design: this approach involves targeted mutations in specific regions of the AAV capsid gene based on knowledge of its structure and function to generate a library of variants with specific characteristics. For example, chimeric capsids engineering is one rational design approach that involves combining different parts of AAV capsids from different serotypes to create chimeric variants with improved properties [17].

2. Directed evolution: this approach introduces random mutations or DNA shuffling throughout the AAV capsid gene to generate a diverse library of variants for screening. AAV variants with desired properties are then identified from the large library through iterative rounds of screening and selection [9–11,18–20].

Scientists at PackGene have combined these approaches to generate more diverse and targeted AAV capsid libraries.

FIGURE 2

π-Icosa system capsid engineering workflow.

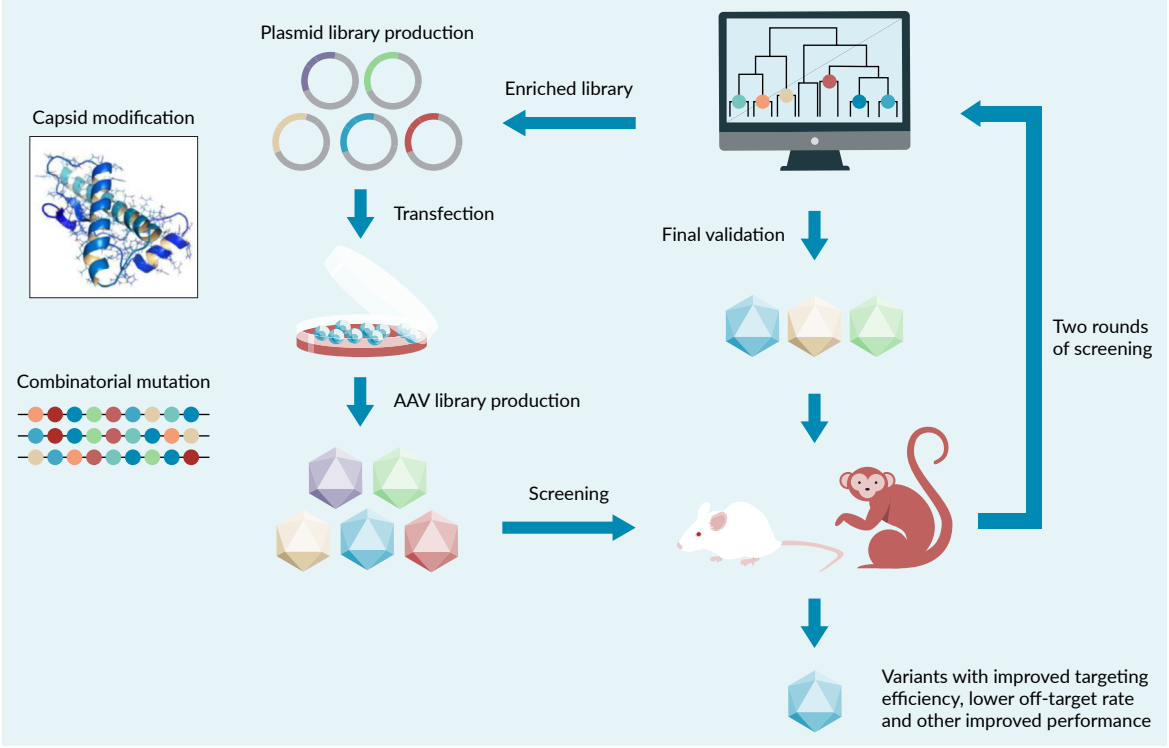
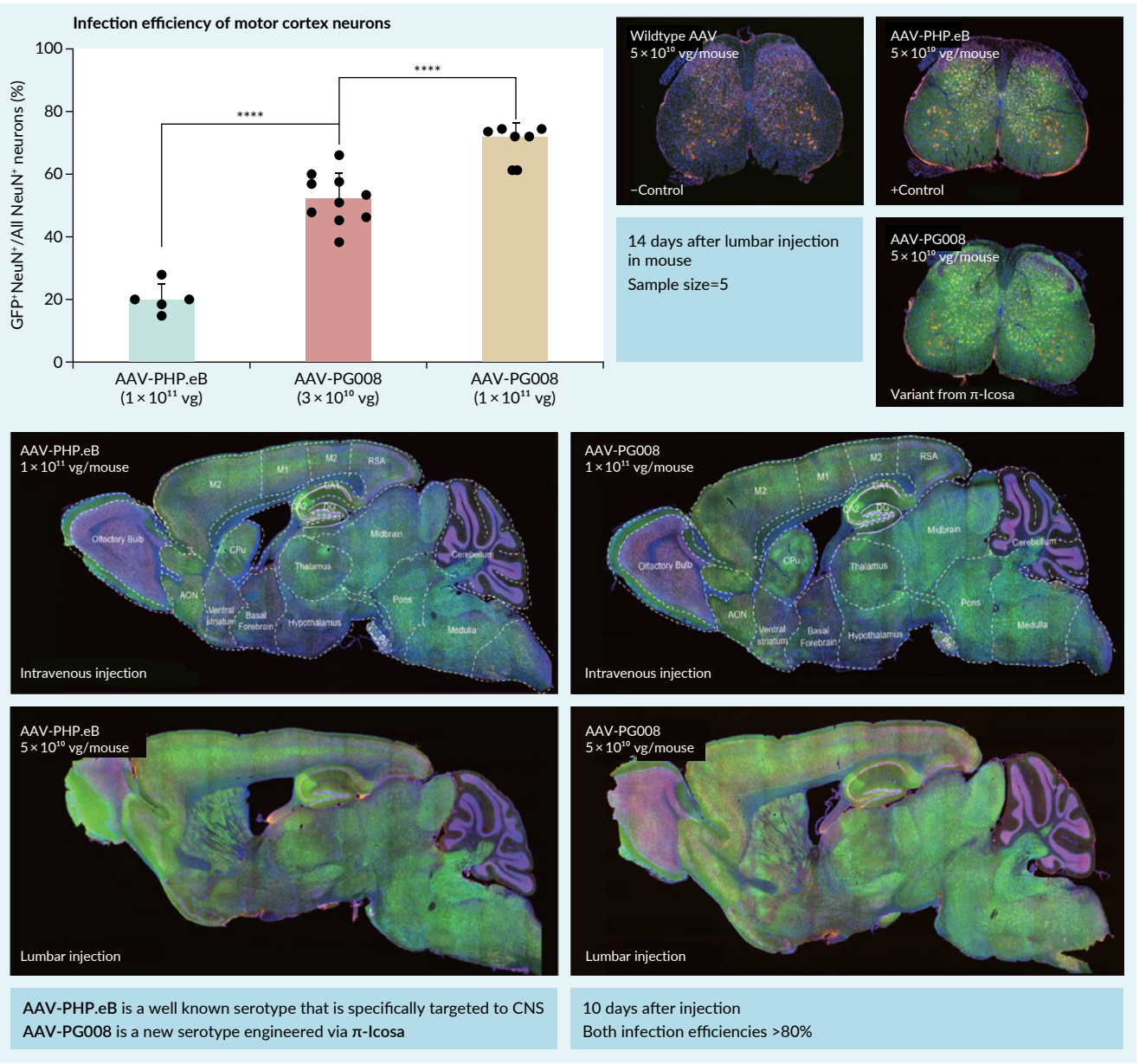


FIGURE 3

AAV-PG008: AAV9 variant engineered via π-Icosa system shows CNS targeting.



RESULTS

Enhancing tissue infectivity through random peptide insertion in capsid protein

AAV-PG008 is a novel serotype that was engineered to target the central nervous system using PackGene's π-Icosa system. The capsid gene library was generated through random peptide insertion into the AAV9 capsid, packed into AAV, and underwent two rounds of animal injection, tissue extraction, and tissue enrichment followed by NGS analysis [21–23]. The top ten variants were individually verified in multiple animals along with positive and negative control serotypes. Quantitative measurements of reporter gene expression were taken using qPCR and Western blot [24]. Figure 3 displays the reporter gene GFP expression 14 days after lumbar injection in mice. AAV-PG008

demonstrated better spinal cord and cortex transduction compared to AAV-PHP.eB, a known serotype that specifically targets the CNS [25–28].

PG008's CNS tropism was observed only in mouse studies. However, when tested in non-human primates (NHPs) through intravenous injection, PG008 failed to target the CNS, highlighting a common translational challenge. This discrepancy emphasizes the limitations of relying solely on mouse models for capsid screening, particularly in the context of human gene therapy. By utilizing the π-Icosa platform and integrating artificial intelligence to analyze data from both species, including ongoing NHP screening, we can optimize the search for capsids with improved translatability. Furthermore, selecting highly conserved cross-species receptors and designing targeted libraries can significantly enhance the discovery of novel capsids that effectively bridge the gap between species.

Enhance tissue specificity and reduce off-target and liver toxicity with a combination of rational design and directed evolution

In some cases, the administration of adeno-associated viral vectors (AAVs) used in gene therapy can trigger an immune response in the liver [29]. The occurrence of elevated liver enzymes has highlighted liver toxicity as a significant obstacle in the systemic delivery of AAVs. Consequently, researchers have been striving to identify

FIGURE 4A

AAV-PG007, a variant of AAV9 and AAV2 chimera, screened via π-Icosa system shows a significant increase in muscle targeting while reducing the off-targeting to the liver in both mouse and monkey.

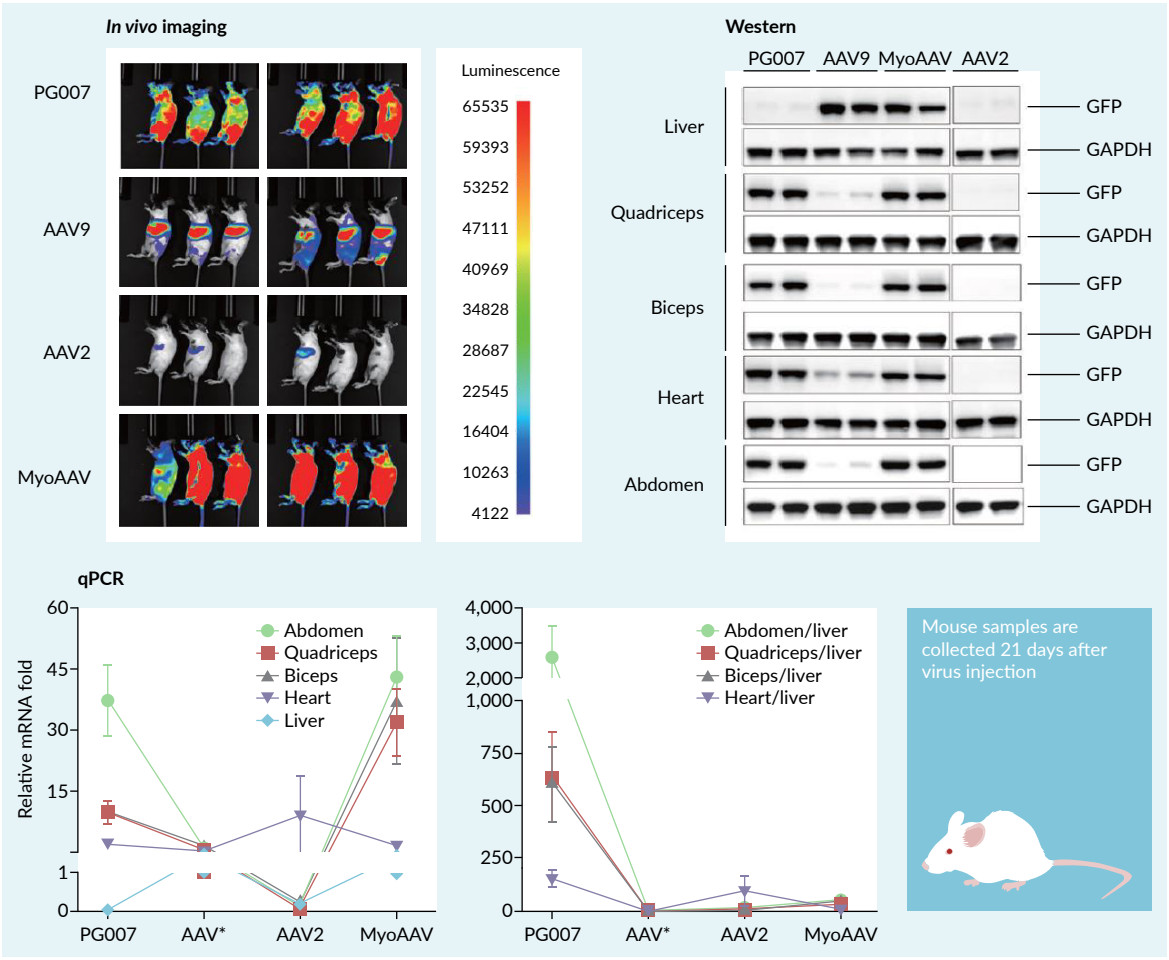
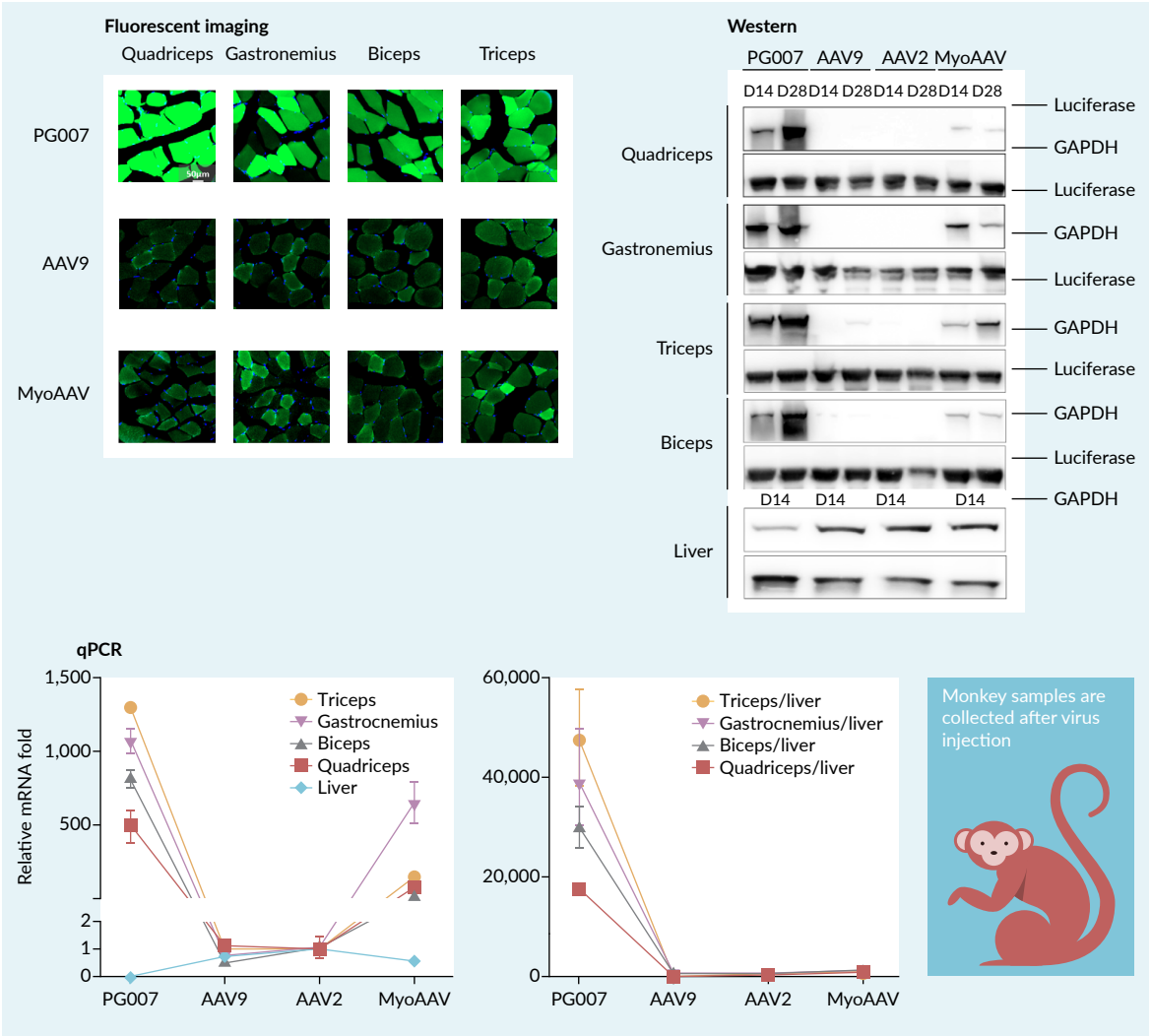


FIGURE 4B

AAV-PG007, a variant of AAV9 and AAV2 chimera, screened via π-Icosa system shows a significant increase in muscle targeting while reducing the off-targeting to the liver in both mouse and monkey.



capsids that can detarget the liver, especially for indications where the liver is not the primary tissue target [30–33]. To enhance tissue specificity and reduce off-target effects, we used a combination of directed evolution and chimeric capsids [34].

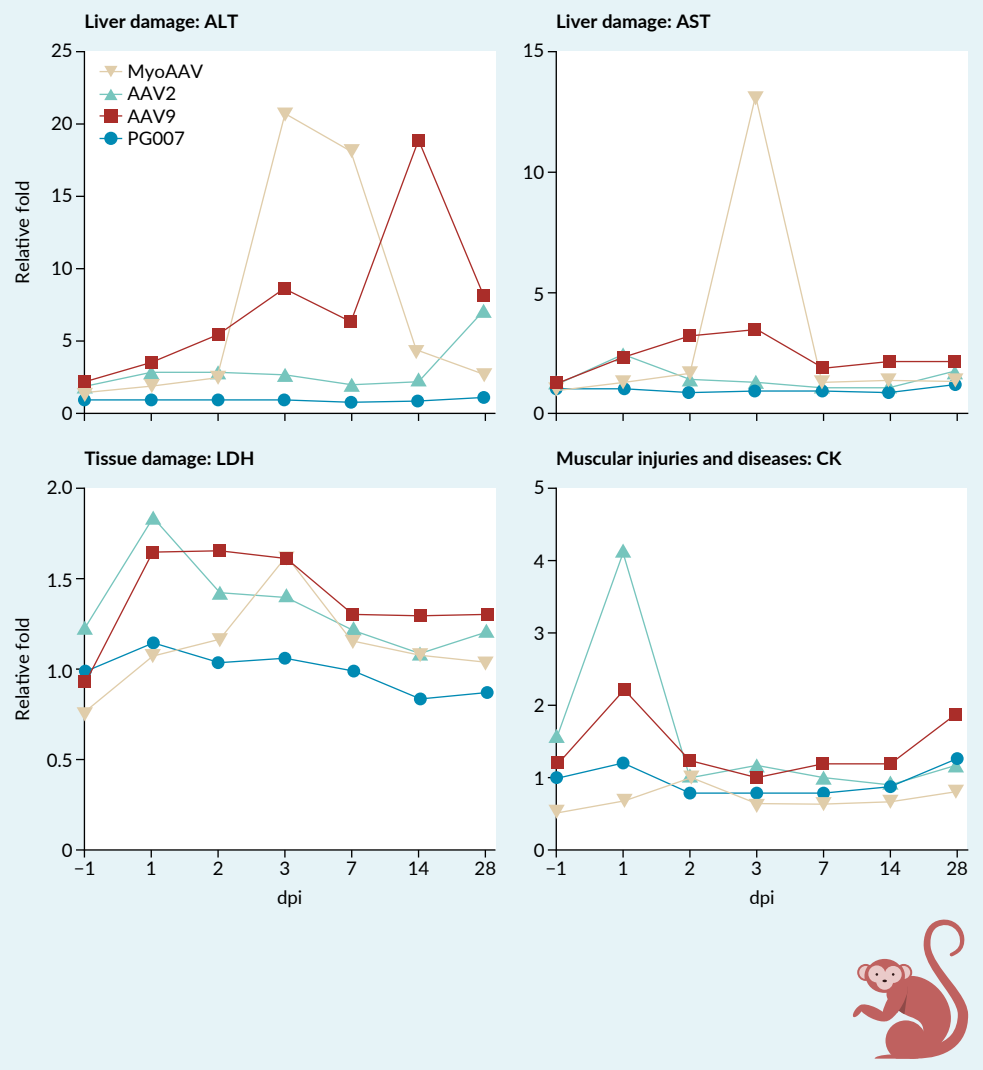
Recombining select capsid domains from different serotypes may generate novel tissue targeting profiles. Using principles of structural biology to guide rational design, we aim to create AAV chimeras that target muscles while reducing off-target effects, particularly in the liver.

Although some of the chimeric serotypes we generated showed reduced liver targeting, we did not observe significant improvements in muscle targeting (data not shown). We incorporated RGD-YNSL, a 7-amino acid peptide derived from MyoAAV 4A [17,20,35-37], into the chimeric serotype designed to reduce liver targeting. The resulting capsid, AAV-PG007, demonstrated significantly enhanced muscle targeting while retaining its liver-detargeting properties in both mice and monkeys.

The efficacy of AAV-PG007 was evaluated through *in vivo* imaging of GFP expression, qPCR analysis of mRNA expression, and western blot analysis of reporter gene expression in different tissues. Results showed that

FIGURE 5

AAV-PG007, a variant of AAV9 and AAV2 chimera, screened via π-Icosa system shows a significantly less liver damage.



AAV-PG007 exhibited muscle targeting similar to the known serotype MyoAAV but had much lower liver transduction compared to MyoAAV in both mice and monkeys (Figure 4A and B).

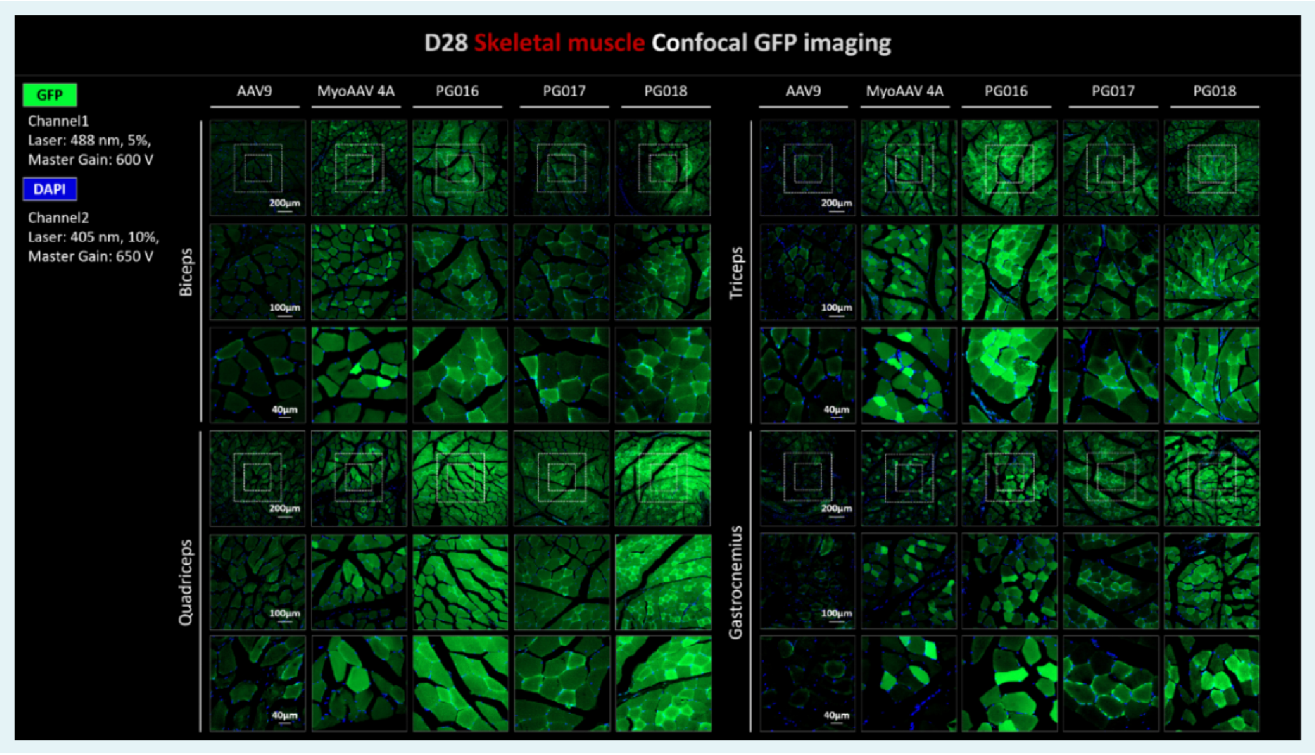
Moreover, we assessed liver and tissue damage in monkeys, and AAV-PG007 demonstrated significantly lower liver damage compared to MyoAAV, AAV2, and AAV9 as indicated by ALT and AST tests (Figure 5). While AAV-PG007 also showed slightly lower LDH and CK levels, the other three serotypes did not exhibit significant tissue damage in these tests either.

MyoAAV, AAV2, and AAV9, as indicated by ALT and AST tests.

Using the same PG007 liver-detargeting backbone, we conducted another round of screening by incorporating random peptides into the backbone and directly screening them in Cynomolgus monkeys. Through this process, we identified three new capsids that demonstrated enhanced tissue specificity in muscles 28 days post-AAV injection,

FIGURE 6

Confocal images of skeletal muscle tissues from cynomolgus monkeys 28 days post-AAV injection with three novel muscle-targeting capsids.



as observed in confocal imaging. Further characterization and identification of these novel capsids (PG016-PG018) are ongoing (Figure 6).

Screening novel AAV serotypes for enhanced T-cell specific transduction rate: implications for *ex vivo* T-cell therapy

In addition to screening in animal models, we screened novel adeno-associated virus (AAV) serotypes in primary human T cells. Our aim was to identify candidates that could be employed in *ex vivo* T-cell therapy with improved T-cell-specific transduction rates. Through targeted engineering, we developed several novel capsids exhibiting a 10x enhancement in primary human T-cell transduction rate. By microscopy and flow cytometry we revealed that the novel capsids, namely PG010, PG012, PG013, and PG014, exhibited comparable transduction rates at a dose of 1×10^4 when compared to wildtype AAV6 at a dose of 1×10^5. Ongoing optimization efforts on these novel capsids hold promising prospects for their application as superior tools for AAV-based gene delivery in engineering T cells (Figure 7).

DISCUSSION

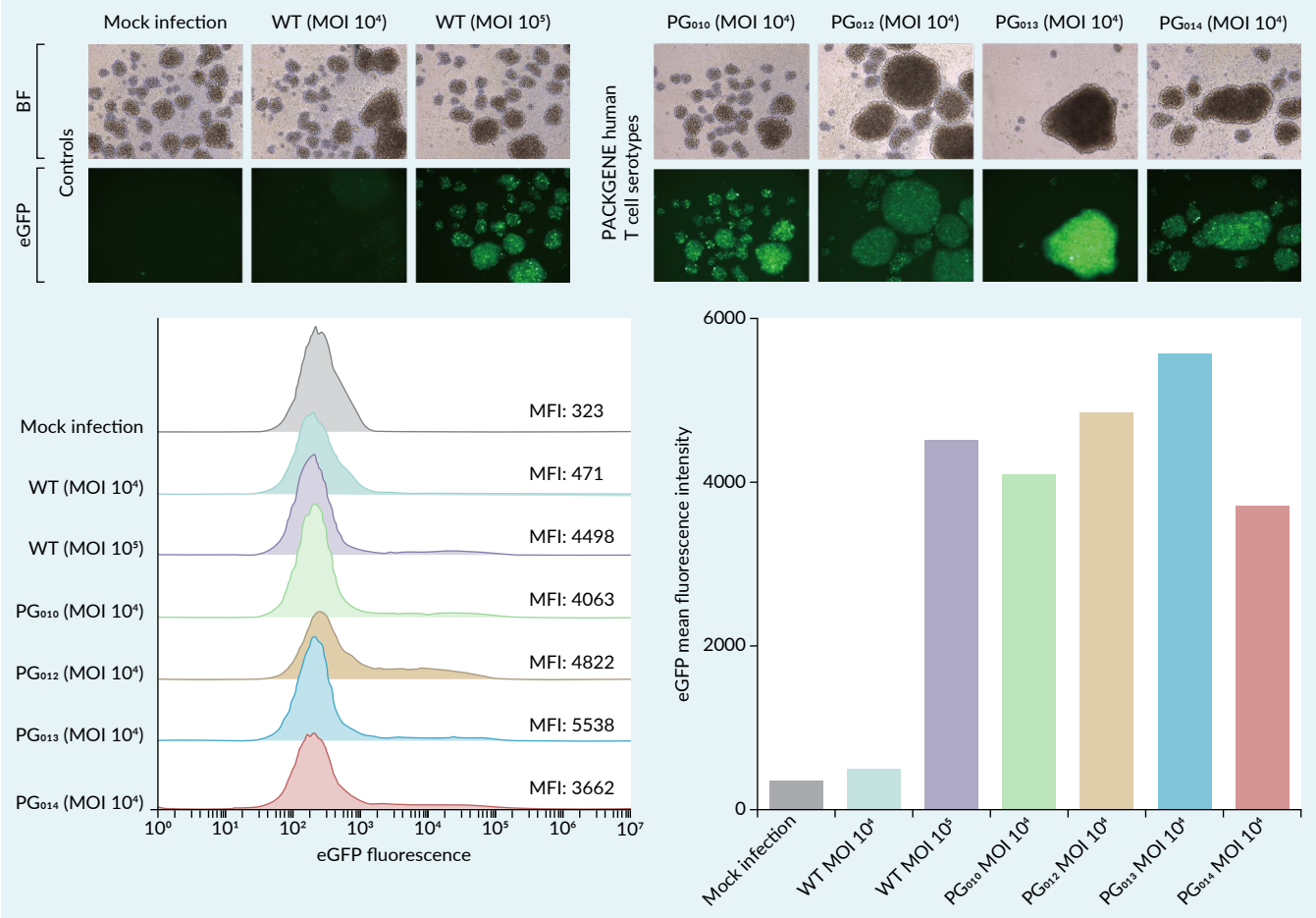
Key success factors for novel capsid screening

Start with a highly diversified and uniform capsid gene library

To construct a plasmid library encoding capsid variants, there are various methods available, including random peptide insertion or computational design. Regardless of the method used, however, it is crucial that two distinct features are closely controlled. The first of these features is library diversity,

FIGURE 7

Transduction of quiescent primary human T cells with novel PackGene's π-Icosa serotypes from AAV6.



which indicates the number of unique capsid sequences included in the library.

The second is library uniformity which indicates the relative amount of each unique plasmid that is contained within the library. An ideal plasmid library is both highly diverse and highly uniform. Tight control and optimization of diversity and uniformity ensure that numerous variants are covered with equal representation and make it easier to identify capsid sequences with desirable features.

PackGene offers different methods to achieve this, including deep mutation scanning, random mutation, and proprietary AI-design tools that generate combinatorial mutations based on experimental data. Typically, an AI-designed library is constructed in the second round of screening, leveraging next-generation sequencing (NGS) data from the first round as a learning dataset. This process results in a more concentrated library with higher chances of identifying the desired features (Figure 8).

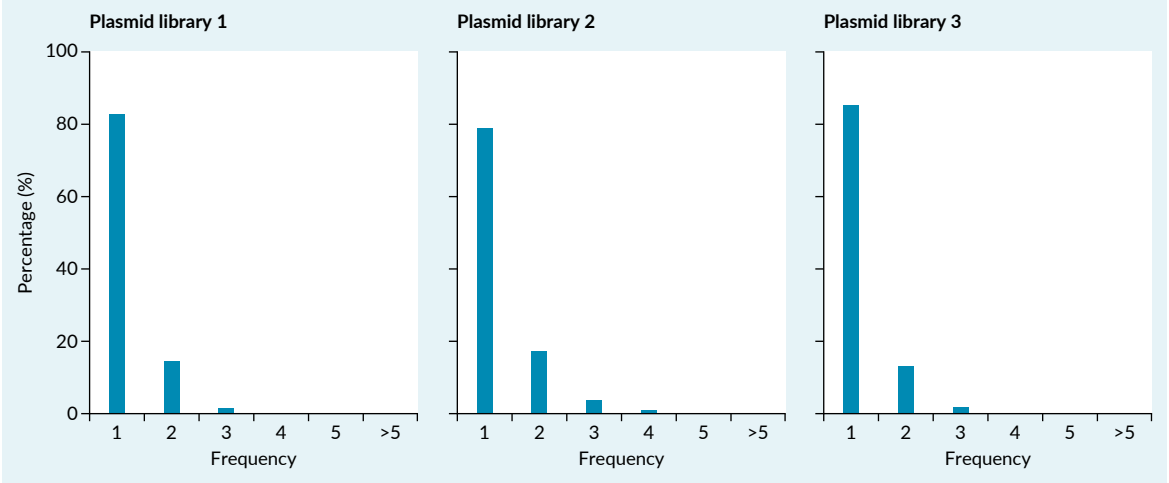
Use specialized AAV packaging method that does not distort the variant distribution and diversity

Packaging a diverse gene library into AAV capsids can be a challenging task. Unlike packaging a single transgene, the capsid gene library is highly variable and requires a different protocol to ensure that the even distribution of the library is maintained during packaging.

To achieve this, specialized protocols are developed that can ensure that the library is packaged uniformly, and no single variant is

FIGURE 8

Diversity and uniformity of capsid gene library created by PackGene.



overrepresented or underrepresented. These protocols involve several critical steps, including the precise calculation of viral particles and genomic DNA to ensure optimal ratios, proper mixing and purification of plasmids to minimize variability, and careful optimization of transfection conditions to maximize packaging efficiency.

Furthermore, the diversity and uniformity of the packaged capsid gene library must be verified before injection into animals. This is typically done through NGS analysis, which provides a comprehensive view of the library's composition and distribution. By analyzing the NGS data, researchers can ensure that the library is evenly distributed and representative of the intended gene pool, minimizing the risk of biased results during animal testing (Figure 9).

Strictly control the mispackaging rate

The capsid gene sequence is a critical component in the effective packaging of the AAV genome into viral capsids. However, during the packaging process, mispackaging can occur, leading to the encapsulation of empty or capsid genes in heterologous capsids. This can result in incomplete representations of the true capsid gene sequence in the NGS data of enriched targeted tissues, potentially compromising the accuracy of downstream analyses.

To ensure the reliability of our screening and validation processes, PackGene employs a rigorous quality control protocol to monitor the mispackaging rate before animal testing. This involves the incorporation of a small amount of capsid sequence with an early stop codon for early termination of translation. The percentage of viruses packed with this early stop codon capsid gene reflects the mispackaging rate, which is carefully monitored to ensure it is kept to a minimum.

At PackGene the mispackaging rate is typically <0.1%, which is significantly lower than rates reported by other labs. This low rate ensures that the NGS data of enriched targeted tissues accurately represents the true capsid gene sequence. By taking these measures, we are able to maintain a highly reliable and reproducible screening process for identifying effective AAV variants for targeted gene therapy (Figure 10).

Avoid potential pitfalls in capsid engineering projects

Capsid engineering is a complex process, but potential pitfalls can be avoided by defining the parameters associated with an ideal serotype candidate, and then carefully designing the capsid engineering program to best cater to those parameters.

FIGURE 9

Diversity and uniformity of capsid AAV library created by PackGene.

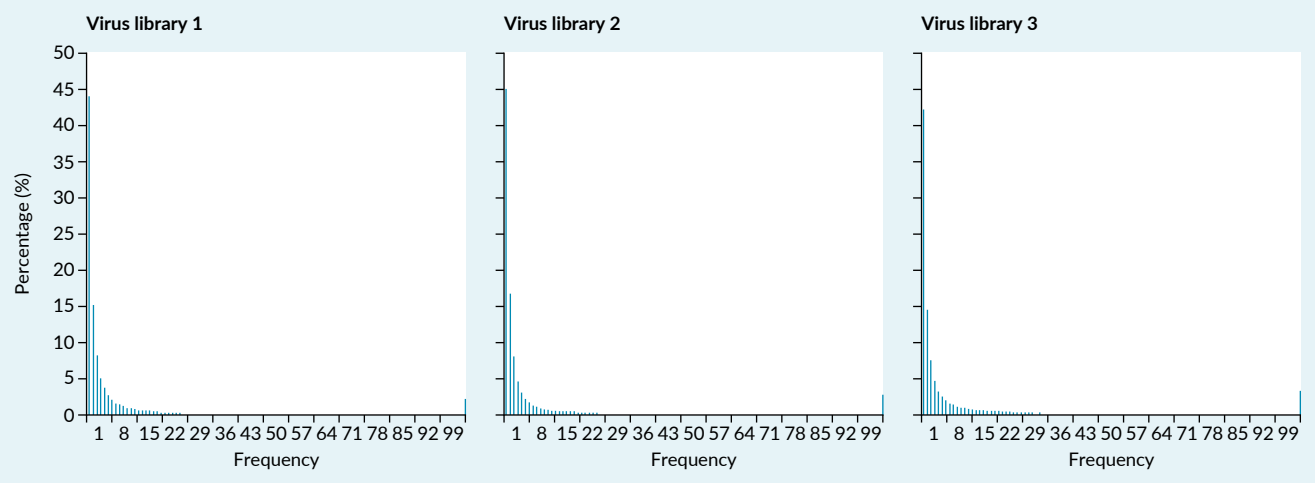
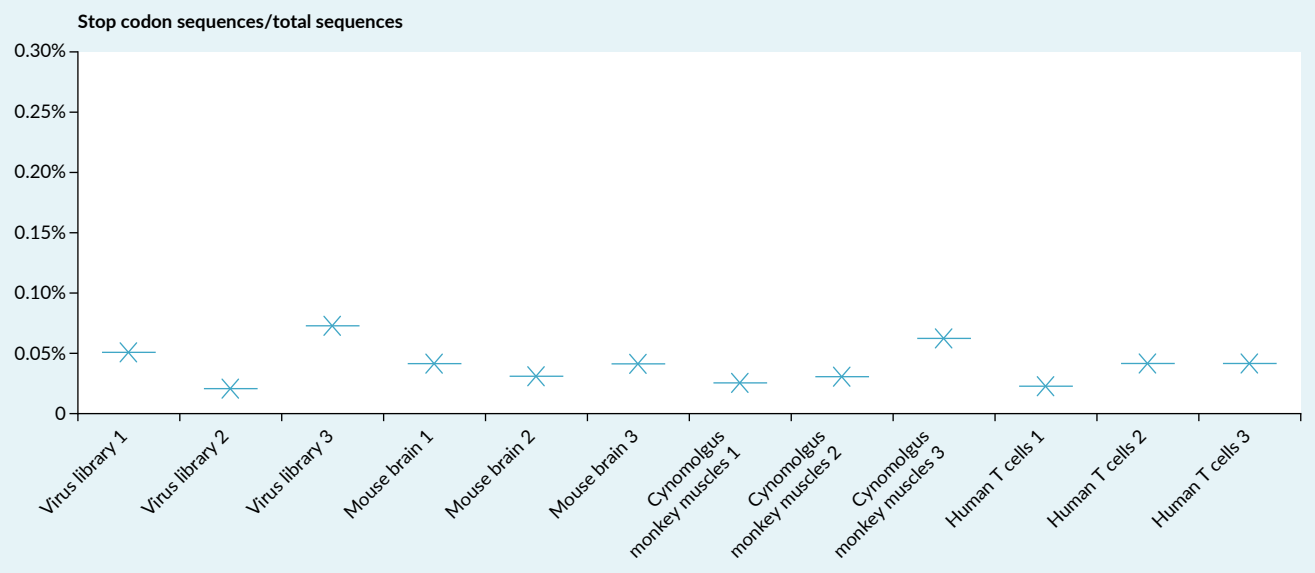


FIGURE 10

PackGene capsid π-Icosa library has an extremely low mispackaging rate, ensuring a much more reliable screening result.



First, it is essential to clearly define the characteristics of an ideal serotype for a particular project. Such characteristics may include but are not limited to target tissue transduction rates, target tissue specificity, specific off-target tissue transduction, immunogenicity, and toxicity. Appropriate verification testing procedures must then be defined to accurately measure these characteristics to help ensure that the AAV serotype that is ultimately selected is safe and effective in delivering the intended therapeutic gene.

It may also be crucial to verify the transduction characteristics of candidate variants across multiple species [19,20]. While *in vivo* studies can be both challenging and costly, this is often outweighed by the benefit in terms of time savings and increased serotype reliability. It is generally recommended to

start with screening in mice due to the lower relative cost.

Nevertheless, subsequent validation in non-human primate models, which have genomes and physiology closer to humans, is often required. This can help identify serotypes that consistently perform well across species and are therefore more likely to result in successful application in human gene therapy.

Finally, selecting an appropriate number of serotype candidates to move into validation following initial rounds of serotype screening will increase the likelihood of successfully identifying an ideal candidate. While the number of serotypes that are selected for validation may be limited by cost and other factors, the selection of too few candidate serotypes may ultimately result in null findings and the loss of valuable time. Thus, by defining the characteristics of an ideal serotype, by carefully measuring these characteristics across different species, and by selecting an appropriate number of candidates, the probability of successfully developing a customized and targeted AAV vector for a client's specific gene therapy needs is increased.

CONCLUSION

The successful development of novel AAV serotypes with enhanced specificity, infectivity, and reduced toxicity demonstrates the significant potential of AAV capsid engineering in overcoming challenges associated with gene therapy. This study has generated promising AAV variants, such as AAV-PG008 and AAV-PG007, which show improved tissue targeting and reduced off-target effects, making them viable candidates for therapeutic applications in the central nervous system and muscle tissues.

The process of identifying these variants relied heavily on key factors in successful capsid screening. Starting with a highly diverse and uniform capsid gene library, we were able to systematically explore a broad range of potential variants. Careful attention to specialized packaging techniques and stringent control of the mispackaging rate ensured accurate and reliable screening results, critical for discovering effective AAV candidates. Additionally, the rational design of capsid libraries combined with comprehensive *in vivo* testing across multiple species provided a solid foundation for identifying serotypes with desirable characteristics for gene therapy.

Overall, these findings support the potential of capsid engineering as a critical tool for developing highly targeted and effective AAV-based gene therapies, advancing the field toward more precise, safe, and efficient treatments.

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AFFILIATIONS

Ye Bu
PackGene Biotech Inc.,
Houston, TX, USA

Yujian Zhong
PackGene Biotech Inc.,
Houston, TX, USA

Yue Pan
PackGene Biotech Inc.,
Houston, TX, USA

Huan Chen
PackGene Biotech Inc.,
Houston, TX, USA

Zhiyong Dai
PackGene Biotech Inc.,
Houston, TX, USA

Youwei Zhang
PackGene Biotech Inc.,
Houston, TX, USA

Ying Fan
PackGene Biotech Inc.,
Houston, TX, USA

Junlin Chen
PackGene Biotech Inc.,
Houston, TX, USA

Keqin Tan
PackGene Biotech Inc.,
Houston, TX, USA

Rui Duan
Laboratory of Regenerative Medicine
in Sports Science,
School of Physical Education
and Sports Science,
South China Normal University,
Guangzhou, China

Min Guan
Research Center for Human Tissues
and Organs Degeneration,
Institute of Biomedicine and Biotechnology,
Shenzhen Institute of
Advanced Technology,
Chinese Academy of Sciences,
Shenzhen, China

Irene Song
PackGene Biotech Inc.,
Houston, TX, USA
(Author for correspondence)
irene.song@packgene.com

Luyan He
PackGene Biotech Inc.,
Houston, TX, USA
(Author for correspondence)
luyan.he@packgene.com

Xin Swanson
PackGene Biotech Inc.,
Houston, TX, USA
(Author for correspondence)
xin.swanson@packgene.com

Paul Li
PackGene Biotech Inc.,
Houston, TX, USA

AUTHORSHIP & CONFLICT OF INTEREST

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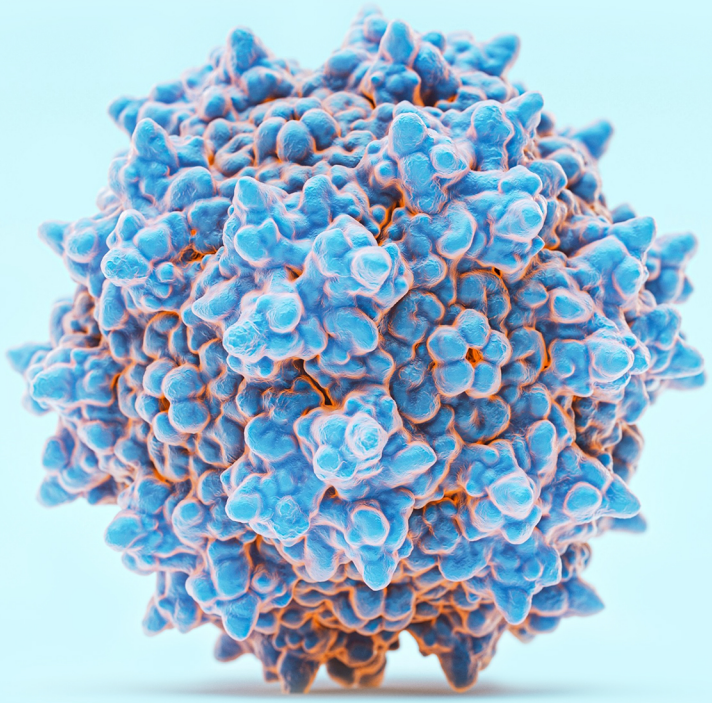

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CELL & GENE THERAPY INSIGHTS

REVIEW OF 2024 AND PREVIEWING 2025

SPOTLIGHT

Industrializing cell therapy: how to bring curative therapies to all patients who need them?

Jens H Vogel
Mirai Bio

"The ideal future of engineering living cells to fight disease will be to completely skip the complex, lengthy and costly *ex vivo* engineering and cultivation of cells..."

VIEWPOINT

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Leveraging living cells to fight disease has shown outstanding potential compared to traditional modalities, as evidenced by the dramatic success of the first generation of CAR-T cell therapies in hematological cancers, with very high response rates and many patients showing durable responses.

However, there are huge challenges with regards to developing and manufacturing cell therapies for all patients who need them.

For autologous cell therapies, starting material is highly variable, aggravated by the fact that patients are often very sick. One batch equals one patient, meaning one can only scale-out rather than scale-up. *Ex vivo* manufacturing process duration can be 2–3 weeks long, which together with the difficult scheduling of manufacturing starts and need for segregation severely limits actual number of batches produced per available clean room. Manufacturing processes are generally highly bespoke, with no consensus platform even for same cell types. Much manual operation is still required, with high pressure on the operations crew (an operating error might mean that a very sick patient will lose his last chance of being treated in time), potentially leading to high personnel turnover and challenges in recruiting and training.

An estimated total of 35,000 patients have been treated with the totality of produced CAR-T therapies in the USA over the last 7 years [1], and patient access still faces limitations. It will be very difficult to expand indications, e.g., to realize the huge potential of autologous CD19 targeting cell therapies in autoimmune diseases like lupus, with approximately 500,000 SLE patients in the USA alone [2]. From a financial perspective, yearly revenues generated from cell therapies have correspondingly been modest in comparison to blockbuster biologics so far, despite a high price per dose. In addition, development costs are currently much higher compared to conventional modalities, contributing to a recent reduction in VC funding of cell therapy start-ups.

HOW DO WE AS AN INDUSTRY UNLOCK THE POTENTIAL OF CELL THERAPY FOR THE BENEFIT OF PATIENTS GLOBALLY?

Speed-up and fully automate autologous cell therapy manufacturing

A crucial step to improving autologous cell therapy supply is to reduce *ex vivo* expansion time to days down from 2–3 weeks, in part by better controlling stem-ness and avoiding T cell exhaustion (thereby shifting much of the expansion process to the patient's body after infusion). Examples include Novartis' T-Charge™ process and Gracell's FasTCAR platform, recently part of a $1 billion acquisition by AstraZeneca.

In addition, processes need to be fully closed and automated, to enable a single advanced robotic system to handle multiple patient batches simultaneously in a small footprint. Capacity can then be modularly scaled out with high consistency by either creating very large farms of robotic units operating in parallel in a single factory, or smaller localized manufacturing units. Cellares is likely the most advanced example overall with their cell shuttle platform. However, Cellares' approach requires developing products on their platform, or a fairly elaborate adaptation into their platform (the latter with corresponding regulatory implications). Another approach is to try to use advanced robotics to mimic the human/machine interface and automate the operation of conventional (or slightly modified) systems already in use throughout the industry, thereby minimizing tech transfer implications. For example, Multiply Labs is pursuing such a modular approach, leveraging existing GMP systems.

Introduce allogeneic cell therapies

Allogeneic off-the-shelf cell therapies could eliminate most of the challenges of autologous cell therapies and enable supply logistics much closer to conventional biologics. Large factories of robotic units making autologous cell therapies could be replaced by relatively small 3D bioreactors and modular platforms making allogeneic off-the-shelf products. Of course, e.g., allogeneic CAR-T therapies require further gene editing of the cells, using tools such as CRISPR-Cas9, to avoid TCR mediated graft-versus-host disease (GvHD) and help minimize HLA related host-versus-graft (HvG) response. There are now many

allogeneic cell therapies in clinical development, with several investigational products such as Poseida/Roche's P-BCMA-ALLO1 and Caribou Biosciences' CB-010 showing promising data. However, the Caribou CB-010 data demonstrated the need for integrating a partial HLA matching strategy to improve progression free survival to levels on par with autologous cell therapies, with 13 different manufacturing batches used to ensure that approximately 90% of patients receive a dose with at least 4 matched alleles [3]. More data will become available showing how well GvHD, HvG, and durability can be managed with allogeneic approaches.

Transition from *ex vivo* to *in vivo*: the future

The ideal future of engineering living cells to fight disease will be to completely skip the complex, lengthy and costly *ex vivo* engineering and cultivation of cells, by creating e.g., patient specific CAR-T cells directly inside the patient's body. For example, Stephan *et al.* have shown in 2020 that non-viral nanoparticle delivery of mRNA can program CAR lymphocytes *in vivo*, causing leukemia regression with efficacies similar to adoptive T cell therapy in mice [4]. A highly tunable mRNA/LNP based approach creating CD19 targeting CAR-T cells *in vivo* also appears particularly attractive in lupus, where long-term persistence is not required to achieve an immune system reset, and the transient, non-integrating approach has clear safety benefits.

Several *in vivo* CAR immune cell programs are now either in the clinic or approaching the clinic in various indications, using lentiviral vectors or LNPs [5]. By 2026, we should see the first clinical read outs and get a first sense of how far out this future may be. In any case, highly effective targeted delivery of information molecules and/or gene editors to specific cell types *in vivo* will be key to the success of this approach, and will ultimately enable drug like economics, reduced development timelines and large-scale patient access.

Even if all three approaches to industrialize cell therapy are successful, they will co-exist for some time and pursuing them in parallel as an industry will ensure that we can treat as many patients as possible, as quickly as possible.

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BIOGRAPHY

JENS H VOGEL has 25 years of leadership experience in biopharmaceutical development, technical operations, and global supply, driving platform innovation and building and transforming successful biotech businesses. Jens has helped file >70 Investigational New Drug applications (INDs), and bring 10 biologics to market. As Global Head of Biotech at Bayer Pharmaceuticals, he covered a US$4 billion commercial biologics portfolio, a multimodality development pipeline, and drove the creation of industrialization platforms for cell and gene therapies. Before that, Jens was President and CEO of Boehringer Ingelheim Fremont, and a member of BI's Global Biopharma Executive Committee.

AFFILIATION

Jens H Vogel
President and COO,
Mirai Bio,
Cambridge, MA, USA

AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

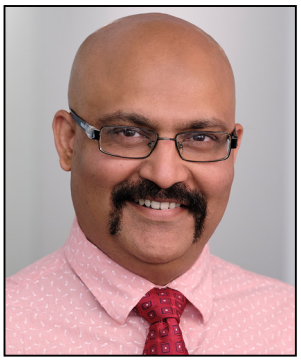
REVIEW OF 2024 AND PREVIEWING 2025

SPOTLIGHT

EXPERT ROUNDTABLE

Preparing for success in gene therapy purification

Xiaotong Fu, Ashish Sharma, Andrew Tustian, and Eugene Sun

In this expert roundtable, four highly experienced industry professionals discuss key considerations in developing an AAV purification process, highlighting the importance of early-stage planning and robust process design. The panelists share insights on QbD approaches in gene therapy development, process intensification, and ensuring sufficient flexibility to adapt platforms for different AAV serotypes while maintaining efficient processes and regulatory compliance.

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"The impact of the gene of interest cassette size must also be considered [when developing an AAV purification process], including the type of vector packaged..." — Ashish Sharma

Q What key issues or considerations should scientists address when starting to develop an AAV purification process?

AS: Firstly, considerations of construct design and capsid serotype are crucial because they significantly impact platform productivity and packaging throughout process development. Secondly, the more we understand which parameters influence potency early on, the better we can design a robust downstream process. Notably, the process can be designed not only to remove impurities but also to eliminate non-functional vector genomes, ultimately reducing required dosage of the final product.

AT: The first step is to define the process goals. It can be beneficial to employ a QbD approach, which involves identifying key quality attributes of the AAV vector and setting development targets based on those. This allows for goal-based development a than developing a process and then measuring the goals afterwards.

For AAVs specifically, it is important to monitor the levels of truncated transcripts in the affinity pool. From my experience, this is largely influenced by the design of the inverted terminal repeat (ITR) region. Sometimes adjustments must be made in that area, as certain ITR-to-ITR sequences may not be feasible due to the presence of hairpin loops and promoters. That's the first thing I like to look at because then I can go back to the team and say "OK, this promoter isn't working—we have a lot of partials—can we redesign it?"

AS: I agree. The impact of the gene of interest cassette size must also be considered, including the type of vector packaged—namely, the difference between self-complementary and single-stranded vectors, and how the type influences the prevalence of functional versus non-functional vector genomes in the final product.

XF: From a CMC perspective, understanding the target quality profile is crucial before starting process development. Using a phase-appropriate approach is key—there is no need to over-engineer the process if the target quality profile has already been met, especially given the current timelines for gene therapy development programs.

ES: When designing a process early on it is imperative to consider scalability. Whichever options are considered upfront, the goal is to accelerate the timeframe to the clinic. In essence, long-term thinking can help developers save time later during clinical and commercialization stages. It is important to consider effectively scalable options to avoid situations where you may need to repurpose, modify, and redesign the process, as it could impede the long-term goals.

Q What major challenges have you encountered when performing a tech transfer or scaling up a process, and what strategies have you adopted to address them?

AT: Scaling up any process presents unique challenges, whether you are working on developing AAVs, monospecific antibodies, or bispecific monoclonal antibodies. Generally, the unexpected issues that arise during scale-up are typically not within the individual unit operations, such as the affinity chromatography step, considering these are well-developed over time. However, a frequently overlooked area is the characterization of processes between the steps. For example, it is essential to consider aspects such as filterability, filter capacity, and sterility between different steps to avoid situations such as where you are halfway through filtering a scaled pool and the filter reaches capacity, causing high pressure.

Additionally, it is important to consider mixing and how material is transferred in and out of the vessels in order to achieve homogeneous solutions. Improper mixing and transfer may cause aggregation, especially if the liquid interface is not carefully managed.

Regarding tech transfer, one significant issue with AAVs involves scaling up the anion exchange (AEX) chromatography step, especially when transitioning from in-house processes to a CDMO. Specifically, pool collection can be challenging. For instance, if you have gradient and specific collection criteria, scaling pool collection requires careful consideration.

ES: It is crucial to understand the intricacies of the equipment used for empty-full capsid separation. Variability is a significant concern here—therefore, knowing the differences between devices at various scales is essential, as flow distribution can change considerably with larger equipment. This may subsequently impact the elution pool volume. In essence, it is vital to understand what you are working with and explore all the options for scaling up effectively.

AS: At Oxford Biomedica, we focus not only on unit operations but also on process robustness. Specifically, it is important to establish small-scale models during the preclinical stage. We also strive to understand process robustness and apply any insights from gap assessments before scaling up to 50 L. This allows us to revisit and tweak certain parameters at the 2 L scale.

Furthermore, we have established specific formulations for shear sensitive serotypes, for enhanced stability, and for appropriate scalability.

To summarize, it is key to connect the 2 L scale development with the scalability and examining individual unit operations—particularly regarding filterability. We found that developing a novel wash on the affinity side significantly improved product filterability due to reduced impurities, improving AEX step robustness on empty capsid removal

AT: The final tangential flow filtration (TFF) step must also be considered, as there can be significant scalability issues, especially in earlier-stage processes. It is important to interrogate which pump is being used, whether it has different shear characteristics and whether there will be many pump passes.

When scaling up, it is essential to avoid air-liquid interfaces in the system and ensure that the pool vessel is adequately mixed so that diafiltration occurs effectively. Additionally, the size of the pool vessel may result in having more or less material from the upstream process than anticipated during scale-up. Therefore, it is imperative to ensure that TFF is robustly sized to handle these variations.

"It is crucial to understand the intricacies of the equipment used for empty-full capsid separation. Variability is a significant concern here—therefore, knowing the differences between devices at various scales is essential..." — Eugene Sun

Q Which tools or strategies can be used in the early stages to shorten development time towards a scaled-up commercial process?

XF: A modular development approach, often referred to as a 'toolbox approach', can be utilized within short process development timelines. For example, when handling different molecules with unique target quality profiles, we focus on identifying the key elements in each unit and understanding how they interact, rather than fine-tuning processes for every single AAV serotype. Most importantly, we assess how these interactions can affect the final output for that unit operation, primarily concerning yield and purity profile.

When a new program arrives with a different target quality profile, we can quickly identify the necessary adjustments to achieve that profile. This approach enhances our efficiency in process development. For example, if an AAV program needs a target purity profile of 90% full capsids, we know there are few levers we can pull: we can adjust the pH, modify microconcentration, employ multiple cycles, or fine-tune the fractionation collection strategy. We have a much greater chance of success in reaching that target purity profile, as we already understand how these elements interact.

AS: We have developed a resin library and screening capabilities at Oxford Biomedica, which have enabled us to conduct rapid proof-of-concept work with various AAV serotypes and genes of interest, facilitating the identification of the correct resins to deliver the highest purity. In downstream processing, we have focused on process intensification, meaning as we achieve higher bioreactor titers, we look for ways to reduce COGs in specific unit operations—for example, removing TFF and enabling direct capture on the affinity side, and introducing novel affinity washes to reduce the impact of product-related impurities on AEX performance

We have generated bench-scale data showing the impact of, and how to control, bioreactor production duration on post-translational modifications, which specifically relates to the potency of the final product.

Q How important is having a platform purification process versus a serotype-specific process? Which parameters do you evaluate when considering either option and why?

ES: There are many benefits to having a platform purification process, particularly if it can achieve the same results as a serotype-specific process. One significant advantage is the ability to reduce the time and resources required for process development and characterization of upcoming serotypes. This is possible by leveraging prior knowledge data generated from past programs that utilize the platform process.

> "...when handling different molecules with unique target quality profiles, we focus on identifying the key elements in each unit and understanding how they interact, rather than fine-tuning processes for every single AAV serotype." — Xiaotong Fu

Testing the platform process to see if it works can save the late-stage development team from needing to revamp the step in question. Additionally, in order to gain prior knowledge, it is important to have more data points. For example, if you are in a situation where you must choose between the platform process and a serotype-specific process, and if the results or parameters fall within acceptable ranges or are close enough, then it is beneficial to utilize the platform process. This logic applies to AAVs, whether for affinity capture using affinity chromatography or for full capsid enrichment typically done with AEX chromatography. If a single affinity resin can be used for multiple serotypes, that would be more efficient than switching out the affinity resin for each specific serotype.

A serotype-specific process may be preferred if the platform process fails to achieve the desired target levels, such as the percentage full capsid or the yield from a process step. If those levels are too low or there is a significant cost difference between the two processes, then a serotype-specific approach would make more sense, as you would primarily need to ensure that the end goals are met.

For AAVs specifically, we have observed that one specific resin may perform better for certain serotypes during AEX chromatography. For example, POROS XQ resins tend to perform better for AAV2 and AAV5 serotypes compared to POROS HQ, which is more suitable for AAV9 serotypes. In these cases, a serotype-specific process for full capsid enrichment may be more advantageous. However, as I mentioned earlier, it is still worth developing the platform processes, regardless of the chosen resin.

XF: From a service provider perspective, having a platform is beneficial, but it is only a starting point, especially since we work with various molecule types. For example, regarding plasmid design, there are times when you do not have the luxury of having a well-designed molecule, which can lead to issues such as truncation. During one program we worked on, the size of the particles was only 2.3 kb, which is only half full for that particular AAV. It simply did not work with our platform because there wasn't enough charge difference to separate full and empty capsids during AEX. At that point, we needed to start tweaking the process as the platform was no longer applicable.

AT: In short, a platform for AAVs of the same serotype is considered beneficial. When moving from one serotype to another, the previous methods used for other serotypes can be utilized as a starting point. However, expecting a full platform to work seamlessly across different serotypes may require too many sacrifices. For example, if you have always worked with AAV8 and are now transitioning to AAV2, some significant changes to the purification process will be needed. While the same order of unit operations can be used, changes in specific areas will still be necessary, such as salt levels in the buffer and the chosen affinity resin.

AS: Establishing a true plug and play platform requires a deep understanding of the purification fundamentals on both upstream and downstream sides. We platform the process

where applicable to reduce the complexity serotype to serotype and streamline tech transfer. Our ability to generate process feasibility data quickly enables us to do serotype-specific processes, particularly around the AEX step.

Q What techniques have you evaluated when developing a process step for full capsid enrichment? What are the advantages and disadvantages of these techniques, and which parameters do you use in evaluating their success?

AT: Traditionally, ultracentrifugation has been used for empty-full separation of AAV capsids. However, it is not a technique we have employed in our laboratory due to scalability and robustness concerns. Instead, we have opted for AEX chromatography, which is known for its scalability and proven robustness in other applications.

Regarding chromatography, four main aspects must be considered. Firstly, developers must determine what pH level provides the optimal capsid separation. Secondly, conductivity must be assessed for efficient separation. Thirdly, one must evaluate which salt type achieves the desired conductivity, whether it is a monovalent or bivalent compound (a common example is the use of sodium chloride gradient along with divalent salt additives, such as magnesium chloride). Finally, it is crucial to decide whether to run a gradient or use isocratic elution. The choice will depend on the resolution and separability of full and empty capsids.

XF: We have had a similar experience to Andrew. One approach that has proven successful for us is fine-tuning the loading conditions during AEX chromatography. By allowing more empty capsids in the flow-through rather than attempting complete removal, the percentage of empty capsids in the eluate can be reduced, while some of them are retained on the column. Subsequently, when regular elution proceeds, a much better resolution of full capsids is observed.

The alternative approach involves using multiple cycles. In the first cycle, the goal is to remove most capsids, while during the second cycle, similar conditions are applied to achieve much better resolution and a higher percentage of full capsids.

Finally, another strategy is to use mixed-mode chromatography, which is not as established as ultracentrifugation or AEX chromatography. This technique is gaining momentum, though, especially for the purification of molecules that are challenging to separate using traditional methods.

AS: We have focused on modulating both loading and elution conditions to manage the empty capsid removal through AEX chromatography. We have done extensive process development to optimize the AEX step with constructs that are known to package poorly. This understanding helped us to make the AEX step more intensified and robust for multiple serotypes.

Additionally, we have conducted a lot of work to understand the impact of bioreactor conditions on capsid charge heterogeneity and AEX chromatography performance. By controlling the generation of negatively charged species and reducing post-translational modifications outside of the bioreactor, the AEX efficiency is improved, which in turn enhances the overall potency of the final product.

"In short, a platform for AAVs of the same serotype is considered beneficial. When moving from one serotype to another, the previous methods used for other serotypes can be utilized as a starting point." — Andrew Tustian

Q How do you justify your target purity requirements, including the percentage of full capsids, for regulatory approval?

AS: During the **Cellular, Tissue, and Gene Therapies Advisory Committee** meeting held on September 2–3, 2021, it was highlighted that most manufacturers either do not remove empty capsids or only partially remove them. We have taken this as an imperative to understand these specific steps and deliver a process to achieve under 10% empty capsids across multiple serotypes, including poorly designed constructs.

Regarding regulatory approval, we are still in a grey area, although the US FDA has indicated that reducing the toxicity risks associated with the final product is considered essential.

AT: The separation of full and empty capsids is a topic of significant debate within the industry, particularly concerning what constitutes an appropriate percentage. Some have suggested that empty capsids may provide a beneficial effect by acting as decoys to remove some of the immunoglobins in the blood against the corresponding AAV. However, there is now a broader consensus that it is best to reduce the number of empty capsids to minimize the immunological burden on the patient, which in turn aims to decrease side effects associated with AAV such as liver damage, complement activation, or dorsal root ganglia toxicity.

According to a paper published by a consultancy group a couple of years ago, it was recommended to achieve a target of 70% full capsids, which attracted significant criticism. It was later noted that it is inappropriate to set a one-size-fits-all target for empty-full ratios in AAV processes. Instead, it should be considered what specific therapy is being developed and what relevant data are available, and subsequently, a quality-by-design approach should be taken to determine the acceptable empty-full capsid ratio.

For example, total doses are much lower if a drug is intended for delivery to an 'immuno-privileged' area, such as the ear or the eye region. In these cases, there may be more flexibility regarding the empty-full capsid ratio. In contrast, for a large systemic dose aimed at achieving high transfection across multiple tissues in the body, such as dosing 1×10^{14}/kg in a patient, a more stringent empty-full capsid ratio would be necessary to minimize the immunological burden on the patient.

BIOGRAPHIES

XIAOTONG FU currently serves as the Head of Downstream process development and pilot operation at Resilience Technology Development Group, Boston, MA, USA. In this role, he focuses on supporting early and lates stage gene therapy programs, building platform processes for AAV and LVV, and managing CMC activities from development to GMP. Prior to his role at Resilience, Fu was leading downstream process development at Biogen, supporting its early and late stage AAV portfolio. Additionally, he led the development of Biogen's

plasmid process team and the high-throughput process automation team. Fu holds a PhD in Chemical and Biomolecular Engineering from Johns Hopkins University, Baltimore, MA, USA and he earned his BSc in Biotechnology from Shandong University, Jinan, Shandong, China.

ASHISH SHARMA is as an Associate Director at Oxford Biomedica LLC, Bedford, MA, USA and is responsible for managing client services and the downstream platform and process development activities for multiple viral vector modalities. He holds an MS in Chemical Engineering from Rensselaer Polytechnic Institute, Troy, NY, USA and has over 18 years of experience in process development, troubleshooting, characterization, and scale-up of purification processes for viral vectors, fusion proteins, and biologics. Sharma also has extensive experience in leading pre-pivotal and pivotal process development, qualifying small scale models, process characterization, in process control strategy, comparability protocols, viral clearance, and IND/BLA submission.

ANDREW TUSTIAN leads the viral vector process development group at Regeneron Pharmaceuticals, Tarrytown, NY, USA, focused on developing bioprocesses for AAV-based viral vectors. Tustian has worked at Regeneron since 2009. Prior to moving to gene therapy, Tustian co-led the purification development group for protein therapeutics, working on Fc-fusion, monoclonal antibody, and bispecific processes. He has worked on the process development for the FDA-approved drugs Dupixent®, Libtayo®, Inmazeb™, Kevzara®, Evkeeza™ and Praluent®, and helped develop the bispecific antibody platform at Regeneron. Andrew received his PhD in Biochemical Engineering from University College London, London, UK and his MSc in Biochemistry from the University of Oxford, Oxford, UK.

EUGENE SUN is the Field Application Scientist for Thermo Fisher Scientific, Bedford, MA, USA and is responsible for providing technical support for POROS and CaptureSelect chromatography resins to various process development customers in the gene therapy, cell therapy, nucleic acid, and antibody space. Prior to joining the Thermo Fisher Scientific Bioproduction Group in 2021, Sun supported Amgen's Pivotal Drug Substance Technologies group in Cambridge, MA and was responsible for the development, characterization, and scale-up support of downstream processes to enable commercial advancement of programs from clinical trials to marketing application. Prior to that, Sun started his career supporting MedImmune/AstraZeneca's early drug discovery/pre-clinical pipeline down in Maryland as a member of the Purification Process Sciences department. Sun has over 10 years of extensive bench-scale experience developing both early and late-stage purification processes across all downstream unit operations for monoclonal antibodies, various formats of bispecific antibodies such as Bispecific T-Cell engagers (BiTEs), and biosimilar programs. Sun earned his BA in Biochemistry and Music from Bowdoin College, Brunswick, ME, USA.

AFFILIATIONS

Xiaotong Fu
Head of Downstream Process Development and Pilot Operation,
Gene Therapy
Resilience Technology Development Group,
Boston, MA, USA

Andrew Tustian
Senior Director,
Preclinical Manufacturing and Process Development,
Regeneron Pharmaceuticals,
Tarrytown, NY, USA

Ashish Sharma
Associate Director,
Process Development
Oxford Biomedica,
Bedford, MA, USA

Eugene Sun
Field Application Scientist,
Bioproduction Group,
Thermo Fisher Scientific,
Bedford, MA, USA

ThermoFisher SCIENTIFIC

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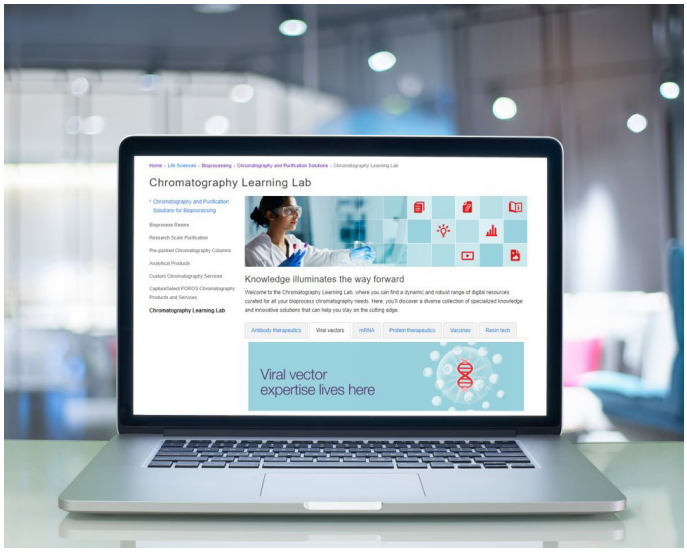

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REVIEW OF 2024 AND PREVIEWING 2025

SPOTLIGHT

EXPERT ROUNDTABLE

Preparing for success in gene therapy analytical development

Yan Zhi, Jing Li, and Andres Castillo



In this expert roundtable, three experienced industry leaders in analytical development share dos and don'ts to set you up for future success in AAV gene therapy product characterization and QC. The panelists troubleshoot a variety of development tasks, including how to select a specific methodology for CQA measurement and potency assay development.

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Q How do you keep up to date with evolving CMC guidance and what are some of the challenges that are associated with that?

AC: Beginning with the challenges, and looking at the industry overall, the pace of innovation in both approaches and techniques makes it challenging to keep up to date. In general, the inherent complexity of gene therapies also poses a major challenge. Then there is also the fact that we are working in a global sector, so there are multiple regulatory agencies with differing viewpoints and guidance to consider.

I think that being region-specific is key—paying attention primarily to any relevant guidance that comes out within your particular region from your local regulatory agency. It is also a good idea to leverage both internal and external networks. For example, at Thermo Fisher Scientific, I leverage both the regulatory and the technical expertise that exists within the company. Then externally, I work with different partners around the world to understand their challenges in terms of their own local regulatory guidance, and to also find out what they are hearing in terms of potential future developments in that sphere.

YZ: I certainly agree with Andres' point about complexity. For one thing, as he pointed out, CMC guidance is not completely harmonized between different countries and regions of the world. Additionally, the current guidance is sometimes open to interpretation, depending on how much you know about your product and how you make your case as a sponsor. It is good practice to not only rely on your own internal stakeholders, but to also communicate with the regulatory agencies and find out whether they align with you in order to reduce risk.

JL: Just to add to what Yan and Andres already said, I would definitely endorse engaging with the major regulatory bodies such as the US FDA and the EMA in order to stay current with the evolving guidance for cell and gene therapy. Sign up for webinars, workshops, or town hall meetings run by the agencies to stay informed.

On the topic of building a strong internal and external expert network, attending industry conferences is worthwhile, as is monitoring recent publications—especially those from companies with strong, long-standing expertise in cell and gene therapy. Also try to establish connections with consultants who have a lot of regulatory experience in this field. Last but not least, try to leverage your internal expertise with your regulatory CMC experts, as well as other colleagues with experience related to regulatory filings for biologics.

Q What are some strategies to generate robust data packages with regulatory compliance in mind?

YZ: As many readers may already know, the ICH Q14 guidance has recently been adopted. This has introduced Quality by Design (QbD) concept to analytical method

"...focusing in on your critical quality attributes and your critical process parameters and building efficiencies into those early on will be important for the future."

development as an enhanced approach. This approach will typically start with the definition of analytical target profiles (ATP) and critical performance attributes (CPA), followed by risk assessment in order to identify the analytical procedure parameters that have a major impact on assay performance. Through the increased knowledge and more extensive, robust data package this type of approach will generate, a control strategy may be further defined, ideally prior to assay validation, and further confirmed, following the completion of assay validation. Such an enhanced approach does require much more upfront work. However, if you do follow this approach, you will arrive at a more robust QC method—and one that requires less effort for life cycle management.

Once an analytical procedure is validated and ready for QC testing, it will be beneficial to try to collect as much representative batch data with that method as possible before you set up specifications or acceptance criteria. In addition, for certain stability-indicating assays, if you expect to have a higher intrinsic assay variability, such as you might with cell-based assay, it may be worthwhile to consider whether you can justify proposing different limits for release and shelf life. This can further reduce your program risk in the long run.

AC: Just to build on Yan's comments and also go back to the previous question, I think engaging with the regulatory agencies early is especially important here. Initiate those pre-IND meetings early to ensure that you are answering the questions they will ultimately ask through your design work and data package. Additionally, really focusing in on your critical quality attributes (CQAs) and your critical process parameters (CPPs) and building efficiencies into those early on will be important for the future.

Q How do you go about choosing a specific methodology to measure a CQA—for example, how do quantitative PCR (qPCR) and digital PCR (dPCR) systems compare in terms of accuracy and efficiency for the quantitation of titer at scale? And in this particular example, are there specific scenarios where one method would be preferred over the other?

JL: CQAs are typically formally established at the pivotal trial stage. By that point, you should already have a relatively good understanding of your analytical methods and of your product itself. In terms of selecting a qPCR versus a dPCR assay, I would say qPCR is the classical, more commonly used method. At the early stage of product development,

> "...once you have a fixed dosage level and a good understanding of the product, I would definitely recommend using a digital (d)PCR assay."

when you don't yet have a thorough understanding of the product, are still producing vector material at various different concentration ranges, and don't necessarily have an alternative assay to predict titer before you submit a sample to the assay, I would suggest using the qPCR method.

However, at a later phase of development, once you have a fixed dosage level and a good understanding of the product, I would definitely recommend using a digital (d)PCR assay. This method is very accurate and precise, and it is capable of detecting minor changes. It also doesn't require a standard curve.

Going beyond PCR assays, at Phase 3, you have a decision to make between methods for characterization versus actual release methods. For some product quality attributes, such as empty-full capsid ratio, you can select mass spectrometry or analytical ultracentrifugation (AUC)-based methods for characterization, which tend to be more accurate and give you high resolution. However, your release assay needs to be robust, reproducible, and easily implemented in the QC lab. In that case, you might want to choose a chromatography-based detection method.

Overall, I think when selecting a CQA, you need to think about future analytical method validation and the assay's suitability for that stage of method development. You need to really be sure that the method you choose can be accurate, precise, and robust—particularly if it is for release purposes.

AC: The only thing I would add here is to also think about phase-appropriate cost considerations and throughput when you are assessing different analytical technologies and their suitability for your product and process. Building a good cost model can benefit you greatly in the future.

YZ: I completely agree with both panelists. I just want to emphasize that if you have the opportunity, please do consider the 'first principle' methodologies such as sedimentation velocity analytical ultracentrifugation (SV-AUC), or absolute quantification methods such as dPCR. Do not underestimate the amount of effort required—and the associated risk—in managing a critical reagent program, particularly around reference materials and reference standards.

This is a headache, because you need the material, you need strong bridging studies, and then you have inventory management to consider. And when you do a tech transfer, that further compounds any concerns. Again, do not underestimate the effort needed to maintain a critical reagent program!

Q What strategies can be employed to ensure consistency and reproducibility in analytical testing across different batches and production scales?

YZ: Firstly, with a QC method, whatever you validate has to be robust and within the state of control, because without that, there is no reproducibility, no consistency. The method must be ready for QC testing. Assuming that it is ready, as we discussed previously, you ideally want to design the assay with a QbD concept in mind in order to make life cycle management a lot easier.

With that said, assuming you have a very strong QC assay with those components already in place, your next task will be to ensure an adequate training program is in place so that your QC analyst can run the assay as intended. And again, you do need a well-managed critical reagent program, particularly for the standard reference material, because that is what your final result is based on.

Additionally, to prepare for the eventuality that you may need to do lab investigations at some stage in the future, you need to plan for a sufficient amount of retains from different batches—ideally, batches produced at different sites. On top of that, I think for most QC labs, data trending is a routine practice. You do need to consider data trending. The amount of information you want to trend may vary from one product to another, but ideally, you need sufficient data to give you confidence that there is no shift during the daily operation of the method.

With all of this in place, I think you have a good foundation to produce consistent and reproducible QC data for your product release.

JL: I just want to add that to establish a very good reference material and characterize well early on, it is important to utilize your control chart for the reference material to monitor the performance of your method. Secondly, it's always very important to develop a sound method and set specifications very early on to establish a strong foundation. Try not to make significant changes to your analytical methods during the later phases of clinical development. If you do have to update your method version, keep track of the version changes and which versions were used for which specific sample batches, and before you implement a change, take care to evaluate the critical time points involved.

Another important factor is equipment. Try to make sure that routinely-used equipment is well maintained and frequently recalibrated. Also try to have multiple sets of equipment. Then, when you need to test a sample with urgent turnaround time, you have an alternative instrument system to help you troubleshoot.

AC: I think that having very clear standard operating procedures (SOPs) and the ability to transfer those within facilities and to partners as well is key. Having methods that have clearly written SOPs in place makes a big difference.

Q Ensuring data integrity is critical in gene therapy development. What best practices can be adopted to maintain data integrity throughout the analytical and manufacturing processes?

JL: Firstly, it is crucial to implement very robust documentation practices. For example, you may have an Electronic Lab Notebook (ELN) system, and that documentation needs to be uploaded to a Veeva or a similar system in a timely fashion. Make sure you utilize well-validated analytical methods, and that you control your user access. Also, it is of course very important to perform regular data backups and make sure your data are stored safely. Thoroughly train your personnel in terms of generating reports or cross reviewing each other's data. And finally, make sure you have a robust data management system with a focus on the chain of custody of critical materials.

AC: You obviously want to make sure that your equipment follows a regulatory standard—for example, that it is 21 CFR Part 11-compliant. As I mentioned earlier, you need to ensure that your SOPs are well written, clear, and that you will be able to communicate them to others.

In general, if you are bringing people into your organization from different—I came into industry from academia, for example—then you need to create a culture where everyone understands what data integrity means and why it's important. Having a really good culture and training environment to be able to maintain those data integrity standards is key, as is having the requisite equipment in place, of course.

Q What are some common pitfalls that can cause delays in the development of AAV gene therapies, and how can one proactively avoid these issues?

AC: We have touched on this already, but it bears repeating that gene therapy is extremely complex. This means there are a lot of pitfalls, from vector optimization at the beginning through to challenges in full manufacturing.

Approaching this from the supply-side perspective, having really good inventory control and management is crucial. So is being able to communicate and consult with both suppliers and regulators as early on in development as possible—that alone can help you avoid a lot of the pitfalls.

Ensure you have continuity of supply—that the assays and reagents you need will be there on time and come from the same lots. It's about maintaining tight control and building the relationship.

YZ: Andres brought up the complexity from the supply and commercial perspectives—there is also complexity from the technical side. We know there is a variety of different AAV particles, and of course, there is also variability in terms of the DNA packaged within. It is not

"...I think you need to consider very carefully before you introduce any changes, from both the process and the analytical sides."

straightforward to understand the changes you make in manufacturing and the impact these have on product quality.

With this in mind, I think you need to consider very carefully before you introduce any changes, from both the process and the analytical sides. This is particularly true once your pivotal trial is complete, when you should only introduce changes if you have a very strong reason to do so and are prepared to conduct additional clinical trials, potentially.

Even when you don't introduce any changes to the process or analytics, you need to make sure you have very tight control of your materials because without it, things can drift over time. This sort of drift in quality and consistency is often difficult to catch.

JL: Developing a potency assay for gene therapy is a common challenge for everybody, because I think the bar is much higher than for other biologics with which we are perhaps more familiar.

At Phase 1, you have an infectivity assay or some initial form of potency assay, then at Phase 2 and Phase 3, there are different requirements, but the endpoint is always to have a functional potency assay. In other words, it's an incremental approach and you have to develop different sets of methods at different phases. Developing these different methods requires you to have a good, robust cell line suitable for your assay, which also requires development—you need to work closely with research to screen and engineer the cell line for your assay. This is a very complicated task, and very different development compared to other types of analytical assays. And ultimately, if you don't have your potency assay ready, this will definitely cause delays in your regulatory submission.

The second key cause of delays that I have seen relates to assay outsourcing. Several AAV-based gene therapy products have reached the market, and a lot of the big CDMO companies now have a great deal of expertise in the field. However, what I'm seeing right now is that very long lead times remain. If you are planning to outsource your manufacturing, or your release testing or characterization, it is vital to talk to potential CDMO partners early on. Make sure you have reached agreement in terms of timeline, and also expect that early on in the method transfer, there may be some troubleshooting required.

Q For those who are new to this space, what advice do you have to help them get started on their analytical assay development journey?

YZ: During the early stage of development, don't be afraid to explore many different technology platforms or types of methodologies to evaluate the same product attribute, just

to gain as much product knowledge as you can before you settle on one. Additionally, while it can be difficult and challenging when you start, try to incorporate QbD concepts into assay development. In the long run, this will not only give you much better assay robustness, but it can also significantly reduce the burden of lifecycle management, as I mentioned earlier.

Again, please do not underestimate critical reagents because in the gene therapy arena, there are very few commercially available standards. Most people in the space end up having to develop their own product-specific reference standard materials, so don't underestimate the amount of work required—the quantity, the quality, and the bridging. It's not an easy task.

Furthermore, do not underestimate the compendial assays. Even if you are just looking for a standard testing panel, you may assume that with a compendial assay, all the labs will know what they are doing. However, it is not that straightforward—for one thing, a compendial method may have different 'flavors' according to different regulatory CMC guidance. Make sure you truly understand the compendial method for yourself - how exactly to set it up, what is the sensitivity, etc.—and then ensure your CRO or CDMO follow that to the letter. Find out if different countries and regions will have different regulatory requirements and whether you will need to harmonize or not accordingly.

JL: Firstly, don't be afraid of developing analytical methods for AAV-driven gene therapy. I think a lot of biologics expertise can be utilized by gene therapy, and it can be a really challenging but also a fun task to develop analytical methods for this arena.

I would again like to emphasize the importance of developing a strategy for potency assay development very early on. Make sure that you understand exactly what you will need at different stages of clinical development so that you will be able to deliver a good method in time.

Additionally, with AAV you are looking at multiple components from an analytical perspective—you are looking at the capsid, as well as the therapeutic payload inside the capsid. In general, I think there are more product quality attributes that need to be evaluated in AAV gene therapy than with most other biologics. So, even though we already have instrument platforms and methodologies from the antibody world, for instance, that can be directly applied to AAV, I would nonetheless encourage everyone to try to develop a gene therapy-specific protocol. Custom methods tend to have better sensitivity and accuracy. They are more closely related to your product. I think it's definitely worth spending that extra effort early in your method development phase as it will really benefit you later on.

AC: Be curious, because the pace of innovation is fierce and there are so many new methodologies and technologies out there. Find out about them all, understand what they are measuring, their sensitivity, and whether they make sense in the context of the platforms that you are building.

I think the other important thing to understand, as productivity becomes a focal point within the industry, is whether there are things within your analytical method development that can be automated. Are there specific technologies that lend themselves to automation, and are you building them in and making those decisions early enough in order to be able to

capture those efficiencies for the future? I think keeping that productivity perspective whilst retaining your curiosity is an approach that can lead to success.

BIOGRAPHIES

YAN ZHI is a Director at CSL Behring, King of Prussia, PA, USA. Yan has over 15 years of leadership experience in biologics testing and cell and gene therapy development. She is currently responsible for the technical performance of Hemgenix to support worldwide markets at CSL Behring, following scientific and analytical leadership roles at Spirovant Sciences, Charles River Laboratories, FUJIFILM Diosynth, and WuXi AppTec.

JING LI is Associate Director of Analytical Tech Transfer, at AstraZeneca Rare Disease, Boston, MA, USA. Jing Li has extensive industry experience in analytical development, CMC, and structural mass spectrometry, contributing to biologics and gene therapy development at companies like Takeda, Bristol-Myers Squibb, and AbbVie.

ANDRES CASTILLO is Global Market Development Manager at Thermo Fisher Scientific, Pacifica, CA, USA. Andres has over 6 years of experience in cell and gene therapy commercialization, specializing in cell therapy manufacturing, gene editing, and pharmaceutical analytical strategies.

AFFILIATIONS

Yan Zhi
Director,
Cell and Gene Therapy Product Owner,
CSL Behring,
King of Prussia, PA, USA

Andres Castillo
Global Market Development Manager,
Thermo Fisher Scientific,
Pacifica, CA, USA

Jing Li
Associate Director of Analytical Tech Transfer,
Alexion,
AstraZeneca Rare Disease,
Boston, MA, USA

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REVIEW OF 2024 AND PREVIEWING 2025

SPOTLIGHT

COMMENTARY

Personalization in advanced cellular ImmunoTherapeutics: activities in Ireland

Athanasios Mantalaris, Nicki Panoskaltsis, and Fiona Killard

Advanced cellular immune therapeutics have transformative potential for an expanding range of diseases, including cancer, autoimmunity, and various immune-related conditions. Despite these advances, significant barriers need to be addressed to achieve improved efficacy, access, safety, and robust manufacturing. In this insight article, we present research activities in Ireland that focus on a) understanding and directing cellular heterogeneity for improved cellular therapeutics through control of metabolism during the biomanufacturing process and b) designing, biomanufacturing, and translating personalized advanced cellular immune therapeutics by creating an All-Ireland Research Center that leverages academic, manufacturing, regulatory, and clinical expertise to deliver a sustainable all-island ecosystem of innovation in cellular therapeutics.

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Adoptive advanced cell therapy (ACT) is defined as the transfer of viable cells from one host to another (allogeneic), or into the same individual, after storage or manipulation (autologous) [1–4]. Advanced cellular immune therapeutics (ACIT) utilize autologous or allogeneic immune cells that are minimally manipulated or genetically engineered from different cell sources to result in unipotent cell types, e.g., T cells, natural killer (NK) cells, tumor infiltrating lymphocytes (TILSs) and chimeric antigen receptor T (CAR-T) cells [1,2,5]. During cell culture, ACIT undergo different types

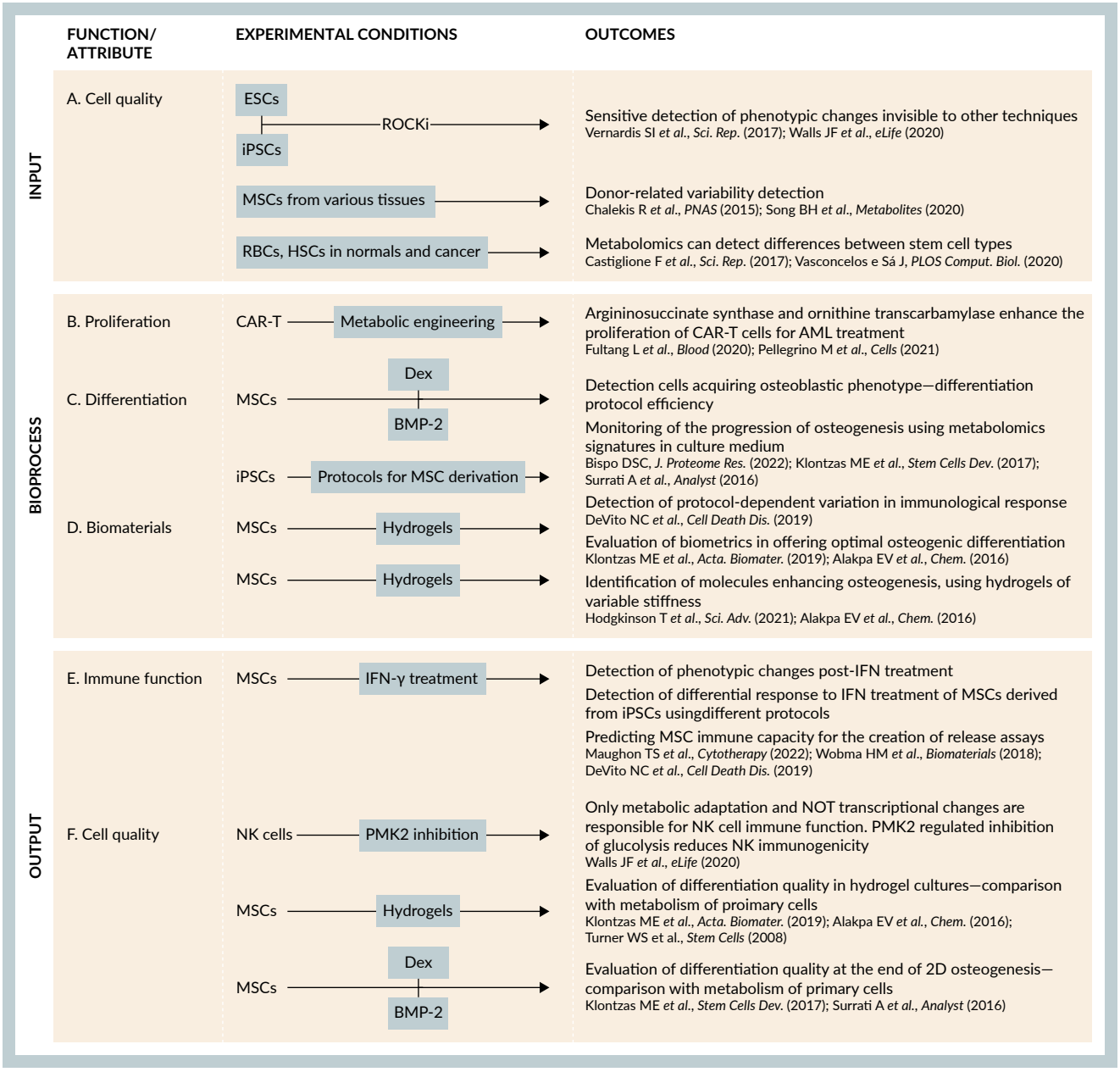
of manipulation, including genetic modification, differentiation, or activation. Despite their transformative potential, significant challenges remain for wide clinical application: (1) identification of robust and stable cell sources; (2) reliable therapeutic action *in vivo*; (3) predictable and defined levels of therapeutic potency; (4) pharmacokinetic (PK)/pharmacodynamic (PD) properties matching therapeutic requirements; (5) acceptable safety, immunogenicity and tumorigenicity profile; (6) balance between treatment cost and patient benefit and accessibility; (7) scalable and robust manufacturing processes; (8) the generation and maintenance of a stable workforce throughout the ACIT supply chain from donor source to patient (vein-to-vein) [2,6]. Currently, the conversion rate from a Phase 3 clinical trial to FDA approval is only ~14.3% for ACTs compared with 48.7% for conventional drugs [1]. Even with successful ACT product launch, clinical demands may outstrip supply in common diseases without addressing biomanufacturing scalability, product robustness and workforce demand early [1,7,8]. With a projected €59 billion global market value (2030), there is an unmet need for robust and reproducible manufacture of ACITs to ensure safety, efficacy, accessibility, supply-chain, value-for-money and financial stability of manufacturers [9]. In contrast to biologics manufacturing and application, ACITs require an established ecosystem of integrated workflows: the bioprocess, cellular product and patient care are interlinked and determine application- and cellular product-specific processes, bioprocess scale, production time(s), supply-chain requirements and patient treatment options/timing, in turn requiring unique, interdisciplinary training [1,2,6,7].

Clinical applications of ACITs have grown exponentially in the last 10 years, particularly in the use of CAR-T cells, driven by clinical trials showing improved outcomes in patients with otherwise incurable cancers, such as acute lymphoblastic leukemia, high grade lymphoma, and multiple myeloma leading to the commercialization of tisagenlecleucel (Kymriah®; Novartis), axicabtagene ciloleucel (Yescarta®; Kite/Gilead), and idecabtagene vicleucel (Abecma®; Bristol Myers Squibb), respectively, amongst others [10–13]. The COVID-19 pandemic has also highlighted that cell therapies may have utility in treatment of inflammation-driven tissue damage, and that immunomodulation with other cell products, such as T-regulatory cells, may be possible in this and other conditions of immune dysregulation [14]. Unfortunately, variability in reported patient responses in many ACIT studies can result from the unpredictable heterogeneity of the infused cell product, highlighting the need for robust and reproducible biomanufacturing [2,15]. This observation has been well characterized for CAR-T cells where improved responses have been identified in patients who received a more heterogeneous product composed of $CD4^+$ as well as $CD8^+$ T cells in defined ratios; products which did not have central memory T cell (T_{CM}) or stem-like memory T cell (T_{SCM}) subsets did not perform as well as those that did due to longevity and cytotoxic effects at the tumor site which can be linked to cell product metabolic fitness [16–19].

There are numerous distinguishing features of ACTs, compared to biologics: the most important being the use of different cell types (with different metabolic needs) for clinical use and the inherent heterogeneity (donor, source, cellular). Cell metabolism is the level of cellular function that allows fueling of intracellular processes through interaction with the microenvironment, and plays a crucial role in proliferation and differentiation, highlighting the fact that manipulation of cellular metabolism can enable regulation of cellular phenotype [20,21] (Figure 1). These changes are exemplified by *in vivo* processes, wherein cellular heterogeneity observed in cancer cells and during the development of specific anti-cancer and anti-viral T-cell subsets are directly related to altered metabolism in response to environmental cues,

FIGURE 1

The central role of metabolism and the efficacy of employing metabolomics for QA and QC of the various steps (input, bioprocess, and output) of the biomanufacturing process.



nutrient availability and oxygen gradients [22–24]. Metabolic heterogeneity of these phenotypically similar cells confer altered function—therapy resistance in the case of cancer cells, and effector vs stemness function in T cells. These data suggest that ACT product heterogeneity could be manipulated based on the understanding and stability of the metabolic changes occurring during the bioprocess. We have shown that metabolism is a more sensitive and accurate assessor of cellular state, and that metabolic signatures change well before genotype and phenotype in response to culture conditions and maturation signals (Figure 1). Robust control of metabolism, including bioprocess and cell culture media design, delivers the essential cellular quality required for clinical applications [8,25,26]. Recognizing the unique metabolic needs and the heterogeneity of the

cell population will enable the development of precision 'personalized' bio-manufacturing strategies, which is the cornerstone of quality by design (QbD).

METABOLISM-DRIVEN PRECISION BIOMANUFACTURING OF CELLULAR THERAPEUTICS

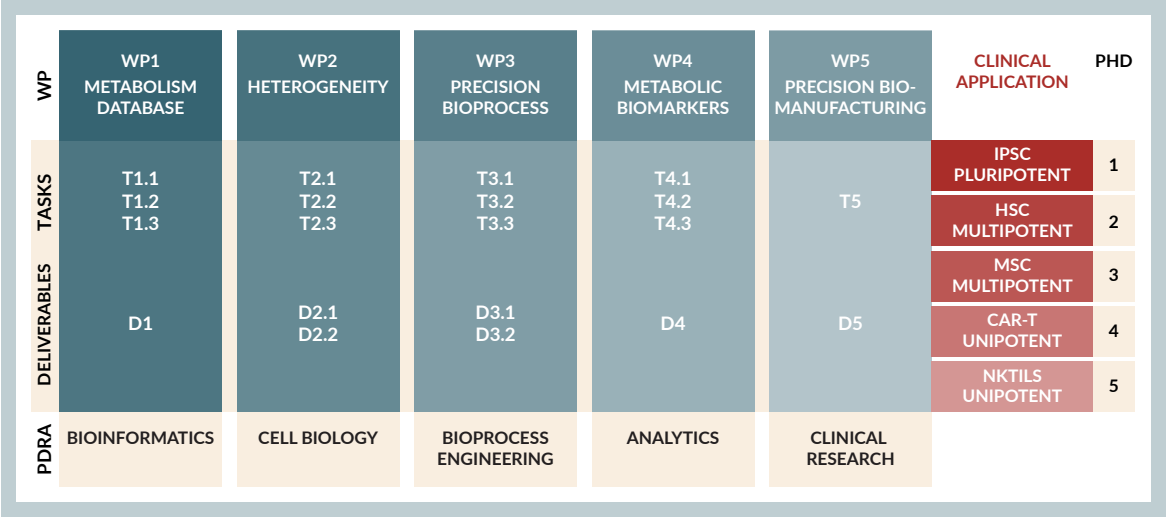
The overall research aim of this Science Foundation Ireland award is to characterize and direct cellular heterogeneity through the understanding and control of metabolism from the input to the bioprocess through to the output in order to develop improved ACTs that can result in improved clinical outcomes. There are five research work packages (WPs; WP1–5) thar run vertically and are integrated with five horizontal cell types, representing the breadth of clinical applications and the expertise in the BioSystems Engineering Laboratory. To ensure that this ambitious research program is delivered, four post-doctoral scientists and six PhD students have been recruited to work as an integrated team that has specific expertise and will work on the five cell types (Figure 2).

Cellular heterogeneity is classically characterized in terms of immunophenotype, genotype and transcriptome expression. Defining heterogeneity of similar cell types derived from different sources and how the bioprocess alters output products is critical to creating a robust platform for ACTs [1,2,15,19,27–29]. Currently, 'homogeneous' cell populations are defined by immunophenotype and/or limited genotype signatures [30–35]. Using these current strategies, maturation efficiencies, product quality and clinical outcomes remain variable resulting in failure of ACTs to gain necessary approvals for more wide-spread clinical use [1,36,37]. The dynamic sensitivity of metabolomic responses to environmental and cellular perturbations, including gene editing, results in heterogeneity during culture, is dependent on bioprocess conditions, and these changes impact epigenetic, genetic and phenotypic qualities of the final output [1,14–16,19,38,39]. Metabolomics analysis has the required sensitivity to capture metabolic shifts associated with genetic and immunophenotypic changes before they occur and can help drive the desired function. We have shown that when hiPSCs, a potential cell source for allogeneic ACIT applications, were exposed to Rho kinase (ROCK) inhibitor, commonly used in differentiation protocols, the expression of pluripotency markers at gene and protein level (TRA-1-81, SSEA3, OCT4, NANOG, SOX2) and stemness were not affected. In contrast, distinct and irreversible metabolomic changes were observed (determined by gas chromatography-mass spectrometry) consisting of reduced glycolysis, glutaminolysis, the citric acid cycle as well as amino acid pools as early as 12 hours, correlating with reduced expression levels of mTORC1 and independent of TP53 and caspase-3 expression (Figure 3) [26]. Thereafter, glycolysis increased with enhanced proliferative activity following 48 hours exposure and with adaptation to the environment during maturation. Similarly, CAR-T cells undergo different types of metabolism throughout the manufacturing process. For example, CAR-T cells which had low or pharmacologically-inhibited glycolytic metabolism during *in vitro* expansion phases had improved formation of memory T cells, longevity and *in vivo* homing, yet the *in vivo* anti-tumor or anti-infective function was best when glycolytic activity was high [16,19]. These data demonstrate the potential use of metabolic profiling in high-throughput workflows, as a tool for monitoring cells and bioprocesses, in directing cellular heterogeneity and becoming an established QA/QC criterion for the bioprocess, optimization and product release for the desired function.

The proposed methodology in our current research program includes de-convolution of culture processes using metabolic profiles with multi-omic characterization of source cells using single cell analysis. This process will enable clonal kinetic evaluation required for dynamic assessment of bioprocesseses,

FIGURE 2

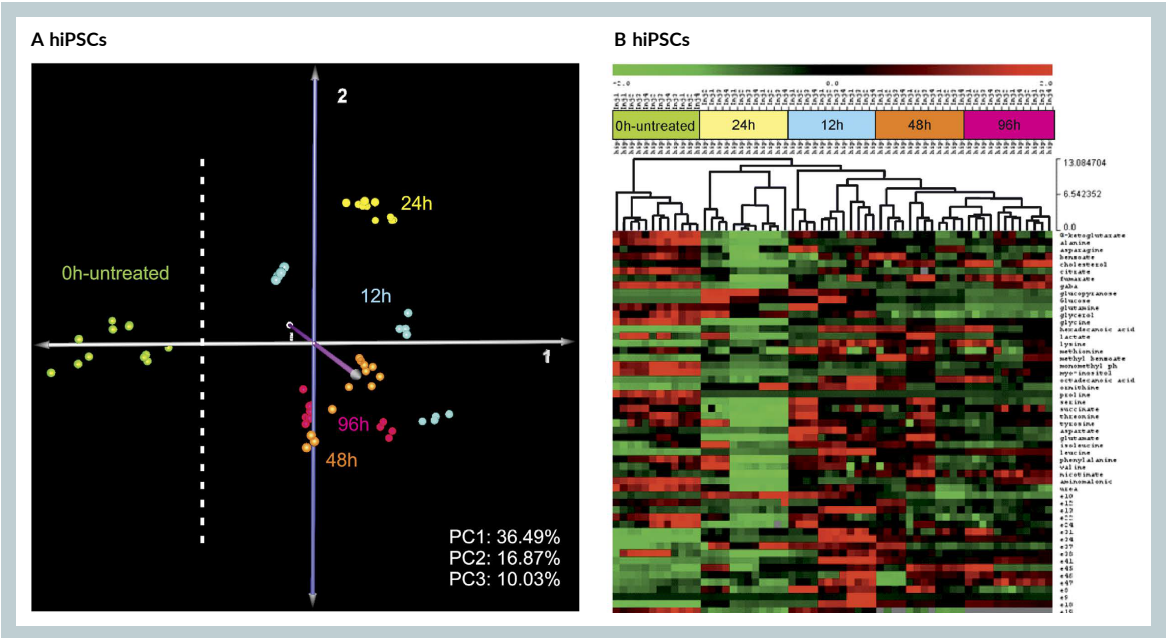
Metabolism-driven precision biomanufacturing of cellular therapeutics work packages.



Research WP1-5 run vertically and integrate with the horizontal cell types used in the majority of advanced cell therapies.

FIGURE 3

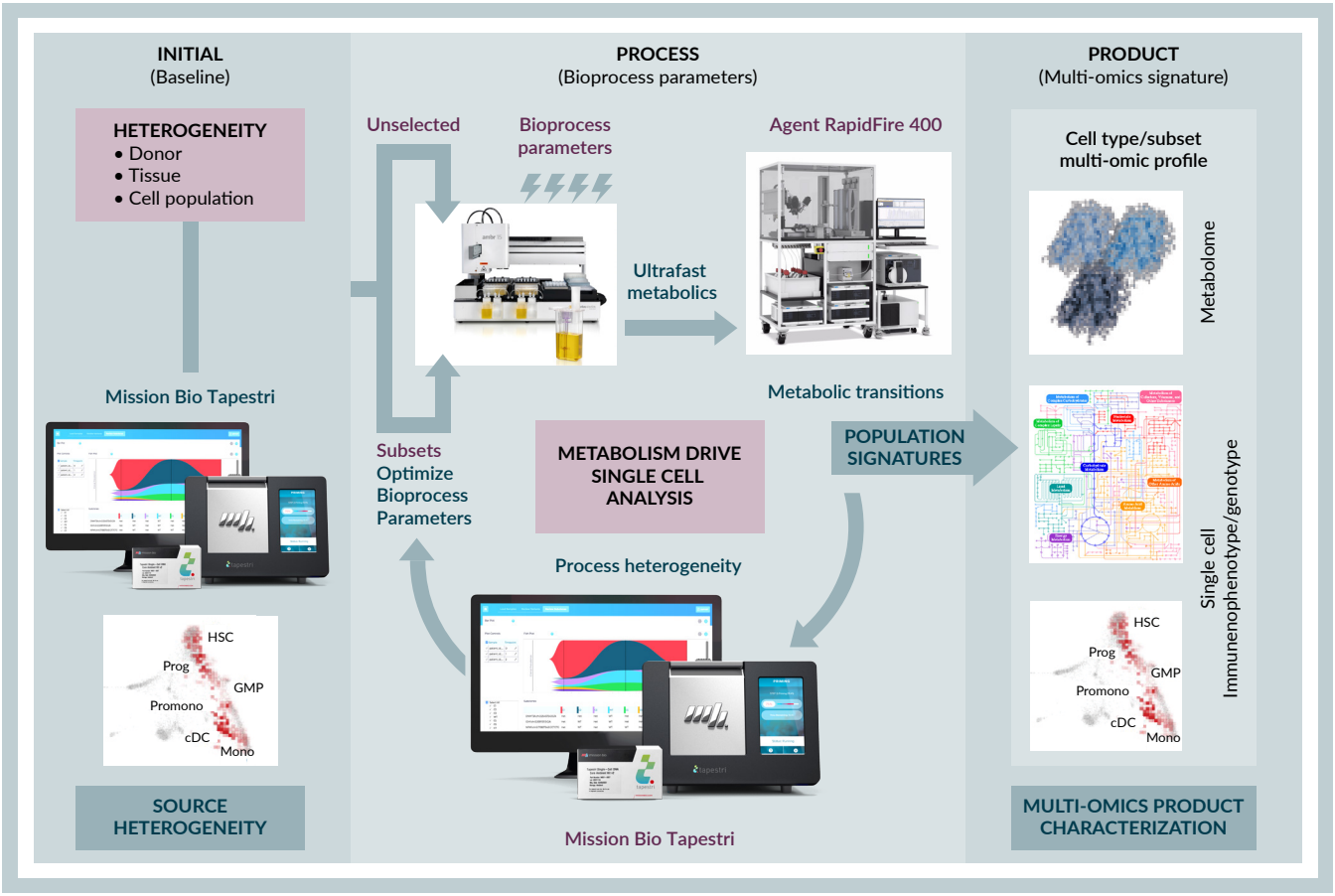
(A) Principal component analysis and (B) Hierarchical clustering show differences of untreated (0 hours) with ROCK-exposed cells (12 hours, 24 hours, 48 hours, 96 hours) in culture [26].



and facile integration from the biomanufacturing workflow to the translational/clinical space in future follow-up of cell products after infusion into patients. Whole cell populations of different cell types (iPSCs, CAR-T and NK/TIL) and sources (allogeneic versus autologous/primary cells) will be cultured under relevant bioprocess parameters to identify metabolic transition points to perform single cell multi-omics to determine heterogeneity. With repetitive rounds of optimization, multi-omic (phenotypic, genotypic

FIGURE 4

Cellular heterogeneity as a function of metabolism.

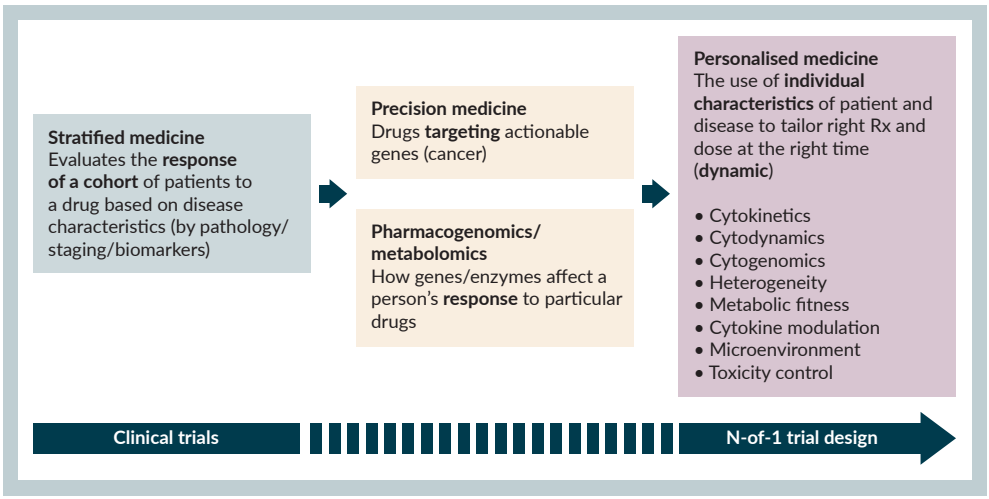


and metabolomic) population heterogeneity signatures will be created (Figure 4). The multi-omics datasets of each cell type under differing bioprocess parameters will be used to characterize the resultant heterogeneity of the output cells. Capturing heterogeneity at single cell level (genotype and immunophenotype) and linking with metabolomic signatures will facilitate characterization, optimization, and manipulation/control of the bioprocess resulting in robust ACT manufacturing strategies with better QA/QC utilizing metabolism as the most dynamic and fundamental defining feature of cellular function. Introducing these multi-omics signatures as part of the benchmark biomanufacturing workflow would accelerate and de-risk novel ACTs for personalized clinical requirements (e.g., defined CAR-T cell subset ratios with low glycolytic function) and outcomes in diverse patient populations in future, supporting rigorous regulatory and authorization processes.

Through application of metabolism-centric QbD principles, we will achieve precision biomanufacturing for the maximization of potency, purity and safety of ACTs. Specifically, the aim is to achieve the desired ACT performance (*in vivo*) by defining cell specifications based on metabolic phenotypes, and optimal process design (*in vitro*) by regulating process parameters for targeted metabolic physiology through precision media design (*in silico*). Finally process controls will be defined based on metabolic characteristics throughout the bioprocess as well as reproducible product quality attributes. Ultimately, a well-orchestrated bioprocess will be tailored to the application enabling rapid regulatory approval, quick adoption by

FIGURE 5

Progression of therapeutic development towards personalized medicines.



industry, readily available cost-effective and non-destructive monitoring at several checkpoints, reduction of the final costs, selection of the appropriate cell source and patient candidates and the delivery of safe, potent, and reproducible therapies that can be monitored and correlated post-infusion for efficacy and outcome measures.

IRISH MEDICINES: CENTRE FOR PERSONALIZED ADVANCED CELLULAR THERAPEUTICS (IMPACT)

IMPACT aims to move the Irish Biomanufacturing Industry up the value chain by creating a sustainable ecosystem for the 'living drug' era. IMPACT will design, manufacture, integrate, engage, regulate, translate, train, and implement personalized ACITs in Ireland and innovate (future-proofing IMPACT): 1) implementing personalized Quality by Design (QbD) by integrating clinical outcomes (incorporating patient and disease heterogeneity and toxicity control) with design and biomanufacturing; 2) delivering an All-Ireland Integrated Innovation Network linking Academia, Manufacturing, the Irish Blood Transfusion Service and Hospitals; 3) replacing sequential pathways (discovery, manufacturing, to translation) with integrated inter-disciplinary teams, for both research and training, to accelerate the process; 4) training a highly-skilled workforce for the whole workflow of cellular therapeutics; 5) developing the essential clinical trials pathway required in cellular therapeutics; 6) enhancing patient access and affordability and ultimately enabling personalized ACITs as first-line treatments; and delivering a New Industry for Ireland. IMPACT aims to deliver the right ACIT product with the right characteristics and phenotype to the right patient at the right time, such that an adaptive manufacturing strategy might be adopted for improved clinical outcomes with minimal toxicity: personalized QbD ACIT, and therefore going beyond precision therapeutics.

ACIT product variables are compounded by the unknowns at the point a patient receives the product, including patient and disease immune cell subsets heterogeneity and the kinetics of immune responses at diagnosis, through treatment and during remission states [17,40,41]. For both autologous and allogeneic CAR-T cell therapies, cells are genetically modified to add or remove functionality, usually achieved using vectors to deliver transgenes of interest [40]. With ongoing clinical trials showing efficacy in larger cohorts of hematological cancers, in other cancers and in autoimmune conditions

(e.g., lupus) [28,42] and burgeoning indications for potential use [43], there is an unmet need to develop safer ACIT, personalized to the individual, to improve outcomes and reduce toxicity, and to ensure rapid and robust product availability. IMPACT will personalize ACITs to target the disease areas of specific interest and of relevant expertise in Ireland: cancer, autoimmunity, infection, and neurodegenerative conditions with improved efficacy and reduced associated toxicities in each of these areas (secondary cancers, immune-related adverse events, infection/cytokine release syndrome, and immune-effector cell neurotoxicity syndrome). IMPACT aims to enhance our understanding behinds the mechanisms of improving efficacy and reducing toxicity by characterizing the heterogeneity of patients and their respective diseases through omics technologies [17], gene delivery methods to dial-in disease targets and dial-out toxicity in specific cell types (T, NK, and M cells) as well as delivering novel approaches to the challenge of Biostatistical n-of-1 clinical trial design methodologies [44,45]. Close collaboration with DESIGN, REGULATE, MAKE, and IMPLEMENT ensures that early R&D is informed to address future GMP manufacturing needs of engineered ACIT through to implementation and that cell engineering tactics are devised to achieve modifications that could enhance therapeutic efficacy and reduce toxicity of ACIT in patient-specific (n-of-1) protocols, i.e., personalization beyond precision (Figure 5).

IMPACT will consolidate the expertise of individual scientists across Ireland, partnered with opinion leaders internationally, to expand capacity to collectively deliver a single national center of research excellence for the future development and utilization of personalized ACIT. The creation of IMPACT is critically relevant in the context of creating a unique consolidated training ecosystem in Ireland for the development of the next generation of scientists, clinicians and allied health care professions for delivery of ACIT to patients and for employment in the national pharmaceutical sector.

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AFFILIATIONS

Athanasios Mantalaris
School of Pharmacy and Pharmaceutical Sciences,
Trinity College Dublin,
Dublin, Ireland
and
National Institute for Bioprocessing Research and Training,
Dublin, Ireland
(Author for correspondence)
mantalaa@tcd.ie

Nicki Panoskaltsis
School of Pharmacy and Pharmaceutical Sciences,
Trinity College Dublin,
Dublin, Ireland
and
Department of Hematology,
St James's Hospital,
Dublin, Ireland
(Author for correspondence)
panoskan@tcd.ie

Fiona Killard
National Institute for Bioprocessing Research and Training,
Dublin, Ireland

AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

REVIEW OF 2024 AND PREVIEWING 2025

SPOTLIGHT

Bio-printing in the context of end-to-end scalable tissue-engineered ATMP manufacturing

Isaak Decoene and Ioannis Papantoniou
KU Leuven

VIEWPOINT

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3D bio-printing technologies have been constantly evolving over the past 15 years in order to address health challenges, such as the need for regeneration or replacement of human organs and tissues. To date, there are no bio-printed tissue engineered products in the EU market and no active clinical trials [1]. Tissue Engineered Products are classified by the EMA as advanced therapy medicinal products (ATMP) [2] and to date only four existing products obtaining marketing authorization (first ATMP approved in 2009) are classified as Tissue Engineered ATMPs (TE-ATMPs) [3]. Out of these four products, two have been withdrawn due to limitations in manufacturing and scale-up capabilities which have been reported as major bottlenecks for these products [4]. Adoption of bio-printing technologies during TE-ATMP manufacture could further support addressing these bottlenecks. In a recent International Society for Cell and Gene Therapy (ISCT) committee report, advice on how to render the ATMP field more attractive to investors, scale-up as well as manufacturing and efficacy have been recognized are recognized as major barriers to further investment increase in the ATMP field [5]. This applies even more so to the complexity of tissue engineered ATMPs.

Tissue defects usually affect more than one cell and tissue type. Therefore, the bio-printing processes should be able to bio-print all the necessary cell types according to the corresponding length scale and complexity of the target tissue defects [6]. To date, bio-printing processes for autologous TE-ATMPs have mostly focused on bio-printing of cells suspended in hydrogels known as bio-inks, but these single-cell-type approaches fail to capture the intricacy of tissue architecture and lack necessary precision [7]. Moreover, bio-printed single cells lack phenotypic stability and, when exposed into large 3D environment such as the post bio-printing case, they differentiate in an uncontrolled manner or initiate apoptosis due to harsh metabolic conditions [8]. However, bio-printing to date has mostly delivered either tissue constructs of small size or larger size with low density due to the fact that it has relied on the bio-printing of cells or organoids that are produced using manual, small-batch operations [9]. Manual operation introduces major sources of variation and negatively affects cell and organoid quality properties and is a major driver for costs in cell therapy and regenerative medicine [10]. For organoid bio-printing to be carried out multiple steps are required such as cell seeding, organoid differentiation, media changes, organoid harvest, bio-ink formulation and organoid delivery to the bio-printer. For example, to bio-print a 1 cm^3 tissue requires 1×10^5 organoids, something that cannot be achieved easily by manual operators.

From the manufacturing perspective, bio-printing complex clinically relevant tissue implants requires a scalable supply of high-quality and robust cells and organoids. A critical barrier to clinical translation to date is that current (manual) manufacturing processes for cells and organoids are inherently inefficient, difficult to scale, resource- and labor-intensive, require skilled personnel, and necessitate significant human intervention, all of which can result in errors, contamination and final product quality variability (and often failure) and high costs [11,12]. Currently various commercial GMP-compliant single stem cell expansion platforms are available can be achieved in bioreactor systems [13] such as Terumo Hollow Fibre bioreactor [14] and the Miltenyi Prodigy [15]. In addition, larger ATMP manufacturing platforms for ATMPs are under development such as robotic pipeline platforms such as the AUTOSTEM [16] and AIDPATH [17] H2020 platforms, as well as the Jointpromise H2020 platform developed for organoids. However, bio-printing platforms are still developed as independent and isolated units of operation lacking predictions for their integration in end-to-end manufacturing pipelines. Hence the question of how can cells and bioinks be automatically loaded within bio-printers mostly remains unanswered.

From the PAT perspective, when assessing current end-to-end manufacturing practice, mostly quality characteristics of engineered ATMPs are registered manually or are distributed in dispersed data repositories [18]. This generates two problems: (i) decisions cannot be made rapidly and hence organoid differentiation and bio-printing processes cannot be rapidly optimized; (ii) these measurements provide only partial insights on generic properties such as metabolic activity and viability. Moreover, when monitoring is applied, various discrete technologies involved in the tissue production pipeline are currently monitored separately and lack a unified control capability. There is limited combination or integration of monitoring data, which hinders overall in-process management of the pipeline. Data storage is necessary for audit and hence the development of an electronic batch record would considerably aid in supporting translation of the bio-printed implants. There are recent considerations of adoption of Quality by Design principles during bio-printing [19] as well as implementation of machine learning [20]; however, there are currently critical gaps on monitoring and control capabilities in existing bio-printing technologies. Besides monitoring mechanical and structural properties of bio-printed constructs, much activity needs to be invested towards integration of biosensors able to measure metrics related to the functionality and efficacy of the bio-printed tissue engineered ATMP. The data from biosensors, imaging and at-line omics analytics is combined in a comprehensive approach to data acquisition, storage and analytics to achieve a data-driven production control for ATMP production [21]. This allows the production control to be 'aware' of both well-established and novel biomarkers and use them to adjust the operation of the production pipeline [22].

In order to develop bio-printed implants with proven identity and potency, a standardized and validated set of metrics (CQAs) that are reflective of cell and organoid functionality but also easy to be measured need to be developed [23,24]. Traditional cell- and tissue characterization relies on invasive, mostly manual, time-consuming methods such as cell counts, microscopy, histological staining, qPCR, and flow cytometry [25]. This results in multiple limitations that constrain the production of commercially viable BTE-ATMPs: (i) the cost of quality control is driven by work-hours; (ii) in-process decisions are delayed by several hours even days; (iii) lack of quality standards and release criteria at the preclinical level block translation. The use of cells and organoids as living building blocks within bio-printing processes requires the integration of non-invasive quality controls and the production of easily measured data sets that can be reflective of bio-printed implant functionality as required by regulatory bodies and quality by design guidelines [26].

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BIOGRAPHIES

ISAAK DECOENE is currently a postdoctoral researcher at the Tissue Engineering Lab of the Skeletal Biology and Engineering Research Centre Department of Development and Regeneration, Faculty of Medicine, KU Leuven, Leuven, Belgium. He holds a PhD in Biomedical Sciences, Molecular Stem Cell Biology at KU Leuven. He is a member of Horizon 2020 'Jointpromise' consortium, where his research activities focus on noninvasive quality metrics, automation, and donor variability in autologous organoid-based tissue engineering approaches, ultimately facilitating clinical and commercial translation of tissue engineered advanced therapeutic medicinal products.

IOANNIS PAPANTONIOU is currently an Associate Professor and head of the Tissue Engineering Lab of the Skeletal Biology and Engineering Research Centre Department of Development and Regeneration, Faculty of Medicine, KU Leuven. He is also the Program responsible of Prometheus, the Leuven R&R Translational Division of Skeletal Tissue Engineering aiming to bring innovation and impact to the real world either as commercial or clinical outcomes. In addition, he acts as a visiting PI at the Institute of Chemical Engineering Sciences, Foundation of Research and Technology (FORTH-Hellas) in Greece. He is the project coordinator of the Horizon 2020 'Jointpromise' consortium aiming at automating the biomanufacturing of organoid-based osteochondral implants while he is also a participant of the H2020 project 'AIDPATH' aiming at the integration of Artificial Intelligence technologies in Cell Therapy Manufacturing. His research activities are geared towards the development of automated and scalable bioprocesses for the manufacturing of organoid-based tissue implants and have received support from national, international, and institutional funding sources. He has published more than 70 scientific articles in peer-reviewed international scientific journals, participated in the writing of three book chapters and holds two international granted patents on organoid technologies for skeletal defect regeneration. He is an elected member of the TERMIS-EU council, serving as the deputy chair of the Communications and outreach committee. He is also member of the board of the Belgian Society for Animal Cell Technologies (BELSACT). He is a regular lecturer at the ESACT summer school series focusing on Manufacturing and Bioprocessing of Cell and Gene Therapies. He has received research fellowships from the National Scholarship Foundation of Greece and the Flemish (Belgian) Research Council. In 2019, he was recognised as a 'rising star' in the field of Regenerative Medicine Manufacturing at the Advanced Therapies Congress, London, an industrial conference attracting more than 1000 peers from both industry and academia.

AFFILIATIONS

Isaak Decoene
Skeletal Biology and Engineering Research Center,
Department of Development and Regeneration,
and
Prometheus, the Leuven R&D Translational Division of Skeletal Tissue Engineering,
KU Leuven,
Leuven, Belgium

Ioannis Papantoniou
Skeletal Biology and Engineering Research Center,
Department of Development and Regeneration,
and
Prometheus, the Leuven R&D Translational Division of Skeletal Tissue Engineering,
KU Leuven,
Leuven, Belgium

AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

REVIEW OF 2024 AND PREVIEWING 2025

SPOTLIGHT

Overcoming the senescence barrier in stem cell expansion

William Ying Khee Hwang
National Cancer Centre Singapore

"Combining strategies like telomerase activation, changing the senescent milieu, and reversal of genetics/epigenetics could enhance stem cell expansion strategies..."

VIEWPOINT

Cell & Gene Therapy Insights 2024; 10(11), 1407–1411

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Cellular senescence, triggered by stressors such as DNA damage and telomere shortening, restricts stem cell proliferation and longevity in culture. Techniques to counteract senescence include telomerase activation, modulation of the senescence-associated secretory phenotype (SASP), and genetic or epigenetic interventions. For hematopoietic stem cells, senescence inhibition has shown promise in supporting cord blood transplantation, where expansion techniques using cytokines and small molecules have improved clinical outcomes, including reduced hospitalization. Mesenchymal stromal cells (MSCs) also face limitations

from replicative senescence, affecting their use in immunomodulation and regenerative medicine. Emerging approaches, such as high-resolution telomere measurement and senolytic compounds, offer promising avenues for extending stem cell utility. Ultimately, measures to counteract cellular senescence could expand stem cell applications, enhancing the range of clinical uses.

Stem cell expansion is crucial for regenerative medicine, but cellular senescence—caused by stressors like DNA damage, telomere shortening, and oxidative stress—limits their proliferation and viability. This review discusses current methods for stem cell expansion and strategies for overcoming senescence, focusing on delaying its onset, enhancing resilience, and extending lifespan in culture.

CLINICAL USES OF STEM AND PROGENITOR CELL EXPANSION

The greatest utility of hematopoietic stem cell (HSC) expansion has been in supporting cord blood transplantation, where limited cell numbers result in delayed hematopoietic engraftment (with the associated problems of infections and thrombocytopenia) and prolonged hospitalization [1]. Various cord blood expansion strategies have been explored, involving cytokines, small molecules, and co-culture with mesenchymal stromal cells [2,3]. Strategies that enhance cell viability by reducing intracellular reactive oxygen species and improving mitochondrial membrane potential are effective in enhancing HSC expansion [4,5]. A successful clinical trial of cord blood HSC expanded in a combination of molecules and cytokines including nicotinamide, reported accelerated neutrophil and platelet engraftment as well as improved survival in patients receiving the expanded HSC product [6]. This product was subsequently approved by the FDA to treat adult and pediatric patients (12 years and older) with hematologic malignancies who are planned for umbilical cord blood transplantation following myeloablative conditioning.

MSCs have been used for their immunomodulatory and regenerative medicine properties. Their immunomodulatory uses include autoimmune diseases and post-transplant graft-versus-host disease. There has also been wide use of MSCs in regenerative medicine, especially for the treatment of knee cartilage injuries. However, MSCs in *ex vivo* expansion cultures are subject to replicative senescence, which limits their potential for benefit to multiple recipients.

The expansion of other stem and progenitor cell populations that have been explored in clinical use include skin epithelial cells for burns and corneal cells to improve or replace corneal transplants. Other uses being explored include neural cells for neural repair and cardiomyocytes for myocardial regeneration.

CELLULAR SENESCENCE AND ITS IMPACT ON STEM CELL EXPANSION

Senescence prevents damaged cells from dividing, impacting stem cell self-renewal and differentiation. Consequently, it affects stem cell expansion by limiting the duration and extent of expansion. Senescent cells exhibit larger size, increased β-galactosidase activity, and SASP factor expression, which create a hostile environment [7]. Two key pathways, p53/p21 and $p16^{INK4a/Rb}$, regulate senescence, often triggered by telomere shortening, which results in accelerated biological aging of the cells [8,9].

STRATEGIES FOR DELAYING SENESCENCE AND ENHANCING STEM CELL EXPANSION

Telomerase activation and telomere maintenance

Activating telomerase extends telomeres, delaying senescence. Controlled activation

is essential to avoid tumorigenesis. However, current methods for telomere measurement are imprecise and not useful for assessing the effectiveness of interventions in *ex vivo* expansion cultures.

A recently reported method for high-throughput high-fidelity telomere length measurement at nucleotide resolution using the PacBio sequencing platform could help identify better methods for telomere maintenance in stem cell expansion [10]. On the other hand, controlling the telomere length of stem cells through telomerase inactivation could improve the safety of cell therapies by reducing the risk of tumor formation [11].

Changing the senescent milieu

Cellular senescence is a stress-induced response that leads to irreversible cell cycle arrest and initiates extensive phenotypic alterations, including the secretion of a bioactive secretome, termed the senescence-associated secretory phenotype (SASP) [12]. Modulating the SASP with novel molecules and proteins could help reduce cellular senescence. Removal of senescent cells with senolytics have not only been explored for improving stem cell culture but have also been used in clinical trials for the retardation of cellular aging [13].

Reactive oxygen species in culture also result in oxidative stress to cells in culture. Hypoxic conditions and antioxidants help mimic natural hypoxic niches and further protect against DNA damage.

Genetic and epigenetic reversal

Reversing the senescent genetic and epigenetic profile of cells can reset their biological clock. Techniques like CRISPR/Cas9 can extend stem cell lifespan by targeting senescence-related genes [14]. Epigenetic changes shift cells to a more youthful state. iPSCs bypass senescence by resetting telomeres and epigenetic markers [15]. However, these manipulations may lead to long-term changes in the cells and pose risks like genomic instability—thus, more study and quality control is needed.

CONCLUSION

Addressing the senescence barrier in stem cell expansion is vital for regenerative medicine. Combining strategies like telomerase activation, changing the senescent milieu, and reversal of genetics/epigenetics could enhance stem cell expansion strategies and expand the scope of regenerative medicine.

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BIOGRAPHY

WILLIAM YING KHEE HWANG is a hemato-oncologist who currently serves as Senior Consultant of the Department of Haematology, Singapore General Hospital and National Cancer Centre Singapore, Co-Director of the Regenerative Medicine Institute of Singapore (REMEDIS), and Senior Advisor for SingHealth. He is the current Regional Vice President of the International Society of Cell and Gene Therapy. In 2023, he was the recipient of the National Outstanding Clinician Award. He previously served as CEO of the National Cancer Centre Singapore (2017–2023), President of the World Marrow Donor Association, Chairman of the Singapore Chapter of Haematologists, and President of the Singapore Society of Haematology.

AFFILIATION

William Ying Khee Hwang
National Cancer Centre Singapore,
Singapore General Hospital,
Duke-NUS Medical School,
Singapore

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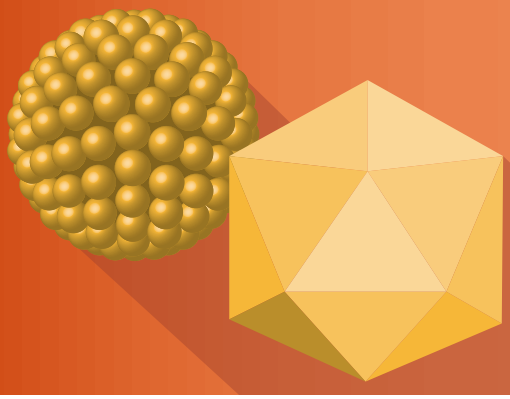

CELL & GENE THERAPY INSIGHTS

VECTOR CHANNEL EDITION
Characterization and validation

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CELL & GENE THERAPY INSIGHTS

INTERVIEW

History and progress in viral vector particle size distribution analysis

Abi Pinchbeck, Editor, *Cell & Gene Therapy Insights*, speaks to **Steven Berkowitz**, Analytical Development Expert, taking a deep dive into the role of analytical ultracentrifugation (AUC) and other methods used in viral vector particle size distribution analysis. They discuss the multi-levels of heterogeneity present in gene therapy products, the history and relevance of AUC as the 'gold standard' of molecular characterization, and the up-and-coming tools and developments in the space.

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Q Can you summarize your career in the gene therapy space, particularly surrounding your work with characterization and AUC?

SB: My experience in the gene therapy area goes back 24 years to the AUC work I did on adenovirus at Biogen in the analytical development department. This work eventually led to the publication of two papers [1,2] with the aim of rekindling interest in the use of AUC in the biopharmaceutical area, specifically to help people recognize, not only the utility that this unique analytical tool played in the early days of molecular biology, but more importantly the

role it could still play in modern drug development. I felt this analytical tool's ability to structurally characterize and analyze macromolecular structures was being overlooked, especially in the growing area of viral vector product development as we entered the 21st century.

My use of AUC on viruses goes back even further to my PhD work over 50 years ago, that I did on the filamentous bacterial virus fd, which involved studies on the biophysical properties and structure of this virus. This work was not associated with what we today call gene therapy; in fact, the concept of gene therapy at that time did not yet exist, nor had the biopharmaceutical revolution gotten off the ground.

In those early days, AUC was the equivalent to day's modern mass spectrometers. Every biochemical/biophysical lab had an analytical ultracentrifuge (Beckman Model E) as a key analytical tool for measuring molecular weight of biopolymers and studying their biophysical properties and interactions. As we approached the 1980s, the use and interest in AUC, unfortunately disappeared. Nevertheless, the technique started to make a comeback as we neared 2000, as a result of developments in computer technology, digital electronics as well as the appearance of a new Beckman (now Beckman-Coulter—the only manufacturer of this instrument) analytical ultracentrifuge.

Between these two time periods, much of my work was involved in applying separation and biophysical sciences to the task of analytically characterizing a wide range of biopolymer and synthetic macromolecules. Over this time, I was exposed to and acquired knowledge on a number of different analytical characterization tools, including electron microscopy, static/dynamic light scattering, calorimetry, chromatographic, electrophoresis, and mass spectroscopy.

Over the last 10 years, although retired, I have remained engaged in science by taking on, from time to time, consulting work with a number of companies where I have helped in training scientists on how to use and apply AUC analysis to their AAV gene therapy products. In doing this work I have also continued to make contributions to this area via scientific publications.

Q Advanced therapy drug products pose unique characterization challenges to developers. Can you outline the challenges here, particularly pertaining to the characterization of biophysical or physico-chemical structural properties, and suggest what necessary characterization and analytical tools could help?

SB: The drug industry has undergone some significant changes over the last few decades, especially as a result of the development of biopharmaceuticals (protein-based drugs). Prior to the expansion of biopharmaceuticals, drugs were typically small organic molecules, generally referred to as pharmaceuticals. Biopharmaceuticals, manufactured via biotechnology using living cells, are much larger and more complex structurally than pharmaceuticals. A key feature of these protein-based drugs is the susceptibility of their folded polypeptide chains, which are predominately held together by a large number of weak interactions, to physical and chemical

"Over the past few years, we have begun to see a further shift in the drug industry towards even larger and more complex biopharmaceuticals consisting of multiple protein subunits that give rise to a supramolecular structure..."

structural changes. Initially, biopharmaceuticals were small proteins; however much larger proteins followed, such as monoclonal antibodies, that now dominate the biopharmaceutical industry.

Over the past few years, we have begun to see a further shift in the drug industry towards even larger and more complex biopharmaceuticals consisting of multiple protein subunits that give rise to a supramolecular structure, e.g., viral vectors that encapsulate a new class of active pharmaceutical ingredient (API)—nucleic acids, the chemical basis of genetic material. Here, proteins play a new critical role as a complex multi-protein structure acting as a delivery system of this new type of API, which is the basis of the modern gene therapy viral vector drug products.

These new very large complex supramolecular drug products have many more potential sources where errors in the drug's structure can occur. These errors, changes, or points of degradation exist at several levels, including not only alterations in the individual proteins themselves (as is commonly encountered in typical biopharmaceuticals), but also in the assembling process and packaging of the genetic material into the drug delivery system. The result is an enormous overall increase in the potential level of heterogeneity in the final gene therapy products that can affect their therapeutic potency and safety. As a result, this heterogeneity must be minimized and controlled in order to yield a consistent finish drug product that can potentially receive regulatory approval.

To overcome this heterogeneity, highly sensitive analytical instruments are required to detect these changes or errors, such as mass spectrometry along with an array of other orthogonal analytical tools, due to the limitations of each analytical tool's ability to detect the various heterogeneous forms of the assembled monomeric viral vector particles and characterize them in terms of their API content. As a result, various size distribution techniques like size exclusion chromatography and AUC with multi-detector capability in combination with newer analytical techniques such as mass photometry (MP) and charge detection mass spectroscopy (CD-MS) are needed. In addition, for the therapeutic payload (API or nucleic acid), more direct and detailed analyses are needed, including high resolution capillary gel electrophoresis (CGE), DNA next generation sequencing (NGS), and genetic mapping techniques to characterize its own heterogeneity.

When the field of gene therapy began to blossom, one old technology, AUC, which was commonly used to characterize viruses years ago, was largely ignored. I felt AUC could bring a lot to the field to help understand the underlining complexity of these drugs. Unfortunately, the technique itself does have some shortcomings, which became more of focal point than its positive attributes. As a result, people started to look for other techniques that could perform

similar analysis quickly using very little sample, such as single particle analysis. However, single particle counting instruments do not have the resolution power that AUC can bring to bear in analyzing these new drugs products.

Right now, most of these instruments I've mentioned for characterizing viral vector size distribution are associated with just one vendor. This can potentially be a problem. If the quality of the instrument and service provided by that vendor is not adequate or the vendor stops making or supporting the instrument, there is no alternative vendor you can turn to. Consequently, developers of the new gene therapy drugs should keep this in mind and be sure to have a back-up plan in place to cope with such situations.

Q A key issue in tackling the complexity of gene therapy products is understanding the multi-levels of heterogeneity that exist within them. Can you explain the heterogeneity of gene therapies?

SB: As previously alluded to, this multi-level heterogeneity is a unique feature of viral vector or nanoparticle-based gene therapy products. Take AAV based gene therapies as an example. AAV consists of protein capsid (or shell) composed of three different proteins, viral protein (VP)1, VP2, and VP3, which can each experience post-translational modifications that can affect the performance of the final AAV drug product (especially AP1 and AP2). These chemical changes constitute the first level of heterogeneity in viral vector gene therapy drug products.

The assembly of these three AAV protein subunits to form the viral vector capsid typically occurs in a specific ratio, e.g., in the case of AAV capsid VP1:VP2:VP3 occur in a ratio of 1:1:10. However, scientists have found after careful analysis that this ratio can vary, affecting the drug's ability to get into cells and release its DNA to perform its therapeutic function. This constitutes a second level of drug product heterogeneity.

Once the protein capsid forms, the API must be packaged into this protein capsid. This process can also be prone to errors, leading to the heterogeneous array of packed monomeric AAV particles where different amounts of the desired genetic material get packaged resulting in drug product particles that are empty, partially-filled, properly filled (full), and over-filled. In addition, the packaged single-stranded DNA, in the case of AAV, could have errors (deletions, insertions, mutations) and even the wrong genetic material, e.g., host cell DNA, can be packaged into the AAV capsid. This leads to the third level of heterogeneity, in terms of the ratio of protein to DNA and the nature of the DNA in the final gene therapy drug product.

Finally, there is also structural fragmentation of the drug product due to the degradation and aggregation of these fragments and the various monomeric viral forms mentioned earlier (empty, partially-filled, full over-filled particles) with themselves and/or each other. This all leads to a fourth and final level of drug product heterogeneity.

Together, these different forms of heterogeneity present a significant challenge to the analytical scientists faced with characterizing the final viral vector drug product, particularly in terms

"The ability of AUC to characterize the sample in terms of different parameters...allows one to assess a much more detailed and informative biophysical picture of the sample's particle size distribution."

of the overall heterogeneity in terms of size, shape, and molecular weights of the drug product particles present. Thus, a particle size distribution analysis of viral vectors is an important parameter to monitor and control.

Q How can AUC be used to solve the size distribution analysis problem of viral vectors?

SB: One of the underlining attributes of AUC, that is not well appreciated, is its ability to conduct analysis on samples in their native solution matrix, thus avoiding any sample processing steps, other than possibly sample dilution. This attribute of AUC avoids introducing any potential perturbing effects on the structure of the viral vector material taken from the vial. This simple attribute is critically important in assuring that the analytical data gathered by the analytical technique accurately reflects the physico-chemical state of the drug product in the vial.

The ability of AUC to characterize the sample in terms of different parameters, e.g., molecular weight, shape, density, and spectral properties, allows one to assess a much more detailed and informative biophysical picture of the sample's particle size distribution. There is also an underlining simplicity in conducting the actual AUC experiment, where no calibrations are required since it is a first principle (absolute) method. At present, this method has the highest resolution over the widest size range (expressed either in terms of physical dimensions or molecular weights) of the methods used to acquire this type of information.

Nevertheless, when AUC is brought up for discussion, many people have focused more on the shortcomings of AUC–high sample material requirement, long experiment measurement time, and low sample throughput. Needless to say, improvements in overcoming these shortcomings of AUC are appearing and being recognized in the literature. For example, an old AUC modality known as band velocity sedimentation requires very small amounts of material, as low as under 5% of what is normally required for classical boundary SV-AUC. This same modality can also be used in interesting ways to look directly at the integrity of the DNA material itself using the intact virus sample as I did over half century ago [3]. There is also a new high-speed boundary velocity sedimentation protocol [4,5], which offers even higher resolution than what is typically seen with boundary SV-AUC, which can also offer some opportunities, given its shorter run time, to increase sample throughput along with the use of pseudo-absorbance [6].

Another older AUC modality, density gradient equilibrium (DGE)-AUC, is also gaining some recognition for its ability to look at virus samples in a unique way in terms of particle

density, which requires relatively small amounts of material. Coupling the information gained by this AUC method with other AUC methods has provided more insight into understanding the complexity of viral vectors, as recently seen in the work by K. Hirohata *et al.* [7]. Similarly, coupling AUC information with complementary methodologies, such as DNA CGE, DNA NGS, and DNA mapping will only further this understanding.

Q Can you compare the other tools and technologies working to solve particle size distribution characterization challenges?

SB: MP and CD-MS are new single particle measurement techniques that are now playing a competing role with AUC. They offer great promise in dealing with the analytical challenges faced in developing gene therapy products by reducing the sample size and analysis time and increasing sample throughput when assessing particle size distribution information. However, they may not have the full capability of AUC in some areas.

Like AUC, MP can analyze viral vectors in their native environment and is not typically influenced by the sample's matrix. However, of particular importance is MP's ability to quickly assess particle size distributions with very little material, making it an ideal analytical tool for monitoring in-process samples during development, where many small samples need to be processed rapidly to make quick decisions. Nevertheless, the resolution capability of this method is inferior to AUC.

CD-MS may also play a similar role to MP, since it requires very small amounts of sample to perform an analysis, although the analysis time is not quite as short as MP. However, given CD-MS's higher resolution than MP, it could better serve areas where material and time limits prohibit the use of AUC. Nevertheless, as this method operates in the gas phase and sample processing is needed to place it in a user-friendly MS buffer, typically an ammonium acetate buffer, means that close monitoring is required to avoid artifacts that could alter the sample prior to analysis.

Right now, AUC is often referred to as the 'gold standard' for viral vector characterization, but its underlining weaknesses allow MP and CD-MS to play useful roles in characterizing the particle size distribution during the development process of viral vector drugs. I see all three methods having a role to play together in a complementary way to help understand the viral vector drug products we are seeing being developed.

Q More generally, where do you see the future of viral vector particle size distribution characterization heading over the next 1–2 years? And 5 years?

SB: Particle size distribution is a critical parameter that needs to be monitored and controlled in making viral vector drugs products. AUC will continue to be an important

analytical tool to use. There are also opportunities and an increasing awareness and appreciation of some of the older and even newer forms of AUC analysis that require less material and can provide even higher resolution in shorter run times (possibly achieving higher sample throughput). Overall, AUC's first principal mode of operation and high resolution make it a key validating tool for ensuring that other analytical tools provide accurate particle size distribution information.

There is also no doubt that the newer particle sizing methods, like MP and CD-MS, will also find greater use in the coming few years for characterizing the heterogeneity of both the intact viral vectors and their encapsulated nucleic acid payload. MP in particular may play an important role in the area of in-process testing, where many samples need to be processed quickly.

In terms of characterizing the nucleic acid payload, CGE, DNA NGS, and genetic mapping methods are important analytical tools that will be combined with MP, CD-MS, and AUC particle size distribution analyses to yield a more complete understanding of how nucleic acid payloads are distributed among viral vector particles in a gene therapy drug product.

It will be interesting to see how the instruments in the particle size distribution space play out in the field. The MP instrument by Refeyn is catching on well and will have a lot of usage in areas where its lower level resolution can be tolerated, but its quick ability to process many samples requiring small sample amounts is its strength. CD-MS can also play a somewhat similar role as MP with a higher resolution capability, but with more complex sample processing. It is presently too early now to see how CD-MS will fit into the viral vector characterization arena given that the first commercial CD-MS instrument just appeared on the market (by Thermo Fisher Scientific and will also likely be offered in 2025 by Waters Corporation). Nevertheless, there is a lot of attention being given to this technique, and it could dominate the whole field.

In general, CD-MS and MP techniques are going to go head-to-head with each other while AUC analysis, for now, remains the gold standard. In the future, it would not be surprising to see all three of these particle size distribution methods play a synergistic role in developing and achieving safe, consistent, and therapeutically effective viral vector gene therapy drug products.

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BIOGRAPHY

STEVEN BERKOWITZ received his BSc degree in Biology from Fairleigh Dickinson University, Teaneck, NJ, USA and his PhD degree in Biochemistry from New York University, New York City, NY, USA where he conducted research on the physicochemical properties and structure of bacterial filamentous viruses. He then spent several years as a post-doctoral fellow at Yale University, New Haven, CT, USA and the NIH, Bethesda, MD, USA where he studied the physicochemical properties and assembly processes associated with microtubules and their interactions with neurofilaments. After his post-doctoral work Berkowitz held various positions at Celanese Research Company (studying the physical properties of a wide range of synthetic polymers), JT Baker (developing chromatography materials for biopolymer separations), and Lederle Laboratories in the bacterial vaccine department (developing purification and analytical methods) before taking a position at Biogen, Cambridge, MA, USA, where he has spent his last twenty year working in the areas of purification, analytical, and formulation development before retiring at the end of 2013. Although retired, Berkowitz has continued to provide, from time to time, technical support to biopharmaceutical companies, mainly in the area of analytical ultracentrifugation analysis. Berkowitz's technical areas of expertise are concentrated in the separation and physical sciences associated with the characterization of a wide range of biopolymers and synthetic polymers. Much of Berkowitz's work has centered on assessing the physicochemical properties, micro-heterogeneity, aggregation properties and higher-order structure of biopharmaceuticals using a range of biochemical and biophysical techniques that has predominately involved the use of light scattering, analytical ultracentrifugation, chromatography, electrophoresis, and various forms of spectroscopy.

AFFILIATION

Steven Berkowitz PhD
Analytical Development Expert

AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

INTERVIEW

Implementing platform-based technologies to scale viral vector manufacturing consistently and efficiently

Although advancements in viral vector manufacturing have enabled significant progress in cell and gene therapies, the field continues to face challenges in ensuring vector productivity and purity as manufacturing scales up. **Abi Pinchbeck**, Editor of BioInsights, speaks with **Xueyuan Liu**, Director of the Research Vector Core at Children's Hospital of Philadelphia, about strategies for overcoming these challenges, including innovations in process optimization, vector design, and advanced analytical tools to enhance the clinical and commercial potential of viral vectors.

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Q What are you working on right now? Can you tell me more about CHOP's Research Vector Core?

XL: I am currently focused on developing and manufacturing preclinical viral vectors, including recombinant AAV and lentiviruses (LV), for R&D and therapeutic development.

"Sometimes, rapid measurements can be particularly valuable in supporting agile decision-making during manufacturing, enabling timely adjustments to optimize outcomes."

The Children's Hospital of Philadelphia (CHOP) Research Vector Core has a long history, formally established in 2005 when the Center for Cellular and Molecular Therapy was formed. The facility has designated spaces for AAV and LV vector manufacturing, QC, and R&D activities. Over the years, it has supported numerous projects targeting a wide range of diseases, including neuromuscular and neurodegenerative disorders, infectious diseases, hematologic conditions, retinal disorders, and cancers. Our core facility has produced thousands of vector products for academic and industry investigators worldwide.

Q Can you outline the critical quality attributes (CQAs) necessary to characterize during recombinant AAV and recombinant lentiviral vector manufacture?

XL: Three primary CQAs, vector titer, purity, and sterility, are essential for characterizing recombinant AAV and LV vectors during manufacture. These attributes ensure the product's safety, efficacy, and consistency.

Vector titer primarily determines the quantity or concentration of the viral vector. For AAV, the physical titer is measured by genome titer as genome copies per mL or capsid titer as viral particles per mL. For rLV, both physical titer and infectious titer are monitored. Standard methods, like ddPCR, qPCR, and ELISA, are commonly used. Sometimes, rapid measurements can be particularly valuable in supporting agile decision-making during manufacturing, enabling timely adjustments to optimize outcomes.

Purity typically refers to the absence of contaminants. There are two main types of impurities in vector manufacturing: process-related impurities and product-related impurities. Process-related impurities include residual DNA and proteins from host cells, plasmid components, nuclease, or other chemicals used in manufacturing. Product-related impurities are specific to vector manufacturing and include empty particles, partially filled vector particles with truncated genomes or host cell DNA or packaging plasmid DNA, aggregate vectors, and vectors with post-translational modifications. These must be closely monitored and minimized, as they can compromise vector potency, therapeutic efficacy, and safety.

The third key CQA is sterility and endotoxin levels. These attributes are monitored throughout the manufacturing process. The vector must be free of microbial contamination to ensure safety, especially for animal studies and clinical applications. In a GMP environment, sterility is one of the most crucial attributes to guarantee product safety and compliance.

Additionally, other CQAs, such as vector integrity, identity, and stability, are typically assessed at the end of the manufacturing process. These involve more in-depth analyses to confirm the quality of the final product.

Q What do you see as the critical key challenges in the manufacturing of rAAV and rLV?

XL: The number one challenge on my list is scalability. While producing small-scale vectors for R&D is manageable, significant challenges arise when scaling up for clinic use. As manufacturing transitions from research to clinical and commercial levels, maintaining consistency in vector quality and yield becomes a major hurdle. This requires extensive process optimization at every step, including upstream cell culture conditions, cell line selection, transfection method, and downstream purification. In some cases, the entire process needs to be modified or changed to ensure feasibility, with each adjustment requiring careful optimization across multiple steps.

Many vectors struggle to scale due to the complexity of their manufacturing. Another often overlooked factor is vector manufacturability. Not all vectors are equally efficient in production; some perform better than others. The scaling-up process is resource-intensive, demanding significant expertise and investment to execute correctly.

The second major challenge, particularly at the preclinical stage, is in-process analytical characterization and QC. A lack of analytical tools for quick, real-time monitoring of vector quantity and quality complicates the assessment of both products and processes during manufacturing. Without these tools, critical process parameters, and quality attributes may not be fully captured, making scaleup challenging. This limitation increases the risk of variability, making it difficult to ensure consistency and reproducibility in vector manufacturing.

Q How can platform-based technologies be further implemented and established in viral vector production?

XL: When we think about platform technology in manufacturing, whether for preclinical stages or commercialization, many current approaches already rely on platform-based technologies. Vector manufacturing typically involves two main phases: upstream viral vector production and downstream purification. A platform-based approach adds flexibility, allowing the same process to adapt across different products. For example, in AAV manufacturing, a transfection protocol can be standardized for different serotypes or viral products, making it adaptable to various production needs. Similarly, in LV vector production, only minor optimizations may be needed for transfection or purification.

To further implement and establish a platform approach, modularizing the entire manufacturing process is key. Modular systems provide standardization across different stages while allowing for quick adjustments. For instance, upstream production might use systems like HEK293 cells with transient transfection, producer cell lines, or Sf9 insect cells, paired with downstream purification methods. Small-scale production might rely on density gradient centrifugation, whereas large-scale manufacturing can use chromatography or advanced purification technologies.

By modularizing each stage and equipping them with multiple tools, processes can be both standardized and fine-tuned for specific products. This approach enhances flexibility and allows the integration of screening and analytical tools to support high-throughput production. At the upstream stage, for instance, modularity enables the optimization of transfection conditions to improve vector yield and quality, along with real-time process monitoring.

Digitization is another valuable addition, facilitating process monitoring and documentation. Applying QbD principles to modular systems further supports standardization, making technology transfer, collaboration, and regulatory approval processes more seamless.

Advances in production platform technology and analytical tools also drive deeper insights into viral vector manufacturing. Engineers are now developing mathematical models to analyze critical steps in production, providing opportunities to enhance quality and yield. These innovations are paving the way for more efficient and scalable vector manufacturing processes.

Q What are the key processing steps to take to improve repeatability and robustness in viral vector manufacture and reduce batch-to-batch variability?

XL: To improve consistency and robustness in viral vector production, process standardization and automation are critical. There are two key stages that require particular attention.

First is the upstream viral production process, where it's essential to use a well-characterized cell line and establish a reliable cell banking system. Standardizing cell culture conditions and optimizing the transfection process are vital since these steps significantly influence the quantity and quality of vector production from batch to batch.

The second critical stage is the downstream purification process, where a standardized protocol can greatly improve consistency. For instance, implementing chromatography for capturing and separating the viral vector from impurities improves both the consistency and purity of the final product. Automating specific processes and using single-use closed systems, particularly in GMP environments, can also reduce variability and minimize contamination risks, leading to a more robust manufacturing process overall.

Another important step is incorporating process monitoring and real-time quality control at every stage of vector manufacturing. These tools allow fine-tuning processes to ensure

"...vector design improvements to minimize empty and partials are being explored to further increase the yield of full capsids."

robustness and consistency. By closely monitoring the workflow, manufacturers can identify weak points and factors that may impact production, creating a knowledge base to control viral vector production better.

For preclinical work, having detailed and well-defined standard operating procedures (SOPs) is crucial, as is rigorous staff training to ensure adherence to these standardized processes. This reduces batch-to-batch variability and supports consistent viral vector production.

When considering specific vector types, AAV manufacturing often involves a longer and more complex process, which requires close attention to multiple critical steps to ensure success. In contrast, LV vectors, while having a shorter production timeline, can present unique challenges due to their specific characteristics. Regardless of the vector type, success depends on having standardized processes, a thorough understanding of process parameters, detailed SOPs, and well-trained staff to ensure consistent, high-quality, and robust production.

Q What are the keys to successfully scaling empty/full separation in AAV production, including limiting partially full capsids?

XL: There are two key strategies to improve the separation of full, empty, and partial capsids during scale-up. The first is adopting more advanced chromatography technologies. Recent advancements have introduced improved methods for separating empty, full, and partials. Some groups currently use ion exchange or hydrophobic interaction chromatography, while others are exploring multimodal chromatography, which combines these approaches. This hybrid method can significantly enhance separation efficiency, making it a promising solution for large-scale production.

The second strategy focuses on optimizing the upstream production and vector design to reduce the prevalence of empty and partial capsids. By improving the production of full capsids at the upstream stage, the starting material becomes more favorable for downstream purification, simplifying the separation process. Efforts to control transfection conditions have been successful in maximizing full capsids production. Additionally, vector design improvements to minimize empty and partials are being explored to further increase the yield of full capsids.

These two strategies—leveraging advanced chromatography for better separation and optimizing vector design and upstream processes to produce higher levels of full capsids—are essential for enhancing scalability and improving overall production efficiency in AAV manufacturing.

Q What are your key goals and priorities for your work over the next 1–2 years?

XL: In the preclinical stages, one significant challenge is the limited availability of advanced tools to thoroughly characterize vectors during production or at the final stage. This makes it difficult to determine whether a vector is fully optimized for further development. My work focuses on developing tools that enable the creation of clinically translatable vectors. The aim is to enhance their therapeutic efficacy early in development while also improving their manufacturability to ensure scalability for downstream processes, such as GMP production and eventual commercialization, if the vector progresses through the development pipeline.

Looking ahead, my primary focus is to refine these tools and strategies to bridge the gap between early-stage development and clinical readiness.

BIOGRAPHY

XUEYUAN LIU serves as the Director of the Research Vector Core at the Children's Hospital of Philadelphia, Philadelphia, PA, USA. Recognized as an expert in cell and gene therapy, she has successfully established the lentivirus (LV) manufacturing process for multiple pseudotypes and transformed the traditional recombinant adeno-associated virus (rAAV) manufacturing and characterization processes, making them more flexible and scalable. She has supported early investigations that have led to US FDA-approved gene and cell therapies. Additionally, she has training in cellular and molecular biology, human genomes and genetics, cancer biology, therapeutic development, and biomedical engineering. As an innovative scientist with over two decades of leadership experience, she has driven significant progress in vector engineering and capsid modifications to improve the efficiency and specifications of gene delivery. Her work has contributed to the development of new therapeutics, as well as to publications, patents, and related intellectual properties. She and her group continue to seamlessly support investigators across academia and industry around the globe.

AFFILIATION

Xueyuan Liu PhD
Director of the Research Vector Core,
Children's Hospital of Philadelphia,
Philadelphia, PA, USA

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CELL & GENE THERAPY INSIGHTS

INTERVIEW

Navigating the complexities of AAV and lentiviral vector characterization

Abi Pinchbeck, Editor, BioInsights, speaks to **Susumu Uchiyama**, Professor, Osaka University, about the challenges and successes of characterization techniques of AAV and lentivirus vectors used in gene therapies. They also discuss the complexities of viral vector particle composition and biophysical parameters, as well as the development of advanced analytical methods to streamline characterization for clinical trials.

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Q What are you and your laboratory currently working on right now, particularly surrounding characterization for gene therapy products?

SU: I run a laboratory at Osaka University where I work with students and a dedicated team of more than ten staff members who primarily focus on viral vector production and characterization. Currently, one of our main activities in gene therapy is non-GMP,

high-quality vector manufacturing for preclinical proof-of-concept studies and for animal tox studies. We perform extensive vector characterization, analyzing >10–15 key attributes in-house

Additionally, I help to develop GMP-compliant characterization methods for clinical trials, which are also handled in-house. Last year, we also developed analytical techniques for LV characterization, including conventional methods such as PCR and ELISA as well as other techniques for LVs used in clinical trials, particularly in CAR-T cell therapies.

Q Can you outline the critical quality attributes necessary to characterize during AAV and LV manufacturing?

SU: For AAV vectors, it is important to characterize both the primary structure and higher-order structure. The characterization of the primary structure involves verifying that the amino acid sequence is correct as designed and identifying any chemical modifications to the viral proteins (VPs). The stoichiometric VP1:VP2:VP3 ratio is also crucial for ensuring AAV quality, as it relates to the biological efficacy of the vectors.

Among primary structure modifications, deamination has the largest impact. For example, the deamination of asparagine residues at positions 57 and 94 in the VP1 region can notably affect AAV activity.

Genome purity is another key factor because sometimes shorter DNA fragments, such as plasmid remnants or truncated genomes containing an inverted terminal repeat may become encapsulated together with the full-length DNA, especially if the full DNA length is <3 kb. These fragments can co-pack within single particles, affecting purity.

Regarding genome impurities, the characterization of LV is even more challenging. For example, it is uncertain which specific proteins must be present to confirm a functional LV. Typically, we focus on p24, a protein closely linked to LV activity, which can be directly evaluated by GFP expression. However, when genes of interest are involved, one can only assess the LV activity after transfection and protein expression on cell surfaces, often using flow cytometry. In essence, p24, and the extent of protein expression upon cell transfection, can be used to reflect LV particle concentrations. We are also investigating additional methods to directly count LV particles, similar to AAV quantification. For this objective, we aim to replace PCR or ELISA methods with a direct counting approach, potentially using nano-tracking analysis or methods that can quantify submicron sized particles.

Lastly, higher-order structure information indicates the arrangement of VPs into a single AAV particle. Despite extensive purification, AAV preparations often contain a mix of empty, full, partial, and overpackaged particles. Therefore, it is important to quantitatively characterize these populations, which includes determining the exact extinction coefficients for empty, full, partial, and overpackaged particles. Quantitative characterization is essential to accurately assess the exact molar extinction coefficients and numbers of each type of particle.

"Currently, we cannot precisely determine the activity of each viral particle with genome in the mixture..."

Q What, in your view, are the key challenges remaining in the AAV characterization space? What tools and technologies are being used to overcome these challenges?

SU: One of the main challenges is that AAV solutions contain a mixture of particles with varying levels of activity. Currently, we cannot precisely determine the activity of each viral particle with genome in the mixture, and confirm whether each particle is a fully intact, highly functional AAV particle with specific activity. Even after purification, some AAV particles are deamidated, have different VP1 and VP2 stoichiometries, or contain genomes of imperfect lengths, which complicates characterization. These different parameters often lead to variability in AAV activity, which is a significant issue.

Another challenge is the lack of understanding of the relationships between different parameters, such as deamidation and VP numbers. Currently, each biophysical parameter is measured separately, but it is unclear how these parameters synergistically or independently impact or relate to biological activity. Hopefully, in future, correlations between different parameters will be discovered, which will streamline the identification process.

Regarding LVs, characterization is even more complex because the full composition of proteins within the LV remains unclear, and each production system can yield a LV with slightly different components. While LV is mostly composed of VPs, its membrane originates from the host cell, meaning it contains host cell proteins (HCPs) that vary depending on the proteome of the host cell. It is still unclear which proteins are consistently present in different LV preparations. Additionally, the exact lipid composition of functional LV is also unknown. In essence, to fully control and understand LV activity from the perspective of biophysical parameters, a comprehensive chemical identification of its components is essential, which includes proteome, partial genome, transcriptome, and lipidome.

Q What is the impact of AAV glycosylation on VP ratios, and overall vector function, and how can this be quantified and characterized?

SU: We have analyzed and published data on AAV6 thus far. Other AAV serotypes are considered to be very similar in their glycosylation profile because AAVs are generally not heavily glycosylated. Only a few rare AAV particles are glycosylated, and, according to our analysis, AAVs are typically not N-glycosylated, but rather O-glycosylated. Typically, O-glycosylation

"...we developed a method called dual fluorescence-linked immunosorbent assay, which detects AAV particles and the genome inside each particle in a single well on the same plate."

occurs specifically in the VP2 region. Once it is fully glycosylated, the AAV particles lack VP1, which compromises the particle's function. In essence, in cases of glycosylation, only VP2 and VP3 are present in the AAV particle, leading to a loss of function. Nevertheless, while glycosylation heavily impacts AAV activity, as mentioned earlier, it is a very rare phenomenon.

For thorough characterization, it is recommended to perform peptide mapping to detect any glycosylated peptides in enzymatically digested AAV. If glycopeptides are not detected in the analyzed peptides, then AAV glycosylation is likely not a concern. In antibody glycosylation analysis, the typical technique is enzymatic N-glycan release using enzymes such as PNGase, which allows released N-glycans labeled with a fluorescent dye to be detected with high sensitivity. However, in AAV samples, N-glycans detected are usually from HCP contamination, not the AAV itself. To confirm this, one can apply lectins that capture glycosylated proteins selectively. For example, if lectins that recognize N-glycans are applied to purified AAV samples, signals are barely detected. Only lectins that recognize O-glycosylated proteins show any indication, confirming that purified AAV particles do not contain N-glycosylated structures.

Q Can you comment on the current tools and methods for measuring the AAV empty-full ratio?

SU: There are two main types of methods: those suitable for in-process analysis and those ideal for drug substance or product analysis. For in-process analysis of the empty-full ratio during purification or production, ELISA and PCR are traditional methods. However, these techniques require splitting one sample into two separate tests, which can bring relatively large variation for the results.

In order to address this hurdle, we developed a method called dual fluorescence-linked immunosorbent assay (dFLISA), which detects AAV particles and the genome inside each particle in a single well on the same plate. This allows simultaneous analysis without splitting the sample into two tests, unlike the ELISA-PCR combination. Additionally, this method can be used for as many as 30 in-process samples at a time. While dFLISA does not have the accuracy of certain rigid biophysical methods, it provides efficient in-process empty-full capsid determination. Mass photometry is another promising option for in-process analysis, though it currently requires affinity resin purification before the analysis.

For drug substance or product analysis, one option is high-performance liquid chromatography-based size exclusion chromatography (SEC), which allows the separation of particles from other contaminants like free nucleic acids and peak visualization of capsids. While empty and

full particles cannot be directly separated by SEC alone, the 260/280 absorbance ratio provides a measure of the empty-full ratio. Furthermore, anion exchange chromatography allows for the separation of empty and full particles. However, both chromatographic techniques have drawbacks if partial or over-packaged particles are present, as neither method can fully distinguish between these types.

Currently, the most reliable method is analytical ultracentrifugation (AUC), as it can be utilized to separate full, empty, partial, and overpackaged particles, and characterize free DNA. While useful for this application, AUC does not provide exact molecular weights. If one needs to determine exact mass of AAV particles, special type of mass spectrometry can be used to accurately determine the molecular mass of particles. For example, charge detection mass spectrometry with ion trap or orbitrap configurations delivers high-resolution mass determination for empty, partial overpackaged, and full particles. However, when the percentage of empty particles is very low, such as 1–2%, any method struggles to quantify the exact count.

If the empty particle percentage is <5%, single-particle analysis, such as cryogenic-electron microscopy (cryo-EM), is a powerful tool. With cryo-EM, every particle is visualized individually, allowing us to distinguish between empty and full particles. We have developed libraries of cryo-EM images for empty and full particles, which can be applied to unknown samples post-purification. Using these cryo-EM libraries and trained convolutional neural networks, a one of machine learning approach, empty and full particles can be identified with high accuracy—even when samples contain up to 99% full particles.

Q Where do you see the future of viral vector characterization heading over the next 1–2 years?

SU: Over the next few years, I believe researchers will extensively characterize and reveal the remaining unidentified aspects of AAVs, achieving complete characterization. Hopefully, within 5 years, we will understand the relationships between various parameters influencing AAV quality, which will contribute to ensure the efficacy and safety of AAV drugs more concretely. In contrast, LV characterization will likely remain challenging, and the development of robust methods for LV characterization may likely take 5 years or longer.

Q Finally, can you briefly summarize your key goals and priorities, both for yourself and the laboratory as a whole, over the next 1–2 years?

SU: We aim to accumulate our experience and gather case studies from clinical trials. Our target is to support 5–10 clinical trials each year, streamlining the characterization process. Additionally, we intend to reduce the number of characterization items for the final drug product, ensuring the quality during process development and in-process analysis.

In order to achieve these targets, it is important to have a deep understanding of the relationship between biophysical parameters and their impact on biological activities. With this approach, we hope to accelerate viral vector characterization and process development for clinical trials. While our primary goal over the next 1–2 years is to support clinical trials in Japan, we are eager to support international trials as well.

BIOGRAPHY

SUSUMU UCHIYAMA is a biophysical chemist with over 30 years of experiences in studying solution biophysics of proteins, protein complexes, and protein-nucleic acids complexes, by using various kinds of biophysical methods such as analytical ultracentrifugation, mass spectrometry, and calorimetry. In 2005, he learned basis and application of native mass spectrometry during 1 year staying at Professor Carol Robinson's laboratory in Cambridge University. He started a company U-Medico Inc. which has been providing analytical services for pharmaceutical companies since 2008. His research interest includes characterization, quality control, and formulation development of therapeutic proteins and ATMPs. Container closure system for biopharmaceuticals and ATMPs with better quality and more safety is also in his research scope. In recent years, he is leading a national project in Japan for the development of characterization and quality control of virus vectors for gene therapy. He has published more than 300 peer-reviewed papers and reviews and edited a book, *Analytical Ultracentrifugation* published from Springer. He contributes to the revision of guidelines for biopharma and virus vectors in different countries.

AFFILIATION

Susumu Uchiyama
Professor,
Osaka University,
Osaka, Japan

AUTHORSHIP & CONFLICT OF INTEREST

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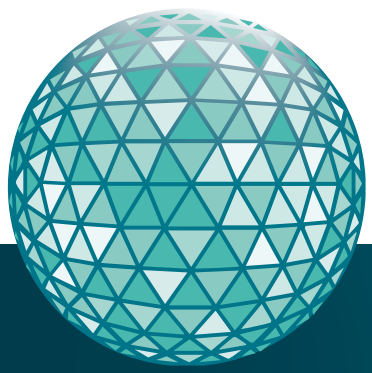

CELL & GENE THERAPY INSIGHTS

EXPERT INSIGHT

‘Winning’ target product profiles for CAR-T cell therapies in oncology: critical success factors for commercially viable therapies

Clare Hague, Louise Street-Docherty, and Frances Pearson

The global CAR-T cell therapy market is predicted to reach US$29.0 billion in revenues by 2029, growing at a CAGR rate of 39.6% from 2024–2029. Despite the attractiveness of the potential market, CAR-T cell therapies have high costs of goods (COGs) due to their complex manufacturing process and a high price, making it crucial for CAR-T cell developers to develop a target product profile (TPP) at an early stage to determine if a commercially attractive product is achievable and hence viable. This article emphasizes the importance of developing a TPP for CAR-T cell therapies and offers best practice guidance on its development. It highlights the strategic role of a TPP in clinical development and the value of adopting an evidence-based approach to developing a TPP, as led by a cross-functional multi-disciplinary team. A CAR-T-specific template is proposed, combining various TPP templates. Finally, we provide clarity on what a ‘good’ TPP looks like, optimal timing for development, governance processes, data and insights needed, and key ‘watch outs’.

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Since 2017, cell therapies have been the focus of immuno-oncology development [1], with over 800 CAR-T cell therapy clinical studies in progress globally (as of September 9, 2024 [2]). The potential for further growth in this sector is evident, with the majority of ongoing studies

still in the early stages of clinical development (Figure 1). From a commercial perspective, market analysis predicts the global CAR-T cell therapy market could reach US$29.0 billion in revenues by 2029, growing at a CAGR rate of 39.6% from 2024–2029 [3].

R&D expenditure on CAR-T cell therapies is not often publicly disclosed. However, it is well understood that due to the complex manufacturing process, CAR-T cell treatments incur high COGs that surpass other cancer therapies [4]. The COGs, together with the hefty investment in undertaking clinical trials and developing a scalable manufacturing infrastructure, mean that it is imperative that CAR-T cell developers have a robust understanding of what constitutes a commercially viable product to yield a return. The latter can be greatly facilitated through the development of a TPP that pre-specifies the attributes of treatment most likely to lead to a regulatory approvable, reimbursable treatment that is highly valued by patients.

The aims of this article are to raise awareness amongst CAR-T cell developers of the importance of developing a TPP at an early stage of product development and to offer best practice guidance on how to go about pulling a TPP together.

First, we outline what we understand to be a TPP based on a synthesis of the published and grey literature, as well as the strategically beneficial role that a TPP can play in clinical development.

Second, we describe how to best go about developing a TPP emphasizing the value of CAR-T cell developers in charging a cross-functional multi-disciplinary team from within their organization with the task of developing this, so that an internally aligned initial draft can be externally validated by patient experts, clinicians, regulators, HTA agencies, and payers. An adapted version of a CAR-T cell specific template is then proposed, which is an amalgamation of different TPP templates reported elsewhere.

Third, we seek to provide some clarity around what might be considered a 'good' TPP whilst providing some recommendations around when a TPP should be developed as well as the frequency at which it should be updated and how the TPP process could best be governed.

Finally, we cover which data and insights are needed to develop a TPP for a CAR-T cell therapy together with the key uncertainties and 'watch outs'.

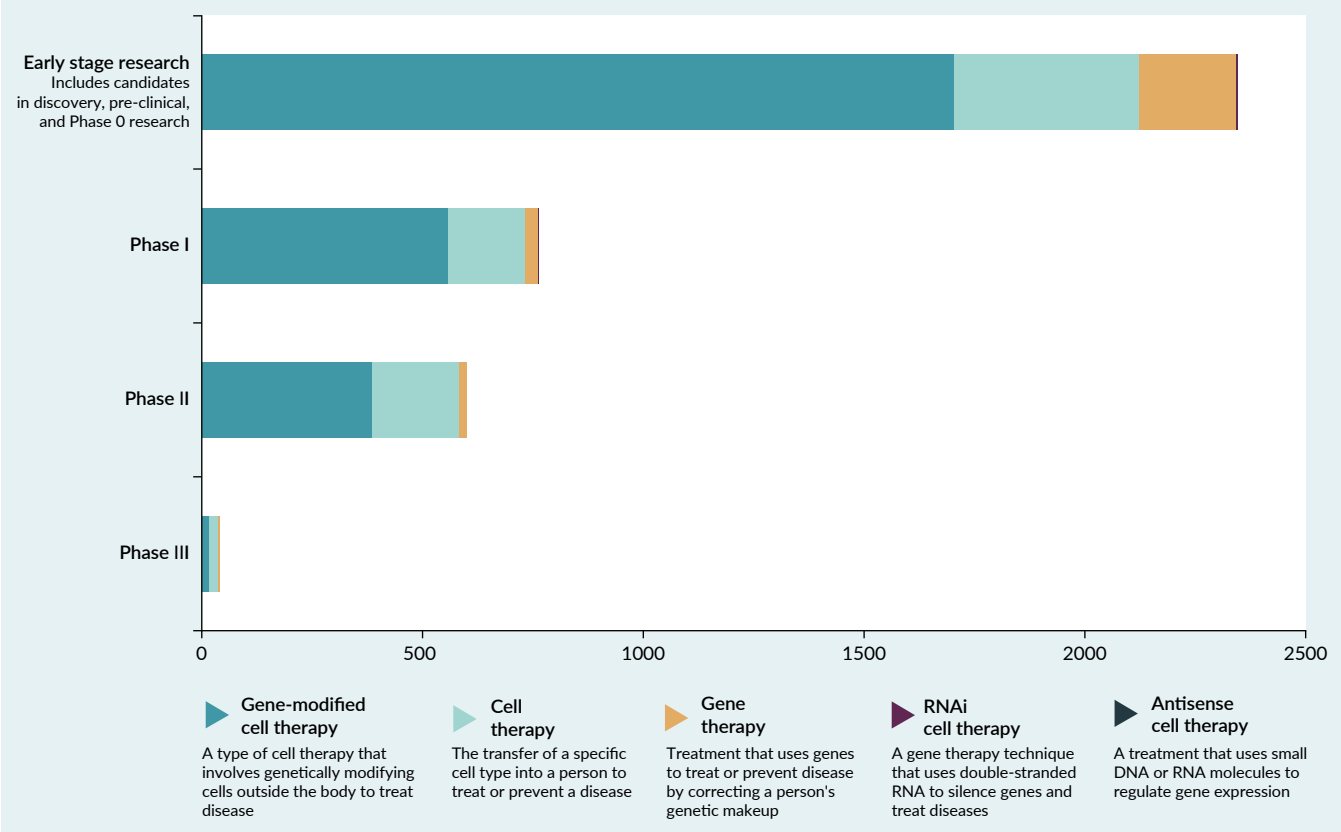
WHAT IS A TARGET PRODUCT PROFILE (TPP) AND WHAT IS ITS PURPOSE?

A TPP can best be described as a strategic planning tool that lays out *a priori* the desired profile of a new therapy that, if realized, can ensure a reasonable probability of regulatory and market access success [5,6]. It includes, but is not limited to, the target indication(s), target patient population(s), and important safety/efficacy/patient-reported outcome (PRO) and health economic characteristics and parameters [7]. 20 years ago, a TPP might have focused on setting out the minimum requirements for regulatory approvable treatments. However, it can no longer be assumed that regulatory success equates to commercial success. This is because following regulatory approval in Europe, CAR-T cell developers need to clear an additional hurdle, i.e., secure reimbursement from Health Technology Assessment (HTA) agencies and payers in different countries which has proven more challenging. The evidence gap between the regulatory requirements with the more stringent HTA and payer requirements is what the TPP needs to mitigate against, and as such is why we consider that broader considerations such as market positioning, differentiation and cost-effectiveness (amongst others) should not be treated separately from the TPP, but instead form an integral part of the TPP development process.

The three key benefits of having a TPP are well described by the National Institutes of Health (NIH) SEED organization, as (1) ensuring enhanced R&D efficiency,

FIGURE 1

The number of CAR-T cell therapies under clinical development by phase of development and disease.



Adapted from [30]. Data not visible: 3× RNAi gene therapies in early stage development and 1× antisense gene therapy in early stage development.

(2) positive regulatory outcomes, and (3) commercial success [7]. It is the authors' opinion that TPPs can also help qualify the intended value proposition and determine how the new CAR-T cell therapy will be differentiated from other therapies and standards of care by time of launch.

A TPP essentially serves as a roadmap for CAR-T cell developers, guiding decisions on clinical trial design, evidence generation strategies, and manufacturing [8]. The TPP may also inform go/no-go decisions at critical milestones as well as guide discussions between developers and regulatory and HTA authorities throughout the drug development process, from pre-IND phases to post-marketing programs and new indications or other substantial changes in regulatory labeling [5]. As HTA now plays a key role in reimbursement decisions at a local level, and it will become clearer in due course how the single, coordinated EU-wide HTA process, known as Joint Clinical Assessment [9] will likely affect access to CAR-T cell and other oncology medicines, the TPP can also be used to inform the development of integrated evidence generation plans to meet HTA and payer requirements [8].

It has also been suggested to the authors that TPPs may be used by health authorities/payers as demand signaling documents on the types of innovations that are needed. This is also a very important benefit of having a TPP that can be used in external interactions between developers and key stakeholders.

HOW DO I GO ABOUT DEVELOPING A TPP?

The WHO [10], EMA [11], and US FDA [5] have all issued guidance documents on TPP

development. The WHO describes a TPP development process that could be applied to a CAR-T therapy, with some minor amends suggested by the authors ***in bold italics.*** It recommends undertaking a needs assessment, appointing an internal ***cross-functional multi-disciplinary*** TPP team to draft an initial TPP, ***eliciting input (into the TPP) from patient expert organizations, clinicians, regulators and HTA agencies through the scientific advice processes,*** revising and finalizing the TPP ***with sign-off from an internal governance committee*** [12]. It is the authors' opinion that the cross-functional team assigned to develop a TPP should consist of representatives from the following disciplines (e.g., project leadership, translational science, pharmacovigilance, product manufacturing, clinical development, regulatory affairs, health economics and outcomes research, epidemiology, real-world evidence, market access, pricing, medical affairs, marketing, and business insights). Note that different functions may be referred to differently within and across organizations and this list should be interpreted as purely illustrative of the types of functions that might be usefully represented. It is not meant to be a prescriptive nor complete list but provided to negate a perception that a TPP is a purely commercial or clinical deliverable. A TPP should be considered as an internally aligned, externally validated and cross-functionally developed deliverable.

This article proposes a template that better suits the unique nuances of CAR-T therapy, building on previous work by Becker *et al.* [13]. The template outlines categories for further treatment attributes beyond those described by the health authorities (Table 1). This template was based on previous work proposed by Becker *et al.* [13] NIH SEED [7,14], and Hettle *et al.* [15] and further adapted to include more specific information on the unmet need, the value proposition, the patient selection strategy including sub-groups, clinical efficacy, PROs, duration of the treatment-free interval, the pricing model, health economics, and patient access. These additional components were informed by the authors' previous experience of developing TPPs for CAR-T cell therapies and the benefits they accrued through having thought through these aspects at an early stage of product development by having them explicitly set out within the TPP.

WHAT DOES A GOOD TPP LOOK LIKE?

Developing a TPP that serves to align developers' and stakeholders' perspectives will be highly valued if designed in the right way, such that it is able to minimize risk, reduce failure rates, anticipate the time needed to generate the necessary evidence and ideally reduce the costs of development [16]. Starting with the end-user in mind is fundamentally important [17]. It is the authors' opinion that early engagement with patient experts can enhance the quality of a TPP through better understanding those attributes of treatment most valued by the end user. This in turn can inform what should be captured in clinical trials to better understand the extent to which a new treatment might alleviate the burden of disease and improve patients' health-related quality of life. Additionally, there are other CAR-T cell specific nuances where early patient expert engagement may be especially valuable, such as eliciting their views on the acceptability of manufacturing turnaround times, site locations for running trials and eventually for commercial use, proposed strategies for adverse event monitoring, and patient education materials amongst others. Stegemann *et al.* propose a roadmap to do this, which is highly recommended [18].

Hettle *et al.* developed two TPPs in their exemplar work on CAR-T cell therapy in hematology which differentiated between 'curative' and 'bridging to stem-cell transplantation' treatment as the primary goal of treatment [15]. This approach may also be very helpful to consider.

As far as specifying the attributes of treatment and the magnitude by which a CAR-T

TABLE 1

Components of the clinical and production sections of the novel target CAR-T cell TPP.

Sections	Components	Target product profile	Data sources/references
Unmet need†	Statement of unmet need	<< >>	<< >>
Value proposition†	Value proposition of the new CAR-T cell therapy	<< >>	<< >>
Clinical	Proposed indication(s)	<< >>	<< >>
	Patient selection strategy (target population including sub-groups†)	<< >>	<< >>
	Target safety and tolerability profile	<< >>	<< >>
	Target clinical efficacy profile†	<< >>	<< >>
	PROs† e.g., benefits in terms of health-related quality of life and symptom palliation	<< >>	<< >>
	Optimal duration of the treatment-free interval†	<< >>	<< >>
Product characteristics	Stability and shelf life	<< >>	<< >>
	Route of administration	<< >>	<< >>
	Dose and conditioning	<< >>	<< >>
	Dosing frequency	<< >>	<< >>
Production	Critical quality attributes	<< >>	<< >>
	Cell viability and vector titer	<< >>	<< >>
	Manufacturability	<< >>	<< >>
	Apheresis logistics	<< >>	<< >>
	Shipping and storage	<< >>	<< >>
	CAR-T cell development	<< >>	<< >>
	Lentivirus production	<< >>	<< >>
	Turnaround time	<< >>	<< >>
Cost of goods	COGs	<< >>	<< >>
Pricing model†	Target price (USA, EU5, rest of world)†	<< >>	<< >>
Health economics†	Cost–effectiveness†	<< >>	<< >>
	Budget impact†	<< >>	<< >>
Patient access†	Managed entry agreements (MEAs), including price discounts, performance-related schemes, and technology leasing†	<< >>	<< >>

Data from Becker *et al.* [13], NIH SEED [7,14] and Hettle *et al.* [15] and †further adapted.

cell therapy has been able to demonstrate its superiority (i.e., the necessary 'hurdle' within the TPP that needs to be cleared), there are different schools of thought on this matter. Some suggest that a TPP should contain a minimally acceptable (essential) hurdle that specifies the minimal parameters for a safe and efficacious drug as well as a higher (ideal) hurdle that specifies the desirable parameters that would allow higher value (access to a larger market, reduced cost of goods, etc.) and hence defines a better commercially viable (optimal) benchmark [19].

We maintain that the latter should be adopted as the 'only' TPP benchmark that developers should focus on, since many developers struggle to determine an appropriate course of action when a product falls short of meeting its optimal benchmark. A minimally acceptable hurdle may, at best, be equivalent to existing therapies, which makes decisions as to what to do next with a CAR-T in development much more difficult as payers tend to assign a much higher value to therapies that can offer patients improvements on standards of care, as opposed to 'me-too' (undifferentiated) treatments. Mobilizing efforts towards delivering to the optimal benchmark simplifies the assumptions underpinning the commercial forecast (e.g., adoption levels, market share, and price) and sets a necessary level of ambition within drug development

teams for success [19]. Importantly, one should be aware of the possibility of trade-offs. This means to say that a different configuration of product attributes from that laid out in a TPP may generate a different value proposition and commercial forecast. Should a CAR-T cell therapy fall short of its TPP in one area, it should be clear whether this is something that can be offset by exceeding another domain within the TPP.

WHAT IS THE OPTIMAL TIMING FOR DEVELOPING A TPP?

Whilst the TPP is considered a 'critical path' tool by the FDA [5], research has found that TPPs are often developed too late in the process, usually at the time of the pre-non-disclosure agreement (NDA) or Biologics License Application (BLA) meeting or following NDA or BLA submission [20]. Companies finding themselves in this position miss out on valuable opportunities to streamline their interactions, which may result in a lengthier and more costly development process [20].

It is the authors' opinion that a TPP should ideally be in place by the time of readout from the Phase 1 study for a CAR-T cell therapy. This is to ensure that the right health outcome data are collected during Phase 2 trials and beyond, and that any competitive and other insights can be factored into the trial design [8]. By preparing a TPP in this timely way, a more productive and fruitful dialogue can take place with health authorities using the TPP to guide discussions around the optimal design of clinical development and/or manufacturing plans for approval purposes.

HOW OFTEN SHOULD A TPP BE UPDATED?

A TPP is crucial in the fast-paced development of CAR-T cell therapies. It is the authors' opinion that it should incorporate the latest evidence and competitive insights, including clinical efficacy, safety, and PROs. It should also include relevant insights from regulatory agencies, patient experts, and payers. The TPP should be considered a 'live' document, updated with new insights and information as they arise, whether they come from competitor data presented at conferences or termination of clinical trials for safety reasons.

WHAT IS AN APPROPRIATE GOVERNANCE PROCESS FOR A TPP?

Once the TPP is drafted, it is important that developers have in place a formal approval mechanism as well as a change control system for any ongoing revisions to the template and to individual TPPs [21]. This preserves the integrity of the TPP and ensures that internal decisions whether to progress a CAR-T cell therapy into more advanced stages of clinical development are justified based on the evidence (i.e., what is being seen in clinical trials) and free from any perverse incentives (financial or otherwise) [18]. Without a governance process in place, a company may unwisely invest in a CAR-T cell therapy that will not achieve their commercial objectives [18] and where the opportunity cost is lost.

WHICH DATA AND INSIGHTS ARE NEEDED TO DEVELOP A ROBUST TPP FOR A CAR-T CELL THERAPY INDICATED FOR CANCER?

Evidence-based insights should inform a TPP through undertaking a robust analysis of the published literature and emerging data on existing therapies as well as scrutiny of emerging competitor therapies under development.

The methodological steps that may be considered are as follows:

1. Develop a protocol that sets out the inclusion/exclusion criteria that guide which studies ought to be included when formulating an appropriate benchmark

within the TPP. The protocol might include certain restrictions on publication dates (to eliminate older studies), the phase of clinical development where later studies are prioritized over earlier phase studies), the line of treatment (where earlier lines are excluded if they do not reflect the target indication), and sample size (perhaps excluding studies with very small sample sizes).

2. Identification of studies that meet the inclusion criteria set out in the study protocol that will likely result in a focused number of regulatory approved treatments and those treatment under development that are reporting promising data for the indication(s) targeted by the TPP from Pubmed/Medline.

3. An analysis of treatment guidelines for the indication(s) identifying the current recommended treatment options for patients to ensure that no treatments have been missed from the list identified in step 2.

4. Extraction of key efficacy, safety, and PRO data on those treatments into an Excel spreadsheet making it clear which phase of clinical development these values relate to, as well as the date of publication. This is important because later stage trials (e.g., Phase 3 and large Phase 2 trials) will often report more mature estimates from a larger sample size.

5. Synthesis of the above data (median and IQR values) can help determine the benchmark within the TPP, against which the CAR-T cell under development can be meaningfully compared at different development milestones.

6. An analysis of reimbursement outcomes for regulatory approved CAR-T cell and other treatments. This will likely uncover important insights on the hurdles other developers face when seeking reimbursement, so that these can be better characterized and mitigated against. For CAR-T cell therapies, these might include the uncertainties arising from non-comparative (single-arm) studies, the immaturity of time-to-event outcomes, trial exclusion criteria that limit the generalizability of data to a broader patient population in clinical practice, amongst others.

Patient experts may offer useful insights around the choice of PRO questionnaires for use in the clinical development program that they consider will best assess the side effects of treatment from a patient perspective and how the efficacy gains translate into an improvement in patients' disease-related symptoms and quality of life. Eliciting insights from clinicians may also prove useful to contextualize the insights obtained from the methodological approach described above and provide guidance to the development of the protocol.

In addition to efficacy, safety, and PRO data extracted on those current standards against which the new CAR-T cell therapy will likely be compared by regulators and HTA agencies detailed descriptive information on patient characteristics may also be valuable, to help inform comparisons of the relative benefit/risk profile of the TPP versus alternative treatments.

Detailed analyses of ongoing and completed competitor clinical trials also help to identify areas where developers can differentiate themselves and at the same time, take advantage of the opportunity to align the design of their clinical studies to others to reduce bias when performing indirect treatment comparisons at a later stage. It is important to bear in mind that clinical practice evolves rapidly, and so developers should be mindful of incorporating older studies into their TPP benchmarks as these may reflect worse patient outcomes that might be typically observed by time of launch.

WHAT ARE THE KEY UNCERTAINTIES AND 'WATCH OUTS' WHEN DEVELOPING A TPP FOR A CAR-T CELL THERAPY?

Like most innovative oncology therapies, there are a lot of uncertainties associated with early data, especially when studying orphan or ultra-orphan populations in a non-comparative manner.

Likelihood of cure

The likelihood of cure or improvement with the CAR-T cell therapy is a key uncertainty because it can take years to demonstrate as a result of having to wait for data maturity and can only likely be determined if no subsequent anti-cancer treatment is administered. For this reason, it is probably reasonable not to allude to an intent to cure within the TPP, but it is very important to be able to identify which patients respond best to treatment and understand the reasons why this might be the case.

Durability of treatment effect

Whilst use of CAR-T cell therapies has historically achieved very high overall response rates the onus is on the developer to demonstrate through adequate follow-up that the impressive responses are maintained over time, such that they translate into significant progression-free as well as overall survival, ideally with an extensive treatment-free window for patients. Trials with surrogate endpoints for overall survival tend to report larger treatment effects than trials using final patient-relevant outcomes, which obviously have implications when it comes to powering later-stage trials and ensuring adequate numbers of patients needed to treat [15]. The pattern and duration of response to treatment can be uncertain if there is limited follow-up data available and so it is important that patients are followed for as long as feasibly possible [22].

Number of infusions and the effect of subsequent treatment on overall survival

The 'one shot and done' ethos of CAR-T cell therapy may not be sufficient for some patients to maintain their initial response and so it is important that developers consider how the economic value proposition of their product stacks up in the event that more than one infusion is needed [23]. The effect of subsequent (non-cellular) therapy may also confound patients' overall survival data, meaning that it is important that developers factor capturing these data into their clinical development plans.

Turnaround times

Manufacturing times should be a key consideration in TPP development to optimize patient outcomes, minimizing the turnaround time as much as possible. Given the potential deleterious impact of patients having to wait too long for their treatment, this should be a key consideration.

Magnitude of treatment effect versus current treatment options

Most CAR-T cell approvals have been granted based on non-comparative data. Consequently, the absence of randomized controlled trial (RCT) versus a standard of care makes it difficult to accurately determine what would likely be the magnitude of *difference* between arms in the event of an RCT. This is because estimates of relative efficacy sought through indirect comparisons (without any linking arms) can be subject to bias and potential confounding [22]. In this situation, developers need to consider how best to generate these comparative data and which standards of care should be studied.

Size of the commercial opportunity

The TPP can also help to create clarity around the likely size of the target population

for the new treatment by describing the proposed indication and where within the treatment pathway it will likely be positioned. Epidemiological data needs to be adjusted to reflect the line of therapy, the requirement of specified prior treatments, and study inclusion criteria (e.g., performance status, age cut-offs, stage of disease, etc.) that may exclude certain types of patients. Figure 2 illustrates the starting point for such a calculation using the example of multiple myeloma. These data would need to be further adjusted to reflect the likely market share of the new treatment over time, given the clinical attractiveness of the TPP, how the TPP stacks up versus established and emerging standards of care and the target price.

Deciding on what determines an appropriate standard of care

Depending on the disease in question, there may be considerable heterogeneity as to what constitutes the standard of care, with wide variation in both the costs and consequences of multiple different treatment options (as was observed in the real-world LocoMMotion study of patients with multiple myeloma who had been exposed to three classes of treatment that was undertaken to support the development of ciltacabtagene autoleucel [cilta-cel]) [24]. This means to say that it is important to isolate what constitutes the dominant standard of care in key sources of business markets guided by robust analyses of the latest treatment guidelines (Figure 3).

FIGURE 2

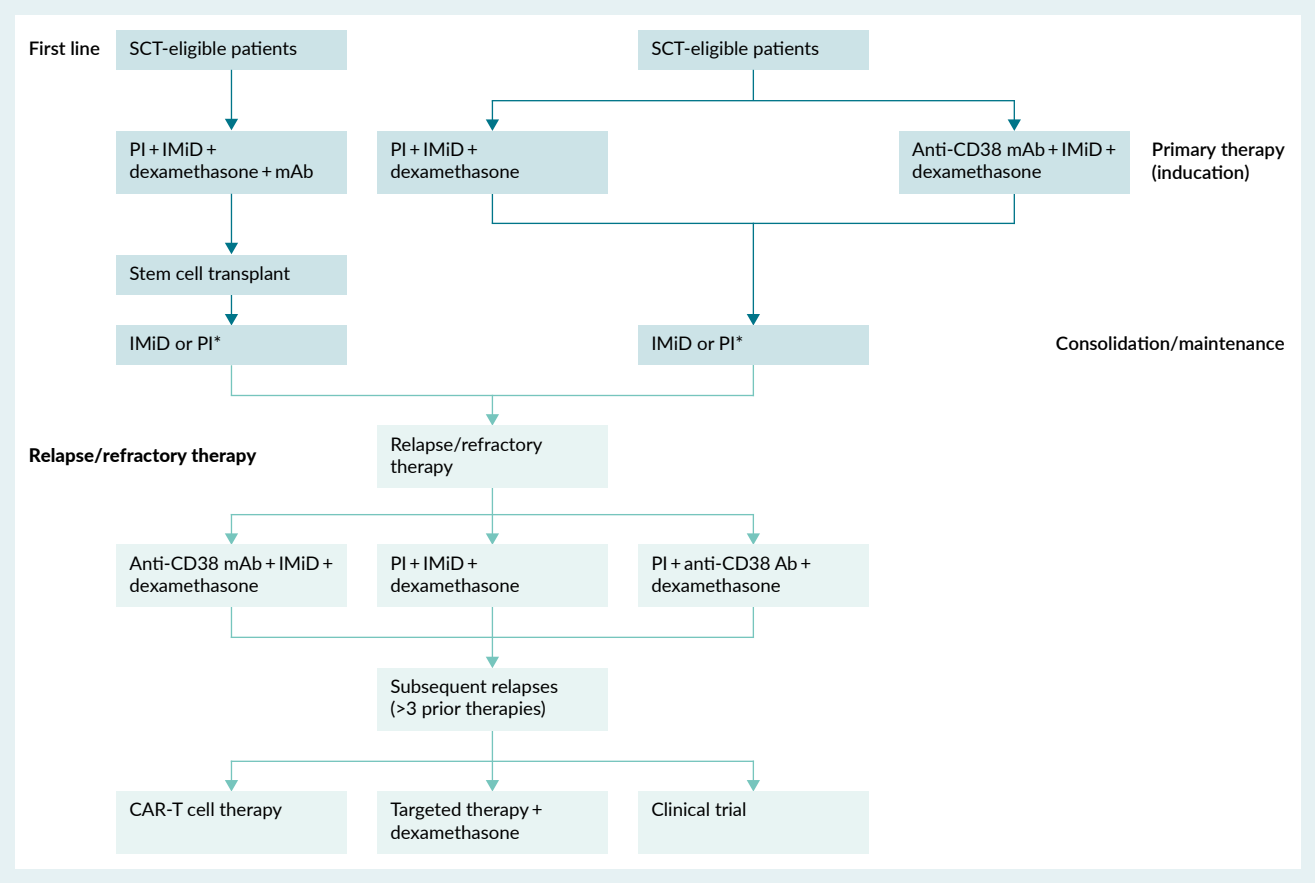
Global incidence and prevalence of multiple myeloma.

	US	UK	France	Germany	Italy	Spain	Urban China	Japan
Diagnosed incident cases of MM (2022)	29,378	6,303	5,689	7,355	6,393	2,865	14,969	7,353
Diagnosed prevalent cases of MM (2022)	15,801	14,019	18,878	23,452	20,532	6,875	20,532	49,196

Adapted from [31].

FIGURE 3

Multiple myeloma treatment algorithm derived from the National Comprehensive Cancer Network (NCCN) guidelines [32].



Immaturity of data for decision-making

A key challenge when developing a TPP for a CAR-T cell therapy is figuring out how emerging data on a CAR-T in early development is likely to stack up versus a competitive benchmark that reflects a more mature estimate of efficacy and safety. It is important to be able to map out the trajectory of data maturity for competitor CAR-Ts and standards of care at their respective stages of clinical development such that meaningful comparisons can be performed for go/no-go decisions. The issue of data maturity serves as an important reminder that not everything can be specified within a TPP. There will likely be both known and unknown unknowns. The key question therefore is what to do when data immaturity is a known unknown? This is to be differentiated from missing data, but clarified as key time to event data from patients who are still benefiting from treatment such that they remain alive, and ideally free of progressive disease.

Landmark analyses can be extremely useful in this scenario [25]. Immaturity of data also poses challenges for HTA agencies that use cost-utility analyses to help inform their decision making since quality-adjusted survival is highly uncertain where both the overall survival data AND utility data are subject to extrapolation (modeling) assumptions, that have a knock-on effect on the robustness of any resulting cost-effectiveness estimates of a CAR-T cell therapy versus standard of care in patients with different health states [26–29].

Hettle *et al.* suggests a way to accommodate data maturity through defining three data sets [15]; however, it is important to note

that one needs to adjust the TPP benchmarks accordingly for each set for it to be meaningful for decision-making purposes.

1. The minimum set: the minimum data considered potentially sufficient for CAR-T cell therapy to be granted conditional regulatory approval.

2. The intermediate set: a variant of the minimum set in which the efficacy and safety of CAR-T cell therapy have been assessed over a longer follow-up period.

3. The mature set: a variant of the intermediate set in which the efficacy and safety of CAR-T cell therapy have been assessed in a larger clinical study but with a similar follow-up period as in the intermediate set.

Model estimates of long-term outcomes, in the absence of observation, are likely to be highly uncertain. For this reason, developers may need to draw on different sources of evidence to inform their modeling assumptions and perhaps consider extending the follow-up period in their clinical trials to be in a strong position to be able to validate their modeling assumptions at a later point in time.

Reliability of comparative evidence in the absence of randomized controlled trial data

Obtaining robust historical 'control arm' data that is a perfect match with the cohort of patients included in the single arm trial can be challenging [15]. Developers are advised to invest carefully in studies that attempt to generate comparable data on standards of care that measure the same endpoints (as captured in their trial), using the same definitions and captured at the same time, as well as ensuring that the patient characteristics are as similar as feasibly possible in terms of their stage of disease, number of prior therapies, performance status and any other important prognostic factors.

Assigning weights to TPP attributes

Not taking the opportunity to externally validate a TPP is a frequent omission of many companies. Furthermore, failing to consider the relative weight of their TPP attributes to commercial success. It is important that companies gain a quantitative understanding of what TPP attributes are the primary drivers of differentiated value that will translate to preferential market shares (and those that are 'nice to have'). The TPP attributes will not likely be valued equally and so ranking them in order of importance could be useful, especially when it comes to making investment decisions at the various stages of development. Different methods can be explored for weighting purposes (e.g., using qualitative means such as Delphi techniques) but the more robust the approach to weighting and valuing individual TPP attributes, the more informed the decision-making process becomes). Ultimately, the pursuit of developing commercially unviable treatments carries with it a significant opportunity cost which should be avoided wherever possible.

LIMITATIONS OF THE PAPER

The authors set out to describe what they consider to be critical success factors for commercially viable therapies and acknowledge that the way they have approached this is from determining what success looks like at launch and working backwards. Success is defined as CAR-T cell therapies that reach those patients that could benefit most from treatment by clearing the market access and payer hurdles through demonstrating a superior offering to alternative treatments where the value-based price can be justified accordingly. The authors accept that some manufacturers may prefer to restrict the focus of their TPP on clearing the first hurdle, namely regulatory approval as it makes for a less complex undertaking. However, incorporating important market access considerations into a TPP at an early stage enables manufacturers

to better understand the higher hurdles that may be involved in securing reimbursement, and hence a return on investment. It also helps to better inform a more reliable and accurate commercial forecast based on a TPP that is optimized in this way.

A further limitation of the paper is that the authors do not allude to the challenges associated with the adoption of CAR-T cell therapies, such as how they might fit within clinical care pathways in different countries, the infrastructure that is needed within hospitals to accommodate these types of treatment, together with workforce capacity constraints and any needed capability (skills) development. These are clearly very important considerations for developers of CAR-T cell therapies.

TRANSLATION INSIGHTS

In summary, there are many uncertainties when developing a CAR-T cell therapy and a TPP aligning developers and key stakeholders can help to minimize risk, failure rates, time needed, and costs. Ideally, a TPP should be in place by the time of initiating the Phase 1 study for internal decision-making purposes, as well as helping to inform discussions with regulatory authorities, HTA agencies, payers, and patients. A formal approval mechanism and change control systems are advised to preserve the integrity of a TPP and ensure that go-no/go decisions can be justified based on the evidence, using the TPP benchmark as a yardstick for enhanced R&D efficiency, positive regulatory outcomes, and commercial success.

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AFFILIATIONS

Clare Hague PhD
Managing Director,
Oncology Access Solutions Ltd,
Cheshire, UK
(Author for correspondence)
chague@oncologyaccesssolutions.co.uk

Louise Street-Docherty PhD
Vice President Strategic Intelligence,
VISFO,
Lincolnshire, UK

Frances Pearson DPhil
Senior Director Strategic Intelligence,
VISFO,
Lincolnshire, UK

AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

From the lab to the bench: scaling up regulatory T cell therapy

Megan K Levings and Hany Meås

Regulatory T cells offer advantages for immune modulation in cell therapies due to their multiple mechanisms of action, including regulation of antigen-presenting cells and T cell effector function. This article addresses the key challenges in the evolving field of Treg-based therapy, emphasizing safety, accessibility, and manufacturing efficiency, and explores strategies such as the implementation of automation and closed systems to enhance operational consistency and minimize contamination risks. The Gibco™ Dynabeads™ Treg Xpander System are showcased for their ability to effectively expand Tregs. Additionally, the Gibco™ Cell Therapy Systems™ portfolio is highlighted for its rigorous safety testing and GMP compliance.

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INTRODUCTION

Addressing challenges in regulatory T cell processing

Regulatory T cells (Tregs) hold promise for modulating immune responses in transplantation, autoimmunity, and autoinflammatory responses. However, manufacturing Tregs poses many challenges, including variability in starting materials, complex scalability, and concerns over product homogeneity. Due to the fact that Tregs are a relatively rare subset of cells, their manufacturing often involves an expansion step. However, Tregs typically deliver low yield after expansion.

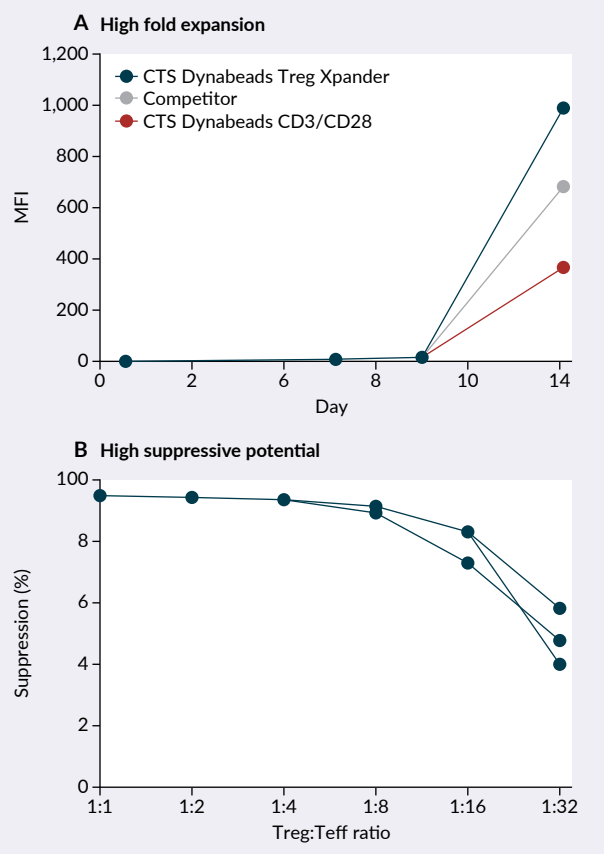
Expansion of Tregs for clinical application could be achieved with the use of the Gibco Cell Therapy Systems (CTS) Dynabeads Treg Xpander System, which consists of 4.5 µm paramagnetic beads covalently linked with anti-human CD3 and CD28 antibodies. The Gibco CTS Dynabeads Treg Xpander System bead is designed specifically for *ex vivo* activation and expansion of Tregs. A key feature of this bead is that it allows for the activation and expansion of Tregs without antigens or antigen-presenting cells (APCs).

Treg Xpander beads deliver efficient, reproducible results with high expansion rates. By restimulating with beads after 9 days, Tregs can be further expanded while preventing non-Treg overgrowth, ensuring the expanded Tregs retain their FOXP3-positive phenotype and suppressive function. The Gibco CTS Dynabeads Treg Xpander beads can be utilized for several days to weeks, and then be readily removed using the Gibco™ CTS™ DynaCellect™ Magnetic Separation System and bead removal kit.

Based on the results of an internal study, the Gibco CTS Dynabeads Treg Xpander beads allowed for over 1,000-fold expansion of Fluorescence-Activated Cell Sorting (FACS)-sorted Tregs over 14 days, significantly outperforming both the Dynabeads CD3/CD28 Magnetic Beads and the competitor product (Figure 1A). Additionally, expanded Tregs were co-cultured with effector T cells (Teffs), and the suppression was measured using CSFE dilution. As seen in Figure 1B, nearly full suppression at Treg-Teff ratios from 1:1 to 1:8 was achieved, and started to decrease at higher Teff concentrations.

FIGURE 1

Comparison between the CTS Dynabead Treg Xpander System, CTS Dynabeads CD3/CD28 Magnetic Beads, and the competitor system for the expansion of Treg cells (A) and maintenance of their suppressive potential (B).



CTS: Cell Therapy Systems.

CASE STUDY

Advantages of allogeneic Tregs in cell-based therapies

Traditionally, Tregs have been isolated from patients' own blood (autologous approach) but they can also be obtained through allogeneic protocols. The British Columbia Children's Hospital Research Institute has been focusing on utilizing allogeneic Tregs.

Allogeneic approaches offer several advantages over autologous methods, including the possibility to screen donors in advance, create efficient manufacturing workflows, and provide off-the-shelf access to large quantities of quality-controlled cells. There are various potential sources for allogeneic Tregs, including the blood of healthy individuals, cord blood, stem cells, and human thymus tissue. As noted above, Tregs are a relatively rare subset of cells, which require *in vitro* expansion. During the expansion, genetic modifications can be introduced as well. The ultimate goal is to infuse a large number of Tregs into the patient, helping to re-balance the immune response in favor of Tregs over Teffs.

Mechanisms of Treg-mediated immune suppression

Tregs are a powerful tool for modulating various immune responses because they operate through multiple mechanisms of action. Over the years, various pathways have been identified that allow Tregs to suppress different immune cells and molecules.

- **Modulation of APCs:** Tregs can downregulate costimulatory molecules and reduce the ability of APCs to present antigens.
- **Induction of T cell tolerance:** Tregs can induce infectious tolerance by reshaping immune responses such that new, peripherally-induced Tregs develop.
- **Immune modulation:** Tregs release various anti-inflammatory cytokines and metabolites that act on many cell types and help modulate immune responses.

Collectively, the activity of multiple immunomodulatory pathways makes Tregs a more potent option for modulating immune responses compared to classical pharmacological agents, which typically target one pathway at a time.

Harnessing thymic Tregs for immune modulation

Pediatric thymus tissue is a relatively abundant source of Tregs. For example, 1 g of thymus contains approximately 500 times more Tregs than 1 mL of blood. In total, one thymus contains approximately 300 million cells.

Thymus-derived Tregs have excellent viability and long telomeres. Compared to blood-derived Tregs, thymic cells are more potent and suppress activated T cell proliferation and graft-versus-host disease (GvHD) *in vivo* more effectively. Furthermore, these Tregs remain stable in the presence of inflammatory cytokines.

FOXP3 expression of isolated Tregs is variable depending on the source. Some types, such as thymic Tregs, exhibit a homogeneous population that consistently retains high FOXP3 expression, while other types may lose FOXP3 levels during expansion.

Addressing the challenges in harnessing thymic Tregs

The thymus is the primary site where T cells, including Tregs, develop. According to flow cytometry data of CD4+ T cells, a distinct group of FOXP3+ and CD25+ Tregs can be identified within this population. However, there are several challenges associated with thymic Treg manufacturing. Firstly, distinct from blood, the thymus contains CD25+CD4+CD8+ cells, which should be removed from the product. Secondly, commercial T cell selection reagents are not designed for thymuses, which necessitates development of a custom process. Thirdly, Tregs expand poorly compared to conventional T cells, and depending on the stimulation method, they can exhibit low viability. Finally, there are logistical challenges with efficient bead removal and concerns about Treg survival during cryopreservation.

In order to address these challenges, a three-step process for the efficient development of Tregs was developed. To begin with, CD25+CD8- Tregs are isolated from thymocytes. Afterwards, the Tregs are expanded and the beads are removed. Finally, the Tregs are cryopreserved to preserve their viability.

The thymic Treg development process

Step 1: isolation

The first step of Treg development is the isolation of CD25+ cells from the bulk cell population. Considering the thymus also contains CD25+ cells within the CD8+ population, bead removal and CD8 depletion is carried out after CD25+ cell isolation. This process yields nearly 100% pure CD4+CD25+ Tregs (Figure 2). Additionally, a population of CD25+ progenitor Tregs without FOXP3 upregulation is also present. However, it was observed that during the *in vitro* expansion process, progenitor Tregs differentiate into FOXP3+ cells upon T cell receptor stimulation.

Step 2: thymic Treg expansion

After isolation, Tregs are expanded in large quantities to meet the demands of clinical

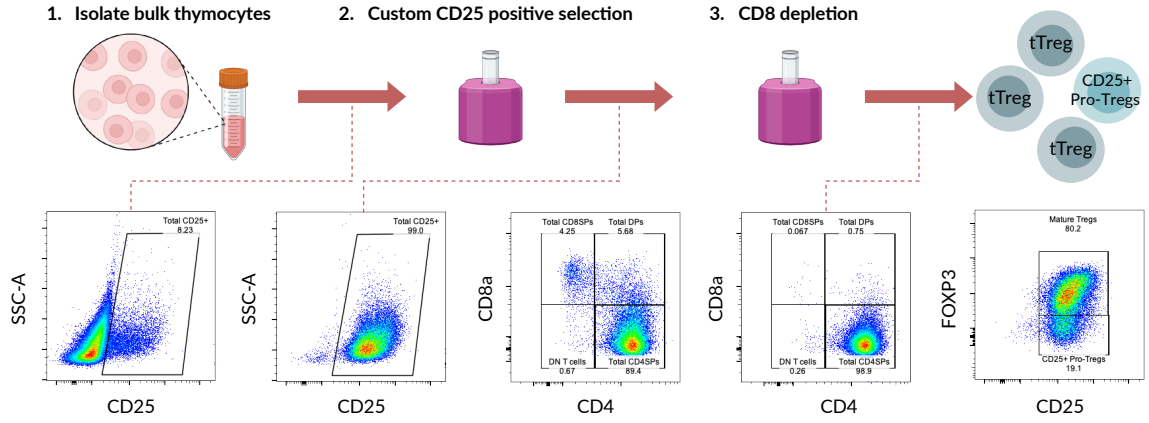
use. Initially, the British Columbia Children's Hospital Research Institute researchers explored different activation procedures for cell expansion. These included methods using artificial antigen-presenting cells, T cell receptor stimulation with CD28 (with and without CD2), Dynabeads Treg Xpander beads, and the TransActTM system. All methods successfully expanded Tregs, but there were notable differences in fold expansion potential, cell viability, and consistency in FOXP3 expression The Treg Xpander system allowed for a 100-fold cell expansion while maintaining cell viability and remarkable consistency in FOXP3 expression. In contrast, the other products demonstrated greater variability in FOXP3 levels at the end of the expansion [1].

Isolated thymic Tregs were also expanded using CTS Dynabeads Treg Xpander and rapamycin to aid the transition to a fully differentiated state. A serum-free media was used for the expansion of cells, increasing the consistency of the entire process. A restimulation with Gibco CTS Dynabeads Treg Xpander beads was performed on day 11 before cryopreservation on day 14. At the end of this expansion process, nearly 100% of the cell population was double-positive for FOXP3 and Helios, two transcription factors characteristic of Tregs. For comparison, blood-derived Tregs often include Helios-negative cells, known as peripherally induced Tregs, while the thymic Treg population consists solely of these double-positive FOXP3-Helios cells.

In order to assess the impact of Treg restimulation density on expansion rates, a study analyzing different cell densities was conducted. Cells that were grown at a low cell density (1×10^5 cells/cm^2) expanded considerably between days 13–15, then stopped growing and their viability fell by day 21. Tregs that were grown at 5×10^5 cells/cm^2 had lower expansion at days 15 and 17, but reached similar fold expansion by day 19 and maintained higher viability than those at lower cell densities at the end of the expansion. Finally, cells that were grown at the highest cell density (20×10^5 cells/cm^2) exhibited poor expansion and their viability dropped during days 13–17, before increasing again by the end of the expansion. In summary, it was discovered that at the highest density, the cells showed a significant drop in viability and expansion, whereas lower densities provided comparable expansion rates and maintained relatively high FOXP3 expression. These data highlight the importance of maintaining a relatively low cell density, such as 5×10^5 cells/cm^2. Therefore, in order to maintain optimal conditions, cell density

FIGURE 2

FACS data of isolating CD25+ Tregs from the bulk cell population.



Tregs: regulatory T cells.

was adjusted every 2–3 days throughout the expansion period.

Large-scale expansion of thymic Tregs

While the thymus provides a rich source of Tregs, it also necessitates large volumes of media during the cell expansion stage of manufacturing. Therefore, one key area of future process development will include testing of different bioreactor systems which would allow for management of large volumes of media in a closed system, reducing the complexity of handling multiple bags of Tregs.

Step 3: cryopreservation of thymic Tregs

The timing of cryopreservation of Tregs is crucial because it significantly impacts the viability of the cells. Tregs are frozen a few days after the second restimulation. In previous experiments, the cells were restimulated with Gibco CTS Dynabeads Treg Xpander beads on day 11 and cryopreserved either 2, 3, or 5 days later. It was discovered that freezing the cells later than 3–4 days after restimulation leads to a notable decrease in viability when the cells were thawed. When cells were frozen 2–3 days after restimulation, they maintained viability upon thawing. However, when the cryopreservation was performed 5 days or later after restimulation, a significant drop in viability, lower FOXP3 expression, and reduced ability to suppress other T cells was observed [1].

Navigating regulatory challenges for thymic Tregs

One of the main challenges associated with thymic Tregs is navigating the regulatory process, considering thymus-derived Tregs possess characteristics of both an organ and a cell. Therefore, a bespoke approach to regulatory compliance is being pursued.

Release assays are also being developed in order to quantify parameters such as recovery, viability, identity, and sterility. Technical approaches used for these release assays include flow cytometry, epigenetic analysis, bacterial culture, and mycoplasma testing. For example, the PureQuantTM assay can be optimized to measure the level of demethylation in the Treg-specific demethylated region. Manual counting or flow cytometry can be used to ensure the product has under 100 residual Gibco CTS Dynabeads Treg Xpander beads per 3 million cells.

Scaling up GMP production of Tregs

Engineering runs for the thymic Treg product are in process. These cells have been successfully expanded in a GMP facility across four process development runs and three engineering runs, achieving expansions ranging from 500- to 1,000-fold using the CTS DynabeadsTreg Xpander magnetic beads. Furthermore, the expansion rates have remained consistent across different scales.

Advancements in clinical applications of thymic Tregs

Currently, the British Columbia Children's Hospital Research Institute is testing thymic Tregs as an allogeneic cell therapy, initially focusing on graft versus host disease (GvHD). Animal models have demonstrated that Tregs can enhance bone marrow engraftment, as well as protect from GvHD. Furthermore, Tregs could also be utilized in autologous cell therapy, especially for children undergoing heart surgery. In this case, autologous cells could be isolated from the thymus that was removed during heart transplantation.

ENHANCING CELL THERAPY MANUFACTURING WITH AUTOMATED SYSTEMS

The primary objectives of cell therapy manufacturing are to ensure patient safety and reduce costs, ultimately making these life-saving treatments more accessible to a

broader range of patients. In order to achieve these goals, automation and closed system operations in production are vital in that they significantly increase efficiency, reduce variability, and minimize the risk of contamination, ensuring both safety and consistency of the final drug product. As the cell therapy field evolves, different treatment types with unique manufacturing requirements will coexist, such as allogeneic and autologous therapies. Therefore, it is imperative to develop flexible, scalable manufacturing platforms to accommodate varying production scales and timelines.

Flexible, scalable, and automated cell and gene therapy manufacturing platforms

Thermo Fisher Scientific aims to address this objective with our Gibco CTS product line, which includes both reagents and advanced instruments designed for cell therapies. These include the Gibco CTS DynaCellect Magnetic Separation System for automated cell separation, the GibcoTM CTSTM RoteaTM Counterflow Centrifugation System for cell processing, the GibcoTM CTSTM XenonTM Electroporation System for cell transfection, and the Thermo Scientific DynaDriveTM Single-Use Bioreactor for scalable cell culture. Each device works independently but can also be digitally and physically integrated into an automated workflow, providing flexibility and efficiency. Tailored for both process development and commercial manufacturing, these devices come with single-use kits and comprehensive regulatory documentation, enabling accelerated process and precision with compact footprints.

SUMMARY

Tregs hold great promise across a range of therapeutic applications. However, as with other immune cell types employed in cellular immunotherapy, developing robust and scalable manufacturing processes that are fit for clinical and commercial purposes is challenging. Integrated automated cell processing solutions, such as the CTS Dynabeads Treg Xpander System in combination with the CTS DynaCellect Magnetic Separation System, hold the key to ensuring patient safety and reducing costs sufficiently to enable broader patient access to life-saving therapies.

Q&A


Megan K Levings and Hany Meås

Q What specific mechanisms do Tregs use to maintain immune balance and prevent an overactive immune response?

MKL: Although it is still an active area of research, some of the main mechanisms of Tregs, as mentioned earlier, involve disrupting the function of APCs and directly suppressing other T cells.

One of the key molecules involved is CTLA-4, which is highly expressed in Tregs. CTLA-4 helps down-regulate CD80 and CD86 on APCs through a process called trogocytosis or transendocytosis, which limits the ability of APCs to activate other T cells.

Another important mechanism is the production of TGF-β, a well-known inhibitory cytokine for T cells. TGF-β not only stops T cell proliferation but also induces *de novo* FOXP3 expression in other T cells, a process known as infectious tolerance.

A third key mechanism is the consumption of interleukin-2 (IL-2). Although Tregs do not produce IL-2 themselves, which is due to FOXP3 suppressing it, they rely on IL-2 from their environment, depriving other T cells of this crucial cytokine.

Q What specific technology is used to optimize the activation signal for expanding Tregs?

HM: The CTS Dynabeads Treg Xpander system is based on CTS Dynabeads CD3/CD28 Magnetic Beads, with the key difference being the coating. The Treg Xpander beads have an optimized ratio of anti-CD3 to anti-CD28 that is specifically designed to ensure optimal activation and expansion of Tregs *in vitro*.

Q What is the significance of achieving a stable FOXP3 phenotype and robust suppressive function in the expansion of suppressive Tregs?

MKL: One of the unique challenges with Tregs is their hyporesponsive proliferation, and in some cases, methods that drive rapid and high expansion can lead to downregulation of FOXP3. Therefore, it is important to find a way to stimulate the cells that balances good expansion and preservation of the desired phenotype. This is less of a challenge with thymic Tregs because they lack peripherally-induced cells, which have a higher propensity to lose FOXP3 expression.

Q Tregs are relatively rare, making their isolation and expansion challenging. How are you addressing this challenge in your workflow development to generate sufficient cell numbers for therapy?

MKL: A major focus is optimizing recovery in order to efficiently isolate as many cells as possible. Another approach is engineering the cells to become more potent on a per-cell basis. For example, we and other researchers are working on different ways to engineer Tregs with CARs or TCRs. By introducing antigen specificity into the Treg product, we can enhance its potency, allowing us to use fewer cells. These studies are still in early stages of clinical testing but ultimately, I believe we will reach a stage in the future where we will no longer need to infuse billions of cells into a patient, making the process more feasible.

Q How does the closed, automated environment for producing Treg-based therapeutics help minimize contamination risks and reduce human error?

HM: A closed and automated environment for cell therapy production significantly reduces contamination risks and minimizes human error by limiting manual handling. This controlled and sealed environment ensures a high level of precision through automated processes. For example, single-use kits ensure sterility while real-time monitoring and standardized procedures enhance consistency, quality, and safety.

Q What research projects is your laboratory currently undertaking, Dr Levings?

MKL: Beyond researching allogeneic thymic Tregs, engineered cell therapy products are another key area of focus. In the past, we demonstrated that it is possible to introduce CARs into Tregs, redirecting their specificity towards any antigen for which the CAR is developed. Our initial work in this area was in transplantation, where we showed that CAR-Tregs

can target HLA-A2—a commonly mismatched class 1 antigen in transplants. This technology has now moved into clinical testing.

Furthermore, we have been using the CRISPR/Cas9 gene editing system to perform gain- and loss-of-function experiments, helping us to understand the mechanisms of action behind these cells. We are continually learning that what works for CAR-T cells does not always work for CAR-Tregs. Therefore, we are trying to understand how to engineer CARs for Tregs for optimized function and use in different patient populations.

Our two main application areas are transplantation and autoimmunity, and many patients we are targeting will already be on different types of immunosuppressive drugs as part of their standard treatment. Therefore, understanding how Tregs would interact with immunosuppressive drugs, and whether we can engineer Tregs to work under these conditions, is another important area of focus for our ongoing research.

Q Are there any ongoing clinical trials or studies exploring the use of CAR-engineered Tregs in human patients?

MKL: There are currently two ongoing studies using the previously mentioned A2-CAR concept. The first one is being led by Sangamo Therapeutics and focuses on kidney transplantation. In this study, an A2-specific CAR-Treg product is infused after the transplant, aiming to reduce the use of tacrolimus, an immunosuppressive agent with many side effects for patients. The second clinical trial is being led by Quell Therapeutics in the UK. It is a similar concept but in the context of liver transplantation, where patients receiving an HLA-A2-positive liver graft are infused with A2-CAR-Tregs to reduce the need for immunosuppression. Several other biotech companies are also working on TCR- and CAR-Treg products for autoimmune indications, and we are likely to see more clinical trials soon.

Q Why is Treg-based cell therapy well suited for treating autoimmune and chronic diseases?

MKL: Tregs are built to suppress harmful autoimmune responses. For example, we know from conditions such as IPEX, a systemic autoimmune disease syndrome that develops in people without functional Tregs, that Tregs are critical to prevent colitis (intestinal inflammation), endocrinopathies (such as Type 1 diabetes), and skin inflammation with elevated IgE levels. Beyond extreme situations such as IPEX, in more common forms of autoimmunity, Treg deficits likely lead to a failure to control certain immune responses. This makes cell and gene therapy-based approaches very promising, as they could potentially restore the defective Treg populations.

Furthermore, recent research is expanding Treg-based therapy beyond classical autoimmunity and transplantation to the treatment of chronic inflammatory diseases, such as atherosclerosis, stroke, and Parkinson's disease, where Tregs seem to be effective at restraining inflammation.

Q What are the main differences in the manufacturing approach when using thymic Tregs versus naïve Treg phenotypes derived from peripheral blood?

MKL: Thymic Tregs come with many challenges, mainly because the commercial products were originally designed for peripheral blood, which is why we have been working to adapt these tools to thymic cells. However, if we manage to isolate naïve blood Tregs, they are functionally very similar to thymic Tregs, which is promising.

One advantage of thymic Tregs is that they are all naïve. Therefore we do not need to introduce a flow cytometric sorting process. In blood, however, to isolate naïve Tregs flow cytometric sorting is needed, making the isolation step considerably more challenging.

Q What optimization steps can be taken to ensure the stability of Tregs and maintain their immunosuppressive function after transplantation?

MKL: The ideal Treg product should be double-positive for both FOXP3 and Helios. Additionally, it is important to assess the state of demethylation at the Treg Specific Demethylation Region locus, which can be measured using the PureQuant kit or similar assays.

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AFFILIATIONS

Megan K Levings
Professor,
Department of Surgery and
School of Biomedical Engineering,
University of British Columbia,
Vancouver, Canada

Hany Meås
Staff Scientist,
Cell Therapy,
Thermo Fisher Scientific,
Oslo, Norway

AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Closed system solutions for cell and gene therapy manufacturing

Anette Funfak

Despite the rapid growth of the cell and gene therapy sector, many challenges remain, including high costs, complex manufacturing processes, and contamination risks. Implementing closed-system solutions can significantly improve efficiency, safety, and cost-effectiveness in cell and gene therapy manufacturing. This article explores the integration of advanced vessels, which allow developers to optimize workflows for cell culture, ultimately enhancing productivity and scalability in cell and gene therapy production.

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INTRODUCTION

There is a need for improved efficiency and more cost-effective solutions for both small- and large-scale manufacturing of cell and gene therapy (CGT). This could involve greater automation of processes, increased capacity for parallel processing within closed systems, shorter processing times, and higher throughput.

Furthermore, it is crucial to ensure consistency and quality between batches and minimize contamination risks. Contamination is extremely costly and time-consuming, which may lead to shortages of therapeutic products for patients in need. Key contaminants include bacteria, viruses, cytokines, mycoplasma, growth factors, unwanted mammalian cells, endotoxins, and other components [1]. In order to tackle this hurdle, it is important to implement early integration of closed systems in CGT manufacturing, which not only reduces contamination risks but also allows for easier automation and may help to reduce environmental costs. Additionally, multiple controls throughout the manufacturing process and higher quality requirements for raw materials and consumables can reduce contamination risks.

KEY STEPS IN CELL-BASED THERAPY MANUFACTURING AND THE ROLE OF CLOSED SYSTEMS

A typical workflow of cell-based therapy manufacturing commences with the

isolation and activation of cells. In some processes, the cells are then reprogrammed using viral vectors or other modalities. Subsequently, the cells are expanded. Next, the cells are harvested, washed, and prepared for downstream processes, such as QC testing, cryopreservation, and cell characterization. The final product is then ready to be transferred to the patient.

Closed system solutions can play a crucial role in upstream processes, including both small- and large-scale cell and viral vector manufacturing. For example, a broad range of standard Corning® products including Erlenmeyer flasks, storage bottles, centrifuge tubes, and stacked cell culture vessels and accessories featuring MPC, Luer, and AseptiQuik® connectors can be used for safe and efficient cell-based therapy production. Other tubing manifolds and closed system adapters offer flexibility for workflows tailored to specific applications and manufacturing needs.

OPTIMIZING CELL CULTURE WORKFLOWS WITH CELLSTACK® VESSELS AND CLOSED SYSTEM ACCESSORIES

A closed system workflow with CellSTACK® stackable cell culture vessels can be applied for various processes, including cell seeding, media exchange, infection, transfection, and ultimately cell harvest or lysis, depending on the intended application. Furthermore, this workflow can be fully assembled outside a safety cabinet when using a sterile connector such as an AseptiQuik connector or tube welding.

CellSTACK cell culture vessels offer a relatively large cell culture surface while maintaining a small spatial footprint. These vessels are available in a range of sizes from 1 to 40 layers, with cell culture surface areas of approximately 600–25,000 cm^2. Furthermore, CellSTACK vessels are available with either tissue culture treatment or Corning CellBIND® surface for enhanced attachment of cells. The CellSTACK vessels are also compatible with aseptic transfer caps and can be used with different closed system accessories with MPC, Luer, or Aseptiquik® connectors. The closed system setup needs to be assembled with the CellSTACK under a biosafety cabinet and can then be handled outside the cabinet for further processing.

Furthermore, a closed system tubing manifold designed for handling smaller volumes of media and cell suspensions enables various process steps, such as cell seeding, washing, and cell harvesting. The manifold also allows a flexible connection of bags and liquid handling accessories due to its multiple connection ports, including Luer lock ports which enable repeated connections and disconnections, spike coupling options, as well as MPC and Aseptiquik connectors.

Finally, the pre-assembled CellSTACKS with aseptic MPC or AseptiQuik connectors can be connected to the standard liquid transfer vessels or novel closed system modalities, enabling connection and manipulation entirely outside of the biosafety cabinet. This allows for a faster and easier setup of workflows and handling of cell culture vessels. Additionally, tube welding can be used.

ENHANCING CELL CULTURE EFFICIENCY WITH HIGH-YIELD PERFORMANCE (HYPER) TECHNOLOGY

HYPERFlask® cell culture vessels use Corning's High-Yield PERformance technology, providing significantly more cell growth surface area compared to traditional cell culture vessels of comparable footprint. The HYPERFlask vessel, for example, is the same footprint of a T-175 flask but provides 10X the cell growth surface area (1,720 cm^2).

This increase in yield is achieved by stacking ten thin layers of rigid polystyrene, each paired with a unique ultra-thin gas-permeable

FIGURE 1

Integration of CellCube® system for large-scale cell expansion.

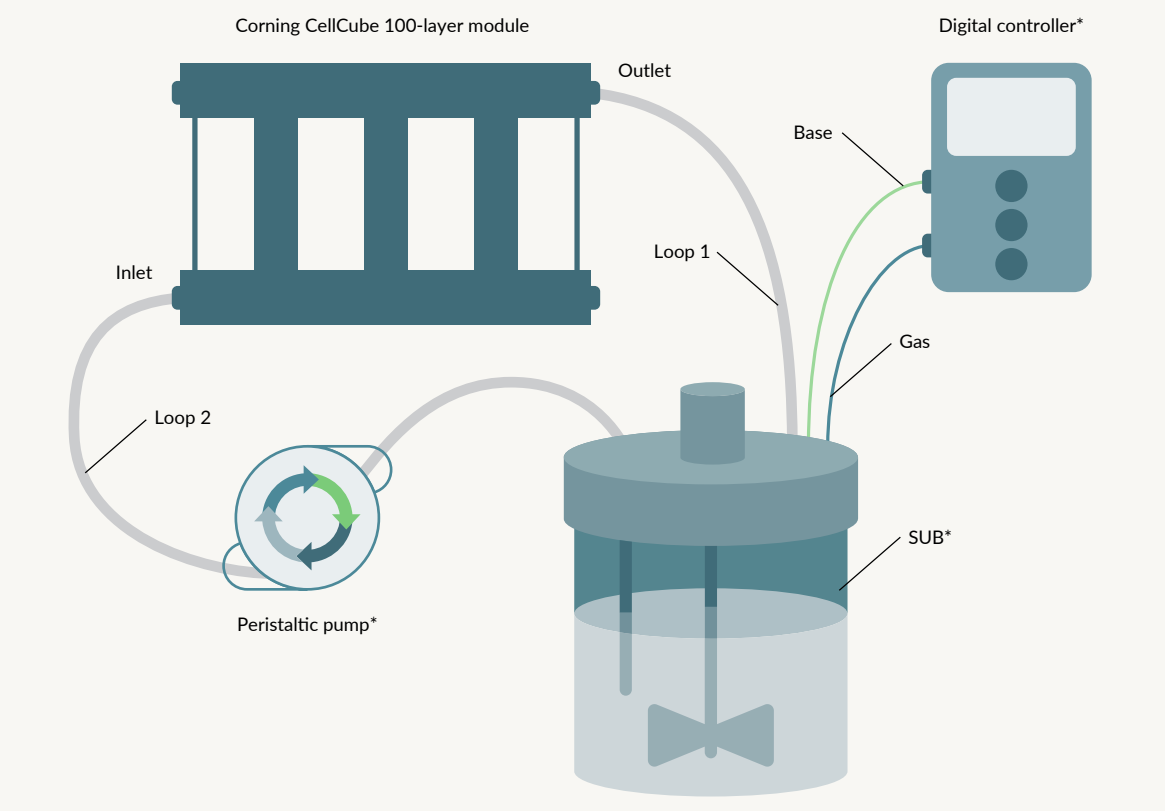
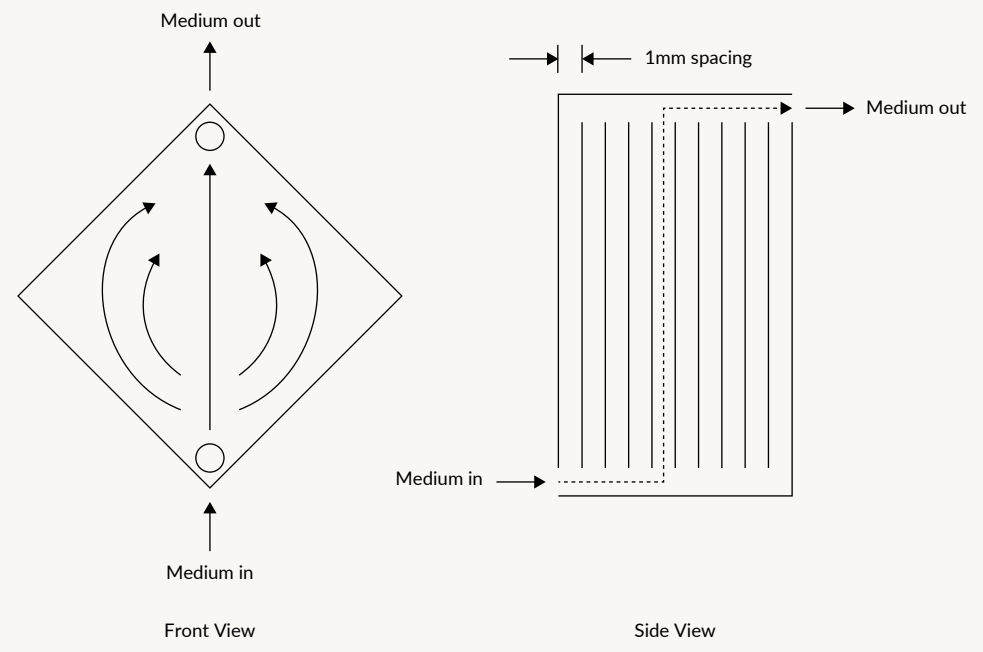


FIGURE 2

In CellCube® modules, cells are grown on both sides of polystyrene plates positioned closely together.



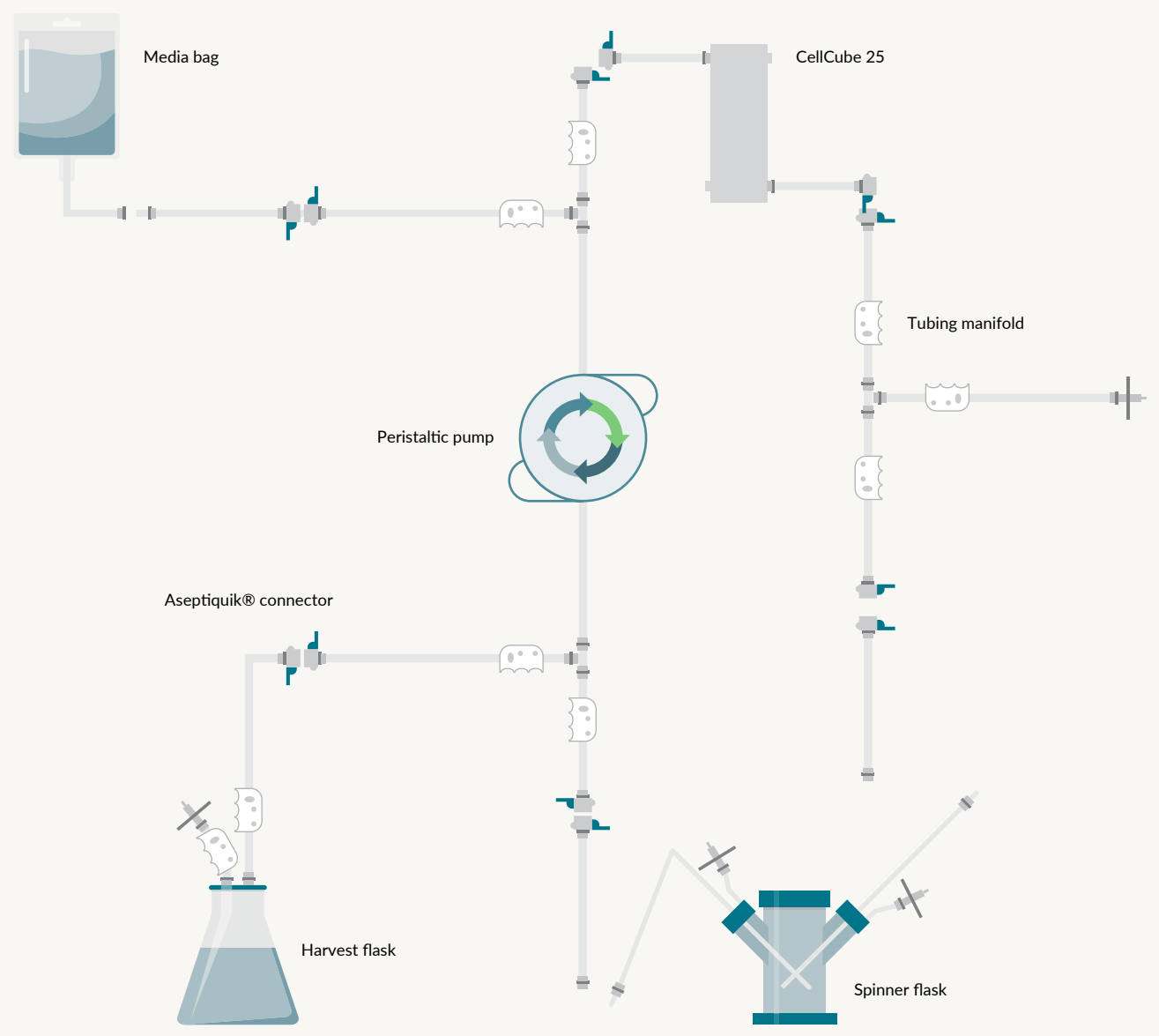
film, The gas-permeable film allows for homogeneous gas exchange.

MAXIMIZING CELL CULTURE CAPACITY WITH HYPERSTACK® VESSELS AND MANIFOLD CONFIGURATIONS

In order to achieve an even higher cell culture surface, HYPERStack® cell culture vessels can be utilized. These vessels use the same gas-permeable film to achieve a high density of cell culture surface and a small footprint. For example, the HYPERStack 12-layer vessel provides 6,000 cm^2 of cell culture surface, while the HYPERStack 36-layer vessel offers a surface of 18,000 cm^2, both within the footprint of a traditional stacked vessel, such as the Corning CellSTACK vessel. HYPERStack vessels are designed as closed systems, featuring a fill line with MPC connector and the option for direct tube

FIGURE 3

Cell therapy manufacturing workflow using a 25-layer CellCube® module connected via tubing manifolds to a spinner flask with two aseptic transfer caps.



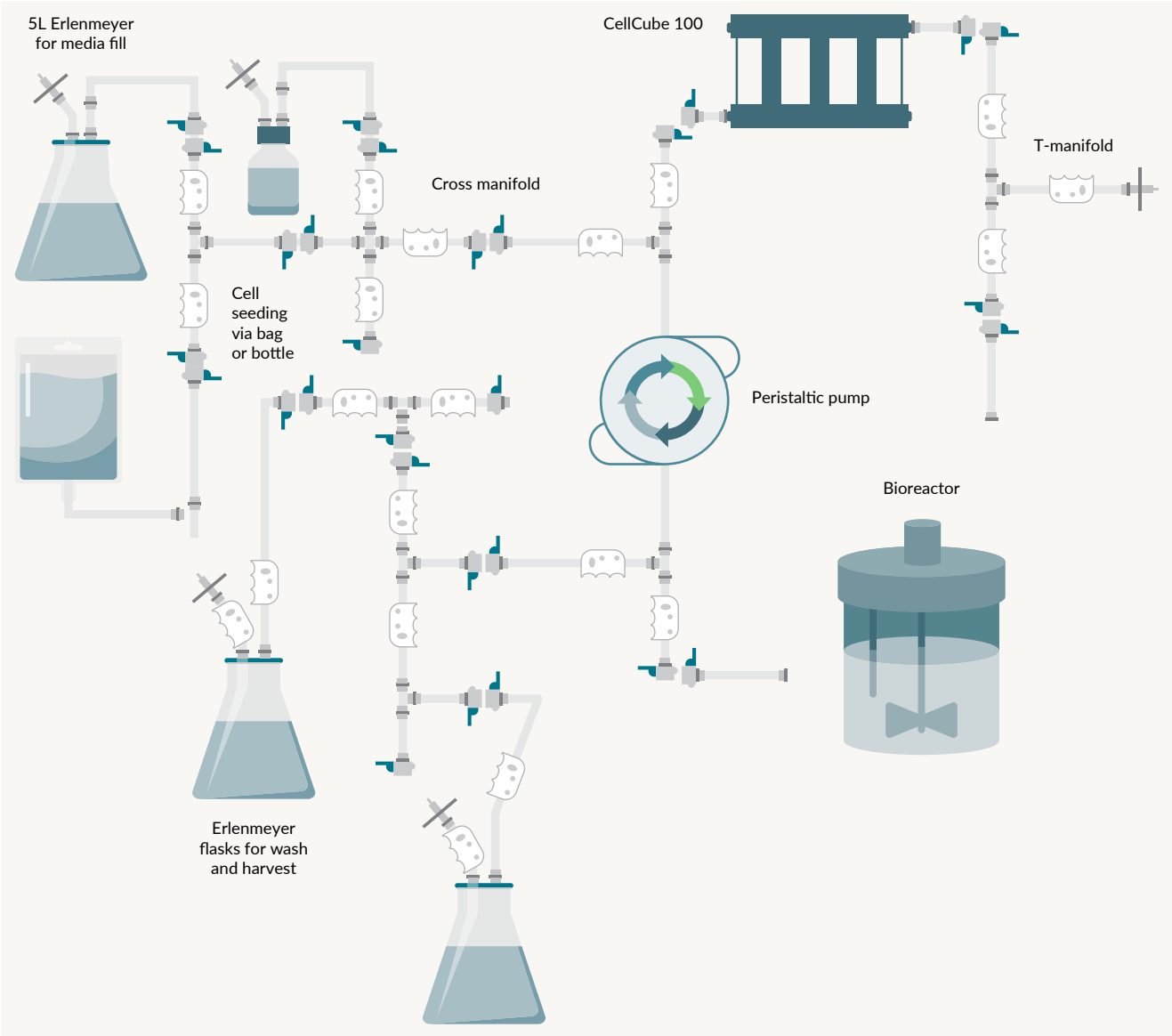
welding, allowing for complete independence from a biosafety cabinet when necessary. Additionally, these instruments are manufactured under cGMP conditions.

The HYPERStack vessels provide even more cell culture surface by utilizing a 'scaling out' approach, where multiple vessels are used simultaneously. Closed system manifolds, such as the five-arm tubing manifold, allow for the connection of up to five HYPERStack 36-layer vessels, offering 90,000 cm^2 of growth surface. This enables an easy parallel manipulation of cell culture vessels also in combination with manipulator platforms for automation.

The closed system workflow utilizing the HYPERStack vessels enables cell seeding and expansion, media exchange, infection, supernatant harvesting, and lysis. This can be achieved by integrating various closed system solutions or accessories, including Erlenmeyer flasks and storage bottles with AseptiQuik transfer caps, AseptiQuik manifolds, MPC

FIGURE 4

Cell therapy manufacturing workflow using a 100-layer CellCube® system connected to a bioreactor using closed system manifolds.



adapters, and standard bags for connecting to HYPERStack vessels.

INTEGRATING CELLCUBE® WITH BIOREACTORS FOR HIGH-DENSITY CELL CULTURE

Unlike the HYPERStack and CellSTACK vessels, CellCube is a dynamic perfusion or circulation-based system that is combined with a bioreactor (sold separately). As shown in Figure 1, this allows for the setup of a semi-automated and controlled cell culture system. Each CellCube module contains several polystyrene plates positioned closely together; cells can be grown on both sides of each plate, providing a large cell growth surface are in a compact vessel (Figure 2).

There are three sizes of CellCube module—10, 25, and 100 layers, providing 8,500 to 85,000 cm^2 of cell culture surface area. CellCubes are available with two types of cell culture surfaces— tissue culture treatment and CellBIND. Standard closed system accessories, such as tubing manifolds with AseptiQuik connectors and AseptiQuik-MPC adapters, facilitate the connection between the bioreactor and the CellCube modules.

EXAMPLE 1: CELL THERAPY MANUFACTURING WORKFLOW WITH A 25-LAYER CELLCUBE SYSTEM

A possible closed system design includes a 25-layer CellCube connected to a spinner flask via a tubing manifold with aseptic transfer caps (Figure 3). The spinner flask is utilized as a media conditioning vessel, simplifying the bioreactor setup. This design can be utilized for pre-testing the system. In essence, this configuration allows for the addition of bags with MPC connections using AseptiQuik-to-MPC adapters or direct tube welding to the tubing manifold for media filling. The media are conditioned and then perfused from the spinner flask to the CellCube vessel using a peristaltic pump. Cells can be seeded from bags or bottles through the same connection port via MPC connection or direct tube welding. A second connection port is designated for cell harvesting. For instance, Erlenmeyer flasks with an AseptiQuik connection can be used for harvesting, washing, and adding a dissociation agent.

EXAMPLE 2: CELL THERAPY MANUFACTURING WORKFLOW WITH A 100-LAYER CELLCUBE SYSTEM

Alternatively, a more complex and scaled-up closed system workflow can be implemented by connecting a 100-layer CellCube module to a bioreactor using closed system manifolds (Figure 4). In this setup, the media is filled from a 5 L Erlenmeyer flask with an AseptiQuik connector directly into the CellCube module via a tubing manifold. This manifold ensures recirculation of media between the CellCube module and the bioreactor via a peristaltic pump.

Media can be added from or removed to Erls or bags, and cells can be seeded from a 500 ml bottle with an AseptiQuik transfer cap, which is also connected directly to the CellCube module via the tubing manifold. Cell washing and harvesting are performed by connecting an Erlenmeyer flask with AseptiQuik connectors to selected tubing manifolds in the recirculation loop between the CellCube and the bioreactor.

USING PREFILLED MEDIA BAGS FOR EFFICIENT MEDIA EXCHANGE AND CELL SEEDING

An advantageous approach to closed system workflows in media exchange and cell seeding operations is to use prefilled media bags. Standard or specialized media, as well as serum or reagents, can be obtained in prefilled media bags that vary in size, volume, and connectors depending on the developer's needs.

CONCLUSION

Closed system solutions can help address issues in CGT manufacturing by enhancing safety and offering flexibility to developers for both small- and large-scale production. Implementing closed systems in the early stages of manufacturing facilitates easier automation and parallelization of processes.

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AFFILIATION

Anette Funfak
Field Application Scientist Manager,
Corning,
Paris, France

CORNING

AUTHORSHIP & CONFLICT OF INTEREST

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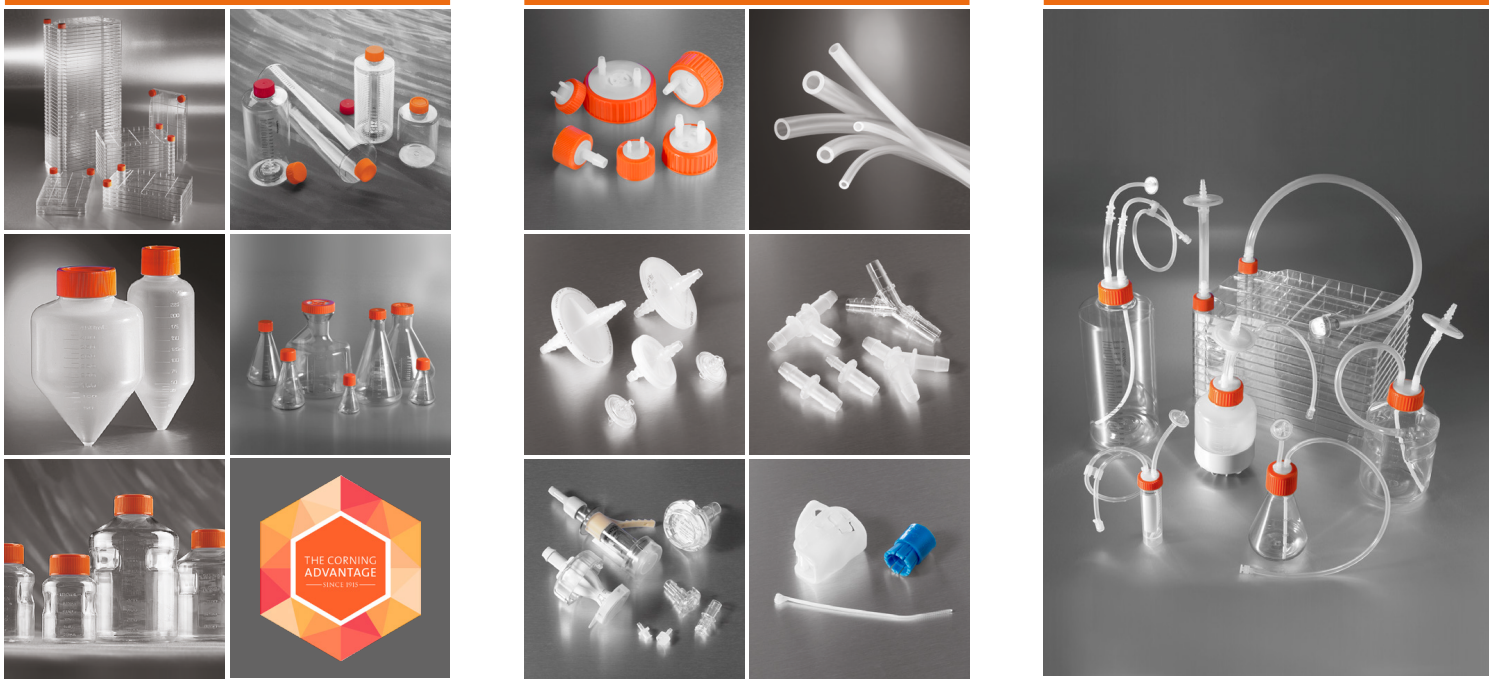

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CELL & GENE THERAPY INSIGHTS

EXPERT ROUNDTABLE

Early-stage analytical development strategies for cell therapy

Ramon Mendoza, Kyle Carter, and Seth Peterson



In this expert roundtable, three highly experienced industry professionals discuss the importance and nuances of phase-appropriate analytical control in early-stage cell therapy development. The panelists share insights on key areas of innovation such as automated solutions, digital PCR, and next-generation sequencing, and share their advice on early engagement with regulatory agencies to help ensure successful outcomes in getting cell therapies to patients.

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"Balancing these two aspects—moving quickly while staying compliant—is crucial for the development of all early-phase clinical analytical methods." — Kyle Carter

Q What does phase-appropriate analytical control look like in the early stages of cell therapy development? How do you determine which analytical assays are necessary?

SP: In the early phases, the optimal strategy focuses on safety, which is necessary and non-negotiable. At the same time, it is important to consider the scalability and transferability of the safety assays. For example, in Phase 1, much less material is produced and there are fewer test points. Thus, selecting an appropriate solution and provider that can support product expansion in later phases is crucial. Additionally, one must assess how easy it is to transfer the methods and technologies to another group or site.

However, one of the main challenges with phase-appropriate analytical control is that while the US FDA encourages it, there is no specific guidance. In the early phases, for example, full method validation from an ICH perspective may not be needed for assays such as potency, stability, or certain impurity testing, considering these can change over time. These assays must be qualified, but full method validation might not be necessary at that stage, in contrast to late-stage development.

KC: One of the key focus areas in our early-phase product development is finding the appropriate level of effort for assay validation or qualification. At that stage, developers are often under tight timelines to advance to the later phases of the clinical cycle. We have worked on strategies that remain aligned with regulatory GMP requirements while also being appropriate for the early phase, providing the necessary flexibility and information to justify the assay for its intended use. Balancing these two aspects—moving quickly while staying compliant—is crucial for the development of all early-phase clinical analytical methods.

RM: One of the key challenges in this industry is that the development process is often not well-defined. Bringing in early robustness and quantifying the variability in the analytical method early on can help developers understand the variability in the pro cess. Nowadays, many developers often wait until later phases to refine their methods and address variability, but locking down those methods earlier can greatly contribute to the future success of the program, especially as the process becomes more stable. As we know, the material itself is relatively variable, making it difficult to determine whether any drift is due to the process or the method.

Q When it comes to QC/QA assays during the cell therapy lifecycle, what are some common challenges or obstacles that developers face? How do you recommend overcoming these challenges to ensure effective quality control and assurance?

KC: From the QC perspective, we primarily focus on life cycle management for commercialized methods and understanding how they evolve to meet product and business needs.

A key challenge we have encountered while working with our analytical development partners and clinical QC teams is ensuring strong collaboration early in the process between these functions. This collaboration is crucial for fully understanding the broad set of measures within the test method that impacts its operational space within the commercial laboratory.

At Kite Pharma, we focus on increasing this collaboration and identifying stage gates for when to involve QC as an advisor in analytical development to ensure that the constraints we face within the GMP space are appropriately assessed and fundamentally integrated into the method early on. When we reach the point of method validation and transfer to our commercial laboratories, the goal is to ensure that the appropriate work has been done, whether that involves assessing critical reagents, setting up multiple suppliers, or establishing the robustness of the assay. This robustness is essential for justifying the assay in a GMP environment, especially given that the frequency of its use in operational settings can impact various parameters over time. These considerations may not be fully addressed early in development with a limited set of runs.

In essence, fostering strong upfront collaboration to ensure the assays are fit for purpose and everyone is aligned on the first intent of any new assay is critical to overcoming obstacles when bringing analytical test methods into commercial GMP settings.

RM: I agree—when an analytical method is run daily, any cracks in that technique will eventually surface, even if these issues are not apparent in the development phase. Therefore, fostering interactions between the development and QC teams early on is crucial.

When a method enters the QC lab and does not perform well, it can create a cycle of mistrust between the method's subject matter experts in development and the QC team when they try to resolve the issues. Additionally, the terminology used in the QC space may often carry different meanings in the analytical development space—regular interactions can help prevent misunderstandings.

How and where can sharp regulatory strategy and innovative analytical solutions address the challenges posed by the growing complexity of engineered cell therapy products? What key factors contribute to success in this area?

SP: First and foremost, it is important to consider the role of regulators and engage with regulatory bodies early and often, especially when working with innovative technologies such as CAR-T cell therapies.

Furthermore, considering the complexity of the analytics, it is critical to view analytical development as a collaborative effort among the internal team, regulatory agencies, and solution providers. It is crucial to look for partners with experience working with these regulatory groups who can provide support throughout the entire product development life cycle. This approach can make a significant difference by reducing the time spent navigating newer guidelines.

As we touched on earlier, the high variability of raw materials is a massive challenge in cell therapy manufacturing. Therefore, it is crucial to develop a plan for the development of your vector in addition to your cell therapy. Notably, the European Pharmacopoeia treats the vector as a separate product, which means it is important to essentially develop two analytical programs, and to seek technologies that address multiple needs. Regarding specific innovations, there are already significant improvements in workflow with novel array-based digital PCR (dPCR) instruments. These can be extensively utilized in the vector workflow for titer measurement or transgene integration. Next-Generation Sequencing (NGS) is also gaining traction, particularly following the recent update to ICH Q5A. While NGS has been around for a while,

it has matured to the point where regulatory groups are starting to approve its use, and I expect to see more progress in that area moving forward.

Lastly, many of the challenges we face are pushing technologies to be faster and more efficient. For example, there is now a demand stemming from the regulators for innovation in safety and sterility testing, which is often performed at risk, but novel technologies are reducing testing times from weeks to days, or even to a single day with quantitative PCR (qPCR) methods that deliver rapid results. These advances also support USP 1071, reflecting some remarkable progress on all fronts.

KC: When considering enhancing efficiency and simplicity in the test methods that characterize cell therapy products, it is crucial to be cognizant of the regulatory strategy required to ensure that any new technologies utilized are viewed as acceptable by regulators.

It is vital to reduce turnaround times in order to deliver the drug product to patients faster. Any reduction in the turnaround time for the testing panels to facilitate drug product disposition and release should be a priority. This effort involves leveraging technologies such as automation. However, one challenge with automation technologies is navigating the heightened increased need for computer system validation due to the integration of both instrument software and external software in a commercial environment. Therefore, it is critical to ensure compliance and maintain control over these novel automated test methods, which are designed to be highly simplistic and utilize cartridge-based technologies. Additionally, it is essential to work closely with the internal regulatory affairs and regulatory CMC teams early and often.

Lastly, beyond just building these technologies, it is important to ensure they operate in compliance, which again involves communicating with regulatory bodies. Maintaining strong relationships with your organization's internal regulatory representatives and directly engaging with the regulatory bodies is critical.

Q How do developers typically choose their QC/QA assays during the cell therapy lifecycle? Are there any key factors or considerations that influence the decision-making process?

KC: Method developers usually begin by assessing the critical quality attributes (CQA), which drive the testing panel necessary to demonstrate that the product is safe and efficient. From this perspective, it is critical to focus on establishing a platform approach. Prioritizing simplicity not only in executing the test method but also in setting up the laboratory for its implementation during the operational phase should be a priority. However, the developers are often constrained by the physical space within the laboratories. In addition, the more equipment that is needed for various tests, the more complex it becomes, which requires additional analysts and specialized expertise.

This complexity can extend to the number of commissioning, qualification, and validation personnel, quality engineers, and other teams needed to maintain the laboratory. Therefore, when considering new technologies or establishing a testing panel, it is crucial to design a platform approach as a first intent. If that initial intent is not feasible, other opportunities to meet the requirements for identifying the CQA in question should be explored. In the QC team, we are particularly focused on consistency across the instruments, as this reduces the need for extensive training, allowing us to leverage a broader range of specialists in the laboratory. For example, highly complex flow methods require personnel with a high level of training and

"In the cell and gene therapy (CGT) space, the regulators are learning alongside product developers. There are two areas that I see evolving simultaneously with our combined understanding—comparability and potency." — Ramon Mendoza

education to run the tests. In contrast, implementing simpler systems, such as cartridge-based setups that reduce the need for pipetting and minimize the number of required steps, can increase automation. And by reducing reliance on manual activities, one broadens the pool of individuals capable of running the assays in the laboratory.

Finally, ergonomic considerations are also significant. Many of our current assays require extensive physical pipetting, which can lead to ergonomic issues and create resourcing challenges. Looking beyond the CQA itself at these broader, operational aspects is critical when deciding which assay to use.

RM: The entire industry is focused on automation and closing the loop earlier—by moving some decision-making analytics to AI platforms, for instance. Having an integrated control strategy will drive the development of a testing panel. If you can control an attribute upstream or inline enough to keep it off the release panel, that can sometimes become a negotiation you can have with the regulatory agency in the later clinical phases.

Ultimately, the focus should be on patient safety, which means ensuring that developers have control over the process while being confident that the methods they are using are sensitive enough to detect any issues. This is often a challenge due to the variability of the incoming material being much higher than what the given method can handle.

When transitioning from the clinical to the commercial space, developers no longer have the same level of control over the incoming population compared to clinical studies. The assumptions and observations from the clinical phase may not always translate into the commercial space. Therefore, it is essential to have a plan in place when launching the product commercially, as this is often difficult to anticipate.

Q How do you see regulatory CMC compliance strategies evolving to adapt to the increasing complexity of engineered cell therapy products? Are there any specific areas of focus or changes you anticipate in the near future, and what should developers be thinking about implementing now that can pay dividends moving forward?

RM: In the cell and gene therapy (CGT) space, the regulators are learning alongside product developers. There are two areas that I see evolving simultaneously with our combined understanding—comparability and potency.

Regarding comparability, traditional methods of assessing process changes are important, such as analyzing historical batch data or comparing the final product. It is critical to establish a baseline for the material entering the process to understand what that change means or looks like. This involves not only examining the final drug product but also understanding how it

"Generally, there have been some remarkable technological innovations [in CGT manufacturing] but there have not been sufficient advancements to allow us to consider most aspects of cell therapy as being fully platformed." — Seth Peterson

arrived at a particular attribute range. Not all materials entering the process will start from the same point—it is like a race where everyone begins at different starting lines, potentially finishing at different points. However, that does not necessarily mean that the outcomes will be dissimilar. Due to the variation between the start and end of the process, you might find that the products are similar enough in the end.

Potency is another highly charged concept within the CGT field. Most potency methods rely on complex cell-based assays involving living organisms in an in vitro space. Typically, potency has been assessed in terms of predicting safety and efficacy. However, expecting a potency method to achieve both for CGT products is a significant challenge. The method must be sensitive enough to detect any process drift, which indicates stability, while also establishing a connection to clinical outcomes. There may be room to maneuver around the concept of potency as it relates to both the process and the product, as well as clinical efficacy and safety outcomes. Recently issued draft guidance regarding potency assurance indicates that the FDA is beginning to view potency as a holistic attribute rather than just one or two measurable factors. The FDA is considering aspects upstream of the final potency test. For example, it is important to consider whether there are elements within the process that can be monitored and linked to potency, which may not directly be linked to efficacy or safety but can still be integrated into the potency framework.

Q Do you think there has been enough innovation in the analytical toolkit to address the complexity of cell therapy products? What novel technologies or approaches do you find particularly promising in this regard, and how might regulators view these technological innovations?

SP: Initially, implementing new technologies in CGT manufacturing may seem easy, but it is actually quite challenging due to the need for effective regulatory compliance. Generally, there have been some remarkable technological innovations but there have not been sufficient advancements to allow us to consider most aspects of cell therapy as being fully platformed. Luckily, the regulators have been very collaborative in exploring novel solutions to meet the demands of cell therapy specifically.

As mentioned earlier, automated solutions hold significant potential for simplifying processes. However, we have also seen some limitations regarding scalability and adaptability from both manufacturing and analytical perspectives. Despite these challenges, automation promises great opportunities across many markets. For example, the advancements in novel dPCR platforms may offer much shorter workflows and simplified analytics.

Additionally, NGS platforms have become more mature and are now showing their utility. These technologies provide deeper characterization at the single-cell level compared to other

analytical methods and could yield valuable insights when linking potency with efficacy, for instance. Lastly, many developers are also hopeful about real-time technologies, such as in-process process analytics or biosensors to help address batch-to-batch variation and choose which parameters should be dialed up or down in real-time.

From a regulatory standpoint, the FDA's Emerging Technology Program is intended to support these innovations, and the regulators have shown flexibility and been willing to discuss many of these novel technologies.

RM: Regarding tools innovation, the 'shiny new things' on the market do not necessarily address the critical gaps. Furthermore, it is essential to consider scalability and how any innovations align with your actual business plan, whether it involves centralized or decentralized manufacturing.

When discussing investment in these innovations, it is crucial to recognize that they have not yet been pressure-tested in commercial settings, and often lack the sustainability required for long-term use. These aspects of innovation are often overlooked but can become problematic during the late stages of the process. For example, relating to difficulties in aligning instruments across different sites.

The goal is to adopt innovations that, while not necessarily simplistic, have fewer failure points. Adopting them early can be beneficial but it is important to keep the commercial model in mind.

KC: There are significant opportunities to capitalize on some of the 'low-hanging fruit' regarding automation in the commercial space. For example, justifying to regulators the automation of repetitive motions and manual techniques prevalent in many analytical test methods should be relatively straightforward. Additionally, these technologies have ergonomic benefits, considering many analysts may develop injuries due to the high amount of pipetting they perform, for instance. Utilizing 'simple technologies' to automate pipetting, such as an automated pipettor equipped with scripts, can greatly enhance operational efficiency. Overall, the approval process for such tools with the FDA is fairly straightforward.

While many developers want to explore cutting-edge technologies such as NGS and digital PCR (dPCR) over traditional qPCR assays, there are existing, established technologies that are also capable of significantly improving operations in laboratories handling patient testing.

Q How do you stay informed about the latest regulatory guidelines and developments in the cell therapy manufacturing space? Are there any specific resources or strategies that you find helpful?

RM: It is important to be proactive in this area and explore resources such as the Alliance for Regenerative Medicine (ARM) and other platforms that are specifically focused on CGT. Furthermore, whenever regulators draft guidance, they invite people for discussions or to make recommendations. I strongly suggest reading public comments from these consultation periods, as they can provide valuable insights into the recurring issues in other companies, and the current gaps between industry and the regulatory agencies. There is also a multitude of podcasts dedicated to harmonizing the industry, which can further increase your understanding. Finally, regularly checking the FDA website for updates may also be beneficial.

Q Do you have any parting words of advice or best practices to share with those working in early-stage cell therapy analytical development?

KC: From the QC perspective, early-stage developers must consider test methods from a full life cycle perspective. As the product progresses through the clinical stages, it is important to refine those methods to ensure the delivery of an effective testing solution for the QC organization, which requires consistent and high-frequency testing. In cell therapy, we often deal with a per-patient, per-lot basis, leading to numerous lots that need regular testing, resulting in a substantial workload. If test methods are not designed to support this demand, you could end up in a situation where you are continuously modifying these methods post-commercialization within a GMP environment, which not only introduces risk but also necessitates repetitive regulatory filings to fully implement mature methods in the commercial setting.

Even after those analytical methods are approved, it is crucial to maintain jurisdictional control during their rollout, which can be labor-intensive and expensive. It can affect a broad set of functions across regulatory CMC, analytical development, and QC.

All things considered, it is important to maintain a balance between speed and completeness. By keeping the end goal of the test method in mind (which is to support commercial operations) and designing it in the context of early-phase work, developers will be in a much better position when it is time to roll that method out.

RM: Firstly, it is essential to follow the ICH guidelines on method development and life cycle management early on, building robustness into the methods, and qualifying them as early as possible. This is particularly important since many CGT development programs are on accelerated timelines. Normally, developers will not have the traditional amount of time to develop methods and conduct cycles of reevaluation as they progress through various clinical phases. Therefore, it is important to expedite as much as possible what might be needed at the later stages. It also enhances the robustness of the data produced in the early stages, potentially eliminating the need to repeat tests.

Secondly, it is critical to consider integrating automation early into an analytical method life cycle. If you know what innovations in this area will be released moving forward, start thinking about how automation can be incorporated into the method development process during Phase 1.

Many analytical method developers entering the space often overlook the opportunity to leverage what is currently known, believing instead that critical tasks are not phase-appropriate and pushing them the back burner. However, by the time these tasks must be carried out, there may not be sufficient time left. Therefore, early commitment to robustness and automation in the method life cycle can pay dividends later.

SP: Long-term planning should begin at the discovery stage. It is important to ensure that development is a collaborative process, and engaging early with regulators and solution providers can benefit everyone involved. Furthermore, ensuring that the support will last into the later stages is just as crucial. When evaluating innovative technologies, it is essential to consider whether there is enough capacity to implement them at later stages. Lastly, regardless of whether you are part of a small or a large biotech company, it is vital to address both domestic and global market interests.

BIOGRAPHIES

RAMON MENDOZA is currently supporting the CGT portfolio by leveraging his ATMP expertise to ensure robust technical strategies are developed. He is also developing holistic control strategies for new modalities in the cell and gene therapy space. Prior to joining Johnson & Johnson Innovative Medicine, Ramon worked for Bristol Myers Squibb (Celgene), Orchard Therapeutics, Rocket Pharma, Bayer Pharma, and Analytical Lab Group (now part of Element Materials Technology). During this time, Ramon had the responsibilities of leading analytical and commercial GMP quality teams, developing process and analytical control strategies for AAV, LV, RNP's, and CAR-T drug products. He has experience supporting CGT programs from early/late-stage development, well into the commercial lifecycle. He has authored many submissions and participated in agency inspections and meetings, leading to successful commercial approvals of new CAR-T drug products. Ramon has a PhD in Cell and Molecular Biology from the University of Washington, WA, USA and held postdoctoral positions at the Fred Hutchinson Cancer Research Center in Seattle WA, USA.

KYLE CARTER is a seasoned biopharmaceutical professional with over 15 years of experience in quality control, project management, and bioprocessing. Carter holds an MBA and an MS in Biotechnology from the University of Maryland, MA, USA and a BS in Biology from West Virginia University, WV, USA. Currently, as the Director of Quality Control at Kite Pharma in Frederick, MD, USA, he leads the Analytical Lifecycle Management for the Global Quality Control organization. In this role, he oversees commercial cell therapy vector and final product analytical testing, including flow cytometry, PCR, and ELISA-based methods. He manages a team focused on laboratory software alignment, global change controls, CAPAs, method performance monitoring, and analyst proficiency assessments. Additionally, he is the Global Business Process Owner of the LabVantage Laboratory Information Management System (LIMS), responsible for the global implementation of a 21 CFR Part 11 enterprise LIMS across multiple commercial and clinical manufacturing sites.

SETH PETERSON, Senior Manager of Application Support at Thermo Fisher Scientific, Waltham, MA, USA, is a seasoned professional in the field of Pharma Analytics. With a dedicated focus on supporting and advancing novel genomic methods, Seth brings a wealth of experience and passion to his role.

Peterson oversees application support initiatives, playing a crucial role in developing and implementing innovative genomic methods tailored for both research and regulated environments within the pharmaceutical industry at Thermo Fisher. His commitment to excellence is reflected in his diverse experience, where he has provided support to teams in Clinical Oncology, Pharmaceutical Manufacturing, and Agricultural biology.

Peterson's career spans over a decade in the field with Thermo Fisher Scientific, during which he has directly engaged with customers, leveraging Next-Generation Sequencing (NGS) applications. His expertise particularly shines in the areas of epigenetic profiling and genotyping methods, showcasing a deep understanding of the intricacies of genomic research and its applications in various domains. With a focus on supporting and developing novel genomic methods, Peterson stands as a dedicated leader and advocate for advancements in Pharma analytics.

AFFILIATIONS

Ramon Mendoza
Director,
Scientific Leader,
Analytical Development,
Johnson & Johnson,
San Fransisco Bay Area, CA, USA

Kyle Carter
Director,
Quality Control,
Kite Pharma,
Frederick, MA, USA

Seth Peterson
Senior Manager,
Application Sciences,
BioProduction Group,
Thermo Fisher Scientific,
Waltham, MA, USA

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This is a transcript of the roundtable
You can also watch the recorded roundtable here:

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WEBINAR DIGEST

Demystifying AAV affinity capture: mapping AAV affinity ligand footprints with cryo-electron microscopy

Nathaniel Clark, Scientist, Downstream Development, Repligen

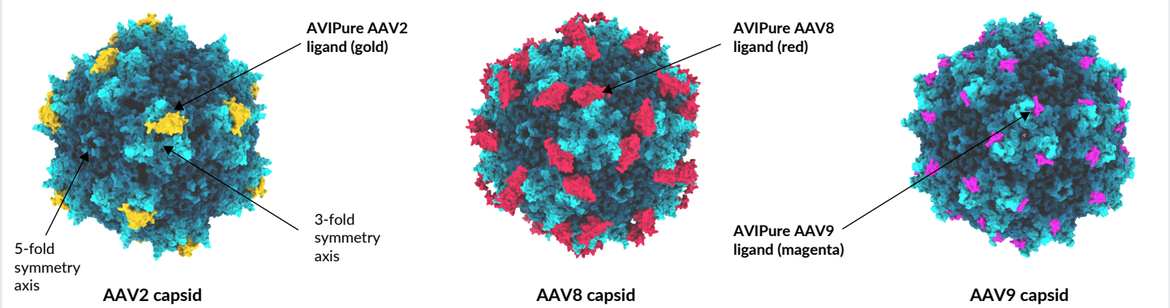
Cryo-electron microscopy (cryo-EM) provides high-resolution structures of AAV capsids and binding partners. Structures of several AAV-affinity ligands with the target capsids elucidate the mechanism of affinity resin capture. This poster delves into how these ligands engage the AAV capsids, and how these results can accelerate process development for these important gene therapy vectors.

CRYO-EM IN AAV AFFINITY RESIN DESIGN

Cryo-EM techniques allow visualization of the three-dimensional structure of AAV capsids. The AAV capsid structure consists of 60 viral protein (VP) monomers, predominantly VP3, which assemble with icosahedral symmetry. To determine where the AVIPure affinity ligands bind on AAV capsids, we determined cryo-EM structures of the ligands:capsid complexes. Identification of the binding site on the capsid helps scientists determine if a given capsid, which often contains proprietary mutations, will bind to a serotype specific AVIPure AAV resin.

Figure 1 illustrates three cryo-EM structures, AAV2, AAV8, and AAV9 capsids bound to their respective AVIPure ligands. These findings reveal that all ligands bind to the capsid's 3-fold symmetry axis. This enables rational pairing of an AAV resin to a specific capsid and enables the selection of capsids that maintain the AVIPure epitopes.

Figure 1. Cryo-EM structures of AVIPure ligands bound to AAV 2, 8, and 9, respectively.

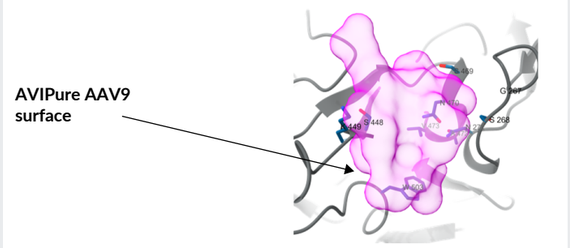


MAPPING LIGAND BINDING SITES

Cryo-EM maps revealed that AVIPure ligands have specific binding "footprints" on each AAV serotype's capsid. The AAV2 ligand binds between lobes of the 3-fold axis, disrupting the axis's natural symmetry. This leads to a highly specific interaction that enhances AAV2 capture and reduces impurities, such as host cell proteins and residual DNA.

The AAV8 ligand also binds at the 3-fold axis but at a different location on the protrusion. Though slightly less extensive than AAV2, this interaction still ensures strong selectivity, which is essential for producing highly pure AAV8 preparations. AAV9's interaction is particularly unique; the ligand is a small 10-amino-acid peptide that binds within the galactose binding pocket on the 3-fold axis, as shown in **Figure 2**. Despite the small size, the surface area of the binding interface is comparable to that of the larger AAV2 and AAV8 ligands. This compact yet highly efficient ligand minimizes the risk of interference from capsid mutations and enables efficient and adaptable purification of AAV9 capsids.

Figure 2. The AAV9 ligand is a small 10-amino-acid peptide that binds with the galactose binding pocket on AAV9 three-fold region.



APPLICATIONS OF CRYO-EM INSIGHTS IN AAV ENGINEERING

One of the key benefits of understanding these binding sites is the ability to predict whether a modified AAV capsid can be effectively purified using AVIPure resins. Compatibility with AVIPure resins can be predicted by comparing capsid sequences with the mapped ligand binding residues. This is valuable for engineered capsids, where specific mutations may affect binding efficiency. Additionally, if this information is integrated into the early capsid engineering and selection stages, the capsids will maintain compatibility with AVIPure capture resins, thus avoiding potential downstream challenges. These cryo-EM insights also support sustainable production. By validating the mechanism of AVIPure affinity capture, producers can be confident in the selection of these resins, which exhibit high stability in sodium hydroxide, and can be reused multiple times, reducing waste and production costs.

SUMMARY

Cryo-EM technology enabled precise mapping of the AVIPure ligand binding sites on AAV capsids. The data on AAV2, AAV8, and AAV9 provide a foundation for developing robust, selective purification methods tailored to gene therapy vectors, supporting advances in gene therapy manufacturing and helping meet growing demands for high-purity viral vectors.

Watch the video and view the poster here

CELL & GENE THERAPY INSIGHTS

Cell & Gene Therapy Insights 2024; 10(11), 1605 • DOI: 10.18609/cgti.2024.184

IN PARTNERSHIP WITH REPLIGEN INSPIRING ADVANCES IN BIOPROCESSING

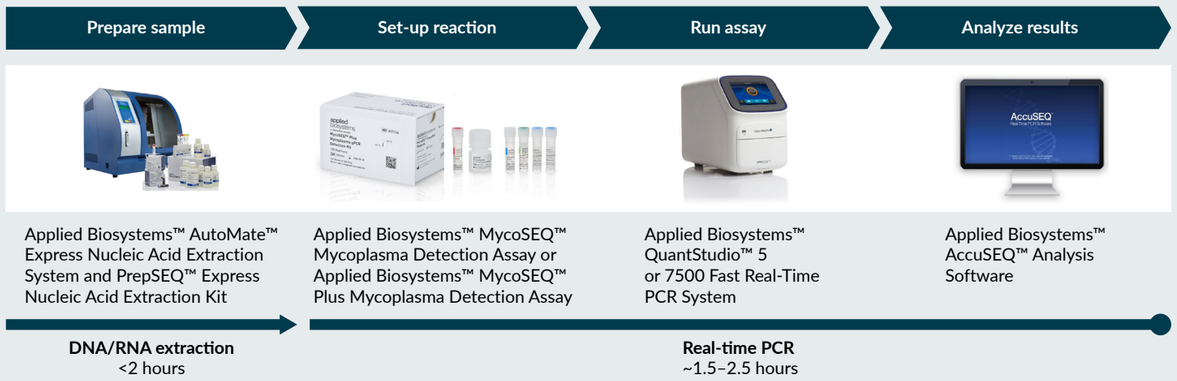
FASTFACTS

Balancing precision and efficiency in cell therapy assays: low volume sampling for mycoplasma detection

Sharon Rouw, Senior Product Manager, BioProduction Group—Pharma Analytics, Thermo Fisher Scientific

Rapid, sensitive, and specific mycoplasma detection, which can be achieved through PCR-based techniques, is essential for ensuring the quality and safety of cell therapy products. This poster presents the Applied Biosystems™ MycoSEQ™ Plus workflow for accurate and rapid detection of mycoplasma DNA in cell cultures, including cell-based therapies. This case study demonstrates the kit's sensitivity with low sample volumes across different sample matrices.

Figure 1. MycoSEQ assay workflow



With the increasing demand for cell therapies and their potential to revolutionize patient care, ensuring the safety and quality of these novel products is paramount. Mycoplasma, a contaminant of cell cultures, poses significant risks to both the efficacy and safety of cell therapies, and thus it is critical to employ robust and efficient mycoplasma detection techniques.

The MycoSEQTM Plus Kit is a qPCR assay that leverages Applied Biosystems™ TaqManTM chemistries, and utilizes a multiplexed pool of primers and probes for amplifying and detecting multiple target species, including mycoplasma.

The MycoSEQ assay workflow, shown in **Figure 1**, involves four key steps: sample preparation with Applied Biosystems™ AutoMate Express and PrepSEQ Express kits; reaction setup with MycoSEQ Plus assay; PCR reaction using the Applied Biosystems™ QuantStudio 5 or 7500 Fast Real-Time PCR system; and results analysis with GMP-compliant Applied Biosystems™ AccuSEQ™ software.

TESTING METHOD SENSITIVITY

This study tested a method designed for situations where a high level of mycoplasma detection sensitivity is required, but only a small amount of testing material is available.

Firstly, the mycoplasma species were spiked into 1.5 or 2 mL of spent T cell media containing 1×10^6 cells at 10 genome copies (GC)/mL. Samples were then processed using the PrepSEQ AutoMate Express workflow followed by ethanol precipitation, and the MycoSEQ Plus kit. The results were then analyzed using AccuSEQ software.

As illustrated in **Figure 2**, all data points were below the cycle threshold (Ct) cutoff value of 38, which indicates that the sample is positive for mycoplasma detection and demonstrates the method's ability to detect 10 GC/mL using 1.5–2 mL of sample material.

EVALUATING DIFFERENT SAMPLE MATRICES

In another experiment, genomic DNA from various mycoplasma species was spiked into 1.5 or 2 mL of mock sample before automated extraction and precipitation. Mock sample types included fresh media, spent media, and a cryopreservation medium, all containing 1×10^6 T cells. As shown in **Figure 3**, this method detected 10 GC/mL for all tested species and sample types, as indicated by the box plots being below the 38 Ct cutoff value.

Figure 2. Detection of mycoplasma species into spent T cell media. Here, the Ct values of each replicate are plotted, with red dots representing data points from the 1.5 mL sample matrix and blue dots from the 2 mL sample matrix.

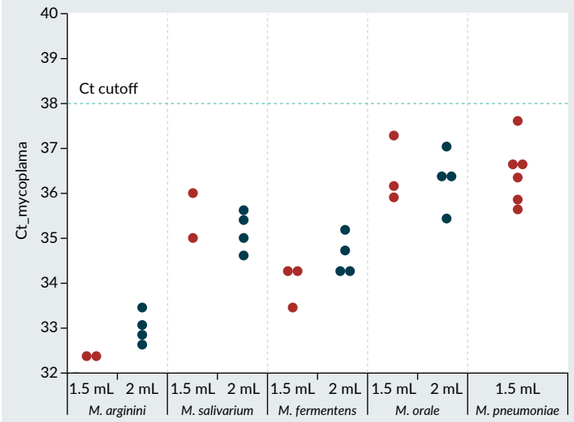
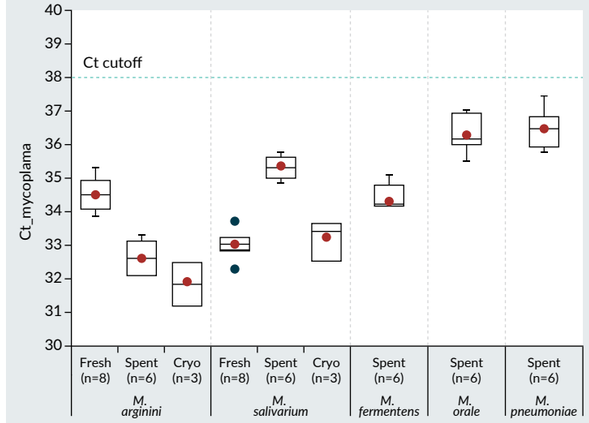


Figure 3. Detection of mycoplasma species into spent T cell media. Here, the Ct values of each replicate are plotted, with red dots representing data points from the 1.5 mL sample matrix and blue dots from the 2 mL sample matrix.



SUMMARY

In summary, rapid and accurate detection of mycoplasma contamination can be achieved with the qPCR-based MycoSEQ Plus assay workflow. Based on the case studies described above, the MycoSEQ system can detect 10 GC/mL using sample volumes as low as 1.5–2 mL, helping to ensure the safety and efficacy of cell therapy products.

Cell & Gene Therapy Insights 2024; 10(11), 1553; DOI: 10.18609/cgti.2024.177

CELL & GENE THERAPY INSIGHTS

CORRIGENDUM

Corrigendum to: Interpreting the new FDA draft potency guidance: an RNA cell therapy perspective

Damian Marshall and Kayleigh Thirlwell

In the version of this article initially published, the title was Interpreting the new FDA draft potency guidance: an mRNA gene modified cell therapy perspective. However, the title should be Interpreting the new FDA draft potency guidance: an RNA cell therapy perspective (as displayed above). This error has been corrected in the HTML and PDF versions of this article as of December 18, 2024. The amended article can be accessed **here**.

Cell & Gene Therapy Insights 2024; 10(11), 1623

DOI: 10.18609/cgti.2024.187

CELL & GENE THERAPY INSIGHTS

CORRIGENDUM

Corrigendum to: Generation of novel AAV serotypes with enhanced infectivity, specificity, and lower toxicity via AAV capsid engineering platform

Ye Bu, Yue Pan, Yujian Zhong, Huan Chen, Zhiyong Dai, Youwei Zhang, Ying Fan, Junlin Chen, Keqin Tan, Rui Duan, Min Guan, Irene Song, Luyan He, Xin Swanson, and Paul Li

In the version of this article initially published, the text on page 1418 read:

We then incorporated random peptide with RGD motif [17,20,35–37] and inserted into the chimeric capsid and subjected the library to two rounds of screening. The top ten performers were individually validated, resulting in the identification of a novel capsid, AAV-PG007, with significantly increased muscle targeting and reduced liver off-targeting in both mice and monkeys [19].

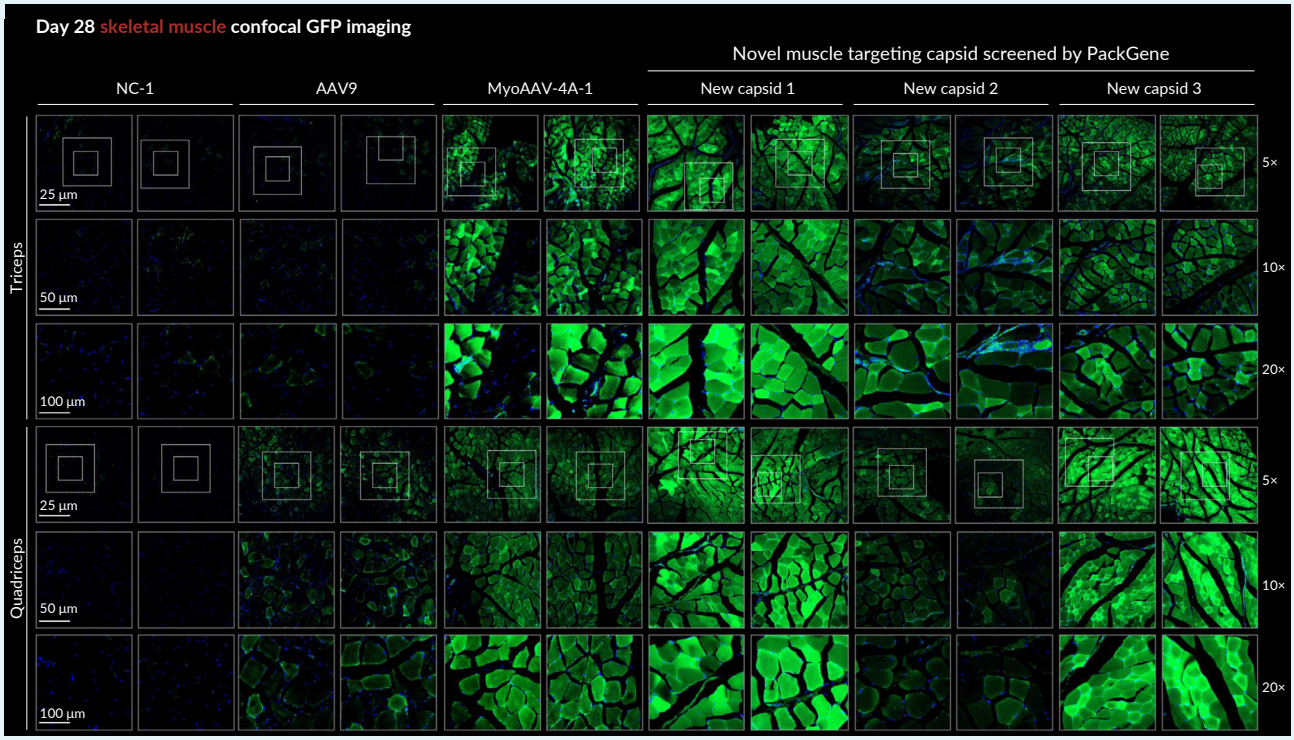
However, this should read:

We incorporated RGD-YNSL, a 7-amino acid peptide derived from MyoAAV 4A [17,20,35-37], into the chimeric serotype designed to reduce liver targeting. The resulting capsid, AAV-PG007, demonstrated significantly enhanced muscle targeting while retaining its liver-detargeting properties in both mice and monkeys.

In addition, Figure 6 and the accompanying text appeared as

FIGURE 6

Confocal images of skeletal muscle tissues from cynomolgus monkeys 28 days post-AAV injection with three novel muscle-targeting capsids.

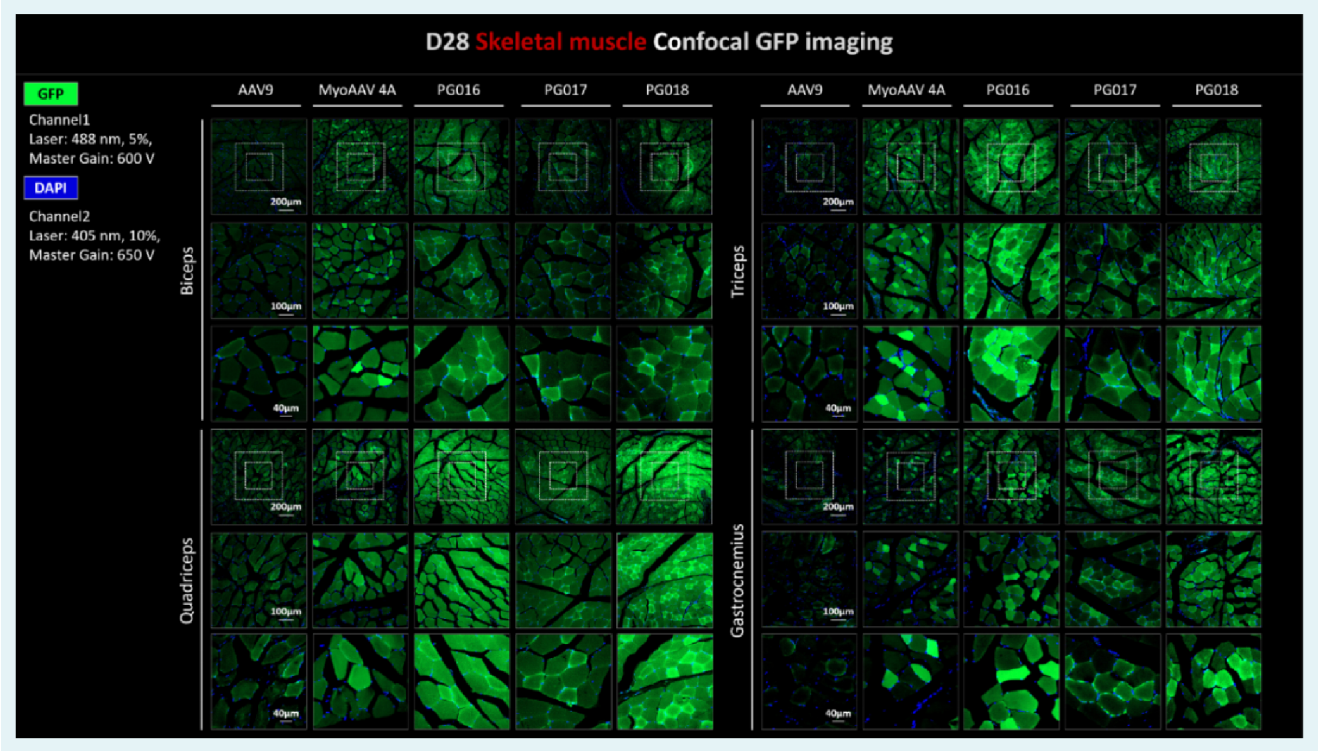


Further characterization and identification of these novel capsids are ongoing (Figure 6).

However, this should read:

FIGURE 6

Confocal images of skeletal muscle tissues from cynomolgus monkeys 28 days post-AAV injection with three novel muscle-targeting capsids.



Further characterization and identification of these novel capsids (PG016-PG018) are ongoing (Figure 6).

These errors have been corrected in the HTML and PDF versions of this article as of December 18, 2024. The amended article can be accessed **here**.

Cell & Gene Therapy Insights 2024; 10(11), 1625–1627

DOI: 10.18609/cgti.2024.188

BIOINSIGHTS

Thank you to all our peer reviewers in 2024

At BioInsights, we use double-blinded peer review to minimize bias and focus the evaluation solely on the quality of the research rather than personal identities. However, despite their invaluable expertise and thoughtful feedback, peer reviewers often go unrecognized and are not formally acknowledged in publications. Their contributions are crucial to our editorial process, helping us assess articles and advance fields such as cell and gene therapy, nucleic acids, vaccines, and immuno-oncology, and we deeply appreciate the time and effort our reviewers dedicate to this process, enabling us to publish content of the highest quality. To honor their contributions, the editors of BioInsights are proud to publicly acknowledge our top reviewers of 2024 and look forward to collaborating with them in the future.

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