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CELL & GENE THERAPY INSIGHTS

SPOTLIGHT ON
Clinical trends of 2023/tools of tomorrow

CELL & GENE THERAPY INSIGHTS

Volume 9, Issue 11

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EXPERT ROUNDTABLE

Fast-tracking gene therapy breakthroughs: discussing the critical role of rapid testing




In this Expert Roundtable, four industry experts, **Rachel Legmann, Garima Thakur, John Long,** and **Seth Levy** debate strategies to overcome analytical testing hurdles in gene therapy development and manufacturing, particularly surrounding gene therapy's need for speed in at-line and in-line testing.

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"A company must constantly evaluate potential new technology that may overcome the challenges facing the scalability and industrialization of vector-based therapies."

Q How and where are rapid/real-time process analytical technologies being applied today in gene therapy manufacturing, and with what results to date—for example, in terms of process optimization, yield improvement, and cost reduction?

RL: Unfortunately, only a few in-process analytical solutions are currently being implemented for vector manufacturing, such as sensors for monitoring dissolved oxygen pH, osmolarity, temperature, and other basic process parameters. Those measurements only provide basic information. Enzymatic patch sensors for glucose pH and basic capacitance-based biomass probes are probably the most widely used process analytical technology (PAT) solutions today.

Most analysis of CQAs is still performed offline with long turnaround times. For more complex analytical monitoring, Raman spectroscopy represents the most mature of the complex spectroscopic PAT tools, although it is difficult to robustly implement into a process and requires dedicated expertise and experimental discovery runs to achieve performance at the required level.

Why have we not yet implemented rapid real-time analytics for gene therapy manufacturing to increase vector productivity and product quality? The reason is that we still need better process and product understanding to make knowledgeable decisions. We must continue developing assays that are high-throughput, sensitive, rapid, reliable, precise, and require smaller sample volumes. They should be able to monitor inline or at least at-line for accurate vector concentration, test for bioburden, identify empty/full capsid ratio, and measure aggregates and other process-related impurities to reduce the risk of low production and low-recovery yield, and significantly reduce the number of failed batches. Furthermore, the industry is currently using available tools that were not specifically designed for gene therapy applications. These may need redesigning or qualification prior to use.

There is a limited understanding of automation, physical techniques, and statistical modeling methodologies. How do we accelerate the development phase, reduce the cost, and meet demand without compromising product safety? I believe that collaborative research and development across the fields of physics, chemistry, cell biology, engineering, computer science, and other disciplines will bring us there faster.

A company must constantly evaluate potential new technology that may overcome the challenges facing the scalability and industrialization of vector-based therapies. Repligen has developed an innovative tangential flow filtration real-time process management technology with integrated viable PAT in-line technology that enables online real-time monitoring with feedback control. This is based on accurate full capsid concentration, plasmid DNA concentration and purity, and mRNA and lipid nanoparticle (LNP) concentrations.

Q Could you share examples of specific challenges or bottlenecks that gene therapy developers face due to the lack of robust in-process at-line testing methods? How can we further encourage the integration of in-process at-line testing into the manufacturing workflow?

GT: One of the main challenges today is that many of the assays that we need to use are slow. Another key challenge is that unlike traditional biotherapeutics, gene therapies require two measurements of concentration: genomic titer and capsid titer. These can vary over the course of the process, so it is important to have something that can ideally tell us both.

In general, DNA-related assays such as droplet digital PCR are time-consuming. For many of them, we simply do not have alternatives. We are also unfamiliar with many characteristics like capsid heterogeneity and others that we simply do not know enough about to be able to develop assays.

Luckily, at the moment, these assays are not required as rapid in-process assays by regulators, but nonetheless, it would be helpful to have information throughout the process where possible. In the upstream process, knowing the transfection efficiency or cell viability would be helpful, as transfection is an important parameter to track. We do not have the tools for this right now.

Another key piece of process information in the downstream process is empty/full capsid ratio. It is difficult to achieve reliable measurements of this without using analytical ultracentrifugation, which is an offline method. There are some interesting tools, like mass photometry, that tell us the distribution of capsids based on weight, but sometimes this does not correlate well with the gold standard of analytical ultracentrifugation.

To push the development of PAT tools in the industry, we need to collect data where possible to build correlations between the sensors that we do have. We should work closely with vendors to implement newer technologies, like mass photometry, in the actual processing space to make real-time decisions. We should collaborate with analytical tool developers to express the challenges that might not be evident for these newer modalities.

JL: To echo what Garima said, leveraging the knowledge of technology innovators or instrument manufacturers is key. As we are working with newer technologies, we should not hesitate to form partnerships with instrument manufacturers.

SL: Often, a bottleneck for any analytical development, whether at-line or offline, is the lack of appropriate reference material and sufficient volumes of that reference material. Hopefully, if we can generate reference standards early and take a QbD approach to analytical process development, it would help us to more quickly integrate at-line testing with reliable analytics in the process.

RL: Do not hesitate to start analytical development at an early stage because validation and implementation take time, and you do not want to get into phase I and phase II manufacturing and then have to return to discovery. Analytical development should be done in parallel to expedite the process.

Q Regarding key recent regulatory guidance relating to gene therapy CMC and analytics (e.g., Annex 1 EU GMP Revision, ICH Q14), what are the points to consider for the field moving forward? How can scientists influence the regulatory landscape?

JL: There have been many recent shifts in the regulatory landscape. One example came out in January 2020 with the US FDA guidance 'CMC Information for Human Gene Therapy Investigational New Drug Applications' [1]. This guidance covers the importance of phase appropriateness. Collecting data and having information available early, even if it is not the full information you need, is encouraged by regulatory authorities within a lifecycle approach.

Other new guidance documents to highlight include USP 1220 and ICH Q12 on lifecycle management. These focus on having the analytics evolve with the process. Additionally, the ICH Q14 Analytical Procedure Development [2] guidance shows that the regulatory authorities are raising the bar on documentation, with the need for developers to state an analytical target profile. If you are going to propose a new in-line or release testing method, capturing and documenting data as early as possible is critical. On the development side, good documentation practices may often not be at the forefront, but the expectation from Q14 is that documentation should begin early in order to give supporting evidence as to why the regulatory authorities should approve your method over a more tried and tested technology.

RL: It is critical to do all the regulatory implementation that John mentioned at the development stage, to significantly reduce the number of development cycles and move faster into the clinic. We cannot wait until the manufacturing stage. This will be especially true when we move into gene therapy 4.0, as we have learned from monoclonal antibodies.

Q In your opinion, how much influence does the industry have in encouraging regulatory bodies to accept new analytical technologies?

SL: To me, it starts with engagement and justification. Engaging with regulatory bodies as early as possible for new technologies and novel methods enables greater collaboration throughout your development lifecycle. This means that as your knowledge and data grow during development, so does the agency's knowledge through your INTERACT submissions, pre-IND submissions, Request for Designation, etc. When it comes down to justification at the end, you will have a better argument to convince them with data that the new technology is robust and helps you reliably deliver safer and more effective medicine to patients. This way, the scientists at the regulatory bodies are much more likely to accept innovative approaches.

JL: The regulatory authorities are actively looking for feedback from industry. One example is that the USP draft guidance on quality analytics for RNA drug products and substances is currently open for comments. This shows the receptiveness that the regulatory authorities have to open those kinds of documents up for commenting and receiving feedback. The US FDA and other regulatory agencies are showing up at conferences and asking as many questions as they are answering. There is strong engagement between regulators and

developers right now, especially in the gene therapy space, which is still a burgeoning field in many ways.

Q What role are automation and PAT playing in enhancing the reliability and efficiency of rapid analytical testing in both upstream and downstream processing? Can share any examples of successful automated testing implementations in the gene therapy field?

GT: There are some interesting examples of automation in gene therapy. In the upstream, there are auto-sampling robots that take periodic bioreactor samples. Often, these are run through two or three analytical columns in sequence, first for an affinity capture and then for an ion exchange to get a good understanding of genomic titer, capsid titer, and percent full capsids in the bioreactor. Along with the standard array of bioreactor probes, such as capacitance, pH, and dissolved oxygen, you can get a good picture of how your bioreactor is doing.

There are some limitations in the sense that these assays are not often specific to gene therapy, so some development is required to ensure they tell you about the attributes in which you are interested—particularly for transient transfection processes. But there are tools out there to do this, and people are utilizing them.

For downstream, there are robots like Hamilton or Tecan auto-sample or sample prep systems for automated droplet digital PCR. Even mass photometry systems now have automated versions, which take samples and perform many different dilutions to get the most accurate reading. Even if a process appears to be working, we want to bring these tools and technologies to the fore to build a knowledge base of data that can help to cut down overall manual effort in the long run.

Online PAT is generally in the early stages for AAV, but looking at the growth in the monoclonal antibody space, particularly as more advanced manufacturing techniques such as continuous processing are implemented, it becomes important to have inline and at-line measurements. Continuous processing can be a great thing for AAV, particularly for clinical manufacturing.

RL: Specifically for the viral vectors market, we must remember that the product is a virus. We cannot do clearance at the end of the process, and safety is the number one concern. Therefore, we must use a closed system continuous process. Continuous testing will reduce and mitigate the risk of contamination, which is critical for this market. Overall, costs must be driven down, and automation and PAT is one of the ways to do this.

Q How will we continue to move towards automating data analysis and enhancing our predictive abilities as a result?

SL: Before we can be in a place to predict data, we need to firstly understand the data that we already have. To me, the first step is utilizing the statistical software that most people already have in their labs for more than just DoE optimization. This software should be used for the analysis of as much data as possible to help you understand all your historical data. Then, as you begin to implement PAT piece-by-piece, you can analyze that data to identify patterns

and trends. Eventually, you can get to a place where you can make meaningful decisions and analyze data in real-time to accelerate development. This is certainly a step-by-step process.

Q AAV and Lentivirus have been the most common viral vectors used in gene therapy over the past decade—what viral vectors may come to the fore in the space in the next 3–5 years, and how might this evolution change the process analytics landscape?

RL: I strongly believe that pharmaceutical companies will continue investing in both AAV and lentivirus moving forward, at least over the next 3–5 years. Having said that, in my opinion, lentivirus will gain in popularity. Cell therapy is growing fast, and I believe lentivirus will become more popular in clinical trials for carrying larger genetic cargo to engineer T cells, addressing patients in many cancer indications.

The major effort in the field right now is to reduce costs without compromising on drug safety. Moving forward, there will be greater efforts to develop other modalities for gene therapy as a strategy to reduce costs. That will include mRNA, other non-viral based vectors, or adenovirus. We need vectors that do not require expensive raw materials like the plasmid transfection reagents used to produce lentivirus and AAV.

The amount of plasmid DNA required for both AAV and lentivirus production and as a template for mRNA is considerable. Therefore, there will be a great deal of effort in implementing PAT within mRNA and adenovirus production. The adenovirus process and the product are different than for AAV and lentivirus, so other analytics will need to be developed, which will require investment and a deeper understanding.

JL: As LNPs come more and more into the RNA space, how much will that spill over into the gene therapy space? There is a lot of innovation going on in the LNP space, primarily in mRNA vaccines, but we will have to see whether the utility is there to get LNPs into cell therapy patients.

SL: Specifically for AAV, I think we will continue to see the development of novel capsids with increased target tissue tropism and transduction, which will drive the need for new analytics. There will be a shift away from capsid ELISAs, and a movement towards size exclusion chromatography with multi-angle static light scattering and mass photometry for empty/full capsid analysis. The other question for AAV and lentivirus will be whether we can move away from plasmids and towards nanoplasmids, doggybone DNA, or closed hairpin DNA. What other plasmid alternatives will emerge to ease that significant cost for all gene therapy manufacturers?

Q What will be some key directions and strategies for the future in terms of further de-risking vector manufacture through analytical technology innovation—for example, in terms of accelerating the development and validation of novel analytical tools such as in-process at-line testing methods?

JL: We need a holistic approach. You cannot simply say "I need a PAT/rapid method." You should take a holistic approach and partner closely with process development to find out the best strategy to control your process. At the end of the day, process control is hugely important. Strong partnership with your analytical space is essential.

For de-risking from a filing perspective, in some cases, you must develop these new technologies in parallel with existing technologies or more tried-and-tested methods. As you continue communications with the regulatory authorities, they will become more and more receptive, especially if other innovators are using similar approaches. The overall aim is to convince the regulatory authorities that these newer technologies are sufficient and provide the same quality of results as more traditional methods.

GT: I agree completely with the need for robust process controls. As we continue evolving the upstream process for novel capsids, engineered capsids, and different methods of transfection, upstream process variability will continue to be seen from batch to batch and serotype to serotype. Even within the same manufacturing process, variation will be there. It is important to link those upstream process attributes to factors that you can control downstream, such as residual host cell protein and DNA. Developing tools to have attribute tracking throughout the downstream process, coupled with knowledge obtained from the bioreactor, will be important as we grow.

RL: I agree that we must communicate between each step of the process. Upstream and downstream are one unit. I cannot agree more with John about the importance of holistic strategy. Each step, even if one step is fully automated, needs to communicate with previous and subsequent steps to expedite the process and ensure a better, more consistent product. The complexity of products within the gene therapy area is challenging but is also why they hold so much potential.

SL: I agree with Rachel that thinking of upstream and downstream as one unit is key. We focus on QbD for upstream and downstream; we need to ensure we also do that for analytics and move towards QbD instead of quality by testing.

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BIOGRAPHIES

RACHEL LEGMANN has more than 20 years of experience in the field of scalable biologics and gene therapy manufacturing of therapeutic products, viral vectors and proteins for gene therapy and biologics. She completed her PhD in Food Engineering and Biotechnology at the Technion-Israel Institute of Technology, Israel. Rachel joined Repligen in 2021 as a subject matter expert offering technical assistance to help customers achieve their technical and operational objectives in their manufacturing of virus-based therapeutics with a focus on gene therapy processes including upstream, downstream, analytics, and scalability. In addition to supporting global customers and building high level networks, Legmann is supporting various internal cross-functional activities and external collaborations. Prior to joining Repligen, Legmann held several scientific and leadership roles at the Department of Microbiology and Molecular Genetics at Harvard Medical School, CRO SBH Sciences, Seahorse Biosciences (part of Agilent), CDMO Goodwin Biotechnology, and Pall Corp part of Danaher.

GARIMA THAKUR is currently a Process Development Engineer in the Viral Production Core team of the Preclinical Manufacturing and Process Development group at Regeneron Pharmaceuticals. Her role includes technology development for novel rAAV-based therapies, production at 2 L, 50 L, and 500 L scale for preclinical trials and toxicology, as well as tech transfer to cGMP. She recently completed her PhD in Chemical Engineering at the Indian Institute of Technology, Delhi. Her work focused on process analytical technology for monitoring and control of continuous manufacturing processes for monoclonal antibodies.

JOHN LONG holds the position of Director, Analytical Methods at Aldevron, and has over 20 years of experience working in analytical methods for large molecule therapeutics including vaccines, biologics, and gene therapy products.

SETH LEVY, Senior Director of Bioprocess Development at Modalis Therapeutics, has built and oversees internal process development and analytics efforts, as well as manages external manufacturing. Prior to Modalis, Seth led teams in manufacturing science and technology and small-scale development for AAV and LV manufacturing at Viral Vectors Services. Seth worked in gene therapy R&D before his time at a CDMO and drove numerous AAV projects including basic biology research, capsid engineering, and translational gene therapy approaches at Sanofi Genzyme and academia.

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You can also watch the recorded roundtable here

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SPOTLIGHT

Key considerations for US biotechs setting up gene therapy studies in the UK and Europe

Chris Moore
Clinical Project Manager, Boyds

"Clinical professionals can support with the logistics ... ensuring the study is set up and executed smoothly, and any issues that arise are quickly resolved."

VIEWPOINT

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In this Viewpoint, Chris Moore, Clinical Project Manager at drug development consultancy Boyds, explains the key considerations for US biotechs when planning to initiate their gene therapy studies in the UK or Europe, and highlights some of the key regulatory differences.

INTRODUCTION

The UK and Europe are globally recognized as attractive locations for gene therapy trials, with the UK alone accounting for over 12% of global cell and gene therapy clinical trials. There are many reasons for this, including the strong regulatory framework and well-established infrastructure for conducting clinical trials. The UK and Europe also have large and diverse populations that can provide a broad range of patients for clinical trials, alongside a strong tradition of scientific research and a high standard of medical care, which can facilitate the conduct of high-quality gene therapy clinical trials.

Moreover, the UK and Europe are home to a number of key opinion leaders, as well as leading gene therapy research centers and academic institutions for conducting the research, which can play a key role in achieving study objectives. For these reasons, US biotechs are increasingly choosing to set up their gene therapy studies in the UK and Europe. But careful planning is key, and there are a number of important things organizations must consider before initiating their study.

STUDY SITE SET-UP

One of the initial challenges that US biotech companies will need to overcome is understanding the differences in study site set-up and initiation in the UK and Europe compared to the standard processes in the US.

Regulatory differences, cultural differences, logistical considerations, and language barriers must all be considered and prepared for in advance to ensure the most efficient site set-up and initiation process and to fully benefit from running clinical trials in the UK and Europe.

SITE SELECTION

The UK and Europe have a mix of academic, public, and private clinical research sites, which differs from the US, where private sites tend to be more common. There is a strong tradition of academic and public clinical research in the UK and Europe, with many large academic medical centers and public hospitals serving as important sites for clinical trials. These academic and public clinical research sites are often affiliated with universities or public health systems, and may have strong connections to government or non-government organizations that support clinical research.

During the site selection process, it is important to consider that academic and hospital sites in the UK and Europe are often used for a multitude of activities, such as patient care and teaching, meaning resources may be less readily available. As such, these sites may incur longer site set-up and initiation timelines, especially in comparison to private clinical research sites in the US.

SITE FEASIBILITY

During the site feasibility process for a gene therapy study, it is crucial to consider the current workload and capacity of the sites, as too much competition for site resources could hold up site set-up activities. The capacity available within the pharmacy department is also a key consideration to make, as the workload for the pharmacy teams is usually significant during the set-up of gene therapy trials, and if the site has multiple advanced therapy trials in set up, this could drain resources and be a rate-limiting step.

Choosing sites that are experienced in gene therapy studies can save a lot of time, especially for countries where additional local risk assessments or approvals are needed. Indeed, EU and UK sites that are required to complete additional submissions and documentation and don't have this experience will tend to require a lot of additional support from the Sponsor to complete these tasks.

The US FDA has issued many guidelines for gene therapy studies focused on product development, preclinical requirements, and trial design. It has also created guidelines

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around specific indications such as neurodegenerative diseases and rare diseases in adult and child populations. The EMA, however, has its own set of guidelines and although there are items where the guidelines are equivalent, it is best practice to identify where the EU guidelines differ prior to completion of a clinical trial authorization (CTA) submission.

KEY REGULATORY DIFFERENCES

Some EU countries also have separate regulatory bodies specifically for the review of advanced therapy medicinal products, such as gene therapies. For example, in Germany, one would submit a gene therapy product to the Paul-Ehrlich-Institut (PEI) rather than the Bundesinstitut fur Arzneimittel und Medizinprdukte (BfArM). It is therefore best practice to consult with individuals that have local knowledge of the regulatory landscape in the countries one wishes to submit to. Having access to knowledge about the new combined clinical trials regulation process in the UK and Europe can also be very valuable, saving both time and money by ensuring key requirements in the EU and UK aren't missed.

In addition to receiving CTA approval from the country's regulatory body, gene therapy trials need to comply with the Genetically Modified Organisms (Contained Use) Regulations, and the appropriate agency must be notified. This process can either be a local or a national one. In the UK, the approval process is focused more locally. Firstly, the sites must be registered with the Health and Safety Executive to perform gene therapy trials and then, for each individual trial, a risk assessment must be completed at the site to ensure the safety of workers and the environment, specific to each project. In the EU, the process for complying with these regulations can differ depending on the member state, so it is important to understand what additional gene therapy approval is required in the specific European country. For example, in France, a GMO application form is completed and goes to the same regulatory body that approves the CTA. However, in Germany, the agency for the GMO Contained Use approval is separate from the CTA regulatory body.

ETHICS

It is essential that US biotech companies are familiar with any additional requirements for ethics committee meetings for gene therapy trials in the countries they are applying for. In the UK, for example, only specific Research Ethics Committees can review submissions for gene therapy products and so to save time, it could be beneficial to manually book the ethics committee meeting in advance of submission. Otherwise, once the submission is performed, it may take a considerable amount of time until the next committee meeting is available to review the gene therapy submission, which could delay the initial submission date.

CONTRACTING & BUDGETING

When the contracting and budgeting process begins, if the country or countries selected have an expected template to be used, it is advisable to use their template where possible. The wording and budget format will be familiar to the sites and so the review process is generally quicker. Whilst it is not mandatory to use the EU or UK Clinical Trial Agreements template or national templates, doing so can help to streamline the negotiation and contracting process and provide a consistent set of terms and conditions for clinical trials across multiple EU member states. For gene therapy studies, one can expect the greatest amount of variance in budgets for pharmacy set-up costs, as this can differ significantly between sites because they all have different facilities and processes in place.

When working with non-English speaking countries in Europe, it is not essential for all documents to be translated into the home country's language. However, it is important to know at an early stage which documents will require translation services. Generally,

any patient-facing document will need translating in the majority of countries in Europe. If required, it is recommended to work with a translation services company that has a medical and clinical research background to ensure medical terminology is translated appropriately. This is especially important for gene therapy trials, which can have complex scientific information that must be conveyed in a way that people with little scientific knowledge will understand. One should ensure that the translation explains the information in an understandable way and that it does not revert to more complex scientific language. Ethics committees in particular pay very close attention to the quality of these translations and will insist on re-translation of documents if required, which can increase costs and impact timelines.

SEEKING EXTERNAL SUPPORT

US companies that may not be familiar with the regulatory landscape in these regions can benefit from working with an external consultancy with strong local knowledge of the relevant requirements in both the UK and the broader EU region, and that is able to provide valuable insight and expertise on issues such as patient recruitment, cultural differences, and healthcare system dynamics. This helps to ensure that the study is designed and delivered in a way that is relevant and acceptable to the local population.

US biotechs should look to identify a consultancy that has an established relationship with a network of UK and European investigators, as this will enable them to provide access and start engagements efficiently, accelerate the study timeline, and improve the quality of data collected. Clinical professionals can support with the logistics such as arranging site visits, managing transactions, and coordinating travel for study personnel, ensuring the study is set up and executed smoothly, and any issues that arise are quickly resolved.

BIOGRAPHY

CHRIS MOORE is Clinical Project Manager at drug development consultancy Boyds. Having successfully led more than 40 phase 1 and phase 2 trials in healthy volunteers and patients, Chris is involved in the day-to-day management of Boyds' clinical projects, from specific task management, such as site set-up and/or REC submissions, to full-service project management for phase 1–3 trials. He has wide-ranging experience across many therapeutic areas including respiratory, central nervous system and gastrointestinal products.

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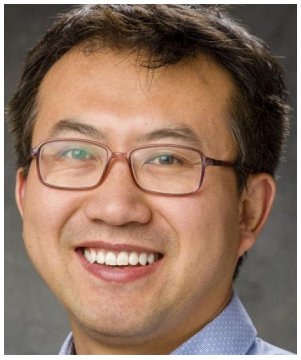
SPOTLIGHT

INTERVIEW

Exploring the burgeoning role of non-viral approaches in the delivery of nucleic acids



David McCall, Senior Editor at *Cell & Gene Therapy Insights*, engages in a conversation with **Zhenghong Gao,** Director, Head of Nonviral Delivery at Asklepios BioPharmaceutical, Inc. (AskBio), exploring the increasing prominence of non-viral delivery in genomic medicine. The discussion delves into the intricacies of incorporating lipid nanoparticles in therapeutic contexts and sheds light on the promises, complexities, and key considerations associated with DNA and RNA payloads.

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Q What are you working on right now?

ZG: My professional expertise lies in the realm of nanotechnology and its applications in biomedicine. Having previously worked in academia, my focus was on harnessing the potential of nanotechnology to address various biological and medical challenges. I am passionate about inventing and bringing new technology and medicine to patients.

Currently, I hold a leadership role at AskBio, where I lead the Nonviral Delivery division. Our primary objective is to lead a talented interdisciplinary team to create a sophisticated new delivery system for RNA and DNA payloads—a sort of nanometer-scale 'Starship Rocket' platform for shipping nucleic acids cargo. Through this platform, we facilitate the targeted distribution of genetic cargo to specific tissues or cell types, enabling the advancement of genomic medicines, including innovative therapeutic gene editing approaches.

Before my role at AskBio, I spent a quite long time at the University of Michigan, Ann Arbor, and the University of Texas Southwestern Medical Center, Dallas, where I worked with several world-renowned scientists and physicians on the translation of cutting-edge integrated molecular medicine tools for early cancer detection and treatment. This work covered a variety of stages spanning target identification, early discovery, preclinical development, and 'first-in-human' clinical trials. Through the years, I have accumulated a highly interdisciplinary scientific background and deep experience in several areas, including drug delivery, molecular imaging, liquid biopsy, and medical devices. As a scientist, I am always humbled to have the opportunity to work with pioneers in the fields. In particular, with all the support I have received from many excellent mentors, colleagues, and teammates, I have received chances to invent several integrated technologies for understanding drug diffusion in the brain extracellular space and improving drug delivery to the central nervous system. These technologies are currently being applied and validated in preclinical models for delivering both viral and non-viral vectors to enable therapeutic gene editing for neurodegenerative diseases and traumatic injuries in the central nervous system [1].

Q AskBio has long been a leader in the AAV vector-driven gene therapy field—can you tell us more about how, where, and why non-viral delivery is gaining a foothold at the company?

ZG: AAV stands out as an effective delivery platform, especially in specific tissues, with several US FDA-approved gene therapy drugs already available. However, despite its success, AAV has its limitations. Notably, patients previously exposed to this virus cannot be re-dosed with AAV. This poses a significant challenge, as immunogenicity from prior exposure can generate neutralizing antibodies against the virus.

The field is actively exploring non-viral delivery (NVD) approaches to overcome AAV's immunogenicity issues. One noteworthy NVD approach is the use of lipid nanoparticles (LNPs), which has demonstrated success in the development of the COVID-19 vaccines. While redosing through local intramuscular injections has proven feasible, systemic delivery presents more challenges, although progress is underway.

It is important to note that NVD approaches, such as LNPs, are not seen as direct competitors to AAV. Instead, they serve as complementary technologies, addressing the specific challenges of nucleic acids delivery.

Taking a deeper dive into the mechanisms, AAV efficiently delivers DNA cargo due to its natural ability to enter the cell nucleus and release DNA after uncoating. On the other hand, LNPs are known for their effectiveness in delivering RNA cargo, but they face challenges when

"It is important to note that non-viral delivery approaches, such as lipid nanoparticles, are not seen as direct competitors to AAV. Instead, they serve as complementary technologies, addressing the specific challenges of nucleic acids delivery."

delivering DNA due to the larger size of the LNP. LNPs can only reach the cytoplasm of cells, not the nucleus. To use LNPs for DNA delivery, the DNA sequence must be engineered to enhance its efficiency in entering the cell nucleus, and significant progress is being made in this direction as well.

Q How would you frame the opportunity of applying LNP technology in the gene therapy setting?

ZG: The landscape of gene therapy, particularly in the realm of *in vivo* gene editing, is a dynamic and emerging field, with LNP technology showcasing great potential. In this context, CRISPR/Cas 'molecular scissors' can be delivered together with guide RNA *in vivo* to create small 'surgical lesions' at the targeted DNA strand, facilitating the insertion of therapeutic genes into the targeted site in the genome. Notably, for this process, only transient expression of the molecular scissors is required. A prime example is the well-known *Streptococcus pyogenes* Cas9 (SpCas9).

When delivered with AAV, SpCas9 provides long-term expression of the molecular scissors in cells. However, this extended expression poses risks, as continuous activity may result in unintended DNA cleavage. On the other hand, delivering SpCas9 via an LNP in a messenger RNA (mRNA) format ensures short-term expression. Once the DNA is cleaved, allowing for gene insertion, the Cas protein will no longer be required, mitigating the risk associated with prolonged activity.

LNPs also find application in RNA therapy. While mRNA delivery, exemplified by the success of COVID-19 vaccines, is established, other RNA formats like circular RNA or short interfering RNA hold promise with LNP delivery post-optimization.

Researchers are actively exploring the delivery of gene editing via nanoparticles, despite the myriad of challenges. Recent advancements in this area are noteworthy, and the overarching goal is to enable high specificity, efficacy, and safety through non-viral delivery methods, opening new possibilities in the field of gene therapy.

Q Can you expand on the specific challenges in implementing LNPs in the therapeutic setting?

ZG: Navigating the challenges in gene therapy delivery is indeed intricate and context-dependent, particularly when considering specific organs or cell types. Notably, the success of local intramuscular injection, exemplified by the COVID-19 vaccine, contrasts with systemic applications where unmodified LNPs tend to preferentially target the liver, homing in on hepatocytes through ApoE-mediated targeting.

In the liver, the efficiency of LNP is evident even without additional targeting elements. However, to enhance specificity, technologies like N-Acetylgalactosamine are employed. N-Acetylgalactosamine, with asialoglycoprotein receptor as its target, which is highly expressed on hepatocytes, facilitates more precise targeted cargo delivery to liver hepatocytes. The advantage lies in the potential to reduce the dose without compromising effectiveness. Nevertheless, there is room for improvement, especially concerning the toxicity of certain ionizable lipids at the high doses required to reach the peak threshold in the liver.

Expanding beyond the liver poses a significant challenge, as the liver tends to act as a drug sink. Noteworthy progress has been made in the lungs, with various labs and companies employing diverse approaches to deliver therapeutics and target specific cell types. However, translation to non-human primates or humans necessitates additional validation.

The spleen, rich in immune cells, is another enticing target. Fine-tuning nanoparticle properties can enable precise targeting of immune cells in the spleen, as demonstrated in the literature [2]. Yet, translating these findings to non-human primates and humans remains to be established. While there are potential targets in other organs, the challenges increase. Special routes of administration or local injections may be necessary in these cases to achieve effective delivery. The quest for optimal gene delivery continues, with ongoing research addressing these intricate challenges.

Q What are the particular challenges and considerations in applying LNPs for DNA as opposed to RNA delivery, and how might they be addressed?

ZG: Foremost among the challenges is the inherent nature of DNA itself. In the realm of *in vivo* delivery, DNA has a historical association with triggering a robust activation of the innate immune response. This innate immune response is mediated through pathways such as the cyclic guanosine monophosphate–adenosine monophosphate synthase–stimulator of interferon genes signaling pathway, wherein cells possess mechanisms to sense foreign DNA. In addition, foreign DNA can also activate toll-like receptors—a class of pattern recognition receptors that initiate the innate immune response.

Addressing the challenge of delivering DNA to the nucleus is another significant hurdle. While nanoparticle delivery systems lack intrinsic capabilities for nuclear delivery, one approach involves engineering the DNA sequence. For instance, adding a nuclear localization signal can enhance the DNA's affinity for the nuclear pore complex to facilitate the transport. However, the therapeutic relevance of such modifications requires experimental validation.

Macrophage uptake of nanoparticles poses yet another obstacle. Upon systemic injection, certain macrophages identify nanoparticles as foreign entities and actively remove a substantial portion from the body. Ongoing research is dedicated to minimizing macrophage uptake, representing a critical area of exploration in the pursuit of effective *in vivo* DNA delivery.

Q How is the analytical toolkit developing with LNPs specifically in mind, and where is further innovation most needed in this area?

ZG: When encapsulating mRNA in LNPs, several considerations come into play. One critical factor is the stability of mRNA over time, particularly when nanoparticles carrying mRNA are exposed to the bloodstream *in vivo*. The assessment of mRNA stability is crucial to determine if it can maintain integrity long enough to reach the desired peak threshold. While tools exist for tracking the integrity of mRNA and nanoparticles both ex vivo and *in vitro*, the ability to monitor these dynamics *in vivo* within a real system remains a challenge. An analytical toolkit for characterizing the 'protein corona' of nanoparticles in a more precise and comprehensive manner is in high demand because this has an important impact on the tissue

and cell tropism *in vivo*. Continued efforts in this direction are essential to enhance our understanding of the *in vivo* fate of nanoparticles and mRNA.

DNA delivery poses even greater challenges. Current endeavors focus on the delivery of multiple cargoes, with the aim of packaging DNA and mRNA together in a single nanoparticle. The ongoing challenge is to establish an analytic toolkit for identifying the optimal ratio of mRNA to DNA within this packet. Currently, technologies are in the developmental phase, working towards achieving this intricate balance in cargo delivery.

Q How problematic are freedom to operate issues in the LNP space, and what is the best approach to addressing them for researchers seeking to work in the field?

ZG: Navigating the competitive landscape of the LNP space, particularly in the context of intellectual property, is indeed complex. A focus on developing new ionizable lipids presents a more accessible avenue within the realm of non-viral vectors.

Additionally, alternative vector options, such as polymeric nanoparticles and virus-like particles (VLPs), contribute to the diversity of choices. Each particle type brings its own set of advantages; for instance, VLPs merge the benefits of non-viral vectors with those of viral vectors, demonstrating advantages in brain and spinal cord delivery.

Beyond the traditional LNPs, the inclusion of polymeric nanoparticles, VLPs, and other vectors broadens the spectrum of approaches to address freedom to operate concerns. The diverse array of vectors within this space will be beneficial for diversifying the non-viral platform. Despite the notable successes achieved thus far, it's important to acknowledge that we are still in the early stages of exploring the vast potential of non-viral delivery systems. The dynamic and competitive nature of the field propels ongoing innovations and advancements.

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

ZG: My primary objective is to propel our liver program forward, leveraging LNPs. With this foundation, advancing this initiative is poised to be an impactful endeavor. Following closely as the second priority is the strategic expansion beyond the liver. This will help us venture into uncharted territories, surpassing the scope of commonly targeted organs. This dual-focus strategy reflects our commitment to pushing the boundaries of non-viral delivery and charting new frontiers in the field. We have a commitment to having non-viral delivery be a pivotal part of the AskBio toolbox serving alongside our AAV platform.

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CELL & GENE THERAPY INSIGHTS

CLINICAL TRENDS OF 2023/
TOOLS OF TOMORROW

SPOTLIGHT

INTERVIEW

Tracking current and predicting future cell therapy analytical toolkit evolution

With complex cell therapy technologies and modalities nearing the clinic—and in several notable cases, the marketplace—the industry's focus remains firmly fixed on delivering an analytical toolkit capable of adequately characterizing these novel products. Here, **Namyong Kim**, CEO of Curiox Biosystems, shares his thoughts on recent and potential future technological breakthroughs that can deliver the requisite improvements in speed, accuracy, consistency, and sensitivity.

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Q How is analytical tool innovation evolving to address the ever-increasing complexity of engineered cell therapy products?

NK: To start off, I would like to contextualize the significance of analytical tools in the realm of engineered cell therapy. It is important to note that unlike traditional therapies, which often rely on small molecules and proteins, engineered cell therapy involves the use of live organisms as therapeutic agents. The challenges associated with manufacturing these living therapeutic entities are unparalleled, thereby making the role of analytical tools indispensable for characterizing and controlling the quality of these engineered cells.

The bioanalytical landscape is experiencing rapid advancements, with numerous players introducing tailored solutions to meet the sector's stringent requirements for quality, speed, and spatial constraints. It is encouraging to witness many collaborations emerging among key stakeholders—from cell therapy developers and contract manufacturers to tool providers and regulatory bodies. This cross-industry collaboration is pivotal for expediting the development and deployment of effective, yet cost-efficient, cell therapy solutions for patients.

Q How would you characterize the state of 'cell sample processing', and its impact on analytical development in cell therapy? And what are the most pressing pain points in the current environment that aren't being addressed?

NK: I am really glad to speak on the subject of 'cell sample processing' as it is timely and warrants attention, especially given the fact that it contrasts with traditional bioanalytical sample preparation. Previously, the focus was largely on analyzers, as the handling of DNA and protein samples required a far lesser degree of sensitivity compared to live cells. The advent of cell therapy has compelled us to reconsider this perspective, placing new emphasis on how cell samples are processed. For instance, the current *modus operandi* for staining cells for analysis is still largely manual, which has several ramifications.

The typical staining process for cell products in flow cytometry involves repetitive centrifugation, supernatant aspiration, and buffer replenishment. Automating such processes demands considerable upfront investment, as well as maintenance costs and space, which have yet to be fully addressed in the industry.

The manual nature of 'cell sample processing' introduces issues of standardization, reproducibility, and accurate audit trails. Although the success of these metrics often hinges on highly skilled personnel, the industry is grappling with a talent shortage.

Q How does Curiox Biosystems address the existing and developing needs of the cell and gene therapy space with its current and future product pipeline?

NK: Curiox Biosystems is acutely aware of the current challenges facing the cell and gene therapy sector, particularly those related to standardization, reproducibility, and auditing. Our approach to resolving these challenges is simple and straightforward—automation. Automated systems inherently provide the consistency and standardization needed for reproducible results, and they ensure meticulous record-keeping for audit trails.

What sets Curiox apart is our focus on revolutionizing the method of automation itself. We have identified that traditional centrifugation-based automation fails to adequately address concerns around cost, spatial limitations, and technical expertise. Consequently, we have pioneered a paradigm shift in the form of Laminar Wash™ technology. This approach eliminates the need for centrifugation altogether, relying instead on Laminar Flow-based buffer exchange, where cells are retained in a well while the assay solution is diluted with a fresh wash buffer.

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> "...we are creating automated solutions that are even more cost-effective, compact, and user-friendly, thereby staying ahead of the evolving needs of the cell and gene therapy industry."

This streamlined methodology simplifies the instrumentation involved and accelerates the processing time.

With Laminar Wash products receiving fantastic feedback from the market, we continue innovating the automation of cell sample processing. At Curiox, we are creating automated solutions that are even more cost-effective, compact, and user-friendly, thereby staying ahead of the evolving needs of the cell and gene therapy industry.

Q What are end users reporting to you about working with Laminar Wash? What are you hearing from them in terms of the key considerations and benefits around Laminar Wash's deployment versus the alternative available technologies?

NK: The feedback we have received from our customers highlights two major advantages of Laminar Wash technology: exceptional data quality and streamlined workflows. There's a palpable sense of relief among users, who appreciate having an alternative to the decades-old, centrifuge-based processes that have long been the only option for cell analysis via flow cytometry. Centrifugation not only subjects cells to stress, thereby introducing variations, but it also complicates the path to automation. With Laminar Wash products, scientists now have an avenue to work around these issues.

What we have heard is that our technology offers a much simpler approach to automation. It eliminates the complexities associated with centrifuge-based workflows, offering a more straightforward, cost-effective path to automating the cell staining process. This leads to cleaner data sets and easier-to-manage procedures, which are crucial considerations for our clients.

Q What are the key next steps towards ensuring that novel cell therapy analytical tools and technologies deliver the required degrees of speed, reproducibility, and precision moving forward? How is Curiox responding to the needs of these customers?

NK: It is still rather early for us to talk about the next steps as we are only in the initial phases of automating and standardizing current manual workflows. Nonetheless, we remain forward-thinking in developing solutions that address the existing and future challenges in bioanalytics.

The immediate next step is the development of a unified automated workstation for cell analysis, deployable throughout the entire cell therapy commercialization pipeline. By this,

I am referring to a singular platform that can be employed across various stages—from early R&D and process development to in-process control and final quality control of manufacturing. This will greatly simplify the transition between different departments and stages, saving both time and resources by eliminating the need for multiple validations and standardizations. Curiox is actively developing such a flexible automated workstation, utilizing a consistent set of reagents and platform technology to cater to these diverse needs.

Additionally, we are in the process of developing a unified workstation capable of automating the full spectrum of bioanalytical assays necessary for cell therapy commercialization. Given that the development of cell therapies involves a wide range of bioassays to ensure product efficacy and safety, a single workstation capable of performing all these assays would be a significant value addition. Leveraging our expertise in automating cell analysis, we are configuring the workstation to perform a comprehensive range of bioassays, thereby serving as a one-stop solution for our clients.

Q Finally, can you pick out one or two specific projects/innovations that Curiox Biosystems is working on that we can expect to see coming through in the foreseeable future?

NK: Certainly. In the short term, we are enhancing the functionality and user-friendliness of our existing Laminar Wash HT and AUTO stations. We are adding automatic maintenance features that will dramatically reduce manual intervention required for the maintenance. Additionally, we are introducing smaller throughput heads for our HT stations to provide users with greater flexibility. With this add-on, users will no longer be restricted to using all 96 channels, and instead have the option to run only 24 or 48 wells, depending on their specific needs.

For the mid- to long-term, we are in the process of developing a flexible and configurable automated workstation that will span the entire cell therapy commercialization pipeline—from early R&D to final quality control assays in manufacturing. This new workstation will utilize a technology that is distinct from our existing Laminar Wash technology and will offer a wide range of throughput options, from single-sample to more than 96 samples.

Our aim is to spearhead the push for global standardization in cell analysis. Curiox's innovative automation solutions will play a key role in achieving reproducible and traceable cell analysis, regardless of the laboratory's geographic location.

BIOGRAPHY

NAMYONG KIM has more than 15 years of experience in scientific research and technical management in the development of biotech instrumentation. He received his PhD in Chemistry from the Massachusetts Institute of Technology (MIT), and his BSc from the Korea Advanced Institute of Science and Technology (KAIST). He holds more than 10 patents and patent applications, and has published a number of scientific publications in peer-reviewed journals. DropArray™ technology was developed by a research group led by Dr Namyong Kim at the Institute of Bioengineering and Nanotechnology (IBN). The group subsequently spun out of the IBN in 2008 and formed Curiox Biosystems, backed primarily by Nanostart AG, a renowned venture capital firm in Germany; and Exploit Technologies, the commercializing arm of A*STAR. The investment of various private equity firms led to Curiox's technology

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and global footprint expanding, with Laminar Wash™ technology at the forefront. Curiox Biosystems was listed in the Korean Stock Market on August 3, 2023, marking the start of the new era for the company and its technologies that will impact the standards of cell sample prep globally.

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CELL & GENE THERAPY INSIGHTS

CLINICAL TRENDS OF 2023/
TOOLS OF TOMORROW

SPOTLIGHT

EXPERT INSIGHT

A straightforward tool for developing a raw material supply strategy for cell & gene therapies

Klara Kulenkampff, Reto Eigenmann, Daniela Karlstetter, Claudia Angenendt, Rene Sielmann, and Scott Probst

Raw materials used in manufacturing processes for cell and gene therapies may carry a supply risk due to the unavailability of GMP-grade material, unique biological and chemical components, or limited supply options. This Expert Insight article outlines a tool that allows an objective and straightforward assessment of supply risk for raw materials and identifies feasible options for mitigating such risk.

Cell & Gene Therapy Insights 2023; 9(11), 1539–1543

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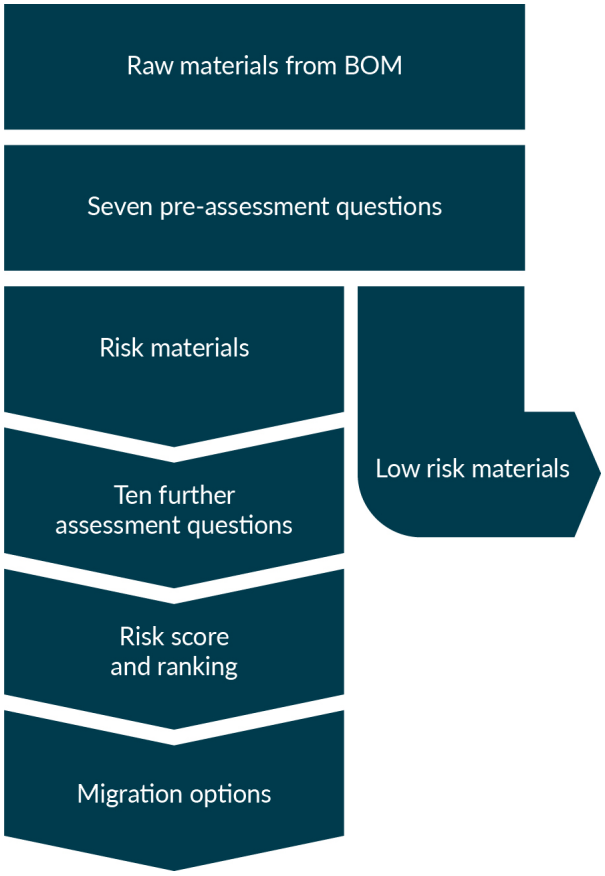
BACKGROUND

Reliable procurement of raw materials poses a challenge for cell and gene therapy development and manufacturing. The manufacturing process commonly requires a wide variety of materials, raw material suppliers may not be familiar with cGMP manufacturing, and second or tertiary supplier sources are rare. Materials often are biological, human or animal-derived, and can present a risk of lot-to-lot variability. Nevertheless, in preparation for launch and commercial production, the regulator requires companies to establish a

robust raw material supply strategy to secure the supply of therapies to patients—even in the case of expedited drug development pathways [1–3]. Because changes in the manufacturing process are difficult to adapt as clinical development progresses, this strategy should be developed early in development. Part of the strategy development is to evaluate materials and manufacturers in terms of their supply risk and, if required, mitigate that risk [4]. Procurement and supply chain teams and adjacent teams such as process development or quality control face the challenge of prioritizing their work with a substantial number of raw materials, potentially across several parallel programs. They require a framework to identify risk materials and define risk mitigation options in an efficient manner. Based on three previously published articles [5–7] we developed a framework that serves as a straightforward tool to evaluate raw materials, including supplier risk scoring, ranking, and proposing mitigation options for the materials.

FIGURE 1

The process flow for the supply risk assessment tool.



BOM: Bill of materials.

CASE EVALUATION

As a first step, we defined the requirements for the tool. The desire was to develop a straightforward and quick-to-use tool that reliably identifies high supply risk materials and ranks them. The tool also had to be unbiased and consistently yield identical results among different users.

The BioPhorum article mentioned above lists different questions to assess supplier risk and thus provided the basis for a questionnaire used in our evaluation [7]. We identified and adapted the relevant questions and introduced a two-step approach to reduce the time required for completing the assessment (Figure 1). In the first step, we wanted to shortlist materials from the bill of materials which carry a minimal risk based on seven pre-assessment questions. We modified the questions to offer binary 'yes' or 'no' response choices, with 'no' suggesting a potential risk. Once one of these seven questions is answered with 'no', the material is flagged as requiring further assessment and ten more 'further assessment' questions were answered to rank the material according to its risk status. Based on the answers given to each of the questions, a score was calculated. Each question also holds a weighting dependent on the impact on risk (Table 1 & Table 2). We have selected a higher weight factor for pre-assessment compared to further assessment questions. The weight factor corresponds to the impact of this question on supply disruption. Thus, each material receives a risk score, and is ranked among all materials.

In the final step, one or several of the following recommendations for mitigation may be given based on the 17 answers provided throughout the assessment (Table 3):

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- Improve supplier relationship, increase governance, or establish a contract with the supplier
- Qualify second source
- Hold increased safety stock
- Build up in-house production capabilities

We identified these four mitigation options as the most common to reduce supply risk in our organization, but variations or other options may exist.

CONCLUSION

In this article, we present an easy-to-use tool, which uses a questionnaire format to calculate supply risk scores for raw materials used in cell and gene therapy manufacturing. The tool also suggests risk mitigation options for high supply risk materials. Using this tool, we evaluated all raw materials used in various programs across our cell and gene therapy platform and thus identified a supplier risk for 30–50% of materials. It is important to highlight that both risk evaluation and recommendations for mitigation rely

TABLE 1

Pre-assessment questions.

	Question	Answer options	Weighting
1.1	Can a risk assessment approach be used to introduce material from a different manufacturer?	Yes/No	10
1.2	Do other supplier(s) manufacture the material (e.g., not sole-source)?	Yes/No	10
1.3	Is GMP readiness of the supplier for this product given?	Yes/No	10
1.4	Is the supplier/manufacturing site located in a low-risk geopolitical region?	Yes/No	10
1.5	Is there a low chance that the supplier may face capacity issues?	Yes/No	10
1.6	Has the supplier been reliable and have there never been major supply issues (e.g., OTIF, complaint rate) in the past?	Yes/No	10
1.7	Is the risk of IP restrictions low?	Yes/No	10

TABLE 2

Further assessment questions.

	Question	Answer options	Weighting
2.1	Does the supplier adhere to certified or regulated quality system standards (e.g., ISO, IPEC, GMP, etc.)?	Yes/No/Unknown	5
2.2	Is the supplier qualified at your company?	Yes/No/Unknown	10
2.3	What is the insolvency risk for the supplier?	Low/ Medium/High/Unknown	10
2.4	How large is the company in terms of sales volume (in USD)?	<5 million/year <50 million/year <500 million/year >500 million/year Unknown	5
2.5	How long is the estimated lead time?	<1 month 1–6 months >6 months Unknown	10
2.6	What shelf life does the material have?	Number of years Unknown	10
2.7	Is the supplier established or new in the market?	Established/New/Unknown	10
2.8	Is your company considered as an important customer?	Yes/No/Unknown	5
2.9	Is the RM custom manufactured on behalf of your company?	Yes/No/Unknown	5
2.10	Is the full supply chain visible?	Yes/No/Unknown	5

TABLE 3

Recommended mitigation options based on answers obtained from the questionnaire.

Recommended mitigation option for further evaluation	Driver/corresponding question
Improve supplier relationship, increase governance or establish contract with supplier	ONE of the following applies: Material cannot be replaced per risk assessment (1.1) GMP readiness is not given (1.3) High risk of capacity issues (1.5) Supplier/manufacturer has been unreliable in the past (1.6) High risk of IP restrictions (1.7) Insolvency risk is high (2.3) Supplier/manufacturer is small company (2.4) Your company is not an important customer (2.8)
Qualify second source	The following applies: Second source is available (1.2)
Hold increased safety stock	The following applies Long lead times (2.5) Long shelf life (2.6)
Build up inhouse production capabilities	The following applies: No second source available (1.2) Supplier/manufacturer is small company (2.4) Low risk of IP restrictions (1.7)

on a simplified model and the outcome may require further evaluation. Teams can, however, prioritize and focus on raw materials ranked as high risk. The risk score can also serve as a key performance index to measure and track supply security defined by mitigation measures. Learnings from one program can subsequently be applied to other drug candidates, reducing risk from early development. Given that challenges often vary for each material along with their mitigation solutions, these options should be further assessed on a case-by-case basis for each specific material.

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CELL & GENE THERAPY INSIGHTS

CLINICAL TRENDS OF 2023/
TOOLS OF TOMORROW

SPOTLIGHT

VIEWPOINT

Enabling cell culture technologies for the intensification & scale-up of viral vector manufacturing

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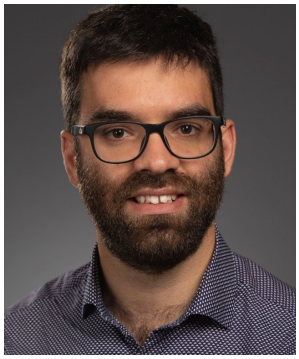

"For as long as the pressure to reduce the cost of goods for gene therapy manufacturing persists, novel cell culture platforms and continuous improvement in the production of AAV and other relevant viral vectors will be needed."

VIEWPOINT

Cell & Gene Therapy Insights 2023; 9(11), 1441–1445

DOI: 10.18609/cgti.2023.187

Recently approved AAV-based gene therapies, as well as positive ongoing clinical trials for new treatments, have raised research interest and stimulated investments. However, these life-changing therapies for the treatment of rare monogenic diseases create challenges regarding affordability for the patients and the healthcare system [1]. As gene therapies are progressing, future treatments might include much more prevalent diseases and/or higher dosage. This will in turn put more pressure on the current manufacturing technologies to improve cost-effectiveness and accessibility, mostly though improving bioprocesses and scale-up technologies. The approach taken by scientists at the National Research Council Canada is to focus on developing QbD biomanufacturing platforms to offset the current bottlenecks for the next-generation of AAV-based therapies. Current platforms are generally based on triple transfection using HEK293 cells. These platforms have limited scalability but can still support the production of clinical material-grade vector in sufficient quantities for the current market needs. However, considering the potential future gene therapies, what would be the bottlenecks to overcome from a cell culture point of view to bolster productivity and manufacturability? This Viewpoint explores some possible approaches that are being explored in order to take viral vector manufacturing to the next step.

PRODUCTION SCALE-UP

While early approved gene therapies often relied on 2D cell culture to produce AAV, the scalability and manufacturability of this type of platform is limited. Cell expansion for adherent cells is time-consuming and labour-intensive, especially when using either cell factories or cell stacks. Fixed-bed reactors for large scale production, such as the iCellis or equivalent, do offer an interesting alternative for a more scalable approach to manufacturing using adherent cells. They offer a large surface area for cell growth (up to 500 m^2) which is sufficient for many gene therapy applications, especially in early-phase clinical trials. Nonetheless, there are obvious limitations in growing cells in 2D related to surface/volume ratio that might be difficult to overcome before scaling up cell culture in fixed-bed bioreactors even further. One option is to scale-out production, resulting in several fixed-bed reactors operating in parallel and pooled together for downstream purification. This approach is often used for large-scale production in single-use bioreactors.

As an alternative, suspension cells grown in stirred tank reactors can be seamlessly scaled up to 2,000–5000 L single-use bioreactors, and even further using the more traditional stainless-steel vessels. The bioprocessing industry has already acquired decades of experience in scaling up cell culture in this format, mainly with CHO cells. It is not only the case that the challenges of scaling up are now better understood, but cell culture can also be done at much higher density that what is often used currently for viral vector production (i.e. over 1×10^8 cells/mL, and even 1×10^9 cells/mL, for fed-batch or perfusion-based processes). While the cell line used for AAV production and the mode of production (infection, transfection) might pose additional challenges, current scale-up knowledge, such as how to deal with oxygenation and CO_2 accumulation issues, may be translated to produce viral vectors in single-use bioreactors.

STABLE EXPRESSION OF THE AAV VECTORS

While cells growing in suspension are fundamentally scalable, the transfection step commonly used for AAV production is more challenging. Polyethylenimine is a widely used transfection agent for AAV production via triple transfection. Polyethylenimine binds to DNA and forms a complex that can transfect the cells with relative efficacy. However, the preparation of the complex is difficult as it is both time- and shear-sensitive. While transfection with these reagents is routine work for small-scale production,

the handling of this critical step is much more complex for large-scale vessels [2]. It is still possible to execute this step at scales of 500–1,000 L and beyond, but it requires the CDMOs involved to develop the appropriate know-how for this type of process to obtain consistent results. The complexity of the transfection at scale have led to the development of next-generation transfection reagent [3], which should be more stable and thus easier to handle. Nevertheless, many issues can arise while the formation of the transfection complex is scaled up: longer transfer time for the reagents, less control of the mixing of the transfection complex, and longer mixing time once the complex is transferred to the bioreactor. While only a small subset of transfected cells produces full capsids with the current technologies available, the intrinsic challenges of transfection can further compromise the transfection efficiency and lead to inconsistencies between batches, and between different bioreactor scales and production sites.

In order to produce functioning AAV, all three plasmids must be successfully transfected, which often results in low effective transfection efficacy and difficult capsid assembly of the AAV [4]. The transfection efficacy limits the cell density at which transfection is effective, often between 1×10^6 to 5×10^6 cells/mL. These values are much lower than the potential cell density that can be achieved in HEK293 cell culture. Despite increasing specific titers (i.e., productivity on a per cell basis) volumetric titers are difficult to improve due to maximal cell density achievable. In comparison, recombinant protein manufacturing has moved from transfection to stable producer in order to achieve consistent, reproducible high titers at high cell density. The generation of virus-free stable producer for viral vector production is more complex than for monoclonal antibodies because of the cytotoxic nature of some viral vector proteins. Packaging cells and stable producers have been generated by placing the cytotoxic protein under the control of inducible promoters. Stable cell lines using cumate, coumermycin or doxycycline gene-switches are becoming increasingly available for lentiviral vector [5] and AAV production [6]. The generation of a stable producer cell line is a long-term and costly investment that delays timelines to clinical trials—it can be hard to justify for early product development and early clinical phases given that triple transfection yields enough material. On the other hand, packaging cell lines for the rapid production of serotype-specific vectors can help improve transfection-based processes since only the plasmid encoding the gene of interest (cargo) is needed. Nonetheless, for therapeutic targets requiring large production capacities, the use of a stable producer cell line is a worthwhile investment. The removal of the transfection step also eliminates the need for costly plasmid as a process entrant. The removal of plasmid from the process also facilitates the logistics of scaling up. In addition, the use of stable producers instead of transfection opens opportunities for developing new and improved processes.

PRODUCTION INTENSIFICATION

To increase productivity of large-scale manufacturing, the titer, scale, and number of batches per year can be improved. While the viral vector production itself spans 2–3 days of the whole process, expanding the cells to generate the inoculum (seed train) can take several weeks. Production campaigns could be shortened, either via the use of high-density cell banking or a perfusion bioreactor as part of the seed train. N-1 perfusion can also be used to for high cell density seed for the production bioreactor, and perfusion can be integrated to the process in cell growth phase to create favorable conditions for high-cell density transfection or infection.

Another alternative is the transition from batch-base production to continuous bioprocessing. This transition would be made possible with stable producer cell lines. Recombinant lentiviral vectors are a perfect

candidate for this type of production because they are cytolitic and thus easy to harvest in semi-continuous modes. Since they are labile, they also benefit from this type of process because of the shorter delay between production and purification. Recombinant AAV is both highly stable and tends to remain in large proportions in the cell cytoplasm after its production. Nonetheless, semi-continuous AAV harvesting could still be a viable option for specific AAV serotypes and constructs that tend to be more secreted. This can be implemented in a relatively simple manner for HEK293 cells using single-use upstream process equipment [7]. For others, the strategy could either be to concentrate both cells and AAV using perfusion in the bioreactor, or even to integrate perfusion and cell harvest with clarification [8]. Overall, upstream process equipment and methods for perfusion exist and are already well-adapted for production of biologics in suspension cells at the commercial scale. In some cases, perfusion and process intensification might be compelling options to improve volumetric productivity while requiring only a small footprint for upstream processing. The challenge is to adapt these approaches to the specificities of viral vector production, including the cytotoxicity of the product and the integration of cell lysis and clarification into the process.

FUTURE OUTLOOK

For as long as the pressure to reduce the cost of goods for gene therapy manufacturing persists, novel cell culture platforms and continuous improvement in the production of AAV and other relevant viral vectors will be needed. Therapies for rare diseases and low-dosage modalities will benefit from more efficient upstream platform processes using transient transfection with one or three plasmids to reduce development costs and accelerate time to clinic. These platforms will rely on robust and predictable generic process parameters. However, as some therapies will target more common diseases and higher dose modalities, the emphasis will be on reducing the overall manufacturing costs per batch for viral vector production. From an upstream perspective, the transition to suspension-based platform combined with the use of stable producer cell lines and intensified processes supported by broader and improved in-line and at-line analytics, will accelerate improvements in cell culture process productivity and product quality.

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BIOGRAPHY

JULIEN ROBITAILLE is currently a Research Council Officer at the Human Health Therapeutics Research Center of the National Research Council Canada (NRC) in Montreal. His research is focused on the scale up and optimization of suspension cell culture in bioreactors. He has led and contributed to the process development, scale up and technology transfer of the upstream production process of many different therapeutic products, including recombinant proteins and viral vector for cell and gene therapy applications. He received his BSc in Chemical Engineering from Polytechnique Montréal, and completed his MSc degree at Polytechnique Montréal in Chemical Engineering also, focusing his research on the metabolomics of cell culture in bioreactors.

AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

CLINICAL TRENDS OF 2023/
TOOLS OF TOMORROW

SPOTLIGHT

INTERVIEW

Looking to a future of multiplex cell engineering

'Multiplexing' has been one of the most prominent buzzwords of 2023 in the advanced therapies space. **David McCall,** Senior Editor, *Cell & Gene Therapy Insights*, speaks to **Marco Alessandrini,** CEO, Antion Biosciences about recent progress with and future targets for tunable multiplex cell engineering platforms.

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DOI: 10.18609/cgti.2023.199

Q As 2023 draws to a close, how would you sum up the year for cell and gene therapy?

MA: Without question, the field has been maturing exceptionally over the last year, with more approvals coming through. The challenges related to novel technologies are becoming clearer, and although there might be some ambiguity around the safety and efficacy of these newer approaches, there is a lot of excitement in the field as we anticipate more patients being cured of diseases that could not be treated otherwise.

Editing technologies, including base, prime, and epigenetic editing, are all evolving in impressive ways. There is a high degree of interest in the space, perhaps increasingly in *in vivo* gene therapy rather than *ex vivo* modified cell therapy solutions. Although there are challenges across the board, there will be wonderful options for patients down the line.

Arguably, cell and gene therapy is currently not living up to its full promise, but with the dedication that we see right now from developers in both the academic and private sectors, there is a tremendous driving force and scope for the application of these therapies. I see no reason why the promise of gene therapy will not be realized with time. For me, both cell therapy and gene therapy are here to stay. I am happy to see that Antion's technology can compete in that space, existing not only as a mere add-on to the field, but also as a potential disruptor to the industry. We feel committed to this journey to see it happen.

Q Can you tell us more about Antion—it's approach, platform, and R&D pipeline—and the progress you have made in 2023?

MA: Antion Biosciences is a spin-off from the University of Geneva. We officially spun out with investment from angel investors in 2019 and then managed to secure a partnership with Allogene Therapeutics. They are invested strategically in the company, with Antion also developing novel gene constructs for Allogene's next generation of allogeneic CAR-T cell products. As a platform technology company that recently stepped out of a university, it has been powerful for us to have such pioneers already looking into the use of our technology. Soon afterwards, we also struck a very exciting collaboration with the Centre for Cellular Immunotherapies at the University of Pennsylvania.

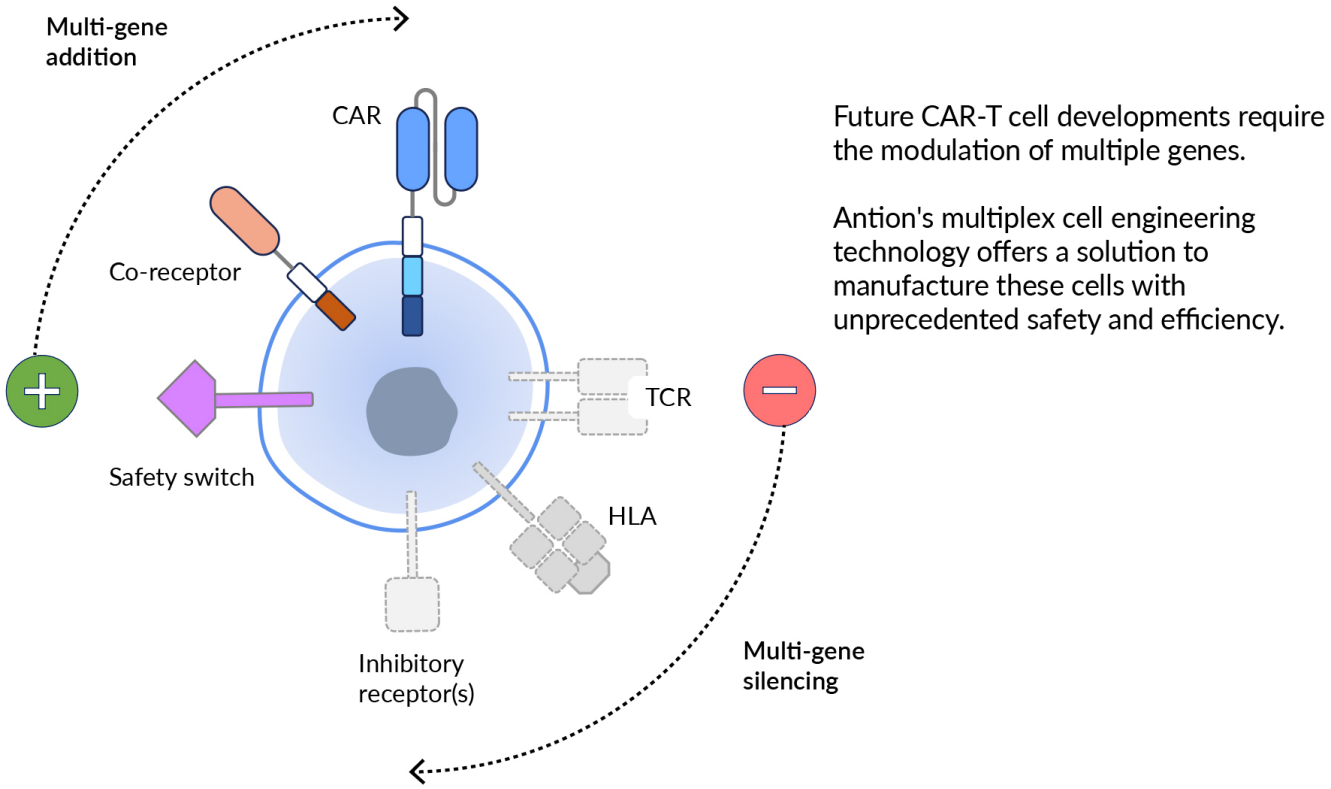
Our technology centers on the ability to multiplex engineer in a modular and tunable fashion. We believe that making cell and gene therapy treatments widely accessible, safer, and more efficacious is primarily an engineering challenge that can only be overcome with high-end technologies that are inherently flexible by design. Our technology has the ability to add genes like a CAR or additional molecules, whilst at the same time—and in the same gene construct—decreasing the expression of others. In other words, both an additive and a subtractive effect, or even multiple additions or subtractions, can be achieved from a single gene construct **(Figure 1)**.

All of the approved therapies on the market are CAR-only therapies engineered with only the CAR molecule. Our technology is a simple bolt-on to this in order to deliver the multiplex capability, not having to alter in any way the manufacturing process. Other approaches generally require the use of two technologies to first add the CAR, and then in a secondary step, to edit out key molecules for the multiplex effect. From a safety point of view, the way we deliver multiplex gene constructs is surprisingly simple and the efficiency of being able to modify many molecules at the same time is very promising. We recently presented work showing we can functionally silence the expression of six genes, in addition to co-expressing two genes at the same time—a CAR and a safety switch. It is a modular approach and by adding different components, we can dial up or dial down the expression of relevant molecules.

The tunability is another advantage. Certain molecules, such as human leukocyte antigen (HLA)-I, benefit from the tuning down of expression. The tuned-down effect might be more efficient than a complete knockout of HLA-I expression when it comes to host rejection of allogeneic cell therapies. With the exception of epigenetic editing technologies, no other editing technology can achieve similar. While HLA-I knockout very efficiently protects allogeneic cells from rejection by host T cells, it renders the cells prone to rampant rejection by host natural killer cells. It is therefore an absolute necessity to co-express natural killer inhibitory receptors, such as non-classical HLA-I fusion proteins. While this offers adequate

FIGURE 1

Next generation CAR T-cell with multiplex gene modulation.



Future CAR-T cell developments require the modulation of multiple genes to achieve prerequisite safety standards and optimize treatment efficacy. The challenge of cell and gene engineering strategies is thus to accommodate contrasting modalities—on the one hand, multi-gene addition to over-express molecules, and on the other to silence the expression of multiple genes (subtractive effect). CAR: chimeric antigen receptor; TCR: T cell receptor; HLA: human leukocyte antigen.

protection, it comes with greater engineering complexity and an increasing genetic payload that compromises gene delivery and manufacturing efficiencies.

From a pipeline point of view, we are now looking to fulfill an unmet need on the clinical side. We have carefully selected our lead indication, which is a T cell malignancy. CD19 CARs are working efficiently for B-cell leukemias and lymphomas and B-cell maturation antigen CARs are working well for myeloma, but the most aggressive forms of leukemia and lymphoma are of T cell origin. It is a small subset of patients but given the current dearth of available treatments, we think CAR-T could be the most efficient way to treat these patients.

From a development point of view, we believe multiplex engineering is essential in this context, and therefore our technology also addresses an unmet engineering need where other technologies suffer poor yields and safety concerns. Antion technology allows the adding of a CAR and a safety switch molecule, in addition to silencing numerous receptors for added therapeutic benefit. We target a molecule called cluster of differentiation 7 (CD7), present on all T cells, which is highly expressed in leukemia and lymphoma patients of T cell origin. Firstly, we silence the expression of CD7 on the T cells and have good data to show that this is essential to prevent fratricide, rendering a higher product yield and healthier CAR-T cell populations. Since there are obvious issues in harvesting malignant T cells from a patient for autologous administration, it is preferable to pursue an allogeneic off-the-shelf product

derived from healthy donors. Therefore, secondly, we need to silence the expression of T cell receptor to facilitate allogeneic treatment. But we also want to make these cells persistent and in the context of malignant T cell populations, greater rejection of the product may be seen as the T cells are present. So, thirdly, we also look to silence HLA molecules as a means of improving the persistence of these cells. Most notably, we are addressing these three arms from a single gene construct.

Q Can you expand on the benefits that multiplex cell engineering approaches bring to the field as a whole?

MA: Being able to multiplex engineer cells and tune them to increase efficiency is the future of engineered cell therapies. The CAR-only therapies are good, but they could be more efficient. If we want to break into a larger space in the industry, we need to address solid tumors. Current treatments are only for hematological malignancies, which covers only about 10% of the total cancer patient population.

We also want to make these treatments off-the-shelf and widely accessible globally, which is an important engineering challenge. Our multiplex approach is efficient in terms of adding and subtracting gene expression in a single construct. We can take the way that the current CAR-Ts are produced and manufactured and simply add our technology to deliver these additional benefits—it is an elegant solution and obviates the need to alter the already well-established gene delivery methods in any way whatsoever.

Modularity in multiplexing is certainly beneficial. As soon as we define a module that works well to silence a molecule, we can add it, remove it, or tune down the expression in our gene constructs. We can create a window of silencing—for example, of 50%, 75%, or even 95% silencing. We have recently shown that functional silencing can be achieved across the entire range. This extra level of tunability, as opposed to basic 'on/off' silencing (knockout), can be beneficial for certain targets.

Q What are the key considerations and challenges for Antion as you prepare to translate into first-in-human trials?

MA: A key challenge is expanding our internal expertise and understanding of what it takes to translate an R&D program into a clinical program. Fortunately, there are many seasoned professionals out there who can support us with this. The key remaining challenges are mainly from a CMC and process development point of view. Upscaling the production of our CAR-Ts is the most significant challenge for us. A natural expansion of the team is required to ensure our CMC and process development is fluent as we interact with regulators. We are preparing for this extensively.

"We want to make [solid tumor] treatments off-the-shelf and widely accessible globally, which is an important engineering challenge."

Q As you look ahead to 2024 and beyond, what will be some important next steps in technology evolution and application to look out for in the engineered cell therapy field?

MA: We are watching technologies mature, including our own, and many are showing exceptional efficiencies. These technologies are powerful in terms of what they can achieve, but the key now is translating them into products.

The editing space will continue to evolve, with gene editing becoming more efficient, along with novel technologies starting to feature more prominently, including base editing, prime editing, and excitingly, epigenetic editing. These are all different tools in the toolbox to help address unmet needs. Certain indications will be best addressed with certain technologies or combinations of technologies. Our technology is one piece of the puzzle, and we are excited to see how it can be applied, whether it be standalone or in combination with any of the editing technologies mentioned above.

The field also needs to consider improving the safety assessment of these technologies. These are all highly novel technologies, so we must ensure we can concretely assess the safety of the products derived from them in their engineering and delivery. Genotoxicity due to genomic re-arrangements and instability can have serious detrimental effects on the industry, which is something we most certainly need to avoid at all costs.

Q Can you sum up one or two key goals and priorities that you have for Antion over the course of 2024 and beyond?

MA: At Antion Biosciences, our main goal is to nail down our technology platform validation. We have already shown the ability to silence multiple molecules efficiently—validation involves creating the allogeneic off-the-shelf versions from an engineered T cell point of view. We are now comfortable in saying that our technology can be used to create allogeneic CAR-T cells with exceptional efficiency.

Another element is to improve their persistence, as I mentioned earlier. This involves preventing rejection, which can be done by downregulating both HLA-I and HLA-II molecules. I hope that in 2024, we will definitively nail down the anti-rejection capability of our technologies and engineer cells that are simultaneously 1) non-alloreactive for off-the-shelf administration, and 2) hypoimmunogenic for improved persistence by preventing rejection from the host immune system. Then, we can address the next most significant key area—improving cellular potency. There is a general drive towards improving the potency (or efficiency) of both autologous and allogeneic cell therapy products. There are many inhibitory receptors that typically regulate T cell functionality—for example, PD-1, TIGIT, and TGF-β receptor. However, there are many other exciting intracellular molecules that could improve the persistence and functionality of CAR-T cells, such as Regnase-1, BLIMP-1, and DNMT3A, just to mention a few. There is a wealth of targets for us to hit in the potency space, which we see as the next realm for us to explore.

The second key thing for us is to get our lead therapeutic candidate for T cell malignancy moving into and through the clinic. This will demonstrate the real benefit of our technology, and we are already seeing how we compete relative to other products. Then, to flesh out our

pipeline, we have one or two interesting solid tumor indications coming through, and we are also looking at an autoimmune disease as a potential target.

Even more broadly, we want to create universal donor cell therapies beyond CAR-T and engineered T cell therapies. Universal off-the-shelf therapies would mean that one day, our technology could play a key role in unlocking arguably the most significant challenge of the cell and gene therapy field—accessibility!

Last but not least, we are looking to leverage our platform technology. We are one of the few companies in this space that wholly owns a proprietary technology, so partnering it out will be a great opportunity for us. Other companies that are developing their own gene-engineered immunotherapies, universal donor cells, or *in vivo* gene therapies could benefit from adding our technology to fine-tune their own. We will actively partner with technology in different areas to complement and enable ongoing therapeutic developments.

BIOGRAPHY

MARCO ALESSANDRINI is the CEO and a member of the Board of Directors at Antion Biosciences, a Geneva-based cell and gene therapy company. He has over 15 years of experience in molecular medicine, and cell & gene therapy developments, and has led Antion's R&D projects since 2016. Prior to Antion, he worked in the Pharma and Biotech industries in South Africa, before returning to academia at the Universities of Pretoria and then Geneva where he started his collaboration with the scientific founders of Antion. Since then, his work has been focused on developing a next generation of universal CAR-T cell therapies using the multiplex gene silencing technology developed at the University. In 2018, Marco started his transition to Antion, and in 2023 he was appointed as CEO of the company.

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AUTHORSHIP & CONFLICT OF INTEREST

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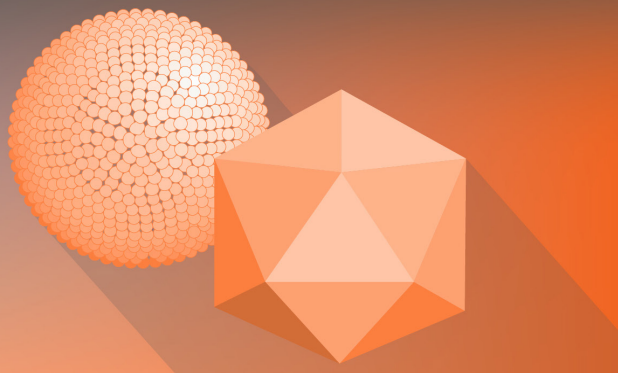
CELL & GENE THERAPY INSIGHTS

VECTOR CHANNEL EDITION

Characterization and Validation



DECEMBER 2023
Volume 9, Issue 11

WEBINAR DIGEST: Regulatory considerations and validation strategies for mycoplasma testing for cell-based therapies
Mike Brewer

CELL & GENE THERAPY INSIGHTS

CHARACTERIZATION & VALIDATION

INNOVATOR INSIGHT

Simplifying residual DNA quantitation and biotherapeutic manufacturing

Ilaria Scarfone

Meeting regulatory guidelines for any biologic depends on robust product characterization, and cell and gene therapies are no exception. The analytical data must prove the safety, efficacy, purity, quality, and potency of the final drug product. A key consideration for the product characterization process is choosing between an in-house developed assay solution or a commercial kit for quantitating residual DNA within a bioproduction workflow. This article will explore the challenges in the development of residual DNA assays and presents a comprehensive commercial solution to help save time and cost in the development process.

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CHALLENGES IN THE DEVELOPMENT & VALIDATION OF RESIDUAL DNA ANALYTICAL ASSAYS

Developing sensitive, robust assays requires expertise and an investment of both time and resources. Typically, residual host cell DNA assays are developed, validated and used as a quantitative test for impurities per ICH Q2 (R1) validation of analytical procedures. Multiple documents must be developed, including standard operating procedures (SOPs) for the test method, critical reagent preparation and qualification, equipment operation and maintenance, method development report, and validation protocols and reports. A key requirement is developing a robust protocol for purification, quantitation, qualification, and storage of quantitative standards must ensure

consistent, accurate performance from lot to lot. Reports for the validation execution are required along with the validation itself.

Quantitation of residual host cell DNA is performed both in development of the purification process and as a required step for lot release due to its potential impact on product quality, efficacy, and safety. To ensure patient safety, the amount of residual DNA contained in the final drug product must meet specific regulatory guidelines. Historically, these guidelines were straightforward with a defined limit of residual DNA per therapeutic dose. Today, the complexity of the regulatory landscape is much greater, with specific regulatory guidelines dependent on the product, process, and geographic region.

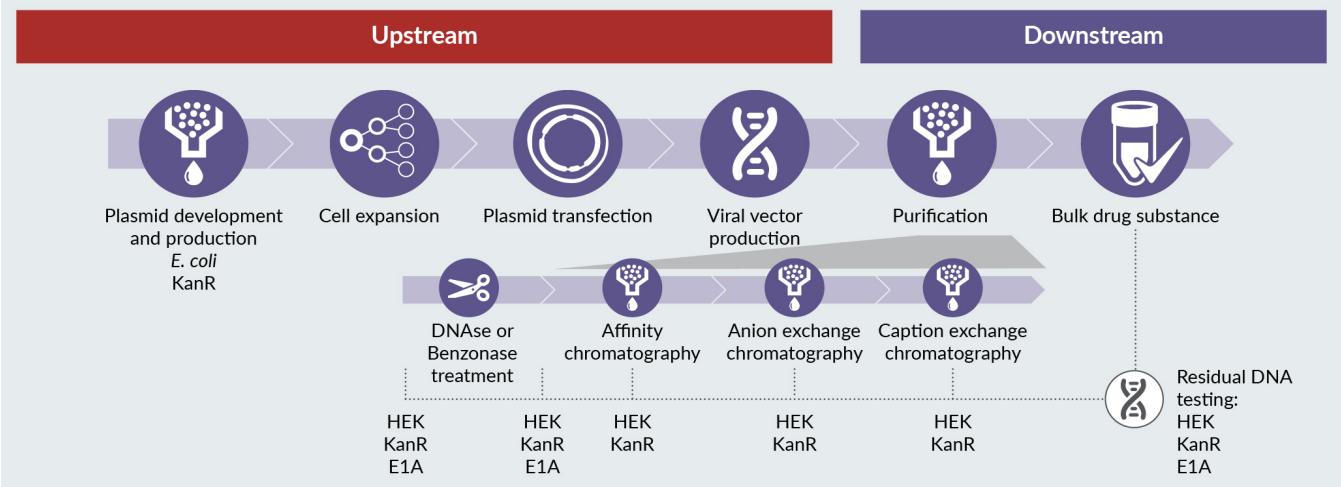
For example, within an AAV workflow, residual host cell DNA testing is performed downstream during the purification as an in-process test for DNA clearance, as well as at final fill-finish for lot release testing. Residual host cell and manufacturing vector DNA, especially in AAV processes, must be well characterized to demonstrate clearance below acceptable levels. Additionally, E1A, an oncogene present in HEK293 cells, that poses regulatory concerns, as the presence of an intact *E1A* gene in the purified product must be avoided. Thus, there is an expectation to take steps to ensure the size of genomic DNA is reduced to below approximately 200 base pairs (bp), and if *E1A* DNA is detected late in purification or in final dosage form, it must be demonstrated that the size is appropriately small. While the regulatory requirement is to demonstrate the residual DNA at lot release, many AAV vector manufacturers test throughout the purification process to ensure DNA clearance (Figure 1). If developing in-house solutions, multiple testing points should be qualified and validated for use with the testing method to ensure high percentage recovery and accurate quantitation at each step, in addition to the final lot release.

RESIDUAL DNA QUANTITATION: SELECTING AN IN-HOUSE OR COMMERCIAL SOLUTION

There are several considerations when selecting an in-house developed or a commercial solution for residual DNA quantitation. The first consideration is to establish whether the expertise and internal development capabilities are present in-house. Staff with specific expertise are required to develop this type of assay. Secondly, there are cost considerations: although commercial kits may seem expensive, there are hidden costs associated with developing an in-house assay. For example, if you develop an in-house solution, the process

FIGURE 1

Testing for residual DNA in the AAV manufacturing process workflow.



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from development through implementation is both resource and time-intensive, especially if recruitment of the requisite in-house expertise is required. Furthermore, if the developed assay proves to be insufficient in meeting regulatory guidelines, this can lead to increased costs and often more importantly, timelines to commercialize the drug product. Finally, the need for process-specific or sample-specific customization must be considered.

To develop an in-house assay, several steps must be taken (Figure 2). Firstly, a target sequence must be identified, so that specific primers and probes can be designed and optimized. Characterization and qualification of quantitative DNA controls/standards need to be performed. Critical reagents such as qPCR Master Mixes should evaluated, selected, and qualified, a sample preparation method that is effective for multiple test sample matrices and product concentrations must be selected or developed, and the overall method performance across the process must conform to regulatory requirements. Then, testing methods must be determined and the analytical testing method must be validated according to specific regulatory guidance.

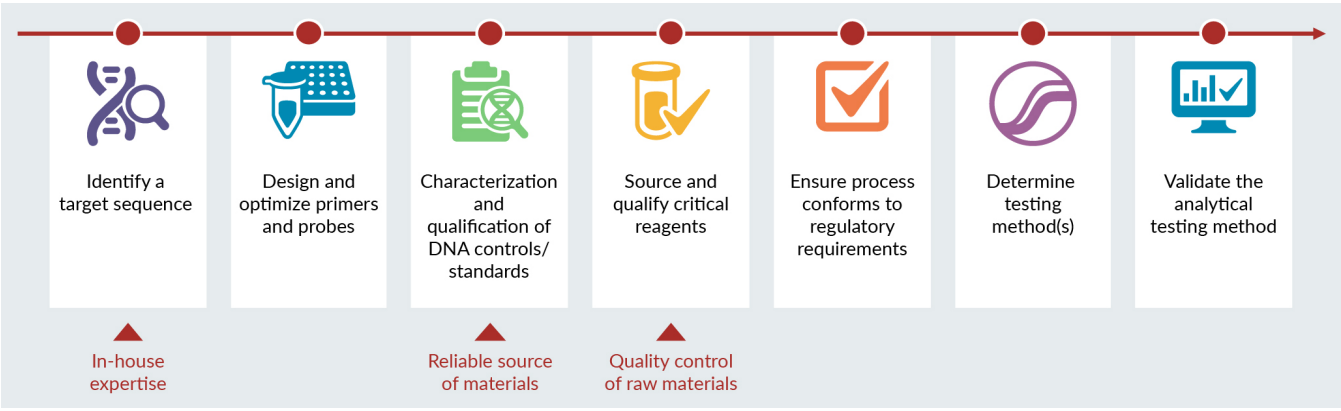
The main challenges in developing an in-house assay lie in sourcing the internal expertise that is needed to identify the right target sequence, and in establishing a reliable source of material. In addition, DNA standard preparation must occur in a specialized lab to ensure there is no possibility of cross-contamination with the routine testing sample. Quality control (QC) of raw materials can also pose challenges for developers, and even post-development, each new lot of critical reagents must be qualified.

Commercial kits for residual DNA testing can dramatically reduce implementation timelines as well as in-house staffing overheads and expertise requirements. This can lower downstream production risks and results in a cost-effective solution overall. The key advantages of integrating a commercial solution include ensuring excellent and consistent performance from kit to kit, lot to lot, and year to year. Fully integrated solutions minimize investment in assay development and optimization, procurement and qualification of reagents from multiple vendors, work on the preparation of standards and controls, and the development of SOPs for critical reagent production and qualification.

Leveraging the experience of a reputable commercial kit supplier can frequently help developers to overcome various challenges, including sample preparation. For example, integrating a Thermo Fisher Scientific solution means benefiting from extensive customer support programs, including specialized training (assay troubleshooting, data interpretation, instrument installation), as well as general troubleshooting and validation

FIGURE 2

Steps and considerations in developing a residual DNA assay in-house.



assistance. Further support is also available from specialist regulatory consultants.

A COMMERCIAL SOLUTION FOR DNA QUANTITATION

The Applied Biosystems™ resDNASEQ™ Residual DNA Quantitation Kit is a fully integrated, real-time qPCR system for the quantitation of residual host cell DNA, including a highly characterized DNA reference standard. It covers the identification of the target sequence, design and optimization of primers and probes, characterization of the DNA standard, and qualification of critical reagents. Specific solutions for the quantitation of host cell, plasmid, and DNA fragment length are available. The resDNASEQ Residual DNA Quantitation Kit is a well-established solution with wide adoption for routine in-process and lot release use at major biopharma companies worldwide. The complete solution includes the Applied Biosystems AccuSEQ™ analytical software for data analysis with features that enable 21 CFR Part 11 compliance. Moreover, flexible sample preparation protocols using the well-established Applied Biosystems PrepSEQ™ Residual DNA Sample Preparation Kit are available, enabling both manual and high-throughput automation.

FIELD CASE STUDIES

The following scenarios which detail real-life issues in in-house assay development, were compiled from regulatory consultants, product managers, and field scientists.

Failure investigation for in-house developed method: qPCR inhibition

- One company developed an in-house assay and observed some inconsistencies with their results. They launched a multi-week, multi-component investigation and found that the root cause was PCR inhibition. The source of the PCR inhibition was further determined to be in the vendor-sourced water—a qPCR inhibitor co-purified with the host cell DNA during DNA extraction.

Failure investigation for in-house developed method: DNA contamination

- Another company was observing inaccuracies in their results. They launched a multi-week investigation and found that the root cause of the inaccurate data was lab contamination. More specifically, the cause was the purification of the DNA standards in close proximity to the routine testing lab. Contamination from the DNA standards interfered with their routine testing.

Workflow efficiency

- A company outlined its process with the Thermo Fisher Scientific field application scientists team and found that its throughput was limited. Through discussing their processes, it was uncovered that the sample preparation step, a multi-step manual process, was highly inefficient, which affected the assay downstream. The solution was to switch to an automated sample preparation solution and equipment, and to requalify their method.

Failure investigation for in-house developed method: inaccurate results

- Another company received inaccurate results and an investigation was required. The root cause was found to be a specificity issue with primer/probe set test sample matrix effects in some sample types, which led to inaccurate results. This impacted the conclusions on purification process efficiency.

SUMMARY

A primary consideration within the process of cell and gene therapy product

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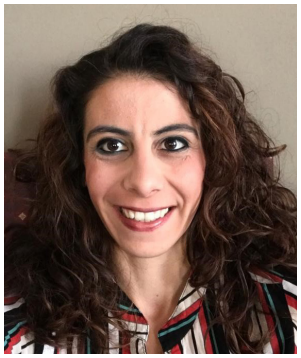
characterization is choosing between an in-house assay or a commercial kit for quantitating residual DNA within a bioproduction workflow. The resDNASEQ Residual DNA Quantitation Kit is a comprehensive solution for residual host cell DNA testing, coming with all standards and reagents ready to use. Furthermore, the resDNASEQ Residual DNA Quantitation Kit allows optimized sample preparation to save time and cost. It has proven high sensitivity and is specific to the target of interest. Performance is guaranteed from kit to kit, lot to lot, and year to year.

Q&A



Ilaria Scarfone

Q For recombinant viruses used in gene therapy treatments, are there additional challenges compared to monoclonal antibody manufacturing processes for developing an in-house test?

IS: For recombinant viruses such as AAV, there are multiple DNA residuals of concern depending on the process, including host cell DNA, plasmid vector, or helper virus DNA. A good size assessment assay is also required as DNA fragment size determination is expected. An additional challenge with AAV is that recombinant AAV has been shown to encapsulate fragments of both vector and host cell DNA.

Q In your experience, how long does it take to develop your own assay versus implementing a resDNASEQ assay?

IS: Typically, it takes 1–2 years to develop your own assay, depending on the expertise and the size of the team. In many cases, you can implement the resDNASEQ assay in 6–12 months, depending on the available resources. Following a period of training that we provide, our assays can be used immediately to generate results and enable method qualifications quickly. It can take some time to prepare the documents, both for method and instrument SOPs, but our user guide provides good templates for this. Overall, it is much more efficient to implement a commercial kit rather than an in-house developed one.

Q How reproducible are the results using the ResDNASEQ solution?

IS: The PCR variation in the qPCR replicates reaction is very low—typically <1%. When considering the entire workflow, including the DNA extraction, the coefficient of variation is typically <10. This variation is usually a result of human interaction, and can be brought down further if automation is incorporated—for example, in the extraction.

Q What is the sensitivity data to support the product?

IS: The limit of quantitation of our resDNASEQ solution, as per the standard protocol, has been determined to be 1.5 pg/mL of test sample for CHO cell line, and 15 pg/mL of DNA for human Taq and *E. coli*. However, we always recommend qualifying the assay sensitivity in your end-to-end workflow during sample-specific validation.

Q Would this kit quantify encapsidated host residual DNA and E1A contaminants?

IS: Yes. This is a typical concern for AAV products. After lysis treatment, our kit can be used for quantitative host cell DNA. We have a kit for residual plasmids with kanamycin resistance and E1A.

Q You mentioned equipment qualification as an advantage for commercial kits. If we use Invitrogen DNA extraction and quantification kits acquired through Thermo Fisher, would that come with support services?

IS: By using Thermo Fisher Scientific products, you will always have broad technical support. We will help as much as we can. The qualification of the equipment itself is done by Thermo Fisher on Thermo Fisher instruments.

BIOGRAPHY

Ilaria Scarfone has been a Field Application Specialist in Thermo Fisher Scientific's Pharma Analytics team since September 2019. She currently lives in Pisa and supports clients in south west Europe. Ilaria completed her PhD in Industrial Biotechnology at the University of Milan in collaboration with the Cell Biology Research Center of Montpellier and subsequently spent 2 years at the Grenoble Atomic Energy Commission as a post-doc. In addition to her academic research background, Ilaria successfully completed an MBA from the IAE Montpellier in 2018, before integrating Thermo Fisher Scientific as a technical specialist for the Pharma Analytics portfolio.

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AFFILIATION

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Field Application Scientist,
Pharma Analytics,
Thermo Fisher Scientific

AUTHORSHIP & CONFLICT OF INTEREST

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WEBINAR DIGEST

Regulatory considerations and validation strategies for mycoplasma testing for cell-based therapies

Mike Brewer, Global Principal Consultant, Regulatory, BioProduction, Thermo Fisher Scientific

Cell therapy manufacturing and quality control workflow incorporates several components including cell isolation, cell selection, genetic modification, cell expansion, cell line authentication, identity and purity testing, potency testing, and microbiological testing. At the microbiological testing stage, mycoplasma detection is particularly important in line with regulatory guidance. In this poster, current regulatory expectations for mycoplasma testing are discussed, followed by review of a sample validation study design and analysis.

CURRENT MYCOPLASMA TESTING REGULATORY GUIDANCE

European pharmacopeial guidance for PCR-based mycoplasma testing details the expectations around validation, which includes those regarding specificity and inclusivity to mycoplasma species. One such expectation is that assays should not detect bacterial species related genetically to mycoplasma. This guidance also addresses the sensitivity or limit of detection (LOD): 10 colony forming units (CFU) or copy equivalent/mL as an alternative to the culture method, or 100 CFU or copy equivalent/mL as an alternative to the indicator cell culture method. Mycoplasma testing is typically performed after the cell expansion phase of the CAR-T manufacturing process, although additional testing points may be added based on process-specific risk assessment. The Applied Biosytems™ MycoSEQ™ Mycoplasma Detection System is specifically designed for both in-process and lot-release testing in a GMP environment, helping to give confidence in your workflow.

Figure 1. An example validation study plan for a qPCR mycoplasma assay.

Specificity	LOD: part 1	LOD: part 2
Part 1 No detection of non-mycoplasma bacteria; data in DMF may be acceptable **Part 2** No interference from customer sample matrix (also addressed in LOD: part 1)	**For each mycoplasma species included in the study** • 6 samples with 100 copies (10 GC/mL) purified mycoplasma DNA spiked into the lysate from 10 mL samples • Run 4 PCR reactions per extraction (24 total PCR reactions)	**This section may be optional depending on discussion with regulators. For each mycoplasma species included in the study** • 6 samples with 100 CFU (10 CFU/mL) live mycoplasma spiked directly into 10 mL samples • Run 4 PCR reactions per extraction (24 total PCR reactions)

Table 1. Specificity of the qPCR mycoplasma assay: external validation results.

Species	PCR reaction number 1				PCR reaction number 2				PCR reaction number 3			
	C_T	T_M	D.V.	+/-	C_T	T_M	D.V.	+/–	C_T	T_M	D.V.	+/–
Hamster	Und.	71.7	0.018	–	38.9049	72.1	0.047	–	39.4103	71.7	0.032	–
Human	Und.	72.1	0.0094	–	Und.	72.1	0.027	–	Und.	72.1	0.0185	–
Mouse	39.8227	72.8	0.024	–	Und.	72.8	0.016	–	38.136	72.8	0.028	–
B. cereus	Und.	70.4	0.0095	–	Und.	72.4	0.017	–	Und.	72.8	0.021	–
B. subtilis	37.7234	75.2	0.0285	–	38.5207	74.9	0.0198	–	37.5753	75.2	0.0325	–
C. albicans	Und.	72.4	0.0113	–	Und.	65.5	0.0076	–	Und.	72.8	0.0088	–
Cl. perfringens	Und.	71.7	0.017	–	Und.	72.4	0.011	–	39.6925	72.4	0.031	–
E. coli	Und.	65.5	0.008	–	Und.	72.1	0.0172	–	Und.	72.1	0.0079	–
St. aureus	Und.	65.5	0.0095	–	39.2726	73.2	0.0385	–	Und.	65.5	0.009	–
St. epidermis	Und.	72.8	0.0125	–	Und.	73.2	0.0123	–	Und.	73.2	0.015	–
Mc. luteus	39.9058	72.8	0.0305	–	39.0225	72.1	0.0285	–	Und.	72.4	0.015	–

A VALIDATION STUDY PLAN: qPCR MYCOPLASMA ASSAY

An example of a qPCR mycoplasma validation study assay based on the ICH Guidance from Q2(R1) on Validation of Analytical Procedures, which specifically outlines limit testing for mycoplasma DNA impurity, is described in **Figure 1**. As per the ICH Guidance for validation of limit tests for the control of impurities, there are two tests that must be performed: specificity and sensitivity/LOD.

The results from the specificity test of the validation study executed at Mycosafe Diagnostics in 2009 are shown in **Table 1**. In this experiment, purified DNA at 10,000 GC/qPCR reaction from a set of off-target species genetically related to mycoplasma, as well as common in-process species used in cell culture manufacturing, were assessed. Three individual qPCR reactions were performed for each species. All species gave a negative result for the detection of mycoplasma and there was no interference in the assay.

Table 2. LOD of the qPCR mycoplasma assay: external validation study using purified DNA, 10 GC/mL using 10 mL test samples.

Mycoplasma species (type strain)	Total number tests/positive reactions	% Positive	Mean C_T (n = 24)	SD	CV (%)
A. laidlawii PG8^T	24/24	100	33.87	0.625	1.8
M. arginini G230^T	24/24	100	30.90	0.99	3.2
M. fermentans PG18^T	24/24	100	32.21	1.68	5.2
M. hominis PG21^T	24/24	100	29.53	0.86	2.9
M. hyorhinis BTS7^T	24/24	100	29.22	0.85	2.9
M. orale CH19299^T	24/24	100	31.85	1.81	5.7
M. pneumoniae FH^T	24/24	100	33.03	0.73	2.2
M. salivarium PG20^T	24/24	100	31.14	0.87	2.8
M. synoviae WVU 1853^T	24/24	100	33.25	0.89	2.7
S. citri R8A2^T	24/24	100	32.79	1.65	5.0

The LOD results from the same external validation study are shown in **Table 2**. In this case, for the 10 mycoplasma species evaluated in the validation, all 24 qPCR reactions were positive. The cycle threshold (C_T) values were well below the positive-negative cutoff value of 36. Analysis of the 24 results showed that the values obtained were very consistent, which is an indication that the lowest LOD of the assay had not been reached.

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Cell & Gene Therapy Insights 2023; 9(11), 1447 • DOI: 10.18609/cgti.2023.188

In partnership with: ThermoFisher SCIENTIFIC

WEBINAR DIGEST

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CELL & GENE THERAPY INSIGHTS

CHARACTERIZATION & VALIDATION

INTERVIEW

Current limitations & potential innovations in developing IND-enabling rAAV- & lentivirus-based gene therapy drug products

Abi Pinchbeck, Assistant Editor, *Cell & Gene Therapy Insights*, speaks to **Rahul Kaushik,** viral vector-based gene therapy expert, who compares recombinant AAVs and lentiviral vectors for *in vivo* and *ex vivo* gene therapy and discusses novel analytical tools in the space, including those to overcome purity analysis, immunogenicity toxicity, and re-dosing challenges.

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Q What are the main viral-based vectors currently used in cell and gene therapy (CGT), and what are the key similarities and differences?

RK: In the field of CGT, two prominent viral vectors currently utilized are recombinant adeno-associated viruses (rAAV) and lentiviral vectors (LV). Currently, seven US FDA and EMA-approved *in vivo* gene therapies rely on rAAV vectors for delivering the genetic payload to target cells. Conversely, *ex vivo* CGT drug products, such as CAR-Ts, primarily utilize

lentiviruses or gamma retroviral vectors. Despite their shared ability to deliver genetic payloads to diverse cell types, including both dividing and non-dividing cells, notable differences exist, making them more suitable for specific applications.

rAAVs, approximately 25 nm in size, possess a non-enveloped capsid capable of packaging a single-stranded DNA (transgene cassette) of 4.7 kilobases or less. The transgene cassette provides therapeutic benefits, while the capsid controls tropism and manufacturing strategy. The single-stranded transgene cassette is replicated inside the target cells and mostly resides as a stable episome, providing long-term stable expression, and minimizing the risk of insertional mutagenesis. These properties make rAAVs suitable for delivering genetic payloads in non-dividing cell types, such as the eye, brain, skeletal, and cardiac muscles, but less suitable for dividing cell types due to the dilution of the therapeutic gene with each cell division cycle.

Conversely, LVs are larger (80–100 nm) and are enveloped with a lipid bilayer carrying transmembrane proteins (usually VSV-G) and two copies of single-stranded RNA. This genetic payload is reverse transcribed, replicated, and efficiently integrated into the host genome with a bias towards active genes. Integration into the genome allows long-lasting stable expression, making LVs favorable for applications like CAR-T therapies. Concerns about insertional mutagenesis are often mitigated in these therapies, as modified T cells are thoroughly screened before reinfusion.

Noteworthy is the lower immunogenicity of rAAVs compared to other viral vectors, reducing the likelihood of triggering an immune response, hence making them well-suited for *in vivo* applications. However, significant challenges persist, with immunogenicity influenced by factors such as dose, route of administration, serotype, pre-existing antibodies, patient-specific factors, and disease-specific considerations.

rAAVs are generally more stable than LVs, which require rapid manufacturing processes to maintain functionality. LVs also face challenges in sterile filtration during manufacturing, a crucial regulatory requirement. A significant difference lies in innovation possibilities; rAAVs with a simple protein capsid allow for considerable innovation in capsid engineering, enhancing precision and reducing off-target effects. On the other hand, the lipid bilayer of LVs poses challenges in controlling tropism or efficacies.

rAAVs, being small, can penetrate tissues deeply in direct delivery, enabling wider biodistribution compared to LVs. The choice between wider or targeted biodistribution depends on the therapeutic goals. Ultimately, these distinctions make rAAVs more suitable for *in vivo* gene therapy, while LVs excel in *ex vivo* gene therapies. Considering these factors is crucial in developing gene therapy products aligned to provide maximum value to the unmet needs of the patients.

Q For the development of the investigational new drug (IND)-enabling rAAV-based gene therapy products, what requirements and considerations are necessary?

RK: Developing an rAAV-based gene therapy product is an intricate and regulated process with key considerations to ensure safety, efficacy, and value for patients. One critical early consideration is transgene optimization, involving the search for an optimal transgene cassette suitable for a specific disease and patient population. This cassette contains DNA elements, including inverted terminal repeats generally sourced from wild-type AAV2 vectors. These 145 bp DNA elements play a crucial role in the application and packaging of transgenes

into AAVs, emphasizing the paramount importance of inverted terminal repeat integrity when developing rAAV vectors.

Promoter and enhancer DNA elements are also vital, controlling transgene expression in the target cell. In early gene therapy product development, ubiquitous promoters were common. However, advanced gene therapy product developers now prefer cell-specific promoters, enhancing drug product specificity. Promoters, varying in size and type, require fine-tuning for long-term stable expression at the appropriate level. Given that these therapies cannot be altered post-delivery, careful consideration in the initial stages is crucial to optimize expression levels controlled mainly by the strength of promoter and enhancer elements.

The third element, the transgene itself, undergoes optimization through methods like codon optimization, CpG island depletion, isotype switching, and functional mutations. Transgene size can impact vector quality and manufacturing, and regulatory elements, such as post-transcriptional regulatory elements, can further enhance expression. A poly(A) signal is necessary at the end for these transgene designs. Transgene cassette design is a versatile and highly customized activity, considering optimal design with trade-offs, such as size, codon optimization, and additional regulatory elements, adding value to expression levels. This is a significant early preclinical activity.

Subsequently, a range of suitable *in vitro* and *in vivo* experiments are conducted for transgene optimization and lead candidate selection in a gene therapy program. Once the lead expression plasmid, along with suitable helper and rep-cap plasmids, is determined, the focus shifts to developing a scalable, robust, GMP-compatible manufacturing process optimized for the disease indication, population, and vector supply. This can be performed with an established CDMO with prior experience in developing such therapies or in-house if capabilities exist. Key CMC activities, including process and analytical development, alongside rigorous quality control measures, ensure the purity, potency, and consistency of the product, along with scalable and robust production

Extensive preclinical studies are then conducted to demonstrate the safety and efficacy of the rAAV-based gene therapy product in relevant animal models. Dosing, toxicity, and biodistribution studies use vectors produced through an optimized GMP-ready process and well-characterized material.

During CMC activities, a critical step is reaching out to regulators such as the EMA, FDA, Paul Ehrlich Institute, and Medicines and Healthcare products Regulatory Agency for early guidance on development and manufacturing plans. They can advise whether assays and studies are fit for purpose, which is crucial for early implementation of changes to meet regulatory requirements.

The next step involves preparing and submitting an IND or clinical trial authorization application to respective authorities, including comprehensive data on preclinical studies, manufacturing, quality control, and a clinical development plan. This plan includes detailed information on the proposed clinical trial, patient eligibility criteria, dosing regimen, and safety monitoring plans. Key inclusions are the design of a well-controlled and scientifically sound clinical trial to evaluate the safety and efficacy of the rAAV-based gene therapy product in humans, alongside robust protocols for patient monitoring and safety reporting throughout the clinical trial.

"[The transgene casette] elements play a crucial role in the application and packaging of transgenes into AAVs, emphasizing the paramount importance of inverted terminal repeat integrity when developing rAAV vectors."

Q How are novel technologies being utilized to overcome immunogenicity, toxicity, and re-dosing challenges?

RK: Immunogenicity of rAAV-based gene therapy products is a complex biological response, which is an interplay of dose, route of administration, serotype, presence of pre-existing antibodies, the patient, and disease-specific factors. The challenge lies in the fact that high doses of rAAV can trigger a robust immune response and toxicity, leading to incidents of patient deaths in cases of excessive doses. Re-dosing becomes problematic, as the initial dose elicits a strong immune reaction against the specific capsid, rendering patients unsuitable for subsequent doses due to the presence of antibodies. Another key challenge is the presence of pre-existing antibodies against various AAV serotypes in patients, which results in their exclusion from clinical trials.

One promising strategy involves engineered capsids with improved transduction efficiency and reduced immunogenicity. This not only allows for a reduction in the required dose, resulting in a milder immune response but also facilitates evasion of pre-existing immune responses. This, in turn, enables the inclusion of patients with antibodies against natural AAV serotypes. However, establishing capsid designs that permit efficient transduction while evading present antibodies across multiple animal models and species, with translatability to humans, remains a formidable challenge.

Another avenue to tackle this challenge is the exploration of innovative delivery technologies, such as lipid nanoparticles or exosomes. These approaches have the potential to decrease immunogenicity and enhance transduction efficiency by leveraging endogenous cell processes for vector uptake.

Moreover, combining rAAV-based therapies with immune modulators, like checkpoint inhibitors and cytokine modulators, is also under investigation to control immune responses and potentially reduce immunogenicity. Tailored immunosuppressive regimens, coupled with advancements in monitoring techniques and the identification of biomarkers, offer potential tools for predicting and managing immune responses in patients undergoing rAAV-based gene therapies.

It is crucial to note that these approaches are currently in various stages of preclinical or early clinical development, and their safety and efficacy profiles are still under evaluation. Overcoming challenges related to immunogenicity, toxicity, and re-dosing in the context of rAAV-based drug products demands a multifaceted approach. Ongoing research and innovation in the field are critical to advancing the safety and effectiveness of gene therapies.

Q What is your assessment of current inline analytical tools available to the vector-based gene therapy space, and where would you like to see further innovation in this area?

RK: Current process analytical technologies designed for the biologics and monoclonal antibody space are insufficient for viral vectors, as they were initially intended for different processes. To enhance manufacturing processes for CGTs, purpose-built analytical tools are essential, yet progress in this area has been limited.

Innovations are needed, including more sensitive and specific sensors capable of monitoring critical quality attributes. These sensors should enable the identification of cell growth behaviour, viral vector production per cell, full versus empty vectors, and media utilization. A critical

requirement is technology that can specifically identify viral vectors and their critical quality attributes early in the process, serving as indicators for efficiency, potency, and purity. Some groups are actively developing advanced sensors with specificity towards viral vectors. Work is also underway on process integration, aiming to seamlessly incorporate this technology into manufacturing without manual handling to reduce contamination risks.

Technologies such as single-cell analysis and metabolomics play a crucial role in understanding cell behavior in a culture system. They provide insights into how nutrients and media components are utilized during viral vector production. This understanding can help identify an optimal growth 'sweet spot' and enable real-time optimization for continuous and efficient viral vector production. The emerging areas of miniaturization and automation are vital, demanding analytical technologies that seamlessly fit into current manufacturing systems without manual intervention.

The integration of process analytical technologies with continuous manufacturing platforms for gene therapies is pivotal for reducing batch-to-batch variability and enhancing efficiency. Additionally, the implementation of real-time quality control methods can identify and mitigate potential product deviations during production. Ideally, the entire process should collect extensive data and generate insights using machine learning algorithms. Detecting initial variabilities in manufacturing with inline process analytics proves cost-effective in the long run. These analytical tools must be purpose-built for the specific requirements of CGT product manufacturing.

Q Can you briefly comment on the current tools and methods for measuring rAAV empty/full ratio?

RK: The concept of full and empty capsids is a significant focus in the current landscape of the gene therapy field. There are established technologies that are the gold standards for identifying empty/full ratio such as analytical ultracentrifugation (AUC). Each technology comes with its own set of advantages and disadvantages. AUC, for instance, is a robust technique for characterizing the size and shape of rAAV particles. It effectively distinguishes between empty and full capsids based on their sedimentation properties. By employing differential sedimentation analysis, AUC can estimate the percentage of empty and full capsids in a sample. However, one downside is that it requires a substantial amount of material.

On the other hand, electron microscopy directly captures images of a product, allowing observation of empty and full capsids. Although this method is more labor-intensive, it serves as a valuable orthogonal approach to validate empty/full values against those obtained through other technologies.

The most commonly used method involves a ratio metric measurement using the genome titer (measured by qPCR or ddPCR) divided by the capsid titer (measured by ELISA). While this is an indirect measurement, it proves to be useful in determining the empty/full ratio. Another method, size exclusion chromatography with multi-angle light scattering (SEC-MALS), provides an advanced means of comparing the mass of the viral vector when it is empty versus full. Emerging technologies, such as mass photometry (offered by Refeyn), directly measure the mass of viral particles, leveraging the weight difference between full and empty rAAV particles. Another innovative technology, Stunner, from Unchained Labs, utilizes UV light and visible absorbance along with dynamic light scattering methods to estimate the empty/full ratio. While different methods generally align to some degree, discrepancies exist. The pressing need moving forward is a high-throughput, sensitive technology requiring less material to deliver a robust and reliable readout. The integration of machine

learning holds promise in extracting the viral vector signal from background noise. I am optimistic that in the coming years, we will witness the development of improved technology for estimating the empty/full ratio.

Q What for you are the most pressing priorities in terms of vector validation and characterization today?

RK: Current gene therapy products encounter challenges in scalability, particularly with the demanding nature of large-scale triple plasmid transfection. A closely associated challenge is the issue of cost, necessitating innovative approaches for scalable, robust, and cost-effective rAAV manufacturing. Assessing vector purity poses another difficulty. To accurately determine the empty/full ratio, multiple assays are currently employed. While various methods are in use, an ideal scenario involves adopting a standardized and reliable method to streamline the calculation of the empty/full ratio.

The complexity of the immune response presents another hurdle. The variation in rAAV dose behavior between two patients remains not fully understood, with patient-specific factors influencing outcomes. In some instances, a patient within a cohort has experienced an unexplained death, emphasizing the need for a better grasp of diseases, patient factors, and product intricacies. Immunogenicity toward AAV remains a critical area requiring deeper exploration.

Long-term safety is a noteworthy concern in this evolving field, as the limited data on the extended effects of gene therapies necessitates ongoing monitoring. As more patients treated with gene therapies live longer, a wealth of data will gradually accrue, contributing to a more comprehensive understanding of long-term outcomes.

Ensuring comparability in manufacturing is a critical aspect. Demonstrating consistency between different production batches and various manufacturers is essential to safeguard product safety and efficacy. Batch-to-batch variability presents challenges in manufacturing similar vectors, compounded by the absence of sensitive viral vector-specific inline sensors for real-time process monitoring. Drawing parallels with challenges faced in the biologics field two decades ago with monoclonal antibodies, advancements are expected to overcome these manufacturing challenges in the next 5–10 years, providing a more nuanced understanding of this technology.

Q Finally, what are the major technological barriers in the areas of rAAV- and LV-based gene therapy drug products and the potential innovations that can help?

RK: Manufacturing with triple transfection poses a significant challenge. A groundbreaking shift towards stable producer cell lines could revolutionize manufacturing by eliminating the need for transfection. This shift not only reduces plasmid costs but also addresses batch-to-batch variability and minimizes expenses related to transfection reagents and consumables. At the top of my innovation wishlist are high-density, serum-free, suspension-stable cell lines capable of producing high-quality viral vectors.

For rAAVs, another challenge is cargo capacity, but this is harder to overcome. Nevertheless, efforts on dual-vector systems, combining multiple vectors and recombining them in the target cell to create the full-length protein, have shown some promise. However, the feasibility of this solution in clinical settings remains to be seen.

Another hurdle to overcome pertains to dosages, especially in disease indications requiring high doses. The broad tropism of rAAV results in uptake by numerous unwanted cells upon injection. Innovations focused on modifying the capsid to make rAAV specific to particular cell types would be highly beneficial, reducing the overall required dose. Additionally, this technology could aid immune evasion by modifying capsids so that antibodies against the wild-type capsid cannot recognize them.

In the case of LVs, the primary concern is ensuring their safe and stable integration into a specific location in the genome. Challenges in LV manufacturing mainly revolve around instability, making any technology that reduces vector loss during the filtration step highly valuable.

Ultimately, a game-changer for all viral vector manufacturing would be advancements in specific and fit-for-purpose process analytics. This innovation has the potential to enhance the efficiency and precision of the entire manufacturing process.

BIOGRAPHY

RAHUL KAUSHIK has over 13 years of R&D experience, with a strong scientific background in viral and non-viral gene delivery technologies and innovations (rAAV, LV, and exosomes), targeting various diseases and tissues (CNS, retina, and liver). As a former group leader at Evox Therapeutics Ltd, he helped develop innovative platform technologies to address challenges related to immunogenicity, toxicity, and repeated dosing associated with rAAV based *in vivo* gene therapy. He led the AAV and ExoAAV production team creating in-house capabilities for the development of gene therapy products and platforms. With over 7 years of multidisciplinary team leadership experience, Kaushik designs scientific strategies and innovative gene therapy solutions. His expertise spans AAV biology, technological advancements, and rare diseases. Previously, he focused on rAAV-based ocular gene therapy for large genes and led rAAV and LV platform technologies for the gene manipulation and editing in the CNS space as Head of the Molecular Biology Unit at the German Center for Neurodegenerative Diseases (DZNE). He specializes in vector design, lead candidate selection, capsid selection, initial process and analytical development, tech transfer, managing contract research organizations and CDMOs. Further, he specializes in designing proof of concept, IND-enabling preclinical rodent and non-human primate studies for dose finding, biodistribution, pharmacokinetic/pharmacodynamics, and toxicity to collect safety and efficacy data. Kaushik has over 20 publications in prestigious journals such as *Science*, *Cell*, *Nature Communications*, *EMBO*, and *Progress in Neurobiology*, which showcase his molecular biology expertise for genetic manipulations. He is deeply passionate about leveraging next-generation engineered viral vectors and enhancing manufacturing processes to develop gene therapy products that can combat devastating human diseases.

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CELL & GENE THERAPY INSIGHTS

CHARACTERIZATION AND VALIDATION

The current viral vector characterization landscape: from navigating evolving regulatory guidance to leveraging analytical tools for process development

Ning Ding

"Peering into the future a decade from now, there is an expectation of significant regulatory evolution in the realm of viral vector development, foreseeing the development of evolving guidelines and increased flexibility in response to emerging technologies and novel therapeutic approaches."

VIEWPOINT

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COMPARING VECTOR CHARACTERIZATION FOR AAV & LENTIVIRUS

AAV and lentivirus (LVs), widely used in cell and gene therapies, exhibit distinctive characteristics in their vector biology. AAV, a non-enveloped virus, carries a single-stranded DNA genome, while LVs, an enveloped virus, carries a single-stranded RNA genome. Notably, AAV has a smaller size, limiting its expression cassette capacity to 4–5 kb, in contrast to LVs, which accommodates inserts up to 10 kb. Despite this limitation, AAV's smaller size provides advantages in infectivity to specific cells and tissues. Consequently, the biodistribution and tissue tropism of AAV and lentiviral vectors differ. Another significant divergence between AAV and LVs is their genetic behavior. Lentiviruses integrate their genetic material into the host genome, enabling stable and prolonged expression. On the contrary, engineered AAVs commonly employed in gene therapy tend to maintain an episomal state, with their genome primarily persisting in the episomal form within the nucleus of infected cells. This impacts the stability of genetic material and the potential for sustained expression over time.

When characterizing these viral vectors, the overarching strategy remains similar due to their shared nature as viral vectors. Key aspects of characterization include the accurate determination of viral vector titer, which denotes the concentration of infectious particles and is essential for dosing and therapeutic efficacy. Additionally, various critical quality attributes (CQAs) such as identity, potency, purity, and safety must be thoroughly characterized to ensure product safety and efficacy for patients.

Due to their biological differences, there are some specific aspects to consider when characterizing AAV and lentiviral vectors. The choice of host cells and production systems introduces unique challenges in the characterization process. Recent publications have underscored the benefits of leveraging diverse host cells, with one option being insect cells like Sf9 cells in AAV production, emphasizing the scalability and ease of growth associated with Sf9 cells. However, this choice requires an examination of the physical and biological characteristics of vectors produced from human cells versus insect cells. One crucial aspect is the distinct profile of host cell proteins and residual DNA in viral vectors produced by insect cells, demanding specific assay designs and the careful selection of antibodies and primers for assay development. Furthermore, a notable difference in the infectivity readout of viral vectors and viral genomes emerges when comparing insect cells to mammalian systems. This difference is attributed to unique post-translational modifications on the viral vector and capsid full/empty ratio in insect cell-produced vectors. Consequently, due to such variations in titer, achieving comparable therapeutic efficacy often requires a distinct dosage when employing vectors produced in insect cells versus human cells. Therefore, it is imperative to discuss specific assay designs and development strategies in advance to accurately characterize these distinctions.

Another difference is their regulatory landscape. The regulatory distinctions between AAV and lentiviral vectors stem from their unique characteristics and intended applications. Despite AAV vectors generally being acknowledged for their lower immunogenicity compared to lentiviral counterparts, the immunogenicity of AAV-based gene products still presents a notable challenge, impacting the efficacy and safety evaluation. Mild to severe adverse events observed in clinical development have been linked to AAV drug product immunogenicity. In the past couple of years, industry leaders and regulatory bodies have collaboratively organized public workshops to deliberate on the current assessment and best practices for immunogenicity testing and mitigation related to AAV gene therapy products, as well as the future direction in regulatory evaluations, indicating their shared commitment to advancing the field.

As for LVs, it was observed that the potential for cellular genome integration by lentiviral vectors presents regulatory challenges for certain biotechnology companies in the recent few years. The US FDA guidance for *Long-Term Follow-up After Administration of Human Gene Therapy Products* [1] delves into the discussion of lentiviral genome integration, highlighting that integrating vectors like LVs entail a potential risk of delayed adverse events. Depending on the perceived level of risk, the guidance suggests considering additional preclinical and clinical assessments, as well as implementing a long-term follow-up strategy. This approach aims to enhance the FDA's comprehension of patient safety over an extended period, ensuring thorough monitoring and understanding of potential implications that are related to lentiviral vector products.

LEVERAGING ANALYTICS FOR PROCESS DEVELOPMENT

Process development can benefit significantly from the strategic application of analytics. There are a few ways in which analytics can be leveraged to enhance process development:

DoE relies significantly on collaborative in-process analytics, where process scientists and analytical scientists work together. This collaboration allows for a thorough exploration of how manipulating process parameters—such as temperature, media ingredients, and reactant concentration—affects the critical attributes of the final product. By systematically collecting analytical data, DoE becomes a powerful tool for identifying optimal conditions and reactions, facilitating informed decision-making for the final process, and ensuring desired outcomes. In more complex processes, where various interrelated parameters influence final product quality, multivariate analytical data analysis comes into play. This approach helps untangle intricate relationships between multiple factors, unveiling hidden patterns and correlations. The insights gained from multivariate data analysis not only aid in explaining potential failures in the experiment but also assist in troubleshooting and ultimately contribute to the refinement of the DoE.

Statistical process control tools play a crucial role in continuously monitoring and controlling various process parameters over time, ensuring they stay within predefined limits. Through the visualization of data on a control chart, this method helps identify any deviations from expected values, signaling when the process is going out of control. Such deviations can be linked to identifying root causes, often attributed to factors like using a different lot of reagents, operator variations, or control failures. Early detection of these deviations allows for prompt corrective actions, maintaining consistency and product quality. Adhering to principles like the Nelson rules (particularly the widely used rule that considers a data point more than three standard deviations from the mean as grossly out of control) provides a systematic approach to recognizing anomalies in the process.

NAVIGATING REGULATORY AGENCIES' EXPECTATIONS FOR VIRAL VECTOR CHARACTERIZATION

Navigating regulatory agencies' expectations on viral vector characterization poses several challenges for developers and manufacturers in the field of cell and gene therapy, including assay customization and standardization, phase-appropriate method development, and maintaining consistency in manufacturing.

Challenge 1: assay customization & standardization

The need for customized assays is driven by the substantial variability in the physical characteristics of viral vectors used by different companies. This diversity poses a significant hurdle in establishing an analytical platform with consistent assay performance. To address this challenge, many companies choose to

develop their customized cell and gene therapy assays in-house. However, despite the diversity in customized assays, a crucial step is standardization during development and validation. This standardization is essential to ensure that the data obtained from these assays are comparable across different laboratories and to facilitate a smoother regulatory review process. Consider the example of a viral vector titer assay, where the testing focus dictates the measurement of various components or functionalities, such as genome content, capsid protein, or infectivity. This diversity in testing objectives results in different titer readouts, including genome titer, physical titer, and infectious titer, respectively. While these customized assays cater to specific needs, they must adhere to a common set of criteria known as system suitability during development. Achieving consensus on standardized methods for viral vector characterization industry-wide can be challenging but is crucial for harmonizing analytical techniques and ensuring consistent and comparable data.

Challenge 2: a phase-appropriate method

Scaling up viral vector manufacturing from phase 1 to phase 2 and 3 clinical trials is crucial in drug development, facilitating larger-scale production, process optimization, regulatory compliance, and preparation for potential commercialization. This ensures that the therapeutic product is not only safe and accessible to a broader patient population but also maintains consistent quality compared to smaller-scale production. However, a significant challenge in the scale-up process is the translation of characterization methods from small to large-scale settings. This often involves modification of assay acceptance criteria, transferring methods to new environments, and revalidating analytical methods to ensure assay performance is sustained at the larger production scale. For instance, phase 3 assays require a higher level of validation stringency compared to phase 1, encompassing aspects such as sample size, assessment of interference and matrix effects, and assay controls. As such, assay development should adopt a phase-appropriate approach, strategically planned to meet rigorous regulatory requirements.

Challenge 3: achieving consistency in manufacturing

Ensuring batch-to-batch consistency and demonstrating reproducibility and reliability across multiple manufacturing runs requires a collaborative effort. The process and manufacturing team plays a crucial role in locking down the manufacturing process, maintaining consistency, performing process performance qualification, and controlling variables. Simultaneously, the analytical team and QC teams are tasked with extensive testing, characterization, release, and documentation to ensure that each batch aligns with predefined specifications. Apart from addressing process variability, attention to analytical method variation is essential. The methods used to characterize viral vectors may introduce their own variability. Thus, it is important to guarantee the reproducibility and reliability of these analytical methods across different runs and batches to achieve consistent and reliable quality assessments. Introducing and monitoring assay controls over time helps understand the trending of method variability, ensuring it stays within a relatively narrow range. This proactive measure provides insights into the stability and consistency of the analytical methods employed.

The CQAs as mandated by the FDA's CMC guidelines include identity, strength/potency, purity, and safety. To meet these requirements, various analytical techniques and methods are employed to assess the CQAs of the viral vector product. Table 1 is an overview of the key quality attributes and the corresponding analytical methods used.

TABLE 1

Summary of analytical tools used in viral vector characterization

Quality	Description	Technology
Identity	Ensuring the intended vector construct and expected properties are present.	• Capsid proteins: SDS-PAGE/CE-SDS, mass spectrometry, immunoblotting, ELISA • Genetic identity: PCR, Sanger sequencing, or next-generation genome sequencing
Potency	Understanding concentration and biological activity of viral vectors. Typically it is accomplished through developing phase-appropriate potency matrix and titer assays.	• Physical titer: gauge concentration based on proteins or genome content. Measured using ELISA and PCRs (qPCR and ddPCR). • Infectious titer: evaluate infectivity using PCRs, FACS, plaque-forming assay, fluorescence foci assay, TCID50 assays • Phase-appropriate potency matrix: evaluate the biological activity of viral vector. Measured using ELISA, MSD, FACS assays and immunofluorescence assays.
Purity	Ensuring minimal contaminants for patient safety.	• Protein purity: assess presence of viral proteins using mass spectrometry, SEC, CE-SDS, ELISA, or HCP. • Nucleic acid contamination: detect residual DNA/RNA, residual oncogenes, residual plasmid with qPCR, ddPCR, and gel electrophoresis. Test fragmented vector genomes with alkaline gel electrophoresis, and long read NGS. • Empty capsids: quantify with AUC, AEX-HPLC. • Process-related impurities: ELISA for detecting residual components from the production process such as affinity ligands used in a purification column or enzymes used during manufacturing.
Safety	Ensuring sterility and absence of specific contaminants.	• Sterility: adhere to EP 2.6.1 and USP <71> standards. • Endotoxin: use LAL method following EP 2.6.14 and USP <85> specifications. • Mycoplasma: detect through PCR and cell culture-based assays. • Replication competent virus: assess with Southern blotting and qPCR. • Adventitious Virus: use *in vivo* and *in vitro* assays to detect infectious agents of human or animal origin.
Stability	Assessing product stability under storage and handling conditions.	• Conducted through freeze-thaw testing, evaluating capsid and genome titers, potency, pH, osmolarity, and the formation of aggregation at designated time points after freeze-thaw.

AEX-HPLC: anion-exchange high-performance liquid chromatography; AUC: analytical ultracentrifugation; CE-SDS: capillary electrophoresis sodium dodecyl sulfate; ddPCR: droplet digital polymerase chain reaction; ELISA: enzyme-linked immunosorbent assay; FACS: fluorescence-activated cell sorter; HCP: host cell protein; LAL: limulus amebocyte lysate; MSD: meso scale discovery; NGS: next generation sequencing; qPCR: quantitative polymerase chain reaction; SDS-PAGE: sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SEC: size exclusion chromatography; TCID50: 50% tissue culture infectious dose.

INNOVATION GAPS IN VECTOR CHARACTERIZATION

Efficient market entry is a critical consideration in the rapidly evolving cell and gene therapy industry, particularly when aiming for a timely first-to-market position. However, the emergence of numerous new and innovative platforms introduces untested processes and undefined specifications, elevating the risk of unexpected technical challenges and delays. One significant bottleneck in this process is that the analytical methods employed often lack high-quality 'real-time' data to inform process development decisions for viral vector processes. The delay between sampling and receiving results from low-throughput assays, which can extend over weeks, poses a significant hindrance to the efficient DoE. The emphasis on process analytical technology within the QbD framework has prompted increased focus on developing

high-sensitivity, high-reproducibility, and high-throughput analytical methods.

Viral vector characterization faces another innovation gap arising from the limited availability of commercially developed kits. The unique attributes of innovative systems newly introduced to the market demand specific assays and specialized kits, contributing to a scarcity of readily available solutions. In this context, analytical scientists often navigate the challenge by creating customized assays, establishing novel cell lines, and developing and validating controls and reference standards from the ground up. Moreover, the market's accessibility to essential components like cell lines, standards, and reference materials is further complicated by intellectual property constraints. Acquiring licenses for cell lines and plasmids from vendors involves legal processes, introducing additional complexity to the overall timeline of development.

Peering into the future a decade from now, there is an expectation of significant regulatory evolution in the realm of viral vector development, foreseeing the development of evolving guidelines and increased flexibility in response to emerging technologies and novel therapeutic approaches. Despite this regulatory evolution, there remains an unwavering commitment to prioritizing patient safety and long-term wellness. Another anticipated change in the future is the widespread adoption of automation and high-throughput screening methods, allowing for faster and more efficient characterization of viral vectors, particularly beneficial in the early stages of development.

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BIOGRAPHY

NING DING is currently a technical expert of analytical development and bioassays in the biotech industry. He earned his PhD in Physiology, with a research focus on molecular cancer biology, from Indiana University Bloomington. He received his postdoctoral training at Massachusetts General Hospital, Harvard Medical School. With a cumulative 11 years of specialized laboratory experience in viral vector development and characterization, including extensive industry CMC experience, Ding has honed his expertise in analytical development for lentiviral vectors, AAVs, and retroviruses.

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CELL & GENE THERAPY INSIGHTS

CHARACTERIZATION AND VALIDATION

REVIEW

Profiling AAV vector heterogeneity & contaminants using next-generation sequencing methods

Ngoc Tam Tran and Phillip WL Tai

AAV vectors continue to be the most promising gene delivery vehicle for treating rare genetic diseases through gene therapy. Understanding vector inconsistencies during the manufacturing process is vital to define batch-to-batch differences, and predicting their efficacies and safety profiles. Although AAV vectors manufactured for clinical use are rigorously tested by several analytical methods, these assays are still not able to provide comprehensive insights into a vector's composition, nor address how or why heterogeneity in vectors emerge. With the power of next-generation sequencing methods, understanding AAV vector composition and why certain designs fail to provide expected potencies can be unlocked.

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INTRODUCTION

AAVs were originally discovered in 1965 as 'virus-like' particles [1,2]. AAVs belong to a class of small, non-enveloped, dependoparvoviruses that rely on co-infection with helper viruses, such as adenovirus or herpesvirus to complete their lifecycles in the host [3]. AAV is single-stranded DNA virus that packages either the plus or minus strand of the genome at equal ratios into an icosahedral protein capsid that is approximately 20–25 nm in diameter [4,5]. The AAV genome has four known open reading frames (ORFs) that encode for

the viral replication genes (*rep*), the capsid proteins (cap), the assembly-activating protein (*AAP*), and the membrane-associated accessory proteins (*MAAP*) [6]. The *rep* gene encodes for Rep40, Rep52, Rep68, and Rep78 [7]. The *cap* gene encodes three viral proteins called VP1, VP2, and VP3, which form the 60-mer capsid at approximate ratios of 1:1:10 for VP1:VP2:VP3, respectively [6,7]. The AAV family of viruses is fairly diverse. Among those that can infect humans and non-human primates, there are seven main clades (clades A-G) [8,9], which encompass AAV1/6 (clade A), AAV2 (clade B), AAV2/3-hybrid and AAV13 (clade C), AAV7 (clade D), AAV8 (clade E), AAV9 (clade F), and AAV4, AAV11, and AAV12 (clade G). AAV5 is the most distinct among the contemporary capsids, and is currently in its own class. Differences in capsid surface antigens have traditionally define viral serotypes; however, among the wildtype AAVs that have been discovered, over hundreds of naturally occurring variants have been identified based on sequence analyses [6]. Importantly, these serotypes and subvariants have different tropism profiles among several mammalian laboratory models that span an array of cell and tissue types.

The AAV genome is flanked by two inverted terminal repeats (ITRs) that are required for rescue, replication, and packaging of the genome. Similar to other parvoviruses, the ITR overcomes the end-replication problem through rolling-hairpin replication [10]. The wild-type ITR from AAV serotype 2 (AAV2) is 145 nt in length and comprises of four internal segments [11]. Its first 125 nt folds on itself to form a T-shaped hairpin with two small internal inverted repeat sequences, named the B and C arms. The stem of the T-shaped hairpin is called the A segment. The rest of the ITR, which is contiguous with the rest of the genome, forms the D sequence. The inverted nature of the ITR is essential for virus genome replication, as it serves as an origin of replication as a self-primed molecule. Embedded within the A sequence is the Rep-binding element (RBE). Together with the RBE, a sequence that is located at the tip of the cross arms, called RBE', serve to recruit Rep68/78, which nicks the terminal resolution site to separate the newly synthesized DNA strand from the template strand [12].

There are several features that make AAV ideal vehicles for gene therapy [11,13,14]. First, they cannot replicate on their own, but require factors expressed by the helper virus. These specific factors, namely those from adenovirus (E1A, E1B, E2A, E4, and viral associated RNA), can be expressed in *trans* to drive AAV replication and genome packaging. Second, AAVs confer low immunogenicity and pathogenicity. In recent years, AAV has been linked to hepatocellular carcinoma and specific cases of acute hepatitis [15–18], but the mechanisms that drive these outcomes are not fully known and are hotly debated. Third, AAV vectors can confer long-lasting transgene expression, since their genomes predominantly persist as circular double-stranded episomes in the host cell nucleus [4].

There have been multiple methods for AAV vector production described throughout the years [19–26]. However, plasmid transfection in HEK293 cells (pTx/HEK293), recombinant baculovirus infection in insect cells (rBV/Sf), and HeLa production cell lines with adenovirus are currently the three most popular production platforms for manufacturing recombinant (r)AAV for basic research, pre-clinical, and clinical use. Unfortunately, the potency of AAV vectors is inexplicably known to be impacted by the manufacturing method [14,27,28]. There are many quality control challenges in producing effective and safe vectors. Purification methods can also vary and impact the quality of AAV vectors. Vector purification chiefly involves obtaining high quality particles that are free from partial or empty particles. Many techniques have been developed for vector purification [26,29–33]; but currently, there is no single method that can completely remove empty particles from preparations. Despite well-established pipelines developed for

obtaining safe and quality vectors, the final product can still contain defective vectors and contaminants [34]. Therefore, characterizing and validating AAV vectors are essential for assuring that the final product meets safety, purity, and quality standards set by the US FDA.

ANALYTICAL METHODS FOR AAV VECTOR CHARACTERIZATION & EVALUATION

Product characterization under GMP must follow guidelines required by the FDA [35]. Different analytical methods are used for characterizing and validating AAV vectors. In general, these assays evaluate a vector's identity, potency, purity, safety, and stability. [35]. These methods have been reviewed extensively [36–42]. The following metrics and the analytical methods that measure them have been industry standards for querying batch-to-batch heterogeneity.

Vector genome titration

The traditional way of quantifying viruses with infectious titers cannot be used for recombinant (r)AAVs, since the highly engineered nature of these vectors make infection a less reliable means of gauging their titers. Therefore, methods to obtain physical titers are favored. The standard means of quantifying vectors relies on the detection of vector genomes in the preparation, for which quantitative PCR (qPCR) has served as the method of choice. However, accuracy of qPCR is dependent on primer efficiencies. Since many research vectors can vary in design, primer/probe sets typically target sequences that are commonly shared, such as the polyadenylation sequence or regions proximal to the ITRs. Digital Droplet (dd)PCR has become more attractive, since the method is not as severely impacted by primer efficiencies as it is with qPCR. The only drawback of qPCR/ddPCR, as with any DNA-based detection method, is that non-encapsidated DNAs (carry-over from production) that survive endonuclease digestion during vector purification steps can be detected, leading to the overestimation of vector titers.

Particle titration

Quantification of vector DNA may not accurately reveal the abundance of vector particles in preparations, since some particles may lack vector genomes (empty capsids). Although empty capsids do not contribute to the overall transduction and potencies, they will impact how the host will respond to dosing, which is typically based on vector genome titers. Particle titers are typically quantified using ELISAs, using a monoclonal antibody that is specific to the fully assembled capsid. Antibodies are typically serotype-specific. In research settings, sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by silver staining or Western blotting is still used. Since silver staining does not rely on antibodies, it is typically favored for the semi-quantitative assessment of VP1, VP2, and VP3 ratios and/or capsid degradation. Although the exact ratios on the single particle scale is stochastic [6], VP ratios that deviate from 1:1:10 tend to be attributed to poor vector titers and/or associated with reduced potencies [20]. More advanced methods based on high-resolution native mass spectrometry can obtain clearer pictures of differential VP ratios in preparations [6]; but how these differences impact transduction is still unexplored.

Detection of plasmid, host cell DNA contaminants, & adventitious virus

Demonstration of vector genome purity is one means of showing that the vectors being produced are free from risks associated with the transfer of foreign DNA. Foreign DNA can encompass any material originating from the production process. This can include DNA from backbone sequences, such as antibiotic resistance genes (e.g., β-lactamase) used

in the production plasmids, viral proteins originating from manufacturing schemes that use adenovirus vectors, and DNA that can originate from the packaging cell line. Importantly, detection of viral sequences not related to the production platform may signify the presence of adventitious viruses that can originate from animal serum found in cell culture media. Adventitious viruses can propagate during the manufacturing process and can elicit strong immune responses in patients, leading to adverse effects and lowered gene therapy efficacies. It should be noted that for commercial manufacturing, production schemes now typically use animal-derived component-free media, thereby limiting adventitious viruses.

The direct method for detecting DNA contaminants is via qPCR/ddPCR using primer/probes that target specific sequences. For example, to detect plasmid contaminants, primer/probes targeting the antibiotic resistance gene can be used; for targeting host-cell contaminants, 18S ribosomal RNA or Alu targets is routinely used [43]; and for adventitious viruses, a panel primer/probes that target a range of viral DNAs are employed [44]. However, PCR-based methods are inherently problematic, since low abundance contaminants can be hard to detect, even under exponential amplification. In addition, only known target sequences can be queried, limiting the detection of host-cell DNA and adventitious viruses.

Full versus empty capsids

A common assessment of vector quality has been the detection of empty capsids in preparations. Since the percentage of empty capsids in final preparations can range widely from 50–90% (depending on the purification method), they are large determinants of vector potency. Transmission electron microscopy is a classical way to visually observe and count the ratio of full-to-empty capsids [45]. However, it cannot reveal information on partial or oversized vectors (e.g., truncated genomes or genomes that exceed the design length). Analytical ultracentrifugation (AUC) can yield sedimentation velocities of particles and relies on the density profiles of empty and full capsids [46]. AUC can reveal species that can deviate from the main empty and full capsid peaks, which can typically point towards the presence of partial or oversized packaged genomes. Unfortunately, AUC cannot further characterize the genomes of these non-unit length species. Direct quantification of DNA and capsid proteins can be measured by optical density using A260/A280 [47]. This method also cannot describe vector genome heterogeneity for preparations. Charge detection mass spectrometry can quantify capsid content by measuring the mass-to-charge ratios unique to empty and full capsids [48]. AUC and charge detection mass spectrometry methods require high amounts of material, have long turnaround times, and require technical training and knowhow. Mass photometry is a fast and label-free orthogonal technique that was developed recently [49]. This technique can be used for multiple serotypes [49], and can also work with low amounts of sample [49]. Unfortunately, all analytical methods mentioned above still lack the capacity to characterize the genomes of non-unit length species nor describe vector genome heterogeneity.

Other recently developed analytical methods and advanced orthogonal approaches, such as size-exclusion chromatography with UV and multiangle light scattering can provide insights into vector genome heterogeneity [38,40,50]. However, these methods do not have the ability to disclose the structure or sequences of truncated or oversized forms, chimeric genomes, and the composition of DNA contaminants. These shortcomings inspired the development of a new class of methods that employ next-generation sequencing (NGS) technology. These bioinformatics-reliant methods have opened the door for gaining insights into AAV biology and vectorology, and have revealed

the types of structures that can be packaged into capsids that are impossible with other methodologies.

The remainder of this review will discuss the challenge of sequencing AAV, and the use of NGS in vector characterization and evaluation. We will also address some of the shortcomings related to these NGS-based methods.

THE CHALLENGE OF SEQUENCING AAV VECTORS

The wild-type AAV2 sequence was the first AAV genome cloned into plasmids [51], enabling genetic studies [52,53]. The ITRs of AAV2 were first sequenced in 1980 by the Maxam-Gilbert method [54]. Since then, sequencing the full AAV vector genome has been notoriously challenging. This problem has been mainly due to the complexity of the ITRs [55]. In addition, the ITR is GC-rich (70%), which makes standard methods like Sanger sequencing, difficult. Substitution of dGTPs with 7-deaza-dGTP during amplification of the ITRs can help to overcome sequencing issues related to GC content [56]. However, such methods are less than ideal. Until recent times, sequencing AAV vectors sans ITRs has been the staple in the AAV gene therapy field. Nevertheless, ITRs are critical for replication, rescue, and packaging; thus, further understanding of these crucial viral elements substantiates the need to develop robust means to sequence AAV vectors with their ITRs.

NGS-BASED METHODS FOR VECTOR CHARACTERIZATION & EVALUATION

Although many sophisticated methods have been used for assessing the AAV vector product, including those mentioned above, they are unable to provide comprehensive insight into the genome compositions of truncated vectors and DNA contaminants (e.g., plasmid backbone DNA, *rep-cap* genes, and adenovirus helper genes) [27,57–59]. Replication-competent AAVs are also another form of contaminant that can alter the safety of gene therapy vectors [60]. Profiling of packaged content in a population of diverse, and low-abundance species, remains challenging with standard methods like qPCR/ddPCR. NGS has been used widely in many disciplines, but has only recently gained use for characterizing and evaluating AAV vectors. NGS-based methods have the ability to reveal the contents of vectors at the level of the DNA sequence, and can identify contaminants that cannot be captured by standard molecular biology methods. Furthermore, it can detect/identify multiple contaminants in a single experiment, as opposed to using multiple molecular assays. Since NGS can achieve read depths of millions to hundreds of millions of sequences, rare DNA species can be semi-quantitatively profiled, and with certain platforms (discussed below), can be quantitatively assessed [57,61].

Since there are no standardized methods to sequence AAV vectors using NGS, investigators usually look for the most efficient way that is best fit for their research goals. Employment of NGS approaches can depend on different factors, such as budget, time sensitivity, accuracy of the results, and technical proficiency.

Short-read sequencing technology & next-generation sequencing-based methods

Illumina is well-known for its popular short-read sequencing technology [62–64]. It is based on a sequencing by synthesis approach that employs cyclic reversible termination [62,65,66]. Currently, Illumina is still the most popular NGS solution [65,67–69]. It has several advantages, including its established technology, high level of cross-platform compatibility, high accuracy, and a wide range of instruments that span low-throughput to high-throughput options [65,67]. However, Illumina has some drawbacks, including its short-read lengths,

high instrument costs, some poor coverage across GC-rich regions, and a tendency towards substitution errors [65,70].

Unfortunately, short-read sequencing has poor coverage at the ITR regions, and fails to capture the full and intact AAV vector genome [27,58]. Nevertheless, they are best for detecting single-nucleotide variants (SNVs) and insertions and deletions (indels), because of their ability to achieve high sequencing depths; and for detecting low-abundance contaminants.

Single-stranded DNA virus sequencing

Single-stranded DNA virus sequencing (SSV-Seq) was the first NGS-based method developed for characterizing AAV vector genomes and residual DNA [57,61], and was developed to address the shortcomings of qPCR. The SSV-Seq method is based on Illumina's short-read sequencing technology. The major steps in SSV-Seq protocol are as follows. First, the preparation is treated with DNase to digest non-encapsidated DNA. Second, DNA extraction is performed, followed by second-strand synthesis with random hexamers to convert ssAAV to double-stranded genomes. Subsequently, the dsDNA template is sonicated into small fragments for NGS library preparation. Next, libraries are sequenced with Illumina HiSeq. Finally, the sequencing data are analyzed by using ContaVect bioinformatics tool. **Figure 1** summarizes the SSV-Seq protocol [57]. A PCR-free version of the method called SSV-Seq 2.0 was also developed for optimizing vector genomes with a high percentage of GC and homopolymers [71]. Although SSV-Seq is successful at characterizing AAV vector genomes including residual DNA, the major drawback of this method is its inability to interrogate full and intact vector genomes. Another limitation is the amount of purified rAAV preparation required for input (2×10^{11} vector genomes of purified rAAV). Furthermore, SSV-Seq cannot provide optimal coverage of the ITRs, since Illumina's short-read sequencing requires amplification of the target using polymerases that have low processivity across the ITRs either at the library preparation stage with PCR, or on the flow cell during bridge amplification steps.

Fast-Seq

The development of Fast-Seq was inspired by the limitations of the traditional Sanger method [72], which requires slow and labor-intensive manual evaluation of sequencing reads. Importantly, the Sanger method it is unable quantitate single nucleotide polymorphisms (SNPs) and indels as a result of low sequencing depths. Fast-Seq relies on a Tn5-based library generation that is compatible with single-strand (ss)AAV genomes [72]. The Fast-Seq approach is an end-to-end method for the extraction, purification, sequencing, and data analyses of packaged vector genomes [72] **(Figure 1)**. Fast-Seq's reliance on fragmentation and simultaneous adapter ligation using Tn5 transposase is inexpensive and relatively easy compared to sonication followed by adapter ligation. In addition, it requires less input DNA, which makes it well-suited for inexpensive and lower throughput instruments, such as MiSeq and iSeq. Furthermore, Fast-Seq provides opensource code with a prebuilt customizable Docker container on GitHub for data analysis. However, Fast-Seq also requires double-stranded genome conversion and it can miss single-stranded genomes that fail to convert. Because it is based on short-read sequencing, it inherits all the limitations described above for SSV-Seq. In addition, Fast-Seq was designed primarily for analyzing variants such as SNPs and indels, but not for analyzing contaminants.

Viral genome sequencing

Viral genome sequencing (VGS) was developed with the aim to overcome the double strand synthesis requirement from the other

NGS-based methods [73]. The VGS method is based on the assumption that rAAV DNA extracts are primarily double-stranded species due to the natural base pairing of complementary plus and minus strands [73]. VGS also utilizes a tagmentation-based library construction approach (Figure 1), and was designed to profile the rAAV genome, as well as detecting the presence of contaminants [73]. Because VGS bypasses the double-strand synthesis step, VGS can save time and costs related to sample preparation. In addition, VGS provides Python scripts for validating serotype and Cre-independent DNA recombination events in rAAVs. However, VGS may miss some single-stranded genomes, because its design is based on the assumption that double-stranded configurations are naturally formed from annealing of plus and minus stranded genomes after DNA extraction. VGS also inherits the limitations associated with Illumina short-read sequencing.

Long-read sequencing technologies & NGS-based methods

For many years, the major limitation with using NGS to sequence AAV vectors has been the need to rely on reconstruction of the genome from small read fragments. Although the approach can be useful in determining SNPs and indels, it fails to reveal the structures of the genomes. AUC analyses and gel electrophoresis can reveal the heterogeneity in vector preparations; however, cannot provide sequence information. An NGS approach that can produce reads that capture targets spanning the entirety of the vector genome would be ideal.

In 2009, the first single-molecule sequencing technology was developed and commercialized by Helicos BioSciences [64]. This approach permitted single-molecule representation of AAV for the first time [74]. However, single-molecule sequencing could not achieve complete coverage of the AAV genome. Fortunately, two sequencing technologies were maturing.

Pacific Biosciences and AAV genome population sequencing

Pacific Biosciences (PacBio) is well-known for its long-read sequencing technology called single molecule, real-time (SMRT) sequencing [64]. This technology has the advantages of achieving long-read lengths (approximately 10–20 kb) and shorter instrument execution times [64,70]. However, its accurate base calling is dependent on the consensus reads of multiple passes across a target template. Therefore, the longer the read fragment, the lower the base calling accuracy. The technology also has high operational costs [70]. Coupled with AAV genome population sequencing (AAV-GPseq) [58], SMRT sequencing can accurately profile genomes that are in double-stranded configurations. Double-stranded genomes can be achieved by annealing plus and minus stranded genomes by heat-treating and slow cooling the rAAV genomes (thermal annealing) [59]. The steps for preparing samples for AAV-GPseq is summarized in Figure 1.

Due to the advantage of covering long sequences in a single read, AAV-GPseq has opened the door for gaining insights into the composition of vector genomes, as well as other packaged elements in the vector product that would be elusive with other analytical methods. The significant feature of AAV-GPseq is its ability to capture the intact vector genome from ITR-to-ITR without the need for bioinformatic re-construction from short reads. AAV-GPseq also requires a significant amount of purified vector genomes for input (1×10^{11}–1×10^{12} vector genomes). Because AAV-GPseq requires the ligation of the SMRT bell adapters to double-stranded genomes, this method can also miss the single-stranded genomes that fail to anneal into double-stranded targets. Due to the lower sequencing depths achieved by SMRT sequencing, the accuracy of SNV and indels is lower than can be achieved with Illumina short-read sequencing. In

contrast, SMRT sequencing can capture full and intact AAV vector genomes, and can cover the ITR regions, since the phi29-derived polymerase has strand-displacement activity, and sequencing in real time efficiently unwinds the ITR structure. High base calling is achieved through multiple passes of adaptered genomes. This overcomes the inherent error of single passes, yielding base calling errors that are approximately 1%. Nevertheless, due to the nature of its flow cell design, its sequencing depth is relatively low (approximately 5–8 million reads can typically be obtained on a Sequel II). Another shortcoming for SMRT sequencing is its bias towards smaller DNA targets. Typically, SMRT reads need to be normalized to a spike in standard ladder such as BstEII-digested lambda phage DNA or calibrated on fragmented bacterial DNA in order to assess relative abundances [58,75].

Oxford Nanopore & ssDNA sequencing

Another well-known long-read sequencing technology is nanopore sequencing. Oxford Nanopore technology can produce the longest read lengths (approximately 2 Mb) [64,67,76]. The MinION instrument is also small and portable, and can be operated with a laptop computer. Running samples using nanopore is quick, relatively easy, and has lower operational costs [70,77]. However, it has high error rates [64,70]. Nevertheless, nanopore sequencing has high processivity through ITRs and it can directly sequence AAV vectors without amplification [78].

The inspiration for developing ssDNA sequencing was to overcome the need to convert single-stranded DNA (ssDNA) into double-stranded templates, which is a prerequisite for existing NGS-based methods. This conversion can again cause bias [78]. Since nanopore uses a transposase that was found to have residual activity on ssDNA, ssDNA sequencing with nanopore bypasses the need for double-strand conversion of the AAV genomes [78]. However, the efficiency of sequencing double-strand templates was still shown to be much higher than ssDNA. Furthermore, similar to the AAV-GPseq method, AAV genomes can be converted to double-stranded templates and sequenced directly as an intact molecule from ITR to ITR [77]. ssDNA sequencing can also detect contaminants and it can reveal the molecular state of vector genomes [78]. Nanopore sequencing has similar capabilities to SMRT sequencing as a long-read technology, but since DNA strands are only covered through a single pass of the DNA, the accuracy of base calling at each position is low [77]. An illustration of the ssDNA sequencing workflow is shown in Figure 1.

TYPES OF NON-UNIT LENGTH GENOMES FOUND AMONG AAV VECTORS

With the development of NGS methods to profile rAAV, the diversity of non-unit vectors has been revealed. In addition, some of the mechanisms by which they arise are being slowly solved.

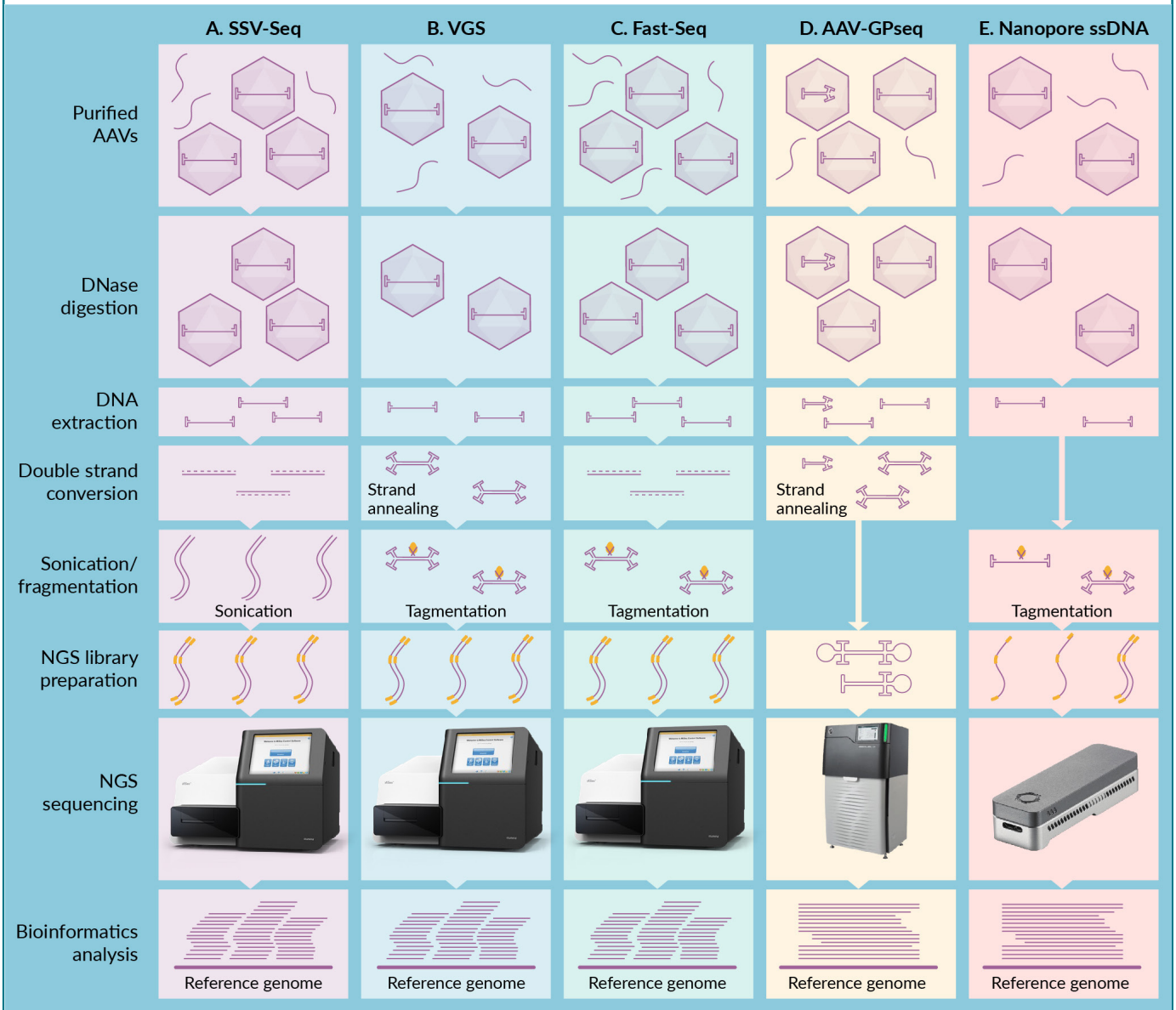
Furthermore, vectors produced by different platforms can have varying degrees of heterogeneity. For example, AAV-GPseq has revealed a diversity of vector genomes including completed genomes, truncated genomes, chimeric genomes, and oversized genomes [27,58,59] (Figure 2). The following section will review commonly identified non-unit length genomes.

Truncated genomes

Truncated AAV genomes were first described with wtAAVs as a hallmark of defective interfering particles [79,80]. Previous studies on the incorporation of short hairpin (sh)RNA or short hairpin-like structures into vector constructs showed that they can lead to truncated vector genomes that have self-complementary configurations. These types of genomes are also commonly called snapback genomes [79,81]. The mechanisms that underpin these events are hypothesized to be due to polymerase redirection or template-switching during viral

FIGURE 1

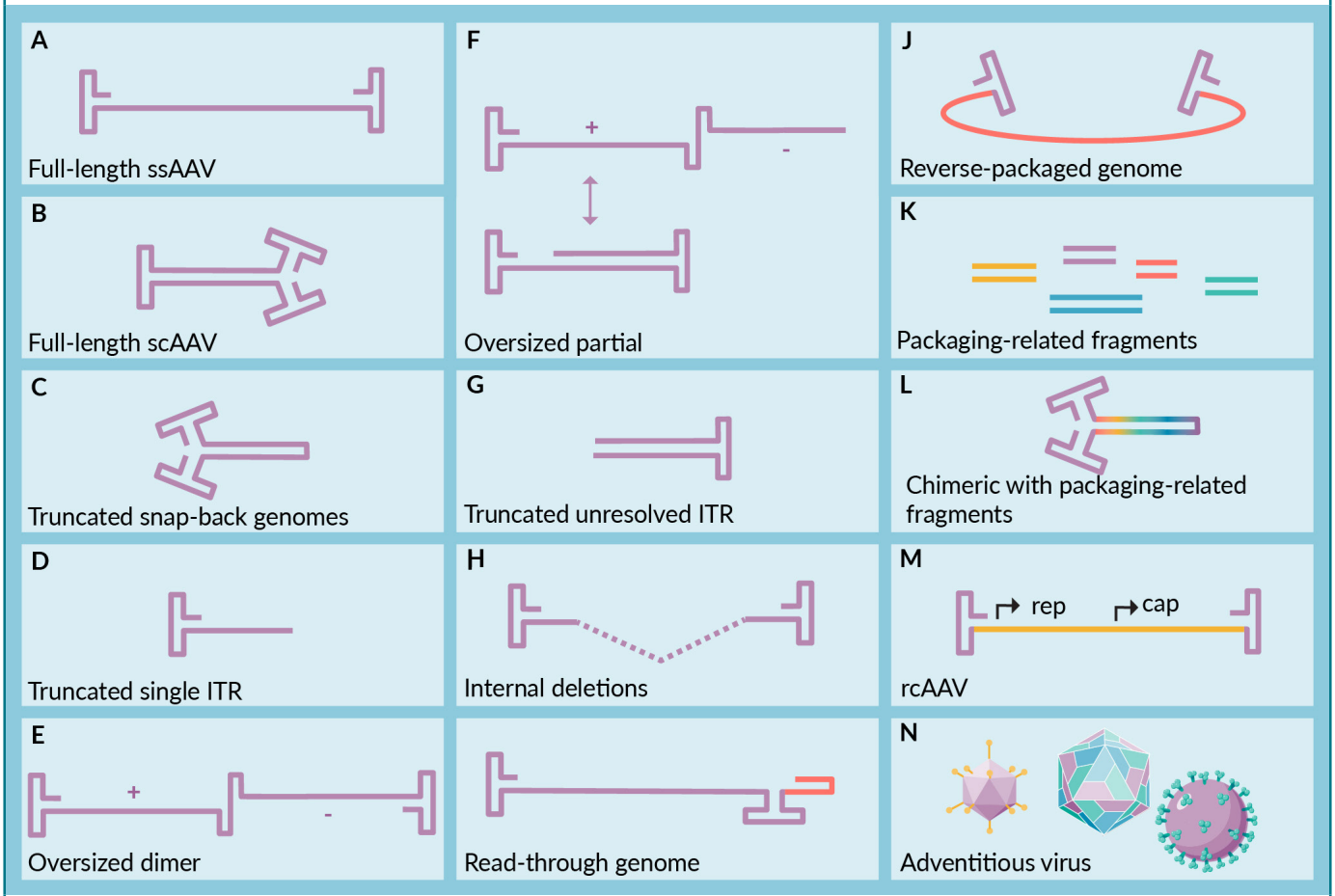
Workflow illustrations for NGS-based methods.



(A) SSV-Seq protocol [57]. The purified particles are digested to remove non-encapsidated DNAs. Next, viral DNAs are extracted and then subjected to second-strand conversion. The double-stranded genomes are then sonicated into fragments for Illumina library preparation. The libraries are then sequenced on an Illumina instrument. Finally, the sequencing data are analyzed by using ContaVect bioinformatic tool. (B) VGS workflow [73]. Purified particles are digested with DNase. Next, viral DNAs are extracted and then subjected to library preparation with tagmentation. Libraries are then sequenced with Illumina MiSeq. Finally, the sequencing data are analyzed with Geneious software and custom Python scripts. (C) Fast-Seq workflow [72]. The purified particles are digested with nuclease treatment. Viral DNAs are then extracted from purified particles followed by second-strand conversion. Double-stranded genomes are fragmented and immediately adaptered by tagmentation with Tn5 transposase. Libraries are sequenced by MiSeq, iSeq, MiniSeq, NextSeq, etc. Lastly, the sequencing data are analyzed with opensource code and a prebuilt customizable Docker container on GitHub. (D) AAV-GPseq workflow [59]. Purified particles are digested by using DNase I treatment. Following digestion, viral DNAs are extracted by using phenol/chloroform. Vector genomes then go through second-strand conversion with heat treatment and cool annealing. Next, vector genomes are prepared for sequencing with SMRT sequencing. Lastly, the sequencing data are analyzed by using custom bioinformatics pipelines. (E) Nanopore ssDNA sequencing workflow [78]. Purified particles are digested with Benzonase nuclease, followed by vector DNA extraction. Next, vector genomes go through nanopore library preparation, which includes adaptering by tagmentation and then sequencing. The sequencing data are then analyzed by using custom bioinformatics pipelines. (NGS: next-generation sequencing; SMRT: single molecule, real-time; SSV-Seq: single-stranded DNA virus sequencing; VGS: viral genome sequencing.)

FIGURE 2

Diagrams of different types of non-unit length genomes and DNA contaminants.



(A) Full-length single-strand (ss)AAV. (B) Full-length self-complementary (sc)AAV. (C) Truncated or snapback genomes (SBG). (D) Truncated genomes with a single ITR. (E) Oversized dimer genome with an unresolved ITR. (F) Oversized partial dimer with an unresolved ITR. (G) Truncated genome with a single unresolved ITR. (H) Truncated genome with deletion between the two ITRs. (I) Read-through genome carrying backbone DNA in red. (K) Reverse-packaged genome carrying backbone DNA in red. (K) DNA fragments from packaging components (ITR-free) (L) Chimeric genome carrying non-vector DNA from multiple fragments following recombination. (M) Replication-competent (rc)AAV with intact *rep* and *cap* ORFs recombined with flanking ITRs. (N) Representation of adventitious viruses. ITR: inverted terminal repeats; ORF: open reading frame.

genome replication [82]. Another means of snapback formation is due to DNA damage [83]. Additionally, truncated genomes are also present in wild-type AAV genomes (Figure 2C) [81]. Truncated genomes with a single ITR can also be observed, but these are rarely identified by NGS. Also directed-repeats are also predicted to cause internal deletions in AAV vectors, but these are also not well represented in sequencing data (Figure 2H).

Oversized genomes

Oversized genomes are those that go beyond the single-unit length of the ITR flanked construct. This type of genome can be a result of abnormal packaging of vector genomes in production or those that are produced from transgene cassettes that are shorter than approximately 5 kb, and are packaged with unresolved ITRs. These types of genomes have been observed with particularly high frequencies with vectors produced by the rBV/Sf system [27], and if designed to exactly half the packaging limit of AAV will form dimers and self-complementary AAV vectors (Figure 2E). Truncated genomes have also been detected with AAV-GPseq from unresolved ITRs. This type of truncated genome was found to be predominantly produced by

rBV/Sf production system, and can possibly arise from partial oversized genomes that have undergone cleavage during library preparation steps (Figure 2F,G) [27].

TYPES OF VECTOR CONTAMINANTS

As mentioned above, overcoming the limitations of qPCR in characterizing contaminants in vector product was among the motivations for the developing NGS-based methods to sequence rAAV preparations. As a result, long-read sequencing in particular has helped to reveal a diversity of packaged genomes that can wind up in manufactured rAAV. This section will review the different types of contaminants that are known to exist.

Vector backbone DNA

The most dominant DNA contaminant comes directly from the vector backbone itself. The vector backbone refers to the construct that houses the ITR-flanked vector genome. For platforms utilizing plasmid transfections into producer cell lines, the auxiliary elements within the vector plasmid, also referred to as the *cis* plasmid, would be considered the backbone. In rBV/Sf systems, the recombinant baculovirus vector would be considered the backbone. SSV-Seq has shown that backbone contaminants can range from 0.84–5.97% with different purification techniques [57]. Identification of these types of contaminants are critical. For example, in plasmid-based platforms, antibiotic-resistance genes such as KanR and AmpR may be transferred to patients, potentially increasing risks related to the spread of antibiotic resistance in microbes or hypersensitivity to antibiotics in some patients [84].

Backbone contaminants can originate from read-through genomes. These genomes are packaging events that are characterized by the encapsidation of DNAs that extend beyond the ITR and into the backbone sequence (Figure 2I). Backbone DNA can also be packaged via reverse-packaging events, whereby genomes are packaged from ITR-to-ITR but encompass product that exclusively spans the backbone (Figure 2J).

Helper DNA

Helper gene contaminants encompass those originating from the helper plasmid. This type of contaminant is less common than vector plasmid contaminants. The common targets for observing contaminant include viral associated RNA, *E2* genes, *E4orf6* gene.

rep-cap DNA

rep-cap DNA contaminants are related to the AAV *rep* and *cap* genes. These genes are required for replication and packaging of the vector genome. These contaminants are also less common, but can be problematic for preparations, as they can hint at the presence of replication-competent (rc)AAV (see below). Expression of Cap can also lead to immune responses in the target tissue, resulting in the loss of transduced tissues.

Host-cell DNA

Host-cell (hc)DNA contaminants are infrequent, but can be problematic. Packaging of promoter sequences or full open reading frames can be transferred to the patient with unknown consequences. However, previous studies have shown that host-cell contaminants are higher in mammalian cell production platforms than with insect cell platforms [34]. It has been hypothesized that regions that bare motifs with sequence similarity to RBEs and are in open regions of chromatin are preferentially packaged.

Chimeric genomes

Typically, contaminants described above are packaged into AAV capsids because they may contain sequence similarity to the RBE

[85], or are packaged passively as fragmented DNA (Figure 2K). However, AAV-GPseq has revealed the presence of chimeric genomes [58], which are contaminants that are contiguous with ITRs, and result from recombination events (Figure 2L). Chimeric genomes would therefore be actively packaged into capsids via the packaging signal within the ITRs. To detect these species, it is important to employ A-tail adaptering methods, whereby the AAV genome is end processed to carry an A-tail, and the SMRTbell adapter is T-tailed. This eliminates false-positives from fragment-to-fragment ligation.

Replication-competent AAV

The formation of replication-competent (rc)AAV is a result of recombination events between the ITR in vector plasmid with the *rep* and *cap* genes during vector production (Figure 2M). These recombination events that generate intact, replicative, and potentially infectious virus-like virions are thought to occur randomly and without sequence specificity [60]. This type of contaminant is very rare and must be detected following amplification in cells in the presence of a helper virus. SMRT sequencing and AAV-GPseq uncovered a diversity of recombination events that provide insights into how rcAAVs can emerge [60].

Adventitious viruses/pathogens

As described above, the detection of adventitious virus by qPCR/ddPCR is complicated by the fact that one has to have foreknowledge of the viral contaminant. With NGS, reads that fail to map to the provided user-defined references can be used to megablast to viral genomes in order to detect any potential viruses in the rAAV preparation (Figure 2N). This approach was taken to validate the purity of plasmid DNA used to generate the vectors used in the first-in-human IND trial for Tay-Sachs disease gene therapy [86].

ITR HETEROGENEITY: TRUNCATIONS, MUTATIONS, & DELETIONS

The wild-type AAV2 ITR is widely used in most vector constructs. SMRT sequencing has also permitted the interrogation of ITR heterogeneity [87], allowing for a more comprehensive understanding of the ITR composition in plasmid DNA and in the vector product. Uncovering the ITR composition is crucial for validating vector design, as well as improving vector quality. Furthermore, there is a correlation between ITR configuration and vector heterogeneity [27]. ITR truncations can occur in different vector production systems [27]. The ITR structures are inherently unstable in the bacteria used in plasmid production and during baculovirus replication [88,89]. The truncation can vary and they can bear several configurations [27]. Deletions can also occur in any region of the ITR. As a result, ITRs can lack the B arm, C arm, or both B and C arms. Trident-shaped ITRs can also result from errors in ITR replication [27]. There is a strong correlation between mutations in ITRs with unresolved AAV genomes, which can lead to higher degrees of heterogeneity [27]. Intriguingly, the phenomenon of ITR repair, which presumably occurs through the copying of the opposing intact ITR, was verified by AAV-GPseq [59].

USE OF NGS IN AAV POST-ENTRY EVENTS

The use of NGS platforms to interrogate the composition of AAV vectors has led to a better understanding for vector integrity, heterogeneity, and risk. However, many of these new concepts have yet to be linked to any functional knowledge related to the potency and safety of vectors. Another significant role that NGS has played in the field of AAV biology and vectorology has been its support in investigating AAV integration. Wildtype (wt)AAV has long been known for its ability to integrate into the human genome following infection. Classical

studies have shown that wtAAV can integrate into several genomic locations including the well-known AAVS1 site on human chromosome 19q13.42 [90–93]. In the early years of AAV integration analysis, molecular methods such as PCR and Southern blot were used to detecting integration events [92,94,95]. Later investigations become more comprehensive, as a result of advancements in NGS approaches [90,96–100]. The integration of AAV2 in host cell genome has been studied extensively, as a result of the concerns for hepatocellular carcinoma found in AAV-positive patients [15,101–104]. In these studies, advanced molecular method and notably high-throughput sequencing have been used for detecting and analyzing AAV integration sites. Recent efforts to understand this potential link continue to reveal aspects of AAV biology that were previously unknown [15,103–106]. Most recently, a comprehensive analyses of human and non-human primates tissues using target-enrichment sequencing and NGS have shown that wildtype AAV and recombinant AAV show preferential integration of respective viral and vector genomes into and near gene bodies of highly expressed genes [107,108]. Such studies have shed more light into the biology and consequences of AAV in gene therapy applications.

Although AAV vectors are considered safe, studies in rodents have shown that AAV vector integration can lead to oncogenesis [97]. At present, there is no evidence that AAV vector integration can cause oncogenesis in humans; although, the FDA now recommends long-term follow-ups after AAV administration. Furthermore, integration analyses in mouse or non-human primates (or other relevant large animal models) are required as part of pre-clinical evaluations of vector safety. For example, a recent long-term study in dogs treated for hemophilia A identified clonal expansion of transduced liver cells [96].

LIMITATIONS & FUTURE DEVELOPMENT OF NGS-BASED METHODS

Unfortunately, there are no standards or universal means of manufacturing AAV. There are also no standardized and universal methods for assessing vector quality control. Recent guidelines from the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), section Q5A(R2) EWG, indicate that NGS is appropriate for viral safety evaluation of biotechnology products derived from cell lines of human or animal origin. Specifically, this pertains to the detection of adventitious viruses. Due to the sensitivity of assay and the breadth of virus detection, NGS can also be used for replacing cell-based infectivity assays [109]. Current NGS-based protocols used to profile AAV have inherit all of the advantages and disadvantages of their adopted NGS sequencing technologies. Therefore, comprehensive interrogation of AAV requires the adoption of both short and long-read sequencing technologies. Long-read sequencing technologies can provide full coverage at the ITR regions to allow a more complete characterizing and understanding of the ITRs in plasmid as well as in vector product. On the other hand, short-read sequencing is more accurate for detecting indels and SNVs in vector genomes. Short-read sequencing can also allow for the detection of very low abundance contaminants. An approach that can encompass the best of both worlds, can achieve reliable quantification of heterogenous populations without biases, requires less genomic input, and can be easily adopted, remains an aspiration for the field. Until such a technique is developed, a combination of long- and short-read techniques may be the most ideal approach for obtaining a complete genomic profiling of AAV-based gene therapy vectors.

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CELL & GENE THERAPY INSIGHTS

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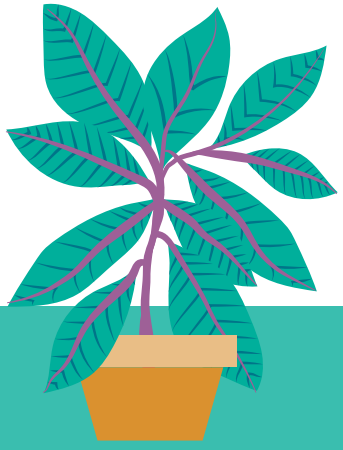

CELL & GENE THERAPY INSIGHTS

INTERVIEW

Creating pathways to necessary innovation in cell and gene therapy

The persistent challenges in the cell and gene therapy space need creative solutions. **David McCall**, Senior Editor at ***Cell & Gene Therapy Insights***, speaks with Nobel laureate and President Emeritus of the California Institute of Technology, **David Baltimore**, about how to successfully foster R&D innovation.

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Q What are you working on right now?

DB: I am retired, so I do not have a laboratory anymore. The structure of my life that was so well established over more than 50 years of research is now in the past. However, I am now working on writing some autobiographical material, and consulting with nonprofit organizations as well as advising various companies. I am leading a very different life compared to five years ago.

Q Cellular immunotherapy developers are finding solid tumors a particularly tough nut to crack—what might be some key next steps for the field to take?

DB: There is some uncertainty about exactly why solid tumors are not responding to immunotherapy–whether it has to do with the tumor microenvironment or things that the tumors secrete…it is all a little uncertain, and so it is hard to know where we are going to go from here. What is happening at the moment is that people are trying everything they can think of—using different kinds of cells, making CARs from different materials, etc.—to see if anything does a better job than the canonical approaches.

Q When you consider the ever-expanding enabling technology toolkit available to cell and gene therapy researchers today, what stands out for you as being potentially the most significant weapon in the battle to understand and overcome the immunosuppressive tumor microenvironment? And what would you like to see coming down the tools innovation pipeline next?

DB: The tools that we have now are not individual solutions—they are contributions to the overall kit that enables research scientists to probe many different kinds of issues. It is a combination of tools that will make a difference. For example, AI is terrific, but I don't think we understand it well enough to know what its limitations are. We have got to keep digging. We must find new ways of building the therapeutic armamentarium to undo the danger that is presented by solid tumors, and tumors in general.

Q How significant will mRNA therapeutics be moving forward, particularly in the cancer space? And what key challenges must firstly be addressed?

DB: We have not seen sufficient investigation of the limitations of mRNA therapies yet. One hint that there are limitations is the relatively short period of effectiveness of mRNA vaccines. They seem to wear off faster than other kinds of vaccines, and we need to understand why that is. It may be that in the long run, mRNA vaccines are a good way of testing vaccines or a good way of delivering them to patient populations to get a quick read on their effectiveness, but they may not be the most effective way of delivering vaccines in the long-term. Some of the more traditional ways of delivering vaccines may turn out to be better.

It may be that we need to go back to the drawing board and develop really long-term effective therapies because with cancer, you cannot allow a single tumor cell to escape.

Q As a high-profile participant in the ethical debate around the advancement and utilization of novel genome editing tools, how do you view the current landscape? Are your concerns around the field assuaged?

DB: No, I think we have only seen the beginning of this development of new ways to modify inherited genomes for therapeutic effect. As innovation continues, we have to keep asking ourselves: are we keeping the treatment under appropriate control, or is it going to become so easy and so widespread that you cannot control it? We do not generally try to control medicines on ethical grounds, but we do on safety grounds. However, as we are getting deeper and deeper into the genetic inheritance of our population, we have to think more and more carefully about what we are doing with the growing knowledge and power to alter it.

Q Can you distil for us some key learnings relating to successfully fostering R&D innovation that you have derived during your storied career?

DB: My secret weapon is finding really creative people and giving them their head. People who are successful often let it go to their heads, and then they look for people they can pass on their insights to. I do not believe in all of that. I really believe that what you want to do is to encourage people to think on their own and to find their own way, particularly young people breaking into the field. Ultimately, they will make the largest contributions to science if they are supported in their work but given the freedom to go in their own directions. That approach has worked very well for me over the years.

Q What issues do you see when you look at the cell and gene therapy research environment today?

DB: What is happening today is that, at least in the US, research is becoming increasingly surrounded by guardrails that limit a researcher's ability to try new approaches. For instance, researchers spend an enormous amount of time writing grant applications or filling out other forms. All of that needs to be looked at from the point of view of what encourages creativity best, because biologics researchers are dealing with really difficult problems. We really need to optimize the brain power that is allocated to these questions, but instead, investigators increasingly find themselves tied up in bureaucratic knots.

After the challenge of bureaucracy, the biggest issues that we have right now are the cost of cell and gene therapy, and the challenge of delivering therapy to defined cell types in the body. We need to find cheaper and more precise methods of delivery. I am optimistic that within the next decade, we will find really creative ways of delivering genes that are both cheap and manipulatable in terms of the target cell population.

BIOGRAPHY

DAVID BALTIMORE served as President of the California Institute of Technology from 1997 until 2006. He is currently President Emeritus and Judge Shirley Hufstedler Professor of Biology at Caltech. Awarded the Nobel Prize in 1975 for research in virology, Baltimore has profoundly influenced national science policy on such issues as recombinant DNA research and the AIDS epidemic. He is an accomplished researcher, educator, administrator

and public advocate for science and engineering and is considered one of the world's most influential biologists.

For almost 30 years, Baltimore was a faculty member at Massachusetts Institute of Technology. Baltimore served as founding director of the Whitehead Institute for Biomedical Research at MIT from 1982 until 1990. An early advocate of federal AIDS research, he co-chaired the 1986 National Academy of Sciences committee on a National Strategy for AIDS and was appointed in 1996 to head the National Institutes of Health AIDS Vaccine Research Committee. Baltimore served as a member of the Independent Citizen's Oversight Committee to the California Institute for Regenerative Medicine until 2007 and on the Board of Directors for MedImmune until 2007, Cellerant until 2008, Calimmune until 2017, Amgen until 2018 and Immune Design until 2019.

He has played an important role in the development of American biotechnology since his involvement in the 1970s, in the formation of collaborative genetics. He helped found Calimmune and Immune Design and he presently serves on the Board of Directors at several companies and non-profit institutions including Altos, Labs, Appia Bio, the Broad Institute, and Regulus Therapeutics. He is a member of numerous Scientific Advisory Boards, including the Broad Institute, PACT Pharma, Ragon Institute, Regulus Therapeutics, Vir Biotechnology and Volastra Therapeutics. He is a Scientific Partner to the venture capital firm, The Column Group, and was a Director of the Swiss investment company BB Biotech through 2011.

Baltimore's numerous honors include the 1999 National Medal of Science, 2000 Warren Alpert Foundation Prize, and 2021 Lasker-Koshland Special Achievement Award in Medical Science. He was elected to the National Academy of Sciences in 1974 and is also a fellow of the American Academy of Arts and Sciences, and a foreign member of both the Royal Society of London and the French Academy of Sciences. He is past-President and Chair of the American Association of the Advancement of Science (2007–2009) and was named a Fellow of the American Association for Cancer Research (AACR). He has published more than 700 peer-reviewed articles.

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CELL & GENE THERAPY INSIGHTS

INTERVIEW

US FDA Perspective: preparing for a bright and busy future for cell & gene therapy

Peter Marks

As a year marked with more ground-breaking advanced therapy product approvals and new guidance from regulators comes to an end, **David McCall,** Senior Editor of *Cell & Gene Therapy Insights*, talks to **Peter Marks,** Director of the Center for Biologics Evaluation and Research at the US FDA, about his reflections on talking points and progress made in cell and gene therapy over the course of 2023, and his plans and priorities for the agency's future activities through 2024 and beyond.

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Q How would you sum up 2023, both for Center for Biologics Evaluation and Research (CBER) and for the cell and gene therapy space as a whole?

PM: Although there has been concern in the community about contraction of investment in the rare disease gene therapy space, it has really been a remarkable year for CBER in the area of cell and gene therapy. We approved our first gene therapies for hemophilia A and Duchenne muscular dystrophy, as well as a gene therapy for certain types of dystrophic

epidermolysis bullosa. We have also approved innovative cell therapy products, including modified cord blood cells for use in stem cell transplantation to reduce the risk of infection, and allogeneic pancreatic eyelet cells to treat patients with type 1 diabetes who have hypoglycemic unawareness.

Overall, we see the pace of progress in the field accelerating. We hope that our actions in leaning into gene therapy and cell therapy development and approvals will address some of the issues that have led some to exit the area, particularly in the area of rare disease gene therapy.

Q How have the twin challenges for CBER around rapidly increasing workload in the cell and gene therapy space and staff sourcing, development, and retention evolved over the past 12 months?

PM: Indeed, we continue to see the number of submissions of investigational new drug applications, Biologics License Applications (BLAs), and supplements all increasing. Plus, the desire for people to have meetings with the agency continues to grow. However, we did anticipate this, and have been on a hiring mission to get sufficient new people on board. Some of this activity has been helped by the fact that the contraction that has occurred recently in the gene therapy space has made it easier to attract people with CMC expertise to the agency. So, I think we have been able to make some headway there, and we will continue to focus on staffing up. We also are benefitting from the reorganization of the Office of Tissues and Advanced Therapies into the Office of Therapeutic Products—a 'super office' in which we now have an office entirely devoted to gene therapy CMC as well as one completely focused on cell therapy CMC. That allows us to be more attractive to those who may want to work with a group of like-minded individuals. It also helps us with having better supervisory to staff ratios, better consistency, and improved responsiveness. I think this has all worked well so far—we will aim to keep it up as we move into 2024.

Q Can you comment on an issue that is currently being felt by regulatory agencies around the world: the loss of key staff members with decades of experience in the cell and gene therapy field?

PM: It is normal to have people with a great deal of experience retire. They do leave a hole when they depart the agency, but that said, we are lucky in that we have many people at the mid-career stage who are ready to move into more senior roles. I think some of those people bring with them a different vision. Perhaps because of their own experiences in this field, they may have a different idea of how to drive cell and gene therapy forward, and I think we can build off that. I also think that the opportunity is there for us to recruit for more people who are both genuinely interested in the field and in coming to work at a regulatory agency.

And although it is hard to see colleagues who have been at the agency for a long time retiring or moving on, it does present us with an opportunity to essentially rejuvenate ourselves with people, some of whom have come of age in a different era. For example, we are now recruiting people who have come of age during a molecular biology-focused period. I straddled two eras—I began my training in the biochemistry era prior to the genetic revolution,

and then had to retool to become a molecular biologist. Molecular biology was around then, of course, and I am not saying I am a dinosaur, but there are now people coming in for whom gene therapy is something that they trained in specifically. I believe it is an exciting time to be recruiting people because many of them have grown up seeing cell and gene therapy as a field of enormous promise.

Q The FDA Office of Therapeutic Products Town Hall on Nonclinical Assessment of Cell and Gene Therapy Products is a recent example of the FDA's ongoing efforts to help sponsors by expediting the pathway to first in human clinical trials. What would you pick out as the key 'take-homes' for cell and gene therapy developers from that meeting?

PM: I have to acknowledge my CBER colleagues here, who have helped me pick out a few key take homes. These include first, that animal model and species selection for the nonclinical studies that are performed should be based on the biological relevance of the intended patient population, and the ability of those models to detect potential toxicity for a given cell and gene therapy product. A second item is that, in cases where the evaluation of the intended clinical product would not be informative in the selected species, testing of an analogous product to the clinical product may be a suitable alternative. In other words, sometimes you might not be able to test the clinical product because you would get a meaningless result, so an analogous product that could provide you with a meaningful result would be appropriate. A third is that specific toxicology and biodistribution study design elements, and things such as study duration, will depend on the persistence and safety profile of the specific cell and gene therapy product in question. These elements are ideally discussed with the agency at a pre-investigational new drug applications meeting.

Finally, we would encourage product developers to explore opportunities to develop alternative testing methods or leverage existing data from related products that can reduce the use of animal studies. Again, these are proposals that sponsors should ideally discuss with us in the early stages of development.

Q There is much anticipation around the new guideline on comparability for cell and gene therapies that is due to come out in the summer of 2024—what are your expectations in terms of how it will help therapeutic developers and manufacturers meet CMC requirements moving forward?

PM: We are excited about comparability guidance because we feel that it is addressing a very important issue in the cell and gene therapy area. The issue is that often, early in development, people use generation one processes that are highly suitable and appropriate based on our current guidance in order to get started and obtain some data on their cell or gene therapy. Then, later on, they have to move to a commercial process. Sometimes bridging that gap and demonstrating comparability is a challenge.

It is our hope that by providing robust guidance in this area, we can help sponsors make plans for how they will move forward from the outset, so that ultimately, we avoid delays at the end of development as people try to catch up and show that the products with which they have completed their pivotal studies are the same as those with which they originally started. I think this will be a really important guidance for industry—we view it as one of the most important things that we see in manufacturing at this point.

Q What will be some other key areas of focus for the FDA in terms of developing new guidance for the cell and gene therapy field over the next 12 months?

PM: We will be spending a fair amount of time and effort on trying to accelerate rare disease gene therapy, including potentially providing guidance on how best to apply our accelerated approval provisions to that space. One aspect of this is leveraging the ability to use biomarkers to help in rare disease gene therapy development. I think there might also be some discussion of how novel study designs may best support advancement in this area.

We will also see continued regulatory policy work in the area of genome editing. That is clearly a very rapidly evolving field, not just with CRISPR/Cas9, but now moving into base and prime editors. It is an area of innovation that we will essentially be looking to stay in step with.

Q Where are you seeing artificial intelligence (AI) starting to impact the regulatory application and review processes, and with what outcomes to date? Where can we expect to see further leveraging of AI in this context moving forward?

PM: I actually wonder whether the question is, where aren't we starting to see AI affecting things? AI is beginning to come into play for everything from helping to assemble submissions, to how people look at manufacturing process optimization, to sorting out adverse events in terms of the signal-to-noise ratio. I think our great challenge right now is to become educated about all the different ways in which AI may be applied in our particular set of circumstances, and to work out how to do so in the most thoughtful manner.

The latter part is key, because we believe there are some things that AI does well and others that AI will not do well for us. I think that over the coming year or two, you will see CBER trying to rise to this occasion, in part through an internal AI working group within the Center that will host lectures and aim to keep up with this field. Again, we anticipate seeing AI permeate all aspects of drug development from manufacturing through to helping understand the ideal nonclinical studies to perform, and from potentially helping in the assembly and interpretation of clinical data to analyzing adverse events. We are not yet sure how far this will go, but we are certain that AI is here to stay, and that we need to learn the extent to which it will come to be incorporated into the various applications that come to us.

Q What are the key points of focus for you as we move into 2024, in terms of fostering international regulatory harmony, particularly related to guidance for advanced therapies?

PM: I am really glad you asked this question because this is actually on my personal list of areas where I would like to see a breakthrough in 2024. I think we have made a lot of important advances in 2023: we have announced some of our internal programs that are moving ahead to help us review things more efficiently; we have announced some external programs to help us give advice to sponsors in a more timely manner. Overall, we are trying to take action that really leans into moving cell and gene therapies forward more rapidly here in the US.

I do think that 2024 really needs to be the breakthrough year in the rare disease space—in particular, in terms of making real progress towards global regulatory convergence in this area of rare disease gene therapy. It is very clear to me that all our different patient populations around the world suffer from minor regulatory differences between jurisdictions. These differences can serve as impediments to rare disease gene therapies developed in one country from being submitted and approved in others.

In my ideal world, in 2024, we would start to pilot some programs whereby we can mutually accept a submission—a common technical document at different agencies across several different countries at least—and then potentially review the submission collaboratively in much the same way as is already happening in the oncology space, through our Oncology Center of Excellence at the FDA.

Q As you mentioned at the start, it has been a year of significant progress in terms of BLA filings and new product approvals across a range of advanced therapy modalities and indications—what will be some specific areas to watch for you over the next 12 months in this regard?

PM: I am hoping that we continue to see a good pace of BLA submissions and see more products making it through the R&D pipeline. We will be looking closely now at how we conduct pharmacovigilance on the wealth of products that are starting to come to market. We need to make sure we are comfortable that we are doing all the requisite safety monitoring that needs to get done.

We will also be keeping an eye on how these products actually get deployed. That is not our primary business, but nevertheless, it will be something that we will be watching and working on with our partners at the Centers for Medicare and Medicaid Services at least, to ensure that approved cell and gene therapy products are able to get to the patients who need them.

As we have discussed, it has been a year of real progress. I think next year promises continued growth in this area. Moreso than I would have said in previous years, I feel that gene therapy is now coming around the corner from being in its infancy to starting to really grow up a bit. We understand now where its limits are, but we also understand where its possibilities are and where we can potentially go with it. I think it is a tremendously exciting time to be in this area and I am really looking forward to what the next 12 months will bring.

BIOGRAPHY

PETER MARKS is the Director of the Center for Biologics Evaluation and Research (CBER) at the US FDA. The center is responsible for assuring the safety and effectiveness of biological products, including vaccines, allergenic products, blood and blood products, and cellular, tissue, and gene therapies. Marks and center staff are committed to facilitating the development of biological products and providing oversight throughout the product life cycle.

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AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Isolating CD4$^+$ and CD8$^+$ T cells for improved drug efficacy and increased productivity

Hany Meås

Isolating CD4$^+$ and CD8$^+$ T cell subsets is critical for controlling the ratio within a final drug product. It also has an impact on downstream steps such as cryopreservation after isolation. Achieving younger, less differentiated T cells is important for optimizing clinical outcomes. In this article, a method to achieve process flexibility characterized by high purity, favorable yield, and desired cell phenotype in both autologous and allogeneic cell therapy processes is explored. A platform technology to enable the isolation of CD4$^+$ and CD8$^+$ T cells with an active release mechanism for either clinical or commercial manufacturing is also described.

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ADDRESSING CELL THERAPY MANUFACTURING CHALLENGES

Current manufacturing challenges in cell therapy focus on ensuring patient safety and reducing costs to expand treatment access to patients. To address these issues, closed automated unit operations during manufacturing increase efficiency, decrease variability, and reduce the risk of contamination. As the field progresses, allogeneic and autologous products will most likely coexist, meaning that a flexible and scalable platform is needed to enable cell therapy manufacturing at a range of scales. The next-generation Gibco™ CTS™ Detachable Dynabeads™ platform is designed to address these challenges.

CTS DETACHABLE DYNABEADS

CTS Detachable Dynabeads support an active release mechanism, allowing users to actively detach the Dynabeads from the cells at any time during the process, including following isolation. This provides the user with fine control over the manufacturing process.

The Gibco CTS Detachable Dynabeads CD4 and the Gibco CTS Detachable Dynabeads CD8 and Gibco CTS Detachable Dynabeads Release Buffer are the newest products in the family, specifically designed for the isolation of $CD4^+$ and $CD8^+$ T cells, respectively. The Dynabeads are optimized for use with the Gibco CTS DynaCellect™ Magnetic Separation System to offer a closed, automated solution that is both flexible and scalable.

CTS Detachable Dynabeads are ~4.5 mm paramagnetic polystyrene beads that utilize CaptureSelect® technology. They are coated with 12–15 kDa single-domain VHH antibodies (derived from llamas) that contain C-terminal variable regions that recognize antigen. Single VHH antibodies have lower binding affinity when released from the Dynabeads, allowing them to detach from the target cells to be easily washed away. The antibodies are animal origin-free to exclude any risk of virus-like particle contamination.

During isolation, the Dynabeads are incubated with material containing mixtures of cells, such as leukapheresis product, and the Dynabeads bind to the target cells. The Dynabead-bound cells are retained on the DynaCellect magnet and the non-target cells are washed away. When the release buffer is then added to the Dynabead-bound cells, biotin interacts with the release mechanism resulting in the detachment of the Dynabeads from the target cells. Using the DynaCellect magnet, the cell-free Dynabeads are captured and the VHHs and release buffer are washed away. The highly pure target cells are then ready for downstream application.

CTS DYNACELLECT MAGNETIC SEPARATION SYSTEM

The CTS DynaCellect Magnetic Separation System is the foundation for the next-generation CTS Detachable Dynabeads platform. DynaCellect is an instrument for automated magnetic cell separation and Dynabead removal, which is intended for use with all Thermo Fisher detachable Dynabeads. The instrument is accompanied by single-use, fit-for-purpose kits for isolation and bead removal.

An automated magnet is situated on a rocker to enable the mixing of cells and Dynabeads during incubation. Automated fluid management is achieved through a peristaltic pump and pinch valves, allowing the transfer of the fluid between bags. Fluid movement and pressure are monitored by bubble sensors and pressure occlusion sensors, respectively. The instrument has an embedded graphical user interface with customizable, 21 CFR Part 11-compliant software that enables flexibility from process development to commercial manufacturing. The instrument can be used standalone or connected to other instruments as a part of a workflow, enabled by Open Platform Communication Unified Architecture (OPC UA) distributed control systems such as Emerson DeltaV™.

CTS DETACHABLE DYNABEADS CD4

The CTS Detachable Dynabeads CD4 is intended for the isolation of $CD4^+$ T cells. To measure cell purity, recovery, and viability achieved with the Dynabeads, isolation performance data was generated on the CTS DynaCellect Magnetic Separation System. The starting material was cryopreserved leukapheresis from healthy donors. The input material contained 400 million $CD4^+$ T cells, and Dynabeads were added at a 4:1 Dynabead-to-$CD4^+$ cell ratio. The total process time was ~115 minutes for both target cell isolation and active release. Flow data was analyzed on the Attune™ NxT Flow Cytometer and cells were counted on the NucleoCounter® NC-3000™.

High $CD4^+$ T-cell purity was achieved with CTS Detachable Dynabeads CD4, as shown in Figure 1. In the starting material, the average $CD4^+$ cell frequency was 43%. After isolation, an average of 95% $CD4^+$ T-cell purity

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was obtained with a very low frequency of contaminant cells present.

After the target cells were isolated and the Dynabeads were released using the CTS Detachable Dynabeads Release Buffer, the target cells in the output fraction were then counted and normalized to the number of target cells in the input material prior to isolation to assess recovery. An average recovery of ~90% was achieved, ranging from 85% to 92%, for the three healthy donors tested.

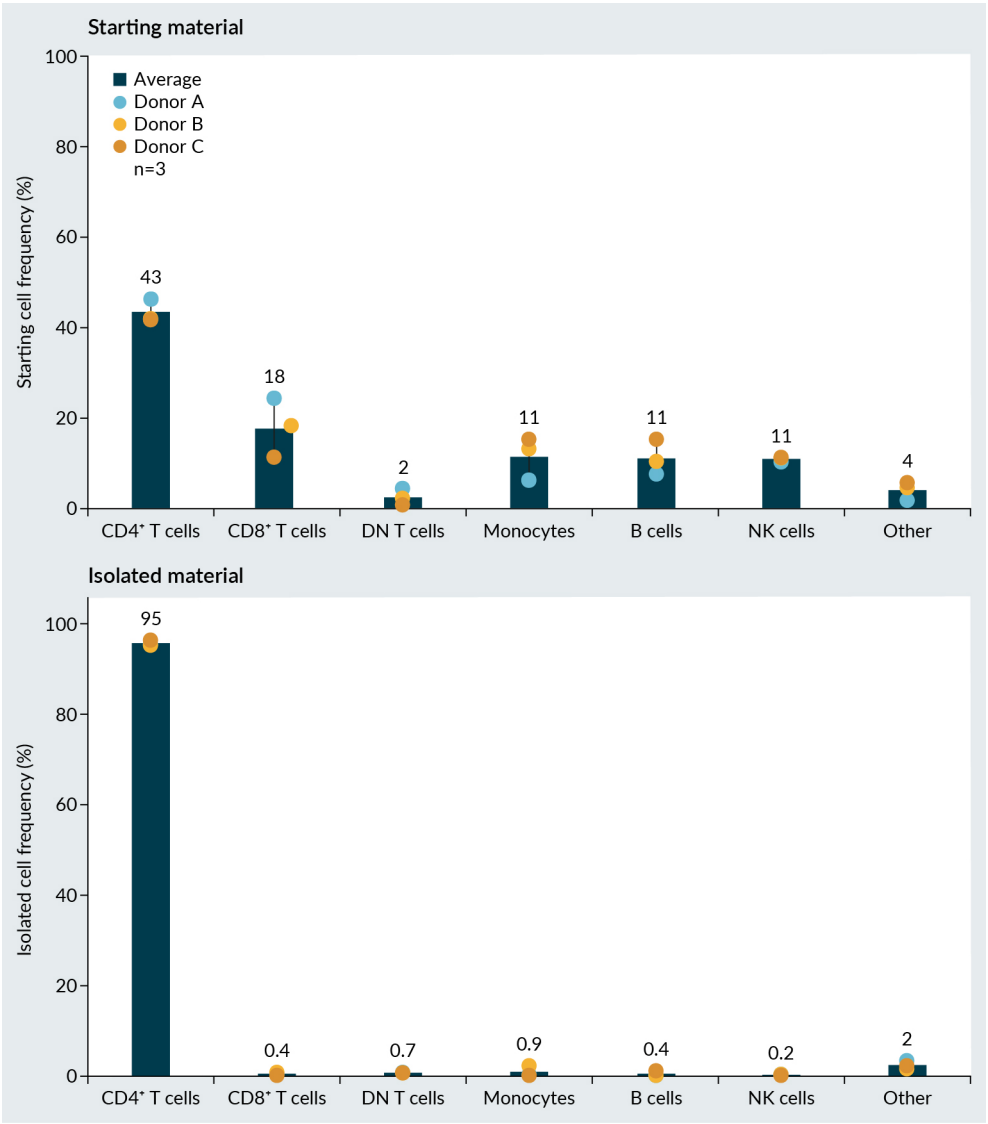
In addition, cell viability remained high—from an average of 93% in the starting material to 98% in the isolated material for all donors. This shows that cell viability is not negatively affected by isolation and release processes when compared to the viability of the starting material. The viability is slightly increased, likely due to the enrichment of viable cells during the washing steps in the protocol.

CTS DETACHABLE DYNABEADS CD8

Isolation data was also generated using CTS Detachable Dynabeads CD8. The protocol

FIGURE 1

CD4⁺ obtained after isolation using CTS™ Detachable Dynabeads™ CD4.



for the isolation of CD8⁺ T cells from leukapheresis material was identical to that for CD4⁺ T cells.

A comparison of cell phenotype in the starting material before and after isolation is shown in Figure 2. CD8⁺ T-cell fraction increased from an average of 25% to 89% after enrichment of the target cells, with low non-target cell impurity observed.

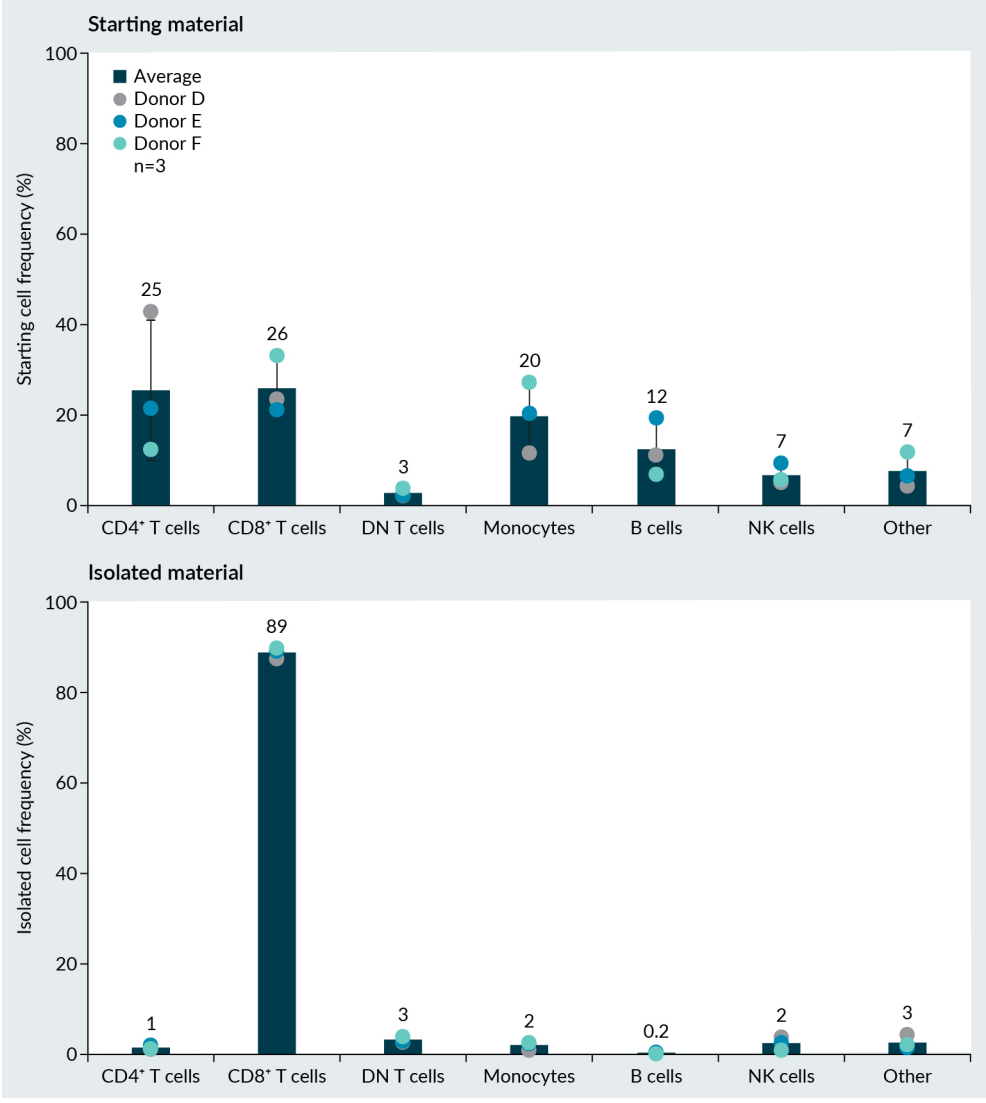
With the CTS Detachable Dynabeads CD8 Kit, an average cell recovery of nearly 90% was achieved, ranging from 85% to 92% for the three healthy donors—identical to the findings with the CTS Detachable Dynabeads CD4. Cell viability remains high after the DynaCellect isolation and release protocol with an average of 94% frequency of viable cells at the end of the process.

USING KITS IN COMBINATION

CTS Detachable Dynabeads CD4 and CD8 enable the user to either run single subset isolations, or to combine the two products in order to obtain an output material containing all the CD4⁺ and CD8⁺ T cells from the leukapheresis starting material.

FIGURE 2

CD8⁺ T-cell purity obtained after isolation using CTS™ Detachable Dynabeads™ CD8.



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Isolation data was collected for the use of both kits in combination. For this process, the protocol used was identical to the previous protocols, with the exceptions that full quarter leukopaks were used as the starting material, and both CTS Detachable Dynabeads CD4 and CD8 were added to the same isolation bag. The process time was also slightly longer (~150 minutes) due to the increase in scale.

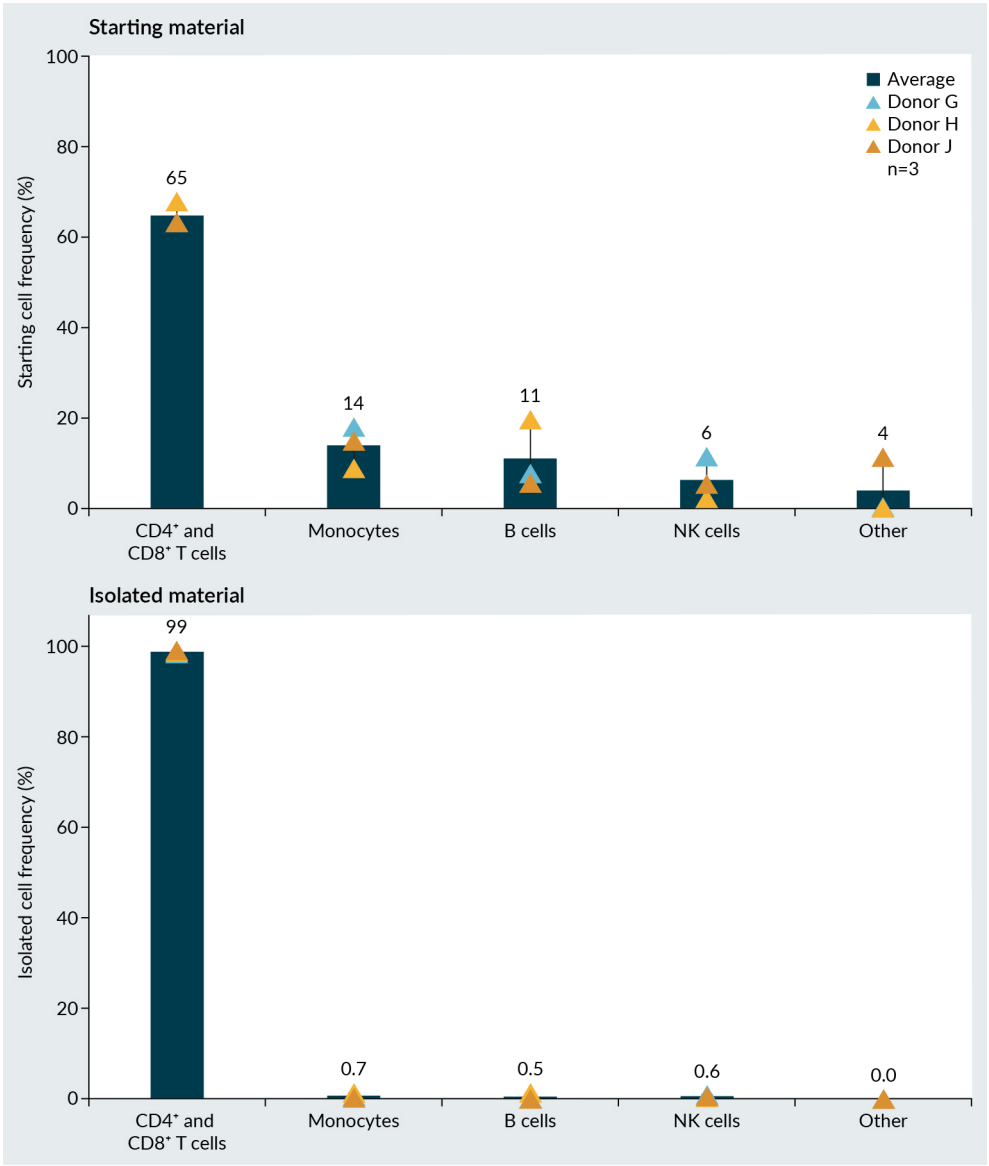
The purity of the isolated cells achieved was extremely high at 99%, compared to the starting material average of 65%, as shown in Figure 3. Consequently, the frequency of non-target cells was very low.

Target cell recovery was calculated separately and the recovery values of both CD4⁺ and CD8⁺ T cells were high, with averages of 94% and 87%, respectively (results for donor CD4⁺ T cells ranged from 92% to 97%, while donor CD8⁺ T cells ranged from 81% to 91%).

Comparing viability before and after the CTS DynaCellect isolation and release process shows an increased frequency of viable

FIGURE 3

CD4⁺ and CD8⁺ T-cell purity obtained after combined isolation using CTS™ Detachable Dynabeads™ CD4 and CD8.



cells and the isolated cell fraction compared to the starting material. Cell viability in the isolated material was at an average of 93% compared to the 71% average in the starting material, ranging from 92% to 94% for all donors. This demonstrates that dead cells were mainly washed away during the DynaCellect protocol.

With the combined use of the CTS Detachable Dynabeads CD4 and CD8, the average ratio of isolated $CD4^+$:$CD8^+$ T cells of 1.7 showed little change from the average starting ratio of 1.6 (donor ratios ranged from 1.5 to 1.7 in the starting material, and 1.7 to 1.8 in the isolated material).

SUMMARY

Isolating $CD4^+$ and $CD8^+$ T-cell subsets is critical for the control of a final drug product in order to improve drug efficacy and increase productivity. CTS Detachable Dynabeads provide process flexibility, scalability, and consistent performance in the cell isolation step. Thermo Fisher's new CTS Detachable Dynabeads CD4 and CD8 achieve high purity, recovery, and viability within a fast and automated process for the isolation of $CD4^+$ and $CD8^+$ T cells. The CD4 and CD8 kits can be used either separately or in a combined protocol.

Q&A

Hany Meås

Q What is the benefit of this product having VHH antibody fragments?

HM: There are several benefits. The VHH antibodies are produced in yeast so they are animal origin-free, thus eliminating the risk of virus-like particles that can be a problem in standard antibodies produced in hybridomas.

Another advantage relates to our release mechanism. As VHHs are tiny, when they are conjugated to the Dynabeads, together they have a very high affinity and are able to isolate the cells. When they are released from the Dynabeads and become single VHHs, they lose some of this affinity, allowing the antibodies to be washed away from the cells.

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Q What is the recommended ratio of Dynabeads CD4 and CD8 Dynabeads to target T cells?

HM: For CTS Detachable Dynabeads CD4 and CD8, we recommend a 4:1 Dynabead-to-target cell ratio. Target cells are cells that express CD4 or CD8.

There is no need to phenotype the starting material using flow cytometry before starting isolation. We routinely use the information we have from the Certificate of Analysis of the leukopak to calculate and determine the bead volumes before isolation.

Q Can I try out CTS Detachable Dynabeads CD4 and CD8 manually with columns rather than with the CTS DynaCellect Magnetic Separation System?

HM: All our Detachable Dynabeads products are intended to be used together with the CTS DynaCellect Magnetic Separation System. They were developed together, and they are optimized for each other. While small magnets can be used to assess the functionality of the product at a laboratory scale, this does not allow the user to see the accurate performance of these products. Testing our CTS Detachable Dynabeads without the CTS DynaCellect system will not result in the levels of yield and purity described here.

Q Can you share more on the method and principle for detachment?

HM: The Dynabeads are conjugated with VHH antibodies that bind to the target cells. Another reagent is used to add to the Dynabead-bound cells, and this reagent will detach the bead from the VHHs that are attached to the cells. When the Dynabeads are detached from the VHHs, the VHHs become single molecules and are easily washed away from the cell surface.

Q How was viability assessed?

HM: Viability is assessed by flow cytometry. We have a flow panel that includes several antibodies for phenotype analysis and a viability dye.

Q Is there any difference in the avidity of the new antibody in the detachable reagent compared to the prior CTS Dynabeads reagent?

HM: The VHHs are small, single fragments that by themselves do not have high avidity. In our case, we coat the Dynabeads with many VHHs to increase the avidity, as this

increases the number of binding sites on the Dynabead that can bind cells. The benefit of this mechanism is that when the VHHs are on the Dynabeads, they have very high avidity and high affinity, but when the Dynabead is released, the VHHs can be washed away.

Q How do you calculate your recovery efficiency?

HM: To calculate recovery, we calculate the number of target cells in the starting material, which depends on the scale. Then, we look at the number of target cells at the end of the isolation. We simply divide these two numbers to give the percentage of cells recovered.

Q Can you co-select $CD4^+$ and $CD8^+$ T cells in a single step? And are there any residual CD4 and CD8 Dynabeads remaining?

HM: You can co-select $CD4^+$ and $CD8^+$ T cells in a single step or use the CTS Detachable Dynabeads separately and isolate only $CD4^+$ or $CD8^+$ T cells at one time.

In terms of residual Dynabeads, the CTS DynaCellect system comes with a Dynabead removal kit that is extremely efficient in removing Dynabeads from the cells. We regularly obtain way below the 100 beads per 3 million cells standard that is accepted in the industry.

Q In the combined isolation release process, is the ratio still 4:1, and do you add equal parts of CD4 and CD8 Dynabeads to reach this ratio?

HM: We do not add equal parts because the ratio of $CD4^+$ to $CD8^+$ T cells in the starting material is not 1:1. The process is to identify the ratio of the $CD4^+$ and $CD8^+$ T cells in the starting material, and then adjust the volume of the Dynabeads to a 4:1 ratio to each kind of cell type. You do a similar calculation of the volume of the Dynabeads as with a single isolation, but in this case, together in a single protocol.

BIOGRAPHY

Hany Meås is an R&D Staff Scientist at Thermo Fisher Scientific and the project lead for the CTS™ Detachable Dynabeads™ and the CTS™ DynaCellect™ Magnetic Separation System. Hany obtained a Bachelor's degree in Molecular Biology from the University of Sheffield, UK and a Master's and PhD in Molecular Medicine from the Norwegian University of Science and Technology, Norway.

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AUTHORSHIP & CONFLICT OF INTEREST

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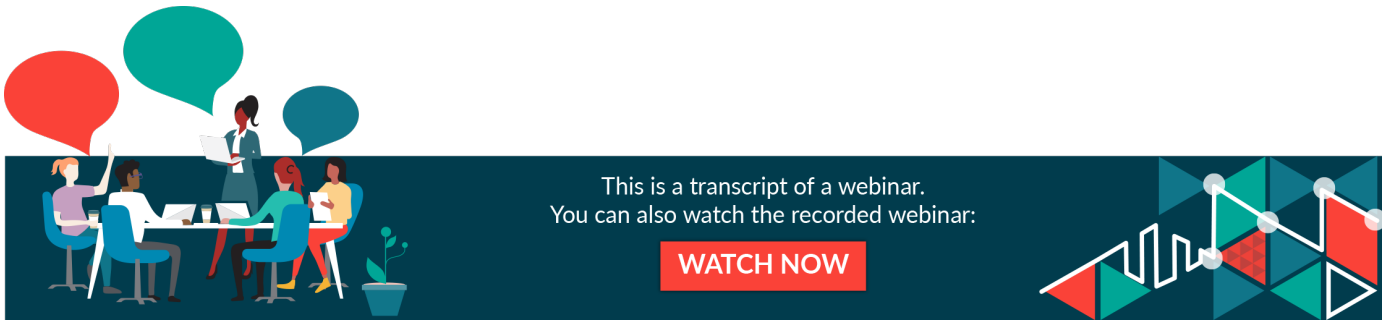


ThermoFisher SCIENTIFIC

EVOLVING

THE FUTURE OF CELL THERAPY MANUFACTURING: CTS DETACHABLE DYNABEADS MAGNETIC BEADS

Cell therapy

Welcome to your flexible process

Meet the next generation of CTS Dynabeads magnetic beads

You no longer need to wait for passive dissociation of Gibco™ Dynabeads™ magnetic beads from T cells or experience the limitations to your downstream process. The next-generation Gibco™ CTS™ Detachable Dynabeads™ magnetic beads enable the active release of CTS Dynabeads beads from your target cells at any point in the process. This flexible technology is meant for autologous and allogeneic platforms, as it enables consistent performance in light of biological variability. For your process, that means optimal isolation, activation, purity, yield, desired phenotype, and cell viability of your T cell population.

Other process improvements include:

- Increased control over target cell isolation and bead removal steps to help you shorten the timing of your overall process
- Increased control over cell activation and persistence of activation signals post-isolation
- High levels of pure T cells free from ancillary material
- Optimal recovery of target cells
- Desired cell phenotypes for downstream gene modification and fold expansion
- Suitable applications for both autologous and allogeneic processes

In combination with the Gibco™ CTS™ DynaCellect™ Magnetic Separation System, this automation-friendly solution is the first within a new platform designed to help accelerate current cell therapy manufacturing processes while driving the next generation of cell-based therapies.

Learn more at **thermofisher.com/ctsdynabeads**

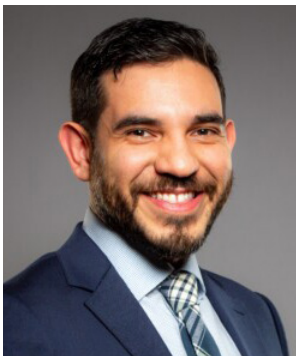
gibco

CELL & GENE THERAPY INSIGHTS

EXPERT ROUNDTABLE

Biological & operational considerations for iPSC-derived cell production

Austin Mogen, Antonio Fernandez-Perez, Jiwu Wang, and Andrew Chammas (pictured from left to right)

While cell therapies offer great promise, questions remain on the most effective tools and processes for transitioning from the lab bench into the production suite. In this roundtable discussion, four experts discuss considerations and challenges for scale-up of induced pluripotent stem cells. Methods and solutions for scaling production will be discussed, including biological and operational process optimizations.

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Q What are the main success factors around iPSC-derived cell therapy manufacturing scale-up from the biological perspective?

AC: Before you reach scale-up, there is a lot of research to be done on the components you need and their performance and stability. The components that are important when you are doing this type of scale-up include the culturing media, the coating substrates, and the vessels.

During our process development stage, we did a lot of testing to ensure that the performance of these components was good and that we were able to passage and culture these induced pluripotent stem cells (iPSCs) long-term. Even when you do all of that testing, there is a lot of line-to-line variation between iPSC lines and there still might be some issues that have to do with the line. You have to do what you can control, and what you can control is thoroughly testing all the components related to iPSC culture.

AM: Regarding the raw materials, one aspect of process development that can help determine whether or not a raw material is going to provide consistency and high quality is ensuring that you have the critical quality attributes set for your cell therapy product. Defining these around marker expression, differentiation potential, and potency for that particular therapy is also helpful as when you are testing these different raw materials for their consistency, you have a standard to which you can compare.

AF: Similarly, the starting material or origin of your iPSC itself is critical. It is important to do your homework and identify the best source for your reprogramming. Additionally, you must consider the technology that you're going to use for reprogramming, to determine if one method versus another is more efficient in your current process development.

When considering your strategy, you have to acknowledge how all these factors are going to play into your end product. By keeping these parameters in mind during process development, you can assess how the modifications will impact differentiation potential or end-product differentiation.

Q What do we know about the stability and variability of iPSCs, and how to optimize these aspects through scale-up?

JW: Firstly, consider a stability issue with iPSC lines. Intrinsically, iPSCs have line-to-line variation that relates to how the cells are made. We have seen cases where iPSCs have a higher tendency to spontaneously differentiate during culturing. That becomes a much bigger problem when you scale up since the culture environment might not be as ideal as when you were working with a smaller population of iPSCs.

If the starting iPSC line is not optimal, it will lead to a bigger problem at a later point. The variability also refers to line-to-line variation in terms of whether a protocol could work with different lines. When starting work on iPSC-derived cell therapy, the protocols should be tried with multiple lines. There have been cases where only one cell line works for a protocol that lasts several months. Different cultures must be tried to ensure the protocol works, and results in the desired end cells.

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During process development, the GMP process should not be overly optimized to a single line—this ensures the protocol can still work if the cell line has to be changed.

The ideal solution is to begin with the right iPSC line, particularly when manufacturing a cell therapy. We use mRNA and have found that the process has been stable, and the lines are completely reprogrammed. The epigenetic memories from the parental cells are erased, which is critical for large-scale therapy.

Q What are the key cell expansion platform opportunities in the iPSC space, both now and in the future?

AF: When expanding iPSCs and generating a future master cell bank, you must confirm that the product is robust from vial to vial or clone to clone. The goal is to maintain high yields and continue to meet your chosen quality metrics, whether at small or large scale. Additionally, safety must be ensured.

One of the platforms that can provide this increased robustness is stacked vessels, and at Corning®, there are several solutions for this: for example, the CellSTACK®, and HYPERStack®. Here, we increased the surface area to boost the yield from one product and one vessel, which can then be scaled out. This allows you to maintain consistency in your production.

When considering higher yields, we have a new platform, Ascent®, a fixed bed reactor that increases the surface area yet further and allows more consistent production of your iPSC culture.

JW: We have previously tried the CellSTACK, we are currently trying the HYPERStack, and we are considering trying CellCube® with different cells produced in our GMP operation. However, we haven't yet found a single platform that will suit all of them. As a developer, you have to do your research to see which platform works the best. So far, I have to say the Corning support has been excellent.

AM: As stated, there is variability in the clones and lines of the iPSCs, but there is also variability in the cells that they further differentiate into. Having the flexibility of different platforms can be helpful, particularly for this application. We have been discussing adherent platforms where the cells adhere to the surface and, generally, that surface is coded for iPSCs.

There have been some transitions to aggregate suspension culture; however, a majority of the literature has been published in 2D platforms. There is a much larger base of data and protocols to pull from utilizing those 2D platforms. There may be some application areas and cell types that the iPSCs would differentiate into that may not be supported in a suspension platform. Again, there is value in having different platforms depending on the end goal of the cell therapy process.

Q How and where are closed systems and automation helping in iPSC processing?

AM: It is well known that there is always a risk of contamination. This is particularly relevant for iPSC-derived cell therapies for two reasons. Firstly, these processes can be longer than other bioproduction workflows so there is more risk of contamination.

Secondly, many of the raw materials, such as the media and reagents, are quite expensive. Closed system solutions help to reduce that contamination risk and can be integrated into the stack vessels (CellSTACK and HYPERStack). Bioreactor processes are generally integrated with tubing sets and bags, which reduces the risk of contamination and allows transfer of volumes utilizing those tubing sets and pumps.

There are a variety of companies implementing automation strategies and Corning offers an automated manipulator platform that handles HYPERStack and CellSTACK units. When it comes to bioreactors, there are some automated pumping systems. For example, the Ascent fixed bed bioreactor has built-in pinch valves that automate the opening and closing of different sections of the tubing, controlling the transfer of liquids to and from the bioreactor, and media vessels.

JW: For operations teams like ours, there may be a need for a particular process and a closed system will be designed accordingly. The design needs to be process-specific, which can be a lengthy process. If there is interaction between a commercial system developer and the end users, that can enhance the final product usability for operations like ours.

Q At what stages are (and aren't) GMP reagents and workflows needed in iPSC master cell bank creation?

AC: There is a lot of investment and commitment to be made in the GMP space due to reagents being expensive. Adopting a GMP mindset at the early process development stage, when drafting the documents and necessary paper trails, has proven beneficial.

It is best to test out the reagents as early as possible and attempt to find the best vendors (with large inventories, quality management systems, and lot-to-lot consistencies), and integrate those in protocols while doing the process development. However, in some cases, there may be materials or reagents that are non-GMP or not available in a GMP offering.

Developing internal qualification processes is good, as it allows an organization to qualify non-GMP reagents and integrate those into the workflow. Generally, this should happen as early as possible. There are financial constraints when dealing with reagents that are more expensive in the GMP offering, but using GMP reagents as early as possible will be of great benefit to the program.

AF: Using GMP ranges early enough depends on process development and workflow, but thinking GMP as early as possible will help avoid future issues. For example, a particular reagent being used in an R&D form may work well, but when you switch to the GMP version, you may have a different potency. In addition to the regular process of development, having that GMP mentality and testing GMP reagents continuously to assess future feasibility is critical.

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Q From the end-user perspective, what do you look for in a raw material supplier for a GMP-compliant process?

AC: Good vendors communicate with clients if any changes are being made and this holds high importance as a lot of documentation is based on product specification and certificates of analysis (COA). This has been an issue with other companies where the product specification or COA is changed, leading to changes in documents, controls, and downstream documentation.

End-users typically use a lot of these reagents, and we like to see a vendor that has a strong quality management system. This ensures a consistent quality of products that are repeatable and reliable. It is critical that the vendor has good quality management, product specification, and consistency of COA. These are all things that make our lives much easier. Thankfully, Corning has been great for communication with the products that we use.

Q What are some key points of focus for iPSC-derived cell therapy manufacture throughout the pipeline as complexity increases in the field?

JW: The stage-specific attributes of the cells are critical when companies move from early development to manufacturing for phase 1, 2, and 3 clinical trials and future market product production. As the later stages of cell culture require more passages, certain problems that were not in the early stage can become major problems later. Pharmaceutical companies who license our iPSCs say that they see the difference between our line and other lines in the field.

Just because a line is available to the field does not necessarily mean it is a good line. Companies may need to spend more time or money to ensure the lines work in different stages. Studies can also be done to assess cell stability in terms of their morphology, post-thawing viability, telomere length, and general genome stability. From firsthand data, the iPSC line quality itself can dictate all of these important attributes further down the line.

The iPSCs are used for differentiating into different effector cells for therapeutic purposes. The desired property of the final product is important and should be included within the study early on to ensure purity and functionality during large-batch production

It should also be noted that genome editing is now becoming part of the iPSC workflow. That requires consideration of selecting single cells as they may have a different genome after genome editing. Our mRNA-produced iPSCs remain very consistent as there are no nuclear events, meaning we do not need to do single-cell selection and can instead use pools.

There are scientific and practical benefits to using a selected pool of iPSCs, but we also ensure that a single-cell process under GMP could be used. Occasionally some projects or partners prefer to have a single clone from a single iPSC. These are the considerations that a team that works on an iPSC-based therapy should keep in mind.

AM: There are some differences in cell biology when going from a small-scale petri dish/T-flask to a bioreactor/stack vessel. It's important to consider the kind of scale-down model that can be utilized and is representative of the final production scale being targeted.

There have been many instances of end users starting at one scale and moving to a much larger bioreactor where the cells do not behave the same after that transition.

AF: Concerning the model and how to extrapolate from smaller to bigger processes, it is important to think about your support system, such as the type of media being used and whether it supports genomic stability. The main goal is to create a robust process that is not as sensitive when there is a formulation change from small to large scale.

Q Finally, can you each briefly share your broad vision and expectations for key future directions in the iPSC field?

AF: I expect that the platform used for iPSC scale-up should be a robust, consistent cell that will be tailored to the process. Whether the manufacturing scale is in a 2D or 3D system, the field is still trying to define the most appropriate model. However, the main thing that I envision is an increased care for the quality of the iPSCs. Taking into account everything mentioned, the important question will be how to have the best starting material for your end-goal therapy.

JW: For the iPSC field, in more general terms, only a couple of dozen players are using iPSC-derived cells for cell therapy currently. However, I am certain that the field will grow. Most companies focus on allogeneic iPSC lines, which is why having a good line is beneficial to begin with. If modifications are needed, they can be done upfront so that you can ensure everything still works for the protocol. I'm hopeful a good line will work for all protocols.

I believe autologous iPSCs will have a bigger place in the industry and that capability will benefit the field as we mature into producing usable cell therapies.

AC: I agree that we are in the early part of this field. There is a lot of focus on the allogeneic approach right now, but I think in the future, autologous could be a major focus point. You are asking for two different things between allogeneic and autologous approaches and conquering the two things will make it a very fruitful field.

AM: Currently, iPSC-derived therapies are the most complicated biological product to produce. There are many steps and inputs in terms of raw materials, and close relationships and partnerships with suppliers will be critical to moving this field forward. Due to the complex nature of the products, this work is very difficult to do in a silo, and having supplier partnerships can provide that support.

BIOGRAPHIES

AUSTIN MOGEN is a Field Application Scientist Manager for Western United States and Latin America at Corning Life Sciences. He gained industry experience as a Senior Scientist in upstream process development, and as a Manufacturing Supervisor for viral vector manufacturing. Since joining Corning, he has worked with academic researchers and process development groups, optimizing cell culture assays, and cellular scale-up conditions for viral production, cellular therapeutics, and biologics.

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ANTONIO FERNANDEZ-PEREZ is a Field Application Scientist at Corning Life Sciences. In previous roles, he worked on process development for iPSC-derived cellular immunotherapies for patients with cancer and autoimmune disorders. Antonio now works with academic researchers, biopharma companies, and advanced therapy manufacturers to optimize cell culture assays and cellular scale-up conditions using Corning technologies.

JIWU WANG is the Founder and CEO of Allele Biotech, an early developer of RNAi, and the first market provider of shRNA reagents. His team constructed the field-leading fluorescent proteins mNeonGreen and mMaple, and he co-invented Allele's mRNA reprogramming method, and established full GMP operations for generating and banking mRNA-induced pluripotent stem cells. He also founded the Scintillon Institute, which conducts research in bioengineering, neuroscience and behavior, immunology, and aging.

ANDREW CHAMMAS is a Process Development Scientist at Allele Biotech with an extensive background in iPSC-related workflows for cell therapy. He has played pivotal roles in innovating Allele's core mRNA-based technology and adapting it to GMP standards. Currently, he manages iPSC-related pre-clinical process development efforts, focusing on the safe and efficient production of iPSC-derived therapies.

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AUTHORSHIP & CONFLICT OF INTEREST

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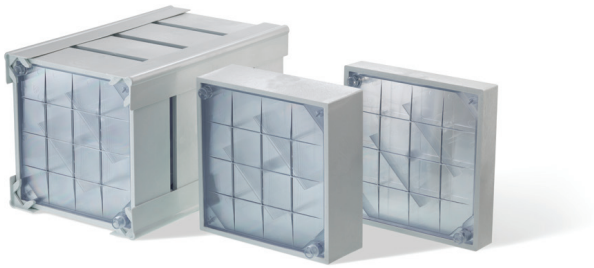

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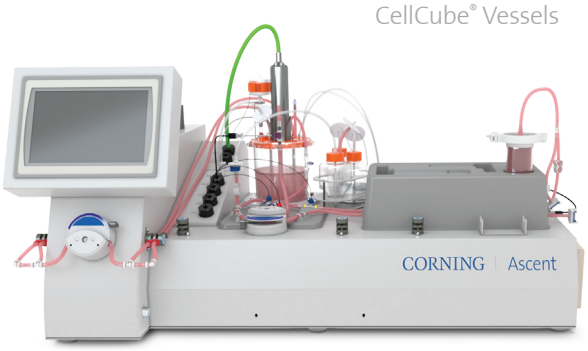

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CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Case study: becoming the first GMP-certified cell and gene therapy manufacturing facility in Thailand

Kitipong Uaesoontrachoon

Roughly 30,000 new cases of leukemia and lymphoma are detected in Thailand every year. Approximately 40% of these cases do not respond to traditional treatment such as chemotherapy. Patients with these unresponsive cancers can then turn to CAR-T cell therapy as a subsequent treatment option. However, there are several challenges associated with the production process for CAR-T cell therapies, including complexity, cost, and regulatory obstacles. Despite these challenges, Genepeutic Bio is able to provide services for CAR-T cell production and is the first GMP-certified manufacturing facility in Thailand to do so. This article explores how Genepeutic Bio is increasing the accessibility of CAR-T cell therapies to people in Southeast Asia through their production of an anti-CD19 CAR-T cell therapy.

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CAR-T CELL THERAPY CHALLENGES

The manufacturing process and supply chain procedures for CAR-T cell therapies are complex, largely because these therapies involve live cells. The functionality of live cells can be affected by external factors, so control of elements such as temperature is vital. Another challenge with CAR-T cell therapy is the high associated cost. The current price for approved CAR-T cell therapies is between US$373,000 and US$475,000.

Furthermore, regulations around producing and approving these therapies present persistent obstacles. The regulatory challenges

can be broken into five categories, as shown in Figure 1. Robust trial design and subsequent strong clinical endpoint data are the first necessities in achieving regulatory approval. In terms of trial design, gene therapies raise important questions surrounding patient consent and may require collaboration between countries to increase the number of patients within cohorts. Clinical endpoint data is used as a sign to determine if a therapy is working, rather than waiting to observe life-long benefits.

However, long-term follow-up is also vital because this is a very new treatment and altering DNA can lead to unexpected adverse effects. The US FDA requires patient follow-up for up to 15 years and the European Medicines Agency (EMA), which Thailand follows, requires patient follow-up for up to 30 years. In addition, evidence generation can pose a challenge because of the personalized nature of the medicine. The drug product cannot be produced in bulk, so variation tends to occur between each batch of production. Obtaining consistent data within a trial can therefore be difficult and requires stringent controls. Lastly, CMC tends to be a challenge since documentation covers the entire upstream and downstream processes, which includes in-process controls.

FIGURE 1

Regulatory challenges for gene therapy.

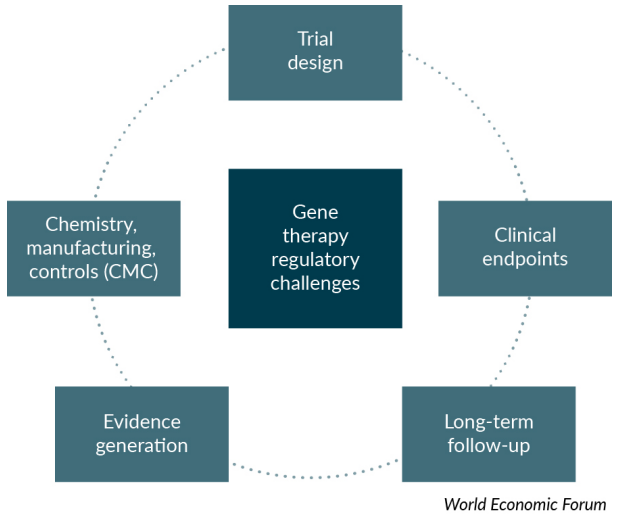
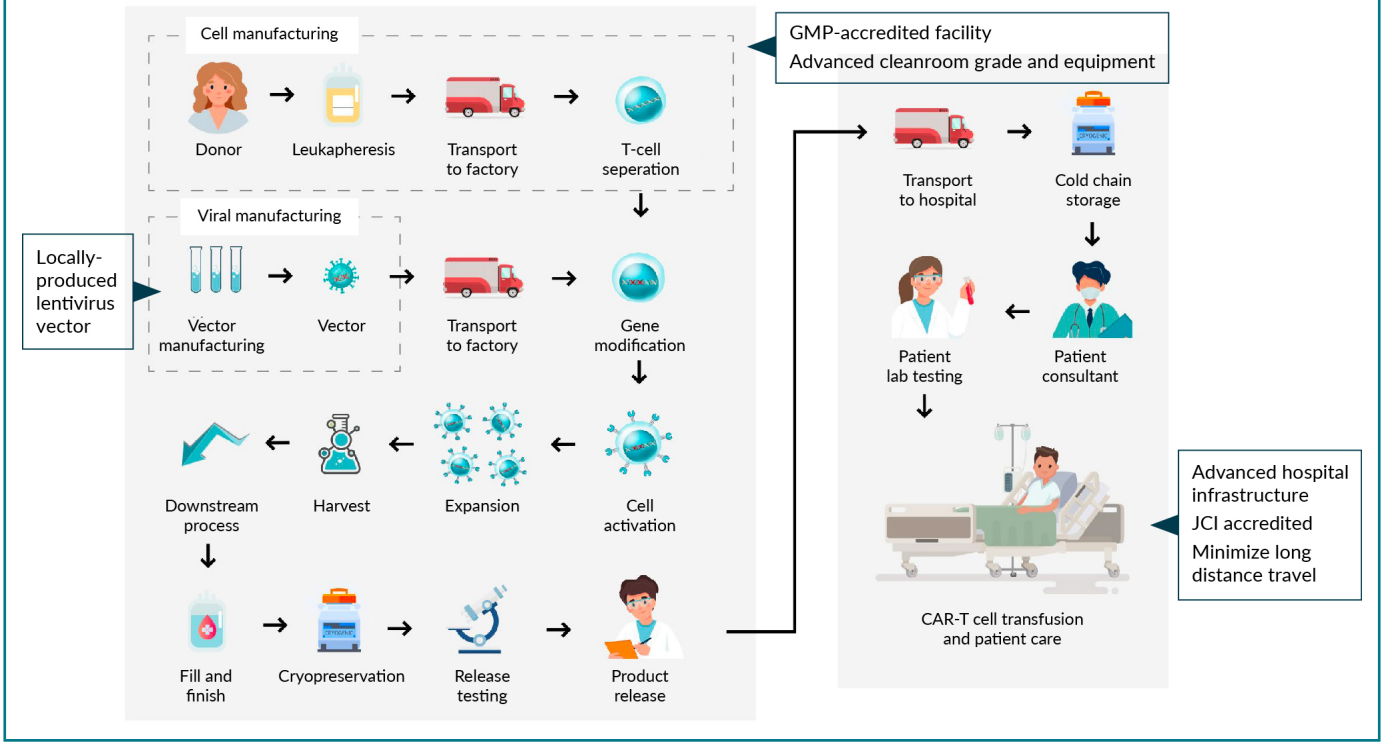


FIGURE 2

Manufacturing process.



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Completion of the CMC document and the review process can therefore be arduous.

IMPROVING CAR-T CELL THERAPY ACCESSIBILITY IN THAILAND

The CAR-T therapies that overcome these obstacles in Europe and the United States are difficult to access in Southeast Asia. Genepeutic Bio, the first GMP-certified cell and gene therapy manufacturing facility in Thailand, was founded to rectify this problem. Genepeutic Bio's mission is to:

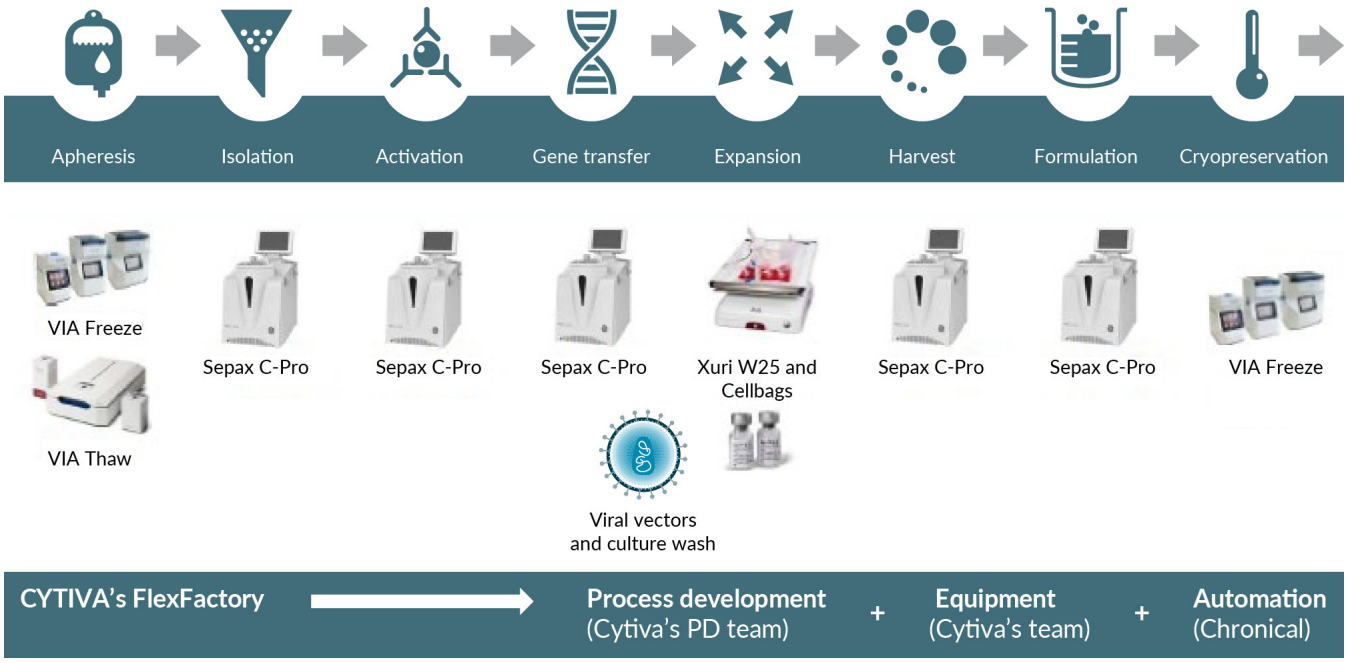
- Facilitate the advancement of the research and development products to commercialization for the treatment of cancers and genetic diseases;
- Provide more accessible and affordable cell and gene therapy for patients in Southeast Asia and globally;
- Support Thailand towards becoming a medical hub in gene and cell therapy that meets international standards.

Genepeutic Bio's facility serves as a CDMO for contracted local and international partners, and develops and licenses technology patents in cell and gene-based medicines in collaboration with local and global partnerships. The manufacturing process is shown in Figure 2.

Genepeutic Bio is able to successfully manufacture CAR-T cell therapies, with plans to locally produce lentiviral vector, a critical ingredient for the production of CAR-T cells. Local production overcomes the challenge of a purchasing backlog, since buying this material from another viral vector producer would introduce delays into the process, contributing to inaccessibility. Genepeutic Bio is also able to achieve success because of its advanced hospital infrastructure, which has been accredited through Joint Commission International accreditation. This infrastructure minimizes long-distance travel of the blood product as well as the final CAR T cell product, thereby limiting logistical complexity. The facility also has government support through the Thailand Board of Investment and the Program

FIGURE 3

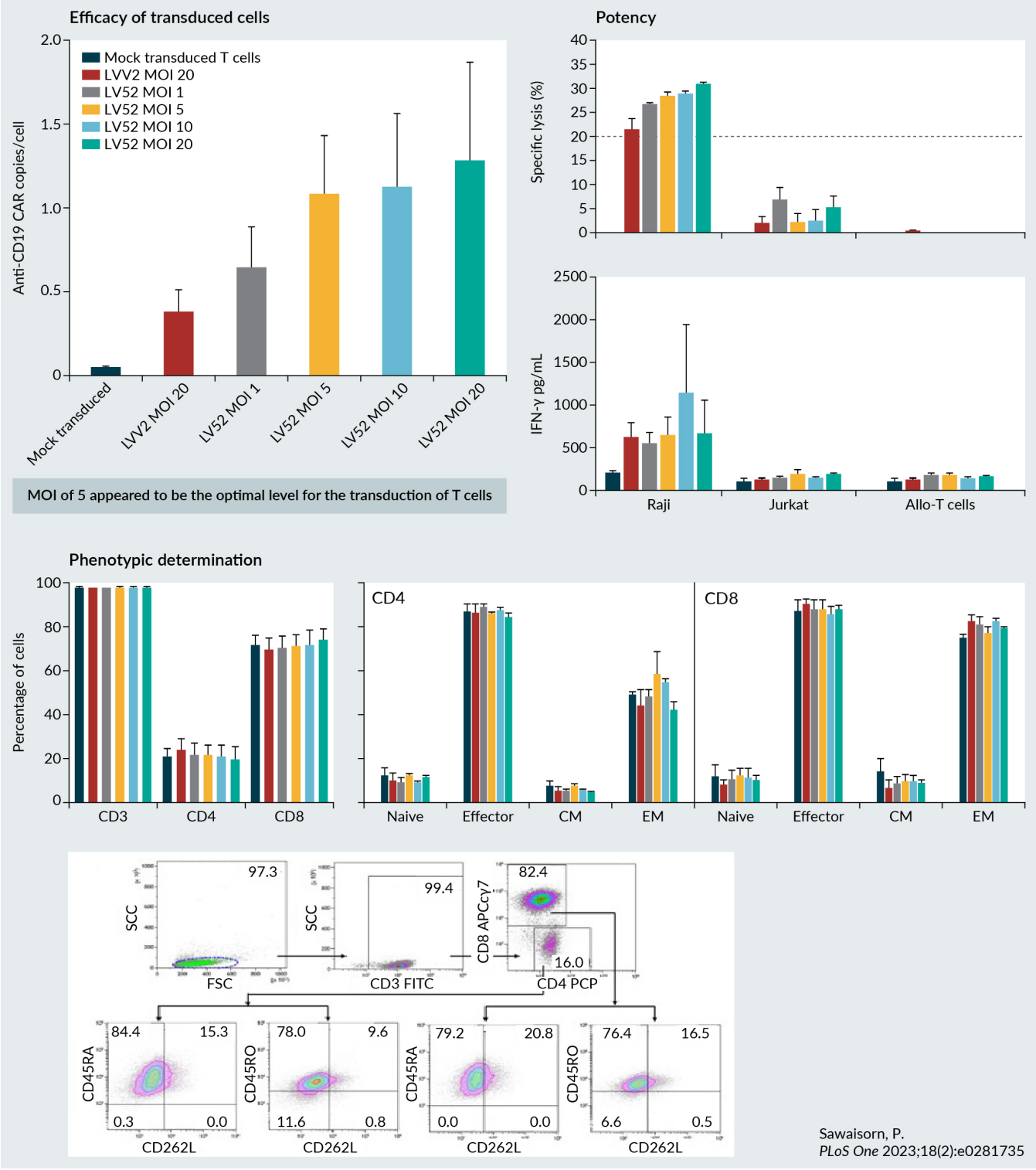
System utilized for CAR-T cell therapy manufacture.



Management Unit for Competitiveness Enhancement. The government of Thailand has set the goal of increasing the rate of R&D to gross domestic product from 1.14% to 4% through a 20-year national strategy called Thailand 4.0.

FIGURE 4

Non-clinical study results.



Sawaisorn, P.
PLoS One 2023;18(2):e0281735

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CAR-T CELL THERAPY PROCESS

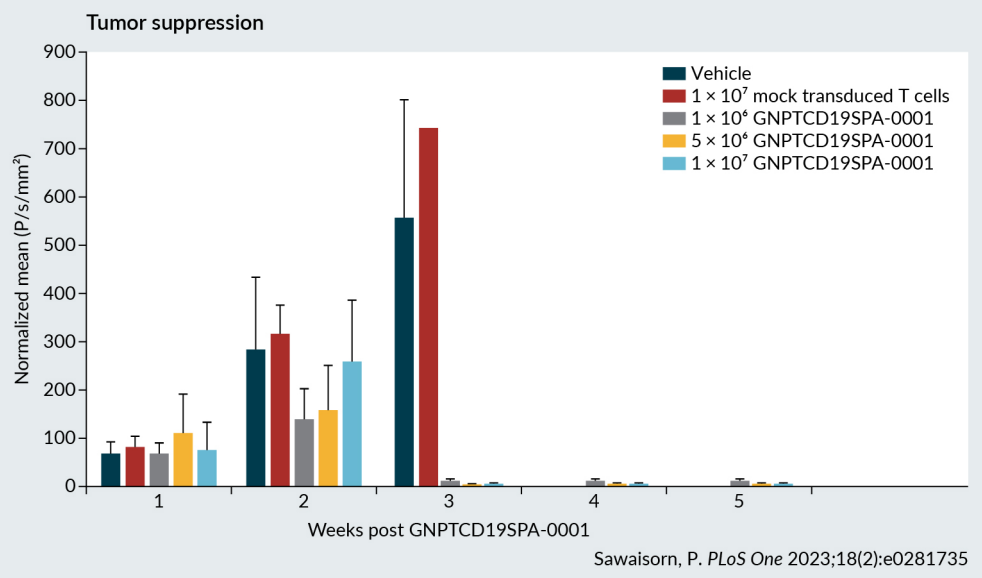
Genepeutic Bio's facility uses a modular automated functionally closed system (Figure 3), adopting Cytiva's FlexFactory process development and Cytiva equipment.

ANTI-CD19 CAR-T CELL THERAPY—INITIAL RESEARCH

Genepeutic Bio's first product was an anti-CD19 CAR-T cell therapy [1]. This product was licensed for the indication

FIGURE 5

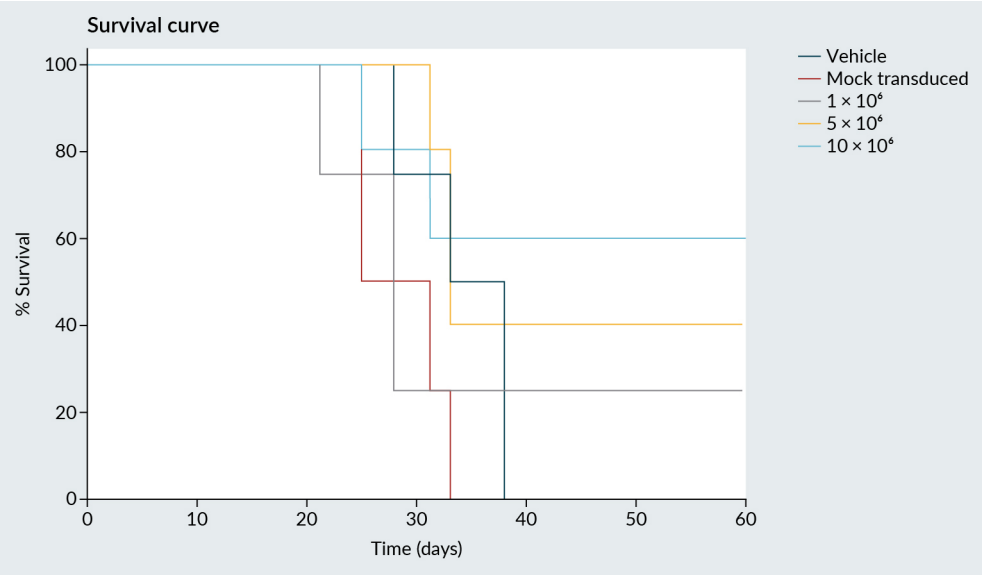
Tumor suppression in animal model.



Sawaisorn, P. *PLoS One* 2023;18(2):e0281735

FIGURE 6

Survival curve in animal model.



of relapsed/refractory CD19+ B-cell malignancy.

As a proof of concept, a non-clinical study was performed in order to determine multiplicity of infections (MOI) (Figure 4). Efficiency of transduced cell, potency, and phenotypic determination were deduced, and an MOI of 5 was found to be the optimal level of transduction for these T cells.

A non-clinical study in an animal model was also performed. Because there is no true biochemical model of leukemia, an immunodeficient animal must be created and toxicity cannot be truly tested (Figures 5–7).

As shown in Figure 5, various doses of CAR T cells were created and injected into immunodeficient mice as the representation of the tumor model to determine tumor suppression capability.

A dose-dependent survival was observed, with approximately 60% of mice treated with the highest dose of CAR-T cells surviving longer than those of the vehicle or those given the mock-transduced CAR-T cells (Figure 6).

FIGURE 7

Toxicity by body weight in animal model.

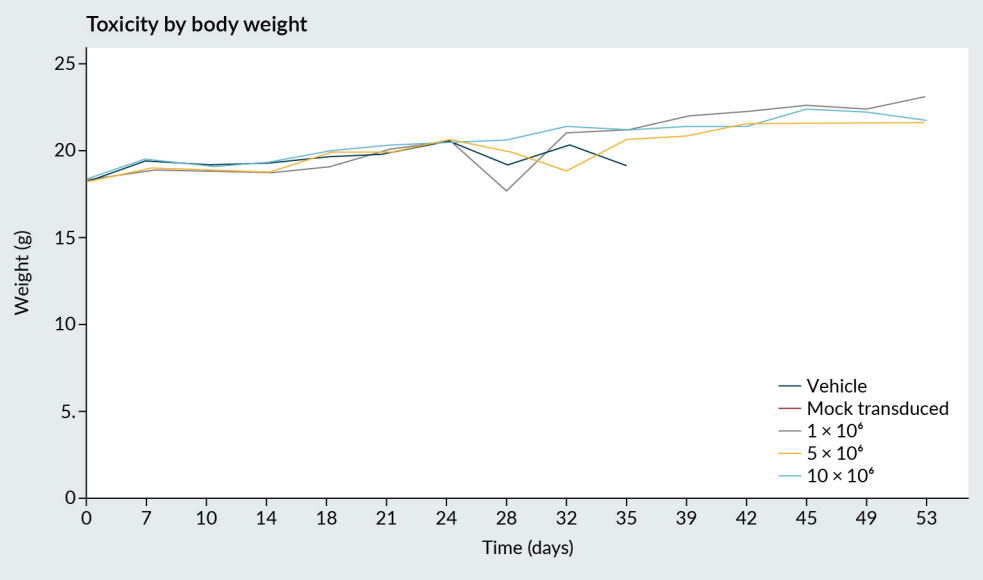
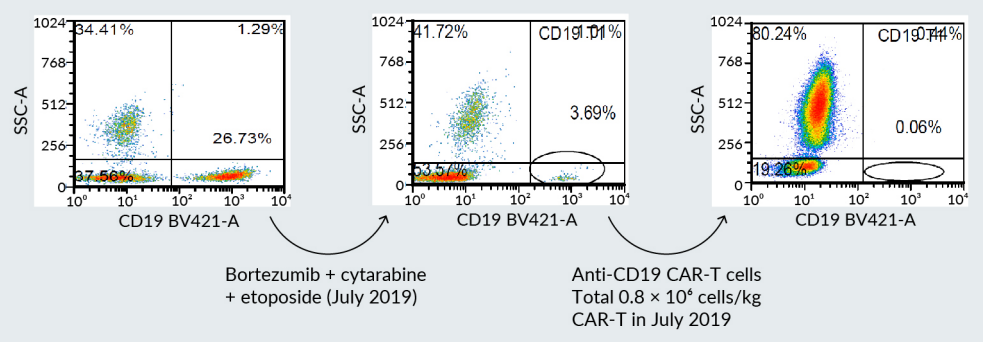


FIGURE 8

Preliminary data in human clinical trial.



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Though true toxicity cannot be tested in immunodeficient mice, the continuous increase in body weight over time (Figure 7), suggests that the CAR-T cell did not induce toxicity.

The current CAR-CD19 T cells were also used in a compassionate case in which a patient with CD19+ acute lymphoid leukemia (ALL) underwent a fourth relapse in the bone marrow. Figure 8 shows the flow cytometry data for the therapies used in the treatment of this patient. The cancer cells at the initial stage before treatment accounted for approximately 30% of the sample. However, after the initial dose of chemotherapy, that figure reduced to approximately 4%. Following the infusion of the anti-CD19 CAR-T cell at approximately 800,000 cells/kg, the cancer cells population was reduced to 0.06%. Ten subsequent compassionate use cases also proved successful in treating ALL.

ANTI-CD19 CAR-T CELL THERAPY—CLINICAL TRIAL PROGRESS

Figure 9 reveals the progress to date and the projected completion dates for the various phases of the anti-CD19 CAR-T cell therapy clinical trial. Phase 1 is currently ongoing. In this dose escalation study, nine patients will receive three different doses (i.e., three doses per patient). The treatment protocol for this phase is demonstrated in Figure 10. Phase 2 will be the efficacy study. (The patient population for this phase 2 is small because ALL is considered to be a rare disease based on the number of those affected). Following completion of this phase 2 trial, accelerated approval by the Thai FDA will be sought to allow marketing to begin.

Establishing manufacturing controls is a vital part of the clinical trial process. The establishment of these controls involves batch record analysis, environmental monitoring, the validation of critical processes, obtainment of a second verification of production processes from QC personnel, following good documentation practice, and implementing quality management.

THE FUTURE OF GENEPEUTIC BIO

Genepeutic Bio will be setting up a viral vector production facility in 2024, with the aim of bringing the facility online by 2025.

In terms of a potential production pipeline, the facility plans to expand the use of its CD19 CAR-T cells from ALL to B-cell

FIGURE 9

Clinical trial progress.

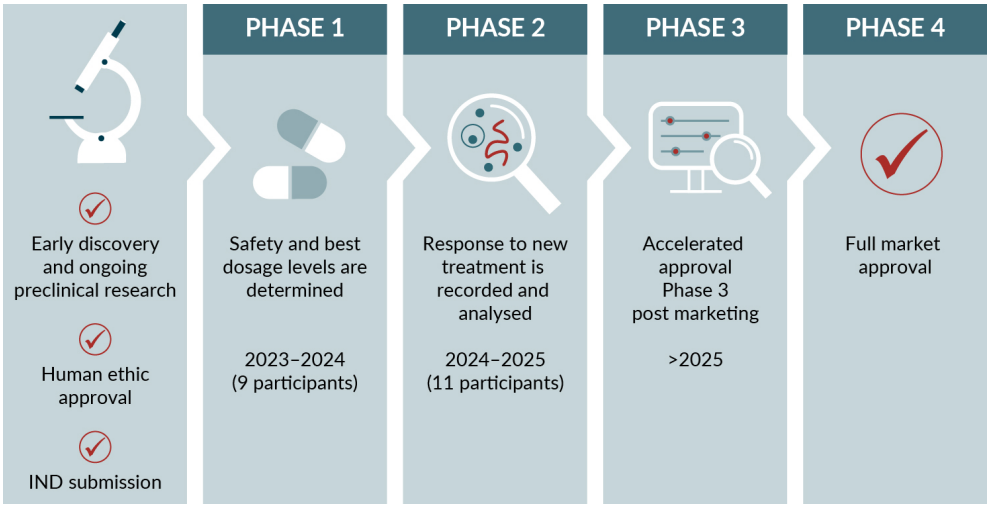
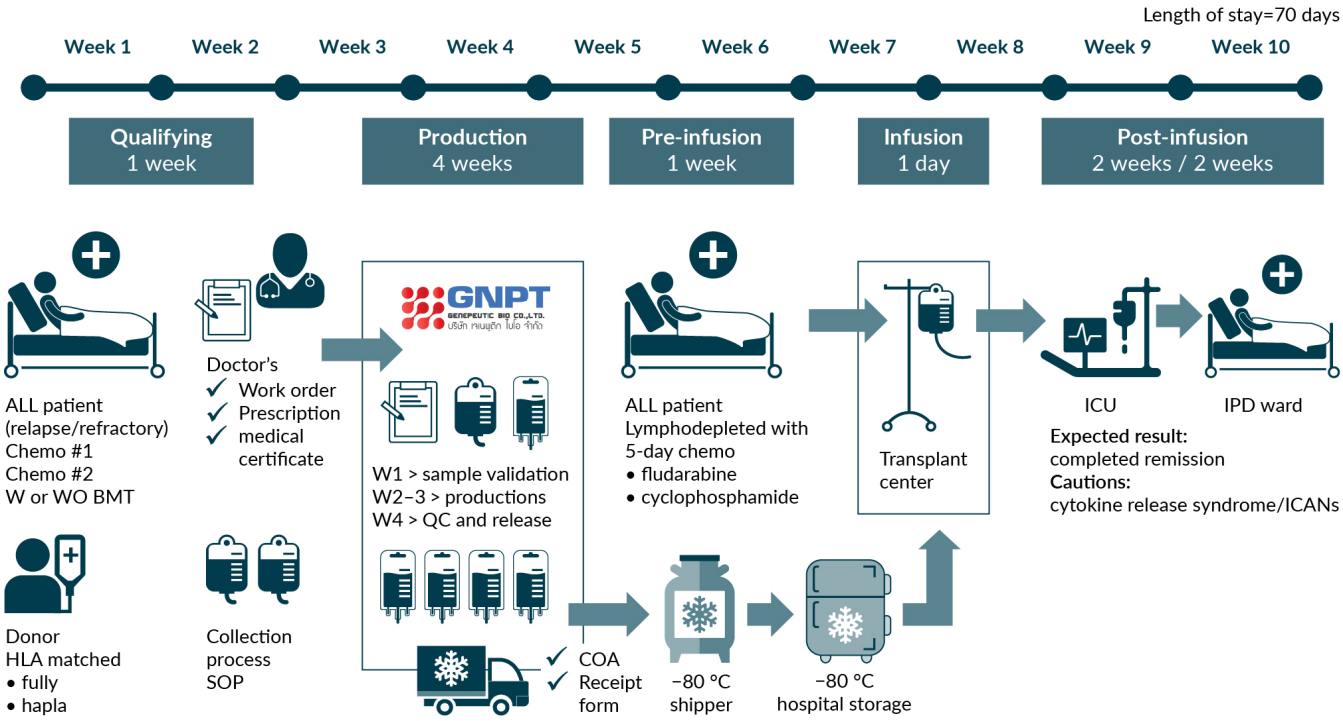


FIGURE 10

Phase 1 clinical trial treatment protocol.



Non-Hodgkin lymphoma. Thereafter, B-cell Non-Hodgkin lymphoma T cells would be used to treat multiple myeloma. In subsequent years, Genepeutic Bio has plans to move into solid tumors such as neuroblastomas and nasopharyngeal cancer.

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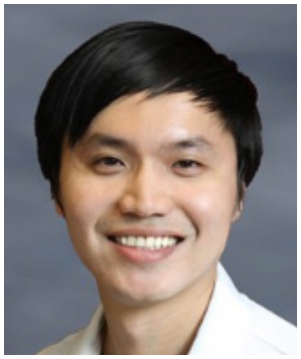
Q&A



Kitipong Uaesoontrachoon

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Q Can you discuss how your collaboration with Cytiva helped you achieve your goals?

KU: Genepeutic Bio's collaboration with Cytiva eases the regulation process, as it encompasses the process development, one of the regulations involved with setting up a GMP facility. Without this collaboration, time would be lost to figuring out the parameters involved in manufacturing CAR-T cells. With the process development availability from Cytiva, along with their easy-to-use equipment, we were able to jump straight into the validation process, becoming a GMP-compliant facility quite quickly.

Q How important are closed and scalable instruments?

KU: Closed systems help to eliminate or reduce the risk of cross-contamination. This, in turn, reduces the need to do cleaning validation, as was necessary after each batch of production in standard steel equipment.

In terms of scalability, being able to scale-up, which is easy with Cytiva's equipment, is vital for controlling budget and increasing patient access.

BIOGRAPHY

KITIPONG UAESOONTRACHOON has been involved in the gene therapy field for more than a decade, starting with neuromuscular diseases. Under his guidance, promising therapies have either entered into various phases of clinical trials or received accelerated approval for use by the US FDA. He received his PhD in Molecular and Cellular Biology from the University of Melbourne, Australia and post-doctoral training in the laboratories for genetic medicines at Children's National Medical Center, Washington, DC. He held an Adjunct Professorship in the Department of Pharmacology at Dalhousie University, Halifax, Canada, and served as the Principal Director of Research at AGADA Biosciences, overseeing their entire pre-clinical trials and clinical testing operations. Currently, he holds a Chief Scientific Officer position at Genepeutic Bio, a CDMO specializing in manufacturing CAR T cells for treatment of various forms of cancer.

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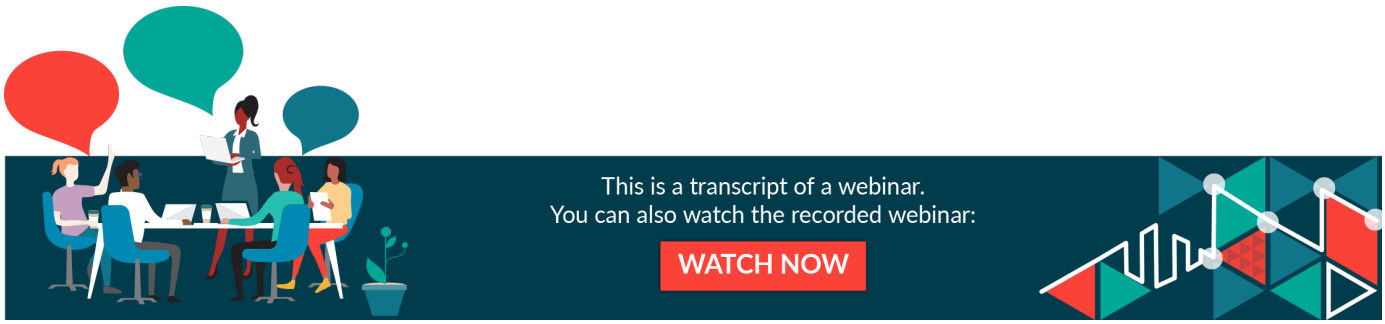

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Achieving flexibility & scalability in cell therapy manufacturing through optimized cell isolation & activation

Eugene Kang

Manufacturing challenges within the cell therapy field continuously center around patient safety, cost reduction, process scalability, and establishing workflows that are both closed and automated. Many cell therapy manufacturers are currently concentrating on autologous CAR-T cell processes. However, as the space becomes more mature, cell therapy will shift its focus to allogeneic off-the-shelf therapies whilst simultaneously continuing to optimize autologous drug efficacy and cost. This will amplify the need for flexible solutions that can support both autologous and allogeneic processes for cell therapy companies everywhere. This article explores one such solution–the Gibco™ CTS™ Detachable Dynabeads™ platform.

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AUTOMATED CELL ISOLATION & ACTIVATION

The CTS Detachable Dynabeads platform possesses an active release mechanism that, after the Dynabeads bind to target cells of interest, allows users to actively detach the Dynabeads from cells at any time within the process. This results in a T cell population with minimal non-target cell impurities, and an overarching process flexibility.

Processed on the Gibco™ CTS™ DynaCellect™ Magnetic Separation System, the Dynabeads firstly isolate target cells of interest. The proprietary active release mechanism then allows for active detachment of

the beads from their target cells. This results in the ability to refine target cell purity, target cell yield, and desired cell phenotype, and subsequently, has benefits for downstream gene-modification and expansion.

The base of the product is a 4.5 micron paramagnetic polystyrene bead. The active release mechanism entails a biotin derivative and a variable heavy domain of heavy chain (VHH) antibody conjugated to the surface of the Dynabead itself. The VHH antibody, a 12–15 kilodalton camelid-derived short-chain antibody specific for various clusters of differentiation (CD) markers, demonstrates tunable specificity and affinity as it binds to target cell surface markers. In addition, produced in yeast, these antibodies are animal-origin free, highly stable, and exclude any risk of viral contamination or adventitious reagents.

The system process is illustrated in Figure 1. After incubation with the CTS Detachable Dynabeads, the target cells are detached by introducing a release buffer that outcompetes the biotin derivative on the surface of the bead. After release, the Dynabeads can be subsequently removed by using the CTS DynaCellect system, which allows for the highly pure T cell population.

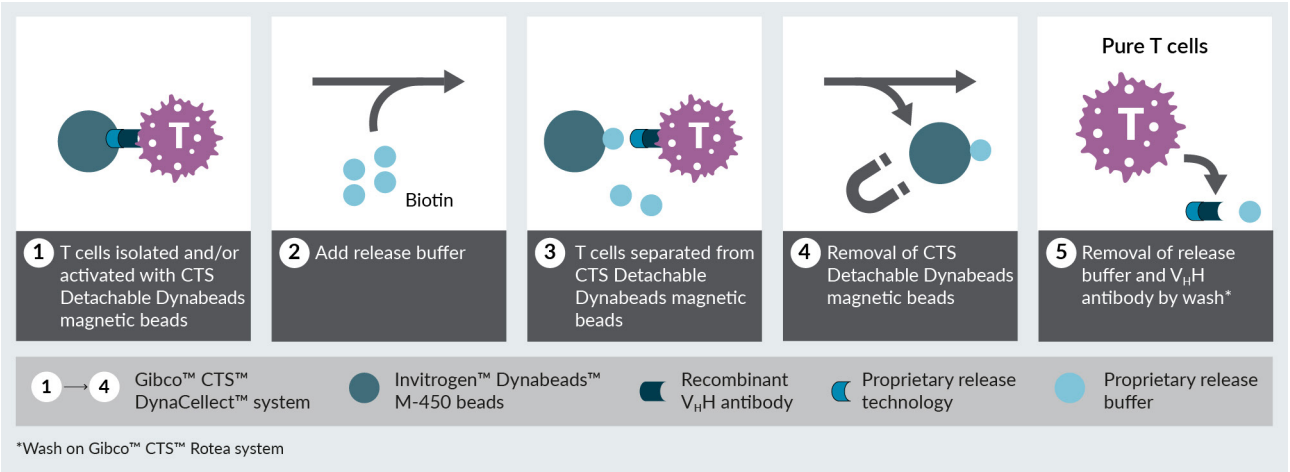
The CTS DynaCellect Magnetic Separation System is a closed, automated device for cell isolation and magnetic removal of Dynabeads. It enables process automation and includes fit-for-purpose single-use consumables for isolation and bead removal. The touchscreen user interface allows for customization of protocols for cell isolation, activation, depletion, and magnetic separation. This instrument can be used standalone but can also be leveraged as a plug-and-play instrument with other instruments within the workflow. This is made possible by an open platform communication unified architecture that is compatible with Emerson's DeltaV™ software.

IMPROVING UPON CURRENT CELL THERAPY MANUFACTURING METHODS

The recently released Gibco™ CTS™ Detachable Dynabeads™ CD3/CD28 Kit allows for control over the duration of T cell activation, thus achieving desired cell phenotype, optimizing the stem cell-like properties of the final cell population, and making for a more efficacious final drug product. In addition, this kit gives researchers the ability to control and optimize activation marker profiles that they experience while obtaining desired central memory phenotypes after activation. Finally, because the CTS Detachable Dynabeads are designed for use with the CTS DynaCellect

FIGURE 1

CTS Detachable Dynabeads magnetic beads product concept and process.



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Magnetic Separation System, the process is automated and scalable.

Although this active-release kit is comparable to the passive-release CTS Dynabeads CD3/CD28 in terms of CD25 expression (Figure 2), fold expansion (Figure 3), CD4:CD8 ratios (Figure 4), and desired cell phenotype (Figure 5), the active-release mechanism does

FIGURE 2

Average CD25 expression for active-and passive-release Dynabeads magnetic beads.

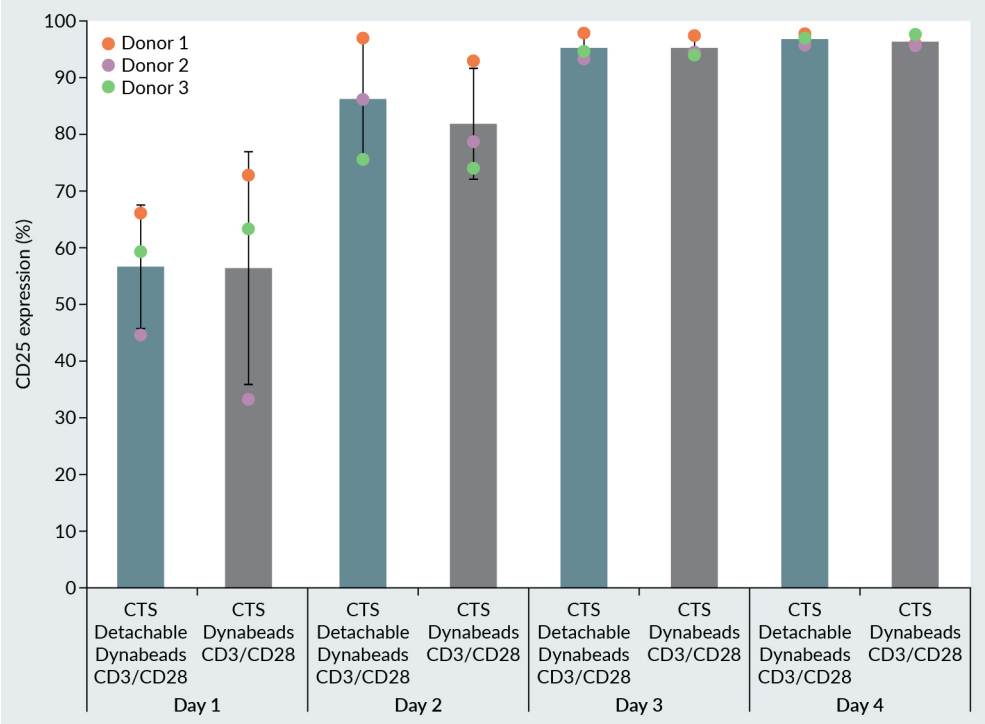
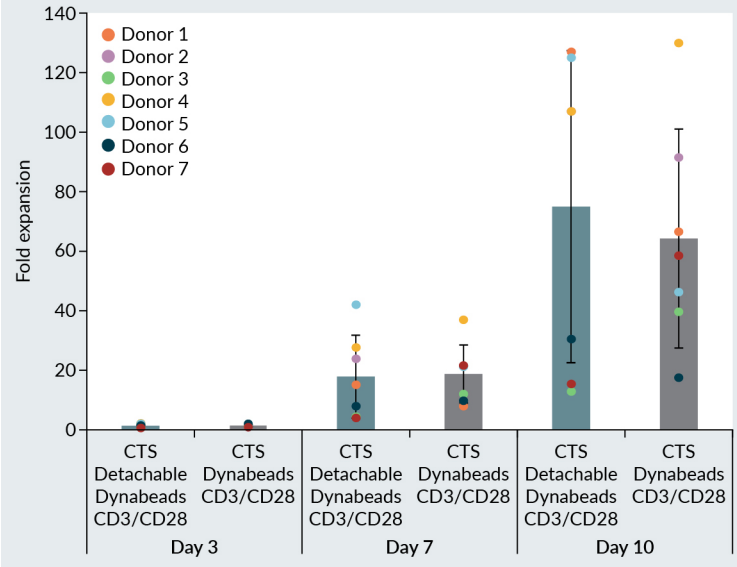


FIGURE 3

Cell expansion for active-and passive-release Dynabeads magnetic beads.



- An average cell expansion of ~20-fold by day 7 was seen for both the active- and passive-release Dynabeads magnetic beads
- An average cell expansion of ≥60-fold by day 10 was seen for both the active- and passive-release Dynabeads magnetic beads
- Individual donor samples drove the standard deviation

provide improvements to the cell therapy process.

One of these improvements involves T cell purity. With the CTS Detachable Dynabeads CD3/CD28 Kit, researchers achieved target cell purity levels of >98% (Figure 6). This consistency in performance is a necessity when the cellular starting

FIGURE 4

CD4:CD8 ratio for active-and passive-release Dynabeads magnetic beads.

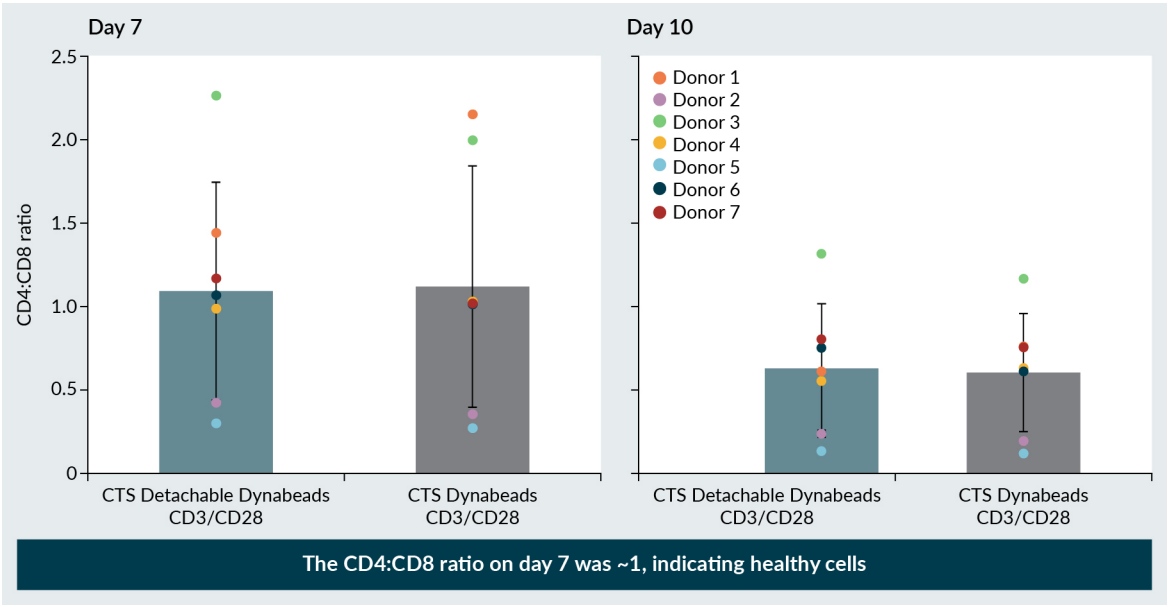
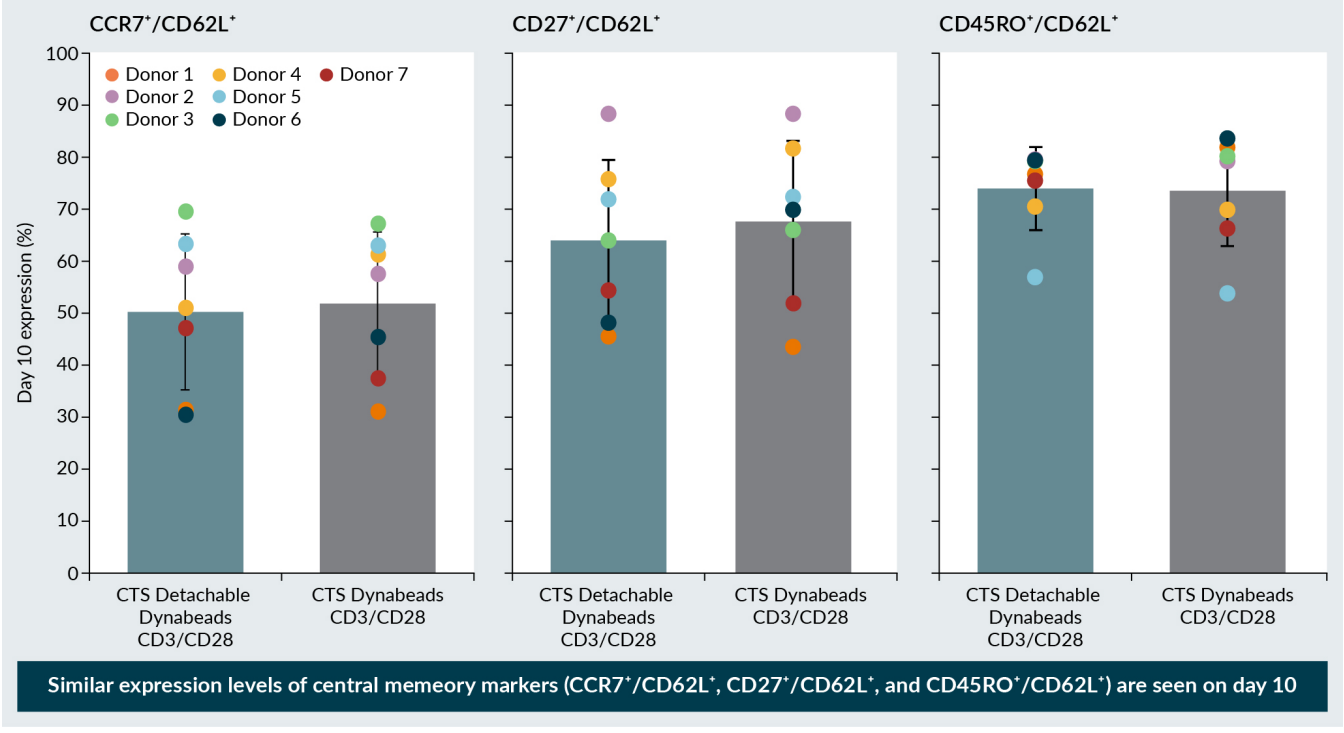


FIGURE 5

Memory T cell phenotype with active- and passive-detachable Dynabeads magnetic beads.



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material is characterized by substantial biological variability. Apheresis profiles are different from one individual to another, but also fluctuate further based on the patient's specific indication and stage of disease. Regardless of the starting material, it is necessary to achieve consistent performance with an isolation reagent.

Another improvement revolves around maintaining healthy cells, which is required for the production of an efficacious therapeutic. As shown in Figure 7, after actively releasing beads from the cells on days one, two and three, viability levels of >90% were observed with flow cytometry. Isolation efficiency was >90%, indicating specificity for early memory cells and revealing the robustness of the VHH antibodies.

Because the CTS Detachable Dynabeads CD3/CD28 Kit is designed for isolation and

FIGURE 6

Features of the iQue advanced flow cytometry platform.

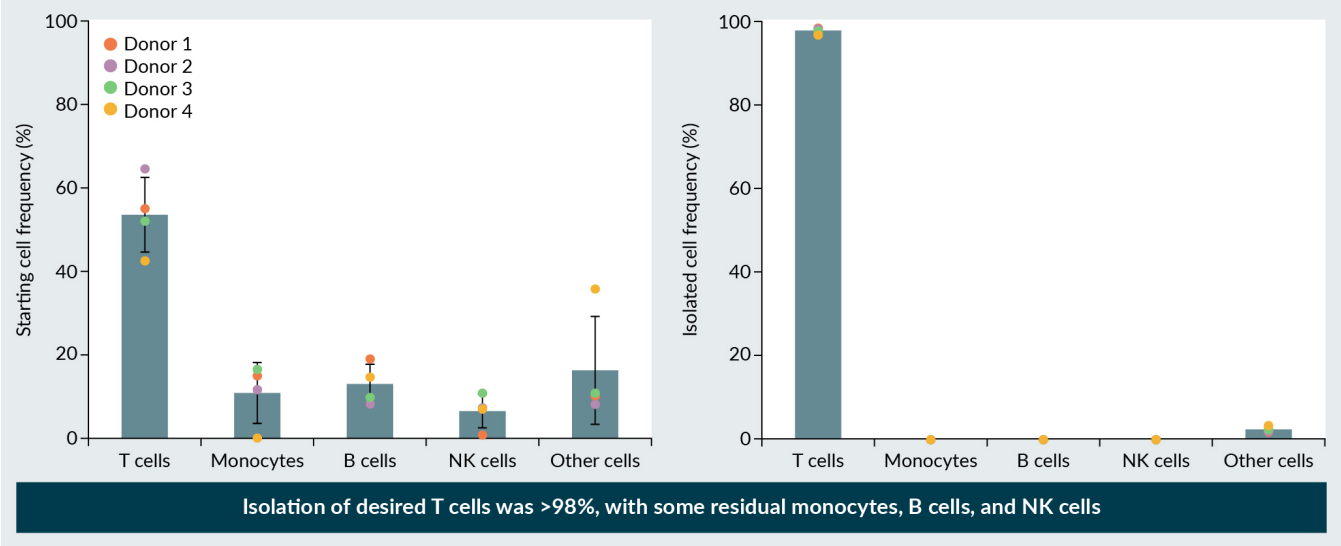
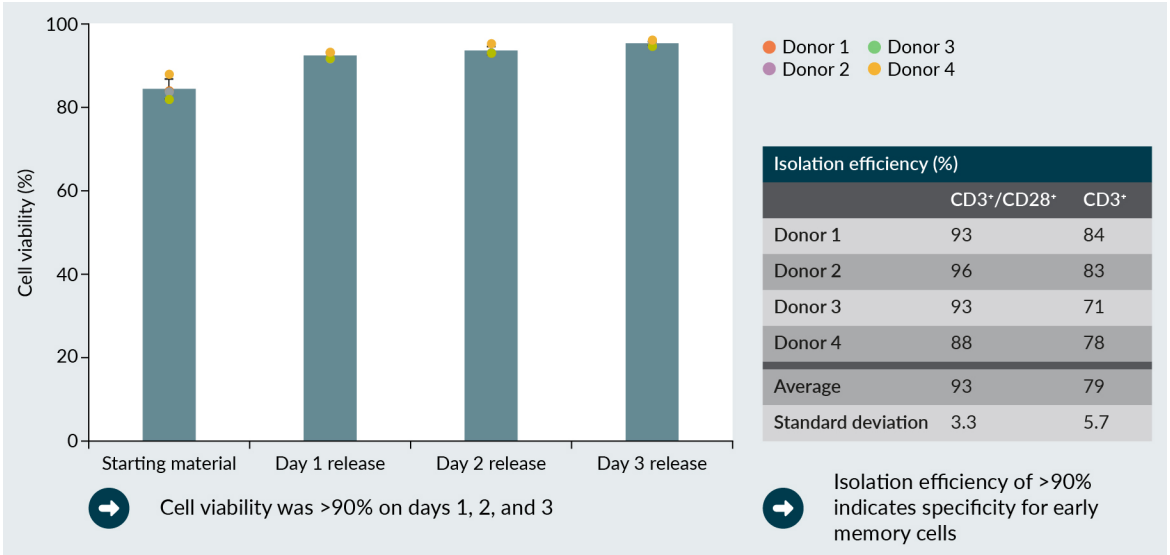


FIGURE 7

Cell viability and isolation efficiency of CTS Detachable Dynabeads CD3/CD28 beads.



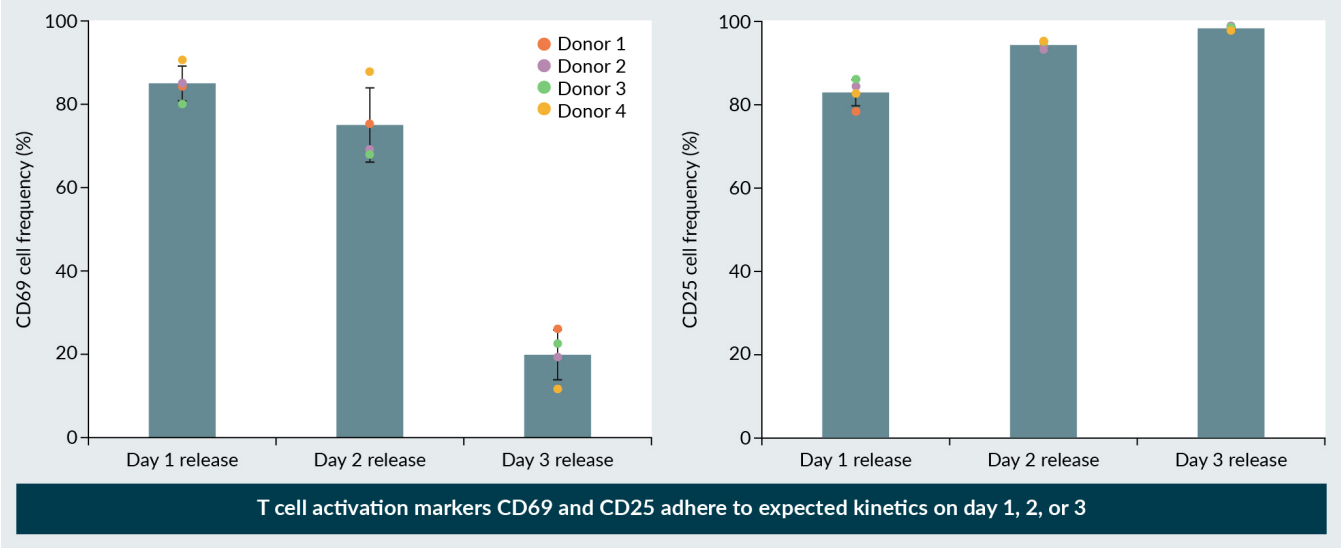
Isolation efficiency (%)		
	$CD3^+/CD28^+$	$CD3^+$
Donor 1	93	84
Donor 2	96	83
Donor 3	93	71
Donor 4	88	78
Average	93	79
Standard deviation	3.3	5.7

Isolation efficiency of >90% indicates specificity for early memory cells

activation in a single step, the activation profile over time was also evaluated, as shown in Figure 8. Flow cytometry was used to assess three different samples, which were analyzed from four different donors. Expected levels of CD69 early activation were seen decreasing through days 1, 2, and 3. Inversely, CD25 mid-to-late activation levels were observed to increase within the same time span. Therefore, T cell activation markers CD69 and

FIGURE 8

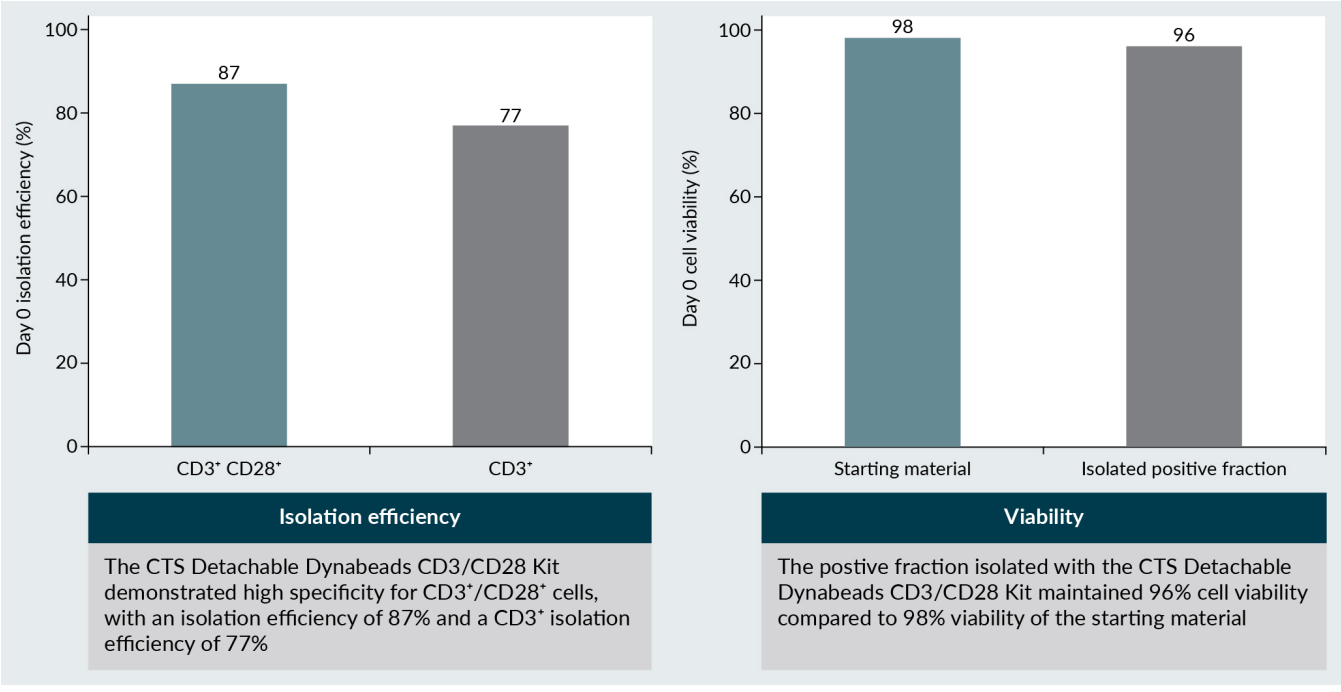
Activation markers for CTS Detachable Dynabeads CD3/CD28 beads.



T cell activation markers CD69 and CD25 adhere to expected kinetics on day 1, 2, or 3

FIGURE 9

Isolation efficiency and viability.



Isolation efficiency

The CTS Detachable Dynabeads CD3/CD28 Kit demonstrated high specificity for $CD3^+/CD28^+$ cells, with an isolation efficiency of 87% and a $CD3^+$ isolation efficiency of 77%

Viability

The postive fraction isolated with the CTS Detachable Dynabeads CD3/CD28 Kit maintained 96% cell viability compared to 98% viability of the starting material

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CD25 adhere to expected kinetics on days 1, 2, and 3.

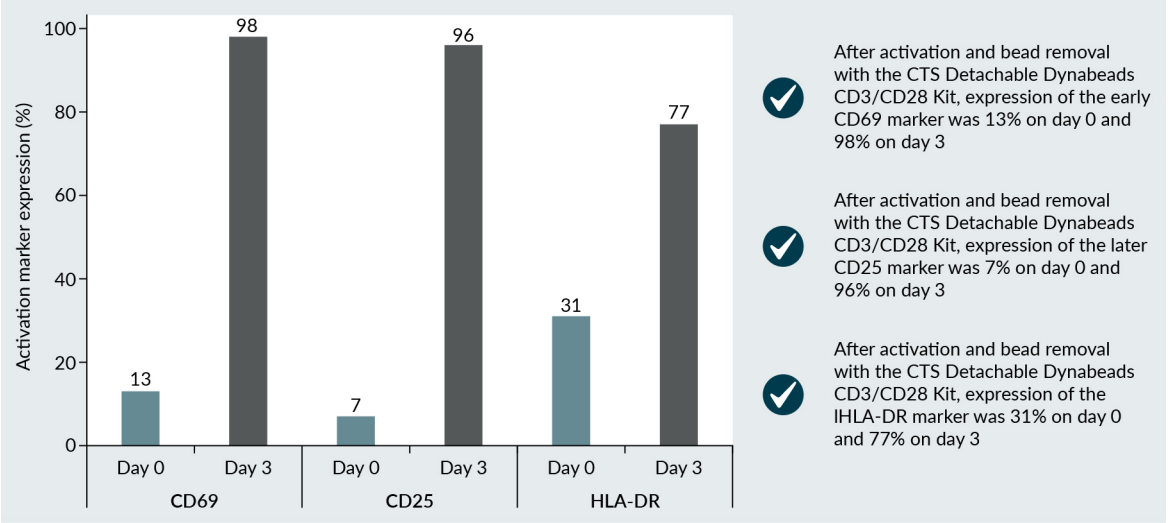
SCALING UP WITH AUTOMATED INSTRUMENTATION

As CTS Detachable Dynabeads have the ability to improve cell therapy in the context of process development, clinical trials, and commercial manufacturing, successful scale-up with the CTS DynaCellect Magnetic Separation System is vital. When scaling up with a variable starting material, researchers must achieve consistent performance with few impurities. When using an automated protocol on the CTS DynaCellect system, post-isolation purity of target T cells was 99.6% with virtually no presence of impurities such as monocytes, B cells, or NK cells. In addition, high isolation efficiency and viability were achieved during scale-up (Figure 9). Furthermore, optimal cell recovery was also achieved during the scale-up process. 106% cell recovery was demonstrated on day 3 after bead removal. The total count of more cells on day three as compared to day 0 was likely due to the fact that cells proliferate after activation begins and before incubation in expansion media. Lastly, after activation and bead removal, activation markers conformed to expected kinetics during scale-up, revealing consistently highly efficacious T cells (Figure 10).

In summary, CTS Detachable Dynabeads will provide the process flexibility, scalability, and consistent performance that cell therapy manufacturers need.

FIGURE 10

Isolation efficiency and viability.



Q&A

Eugene Kang

Q What is the recommended ratio of Dynabeads to target T cells?

EK: When the Detachable Dynabeads CD3/CD28 Kit is being used for isolation and activation in one step, a Dynabead-to-target cell ratio of 3:1 is recommended. I say target cell because the ratio should not be calculated based on total nucleated cells. That 3:1 ratio is based on a series of titration studies performed to optimize the number of Dynabeads, the isolation efficiency, and the activation profile. If using the kit as a downstream activation reagent, a 1:1 ratio is recommended.

Q How do you test bead residuals in the culture post-detachment and at the end of harvest to be sure there are no beads in the therapeutic product?

EK: Because cell therapy is constantly evolving and regulatory agencies are always playing catch-up, there is not yet an official ruling on the number of residual beads that can be in the therapeutic product. However, there is guidance to be followed.

There are a number of clinical trials and commercial manufactured drugs that use CTS Dynabeads in the United States. For those clinical programs, the US FDA's recommended guidance was a threshold of 100 beads per 3 million T cells. With these new Detachable Dynabeads on the CTS DynaCellect system, we have seen residual beads as low as two or three beads per 3 million T cells, which is obviously quite far below that 100 bead threshold.

Safety is a very important focus here. That is why we are minimizing the presence of residual Dynabeads far below recommended levels. On top of that, the Dynabeads themselves are inert during phagocytosis. Any residual beads that are left are not incorporated into the T cells and therefore, the patient.

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Q What are the minimum and maximum requirements for sample volume and cell concentration of the starting material?

EK: Again, it is highly recommended that the CTS Detachable Dynabeads be used with the CTS DynaCellect system. This will vastly cut cost, simplify workflow, and optimize overall performance of the final drug. Having said that, the CTS DynaCellect system can handle up to 1 L of volume from a starting material, with a concentration of 10 million cells per mL. This system has also been successfully tested with a volume as low as 10 mL.

The CTS DynaCellect system can also handle continuous Dynabead removal as long as input and output bags are swapped. This is why it is suitable for both allogeneic and autologous therapies.

Q Does the use of CTS Dynabeads magnetic beads pose any potential risks to patient safety?

EK: As mentioned previously, not only are the number of residual Dynabeads left in the final drug minimized, but the effect of the beads on the patient is also minimized since the beads are not incorporated into the T cells.

The CTS Dynabeads are currently being used in over 200 active clinical trials as of mid-year 2023 and, in terms of safety profiles, they have continued to meet regulatory expectations.

Q What is the duration of activation needed to achieve the desired T cell phenotype?

EK: Every manufacturing process is different, but right now, the norm is activating within a 24–48 h period. Within that timeframe, we are seeing optimal desired phenotypes (like the central memory stem cell phenotype) after activation. However, the duration of activation will really depend on how the protocol is modified and optimized.

Q What is the benefit of this product having VHH antibody fragments for both CD3 and CD28 cells?

EK: With naïve T cells, there are three different activation signals to look for: CD3 for T cell receptor engagement, co-stimulatory receptors such as CD28 for activation, and also cytokine stimulation (e.g., IL2).

Instead of having separate reagents for positive cell isolation and activation, having CTS Detachable Dynabeads with both antibodies on the surface allows you to combine isolation and activation into one step, cutting down cost and the number of steps in the workflow.

More specifically, the presence of both CD3 and CD28 allows for the preferential isolation of CD3 and CD28 double-positive cells. This is important because CD28 negative T cells have been shown to be associated with T cell exhaustion. By controlling that activation period, you can avoid exhausting the T cells and facing cell death.

BIOGRAPHY

EUGENE KANG is a Senior Product Manager at Thermo Fisher Scientific, with a personal desire to establish cell therapy as a first-line treatment worldwide. He holds a Bachelor's degree from the University of Notre Dame, a Master of Public Health from Columbia University, and a Master of Business Administration from Boston University.

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AUTHORSHIP & CONFLICT OF INTEREST

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EVOLVING

THE FUTURE OF CELL THERAPY MANUFACTURING: CTS DETACHABLE DYNABEADS CD3/CD28 KIT

Cell therapy

Welcome to your flexible process

Meet the next generation of CTS Dynabeads magnetic beads

You no longer need to wait for passive dissociation of Gibco™ Dynabeads™ magnetic beads from T cells or experience the limitations to your downstream process. The next-generation Gibco™ CTS™ Detachable Dynabeads™ CD3/CD28 Kit provides one-step isolation and activation, and enables the active release of CTS Dynabeads beads from your target cells at any point in the process. This flexible technology is meant for autologous and allogeneic platforms, as it enables consistent performance in light of biological variability. For your process, that means optimal isolation, activation, purity, yield, desired phenotype, and cell viability of your T cell population.

Other process improvements include:

- Increased control over target cell isolation and bead removal steps to help you shorten the timing of your overall process
- Increased control over cell activation and persistence of activation signals post-isolation
- High levels of pure T cells free from ancillary material
- Optimal recovery of target cells
- Desired cell phenotypes for downstream gene modification and fold expansion
- Suitable applications for both autologous and allogeneic processes

In combination with the Gibco™ CTS™ DynaCellect™ Magnetic Separation System, this automation-friendly solution is the first within a new platform designed to help accelerate current cell therapy manufacturing processes while driving the next generation of cell-based therapies.

Learn more at **thermofisher.com/ctsdynabeads**

gibco

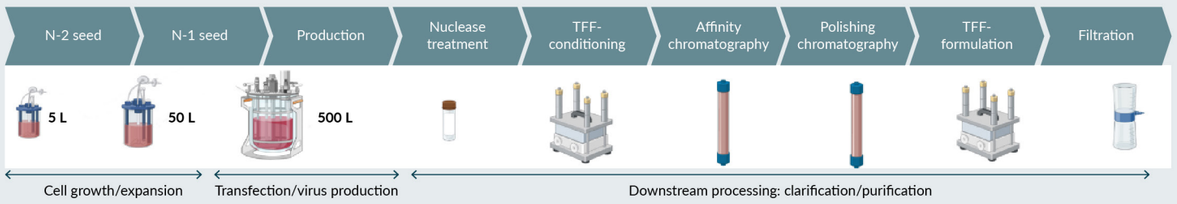
FASTFACTS

Is bigger better? Modeling AAV production to find optimization opportunities

Andrea Vervoort, Technical Lead, Virica Biotech

Within AAV manufacturing, enabling technologies can provide additive increases to yield and additional options for manufacturers to meet target demands. In this poster, it is demonstrated that increasing productivity and reducing scales can coincide through the use of bioprocess modeling.

Figure 1. Overview of bioprocess model configuration.



INTRODUCTION

One of the largest obstacles to the broader application of advanced therapies is the cost of manufacturing. Insufficient yield is arguably one of the most significant contributors as demand continues to outpace supply. Understanding the key cost drivers of production processes is crucial to creating more accessible and affordable therapeutics. Bioprocess modeling offers insights into manufacturing cost drivers to help inform the long-term feasibility of various production strategies. Considering key modeling output metrics can allow for more informed decision-making when considering multiple manufacturing strategies to increase yield.

KEY COST DRIVERS IN AAV MANUFACTURING

Understanding the economic feasibility of various production options is an important consideration when selecting a strategy to address manufacturing bottlenecks. BioSolve Process software (Biopharm Services Ltd) was used to develop a model of transient-transfection based AAV production in suspension HEK293 cells **(Figure 1)**.

The overall yield of the process was modeled as 1×10^{14} VP/L, and the dose size was selected from available information in a product insert from a commercially available gene therapy for spinal muscular atrophy, assuming a 65 kg patient size. Initial cell thawing, expansion steps, and fill/finish steps in the process were not modeled as these are product-dependent and outside the scope of the model.

The main cost driver for this manufacturing process was found to be within the production step, which contributed 50% of the overall cost per dose **(Figure 2)**. This is largely due to the costs of the GMP-grade plasmids needed in addition to high media, labor, and capital costs.

CASE STUDY: INCREASING PRODUCTIVITY OF THE PRODUCTION STEP

As the production step was the key cost driver in the manufacturing process, subsequent modeling aimed to understand the economic impacts of increasing productivity of the production step.

The modeling case study aimed to compare different process configurations to achieve a 4-fold increase in total yield. The baseline process was a 500 L production process operating at 1×10^{14} VP/L. Two options for producing 4 times more vectors were considered **(Table 1)**. Scenario A: scaling to a 2000L production at the same productivity of 1×10^{14} VP/L, or Scenario B, running 2×500 L reactors with optimization strategies to increase the productivity by 2-fold (2×10^{14} VP/L).

A 35% reduction in annual CoGs was achieved by running smaller bioreactors at higher productivity. This increases costs in capital, labor, and other (cleaning, etc.) but this was outweighed by a decrease in costs for materials and consumables **(Figure 3)**. Media consumption in the production step decreases by 49% in Scenario B, and smaller volume requirements mean that fewer plasmids are used. In addition, it is estimated that Scenario B will save over $2 million in associated yearly batch cost losses when a 7% batch failure rate was modeled.

Figure 2. Cost per dose breakdown by process step in AAV manufacturing.

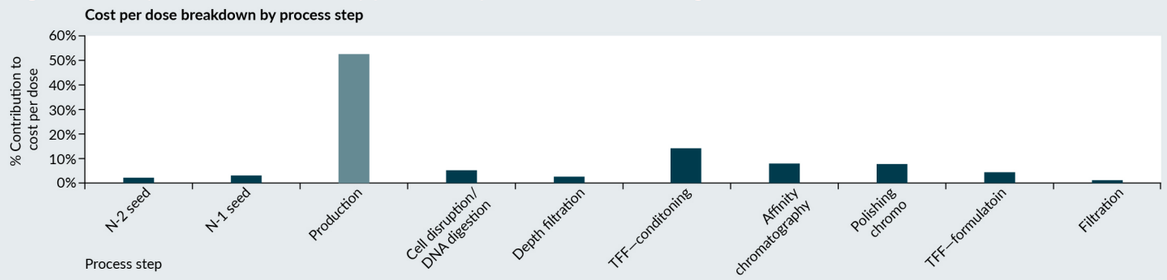


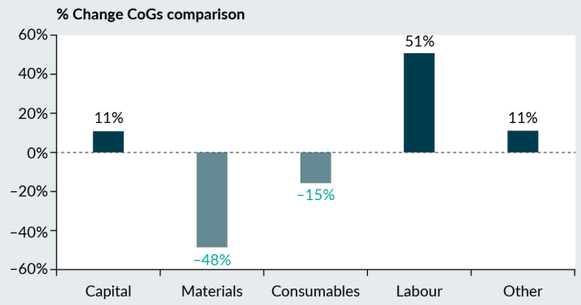
Table 1. Comparison analysis breakdown.

Comparison analysis	Scenario A	Scenario B	% Change
Size of bioreactor (L)	2000 L	500 L	
Number of bioreactors installed	1	2	
Bioreactor yield (VP/L)	1×10^{14}	2×10^{14}	
Annual CoGs (USD)	**94,070,592**	**61,393,848**	**–35%**
Batches per year	17	34	100%
Throughput (doses/year)	207	207	..

KEY TAKEAWAYS

- Bigger is not always better. Increasing productivity and reducing scales can alleviate the need for larger bioreactors.
- Enabling technologies can provide additive increases to yield and are additional options for manufacturers to meet target demands.
- ViricaTM uses Bioprocess Modeling to evaluate the impact of process changes and make informed decisions to drive down manufacturing costs of life-saving therapies.

Figure 3. Percentage change in CoGs breakdown: Scenario A to Scenario B.



CELL & GENE THERAPY INSIGHTS

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In partnership with: virica™

FASTFACTS

Streamlining nucleic acid extraction for biotherapy manufacturing: manual and automated solutions for success

Suzy Brown, Senior Field Application Scientist, Pharma Analytics, Bioproduction, Thermo Fisher Scientific

Nucleic acid extraction is a crucial first step for any downstream QC analysis. Sample preparation kits can enable lab productivity by reducing hands-on sample preparation time, increasing throughput, reducing costs, and improving the quality of sample extractions.

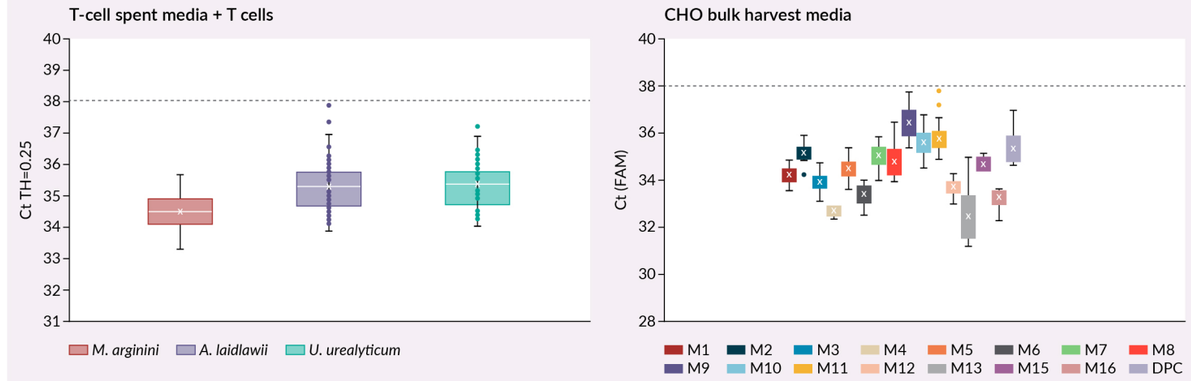
A critical aspect of the bioproduction workflow for complex therapeutic molecules, such as recombinant proteins and vaccines, is analyzing product purity, potency, and safety. At the heart of these analyses is the need to extract high-quality nucleic acids from various starting materials. This step is crucial in helping to ensure that subsequent analytical steps result in accurate data. However, nucleic acid extraction can be a complex and time-consuming process. Thermo Fisher Scientific offers numerous kits to help with nucleic acid extraction, including kits for manual or automated sample preparation.

THE IMPORTANCE OF NUCLEIC ACID EXTRACTION

The first step to implementing any molecular assay for therapeutic production analyses is an optimized nucleic acid extraction process. Downstream, real-time PCR sample quality is critical for any in-process and lot release tests. Therefore, the best sample preparation possible, with high recovery of DNA/RNA from complex matrices, is required. The success of a QC analytical test is dependent on: (i) sample preparation being able to remove non-specific molecules and any possible PCR inhibitory factors; and (ii) the ability to reproducibly extract very low levels of DNA or RNA when applied to residual purity testing or contaminant assays.

Figure 1. The MycoSEQ Plus workflow is a complete sample-to-answer solution.

Figure 2. Sensitivity at LOD demonstrated in two sample matrices.



Thermo Fisher Scientific offers a universal sample preparation solution using Applied Biosystems™ PrepSEQ™ chemistry to enable superior recovery efficiency for applications where consistent absolute quantitation and the highest sensitivity of detection are required. The PrepSEQ kits can be applied to manual or automated processes as needed.

APPLYING NUCLEIC ACID EXTRACTION TO MYCOPLASMA DETECTION

Biotherapy manufacturers need to quickly and confidently ensure their product and materials are free from mycoplasma contamination. The Applied Biosystems™ MycoSEQ™ Plus Mycoplasma Detection Kit is a TaqMan™-based qPCR assay that delivers actionable results in <1 day. This kit fits into the well-established MycoSEQ Plus workflow **(Figure 1)**.

The verification study data for the limit of detection (LOD) achieved using the MycoSEQ Plus kit workflow is shown in **Figure 2**. In this study, 10 genome copies/mL gDNA of each species were spiked into two sample matrices and processed using a typical workflow with lot release protocols. The T cell spent media represents example cell therapy samples, and the CHO bulk harvest represents recombinant protein processes. All data shown is above the positive cut-off threshold, proving the successful extraction of genomic mycoplasma DNA at only 10 GC/mL. The LOD results are in line with the expectations from regulatory guidelines for the validation of nucleic acid amplification techniques.

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In partnership with: ThermoFisher SCIENTIFIC

FAST FACTS

The evolution of gene therapy manufacturing

Rachel Legmann, Senior Director of Technology, Gene Therapy, Repligen

The complexity of emerging therapeutics like mRNA vaccines and viral vector-based gene therapies poses a challenge in achieving cost-effective large-scale production. This poster explores how integrating process intensification by advanced technologies in both upstream and downstream production of viral vectors impacted yield of potent vectors and vector recovery.

UPSTREAM PROCESS PRODUCTION INTENSIFICATION: KROSFLO® TFDF®-BASED PERFUSION BOTH FOR GROWTH & PRODUCTION PHASES

The perfusion technology in upstream production utilizes tangential flow depth filtration (TFDF), combining the advantages of tangential flow for high cell density and filtration for effective transmission through 2-5 µm sizes. The KrosFlo TFDF perfusion system offers substantial filtration capacity, rapid step processing, high flux, and scalability from a 2 L bioreactor to a 2000 L bioreactor. **Figure 1A** illustrates the impact of this technology on HEK293F suspension cell culture in a single-use bioreactor, transfected on day 3 using the Mirus Bio transfection reagent, TransIT-VirusGEN® AAV Kit, for AAV9. Notably, there is a discernible increase in vector yield AAV9 production compared to the control batch process.

Figure 1A. Vector yield of AAV9 in upstream production intensification using the KrosFlo TFDF system and a batch control across a 3-day period post-transfection.

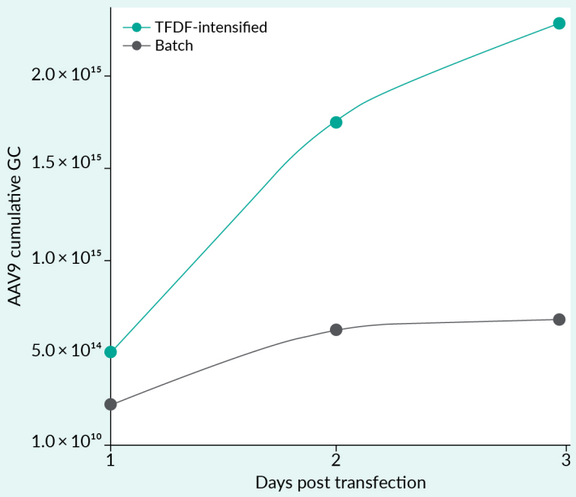


Figure 1B. Fold increase of the vector yield of AAV9 during perfusion process of upstream production intensification with KrosFlow TFDF and a batch control.

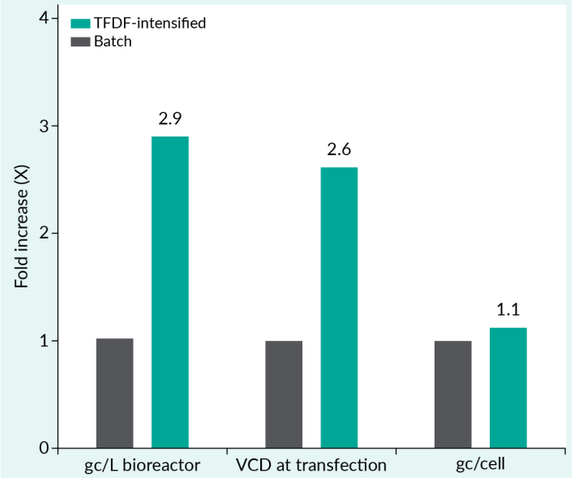


Figure 1B highlights the proficiency of the perfusion process during growth and production, post-transfection. This process enhances the total production yield of AAV9 per batch by almost 3-fold, attributed to higher cell density at the time of transfection and increased cell-specific productivity.

DOWNSTREAM PROCESS INTENSIFICATION: KRM™ CHROMATOGRAPHY SYSTEM SCALABILITY

During the downstream process, the focus shifts to increasing vector recovery yield during the affinity purification step. The KRM chromatography system was designed to address the challenges posed by large and fragile molecules to increase potent viral vector recovery yield. The single-use overmolded flowpath contains consistent internal diameter of tubing dimensions, minimizing connections to eliminate leak potential, and maintains optimal velocities (<2m/sec) to reduce hydraulic forces. Shear, a significant threat to the integrity of more delicate products like lentiviral-based therapies, is mitigated using a gentle quattroflow pump and the elimination of traditional hose-barb connections, all of which ensure higher overall yield, reduced process step deviation, and the protection of the bioactivity of complex viral vectors.

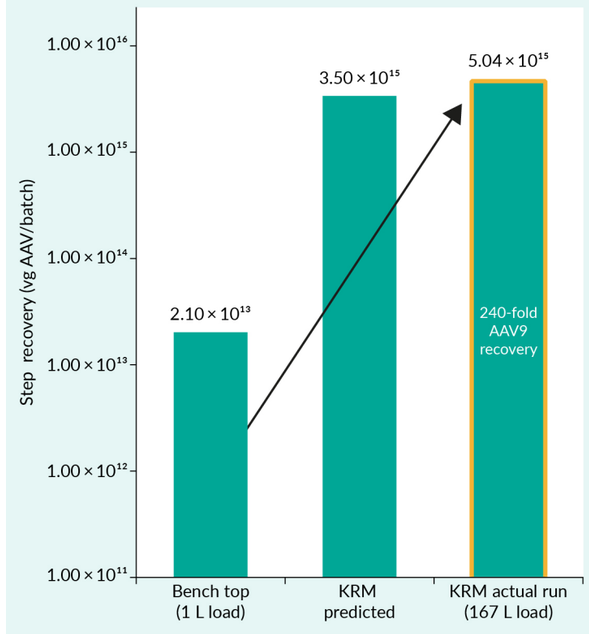
In a collaboration with Forge, a 500 L bioreactor was used with the same source material (AAV) split into a benchtop chrome control system (1 L) and the KRM chromatography scalability system (50 L), scaled up to 167 L, and reproduced. **Figure 2** demonstrates that while scaling up from 1 L to 167 L would traditionally yield a total viral increase of 167-fold, the careful redesign of the system and consideration of the molecule's journey with KRM results in a 240-fold AAV increase.

SUMMARY

During the upstream production process, the KrosFlo TFDF perfusion process effectively increases the number of cells producing virus with better specific viral vector productivity, while enabling virus transmission which allows for continuous perfusion to intensify AAV and lentivirus production. For the downstream AAV intensification process in this case study, the KRM chromatography scalability process successfully demonstrated a linear and better scalability in the overall viral vector recovery due to innovative chromatography system redesigns.

By integrating advanced platform technologies for intensification of viral vector manufacturing, this can help drive down the cost.

Figure 2. Fold increase of AAV vector recovery using KRM chromatography scalability system compared to a bench top control during the step recovery process of downstream intensification.



CELL & GENE THERAPY INSIGHTS

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In partnership with: REPLIGEN INSPIRING ADVANCES IN BIOPROCESSING

FASTFACTS

Unleashing the potential of mRNA therapeutics with high-quality bioreagents and optimized *in vitro* transcription

Jessica Madigan, Director Business Development, Nucleic Acid Modalities, BIOVECTRA

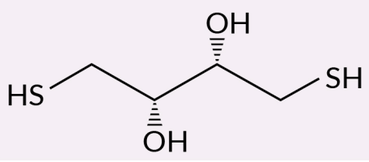
There are several challenges to address in the mRNA manufacturing workflow. For example, verifying raw material quality, performing drug product purity testing, and meeting regulatory standards are all obstacles that stand in the way of manufacturing mRNA cost-effectively, at a large scale, and in a timely manner. This FastFacts poster explores potential solutions to these challenges, including the use of GMP-grade raw materials, ensuring complete plasmid DNA (pDNA) linearization, and utilizing comprehensive analytical tools.

The utilization of mRNA therapeutics poses challenges in terms of mRNA degradation, stability, and delivery of lipid nanoparticles (LNPs), and immunogenicity upon delivery. Manufacturing mRNA therapeutics is complex in and of itself because it is difficult to achieve high-quality and highly pure mRNA with scalable manufacturing processes.

UTILIZING GMP-GRADE RAW MATERIALS

The most straightforward method to streamline manufacturability is to utilize an *in vitro* transcription process that yields the greatest amount of material at the highest quality and then preserves that quality throughout the downstream processing. For example, BIOVECTRA's process relies on GMP-grade raw materials for batch-to-batch consistency, even for early-phase clinical trials. This ultimately creates a consistent process and drug product. Using GMP-grade raw materials also eliminates the risk of needing to switch materials later in the development process, thus reducing the risk of program delays and regulatory concerns.

Figure 1. DTT molecular structure.

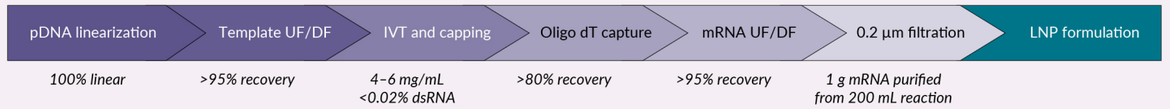


One such GMP-grade material is dithiothreitol (DTT), a reducing agent used to prolong the life of enzymes in a reaction tube by limiting the natural oxidation that would inactivate them **(Figure 1)**. This high-quality material, manufactured by BIOVECTRA and distributed by Thermo Fisher Scientific, is crucial in *in vitro* transcription buffers as it protects enzymes such as T7 RNA polymerase, inorganic pyrophosphatase, and RNAse inhibitors. It facilitates high-yielding reactions and high-quality mRNA transcripts. Furthermore, CGMP compliance of the material is aided by Thermo Fisher Scientific's validated and certified supply chain support and facilities.

pDNA LINEARIZATION

The mRNA transcription process starts with the linearization of a circular plasmid. Ensuring 100% linearization is essential for the prevention of run-on transcription and the production of undesired transcription products. Traditional linearization methods rely on agarose gel QCs. However, this method is only semi-quantitative and is unlikely to possess the required level of sensitivity to confirm 100% linearization. A solution to this lies in utilizing BIOVECTRA's HPLC analysis method, which can efficiently assess plasmid linearization efficiency. After linearization is ensured, BIOVECTRA's *in vitro* transcription process **(Figure 2)** can be followed to achieve high yield, low double-stranded RNA, and high purity full-length species with or without co-transcriptional capping.

Figure 2. BIOVECTRA's in vitro transcription process.



COMPREHENSIVE ANALYTICAL ASSAYS

Throughout the mRNA manufacturing process, comprehensive and GMP-compliant analytical assays **(Table 1)** are required for method development, method familiarization, qualification, and validation.

Table 1. BIOVECTRA In house analytical assays support all phases of clinical development.

Quality attribute	Product attribute		
	Plasmid DNA	mRNA drug substance	PLNP drug product
Content	DNA concentration	DNA concentration	DNA concentration
Purity/integrity	Purity (total plasmid) by agarose gel electrophoresis Purity (supercoiled DNA) by capilllary electrophoresis Residual protein by SDS-PAGE Host cell protein by ELISA Residual host cell DNA by qPCR Residual host cell RNA by HPLC Residual kanamycin	Poly A tail length/homogeneity Capping efficiency Purity by agarose gel electrophoresis Fragment analyzer HPLC Residual protein dsRNA by ELISA Residual DNA by qPCR DNAase contamination RNAase contamination	Polydispersity/size mRNA integrity
Safety	Endotoxin Bioburden Sterility	Endotoxin Bioburden Sterility	Endotoxin Bioburden Sterility
Identity/potency	Identity by restriction digest Sanger sequencing	Sanger sequencing In vitro potency/functional translation	Lipd components
Other	Appearance pH	Appearance pH Osmolality Residual solvents	Appearance pH Osmolality Residual solvents Lipid related impurities

SUMMARY

Using high-quality raw materials to optimize *in vitro* transcription conditions is one method to address common challenges in the mRNA manufacturing process, such as low yields, low purity, and high double-stranded RNA, to help ensure safe and effective drug products. A case in point, BIOVECTRA's GMP-grade DTT, can be sourced through Thermo Fisher Scientific.

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In partnership with: ThermoFisher SCIENTIFIC BIOVECTRA

WEBINAR DIGEST

Addressing regulatory guidance for HEK293 cells and AAV-based therapeutics manufacturing

Mike Brewer, Director and Global Principal Consultant, Regulatory, Thermo Fisher Scientific

Characterization of a biological product's identity and potency via analytical assays is necessary to allow for relevant specifications to be established. However, there are a number of regulatory hurdles associated with the development and validation of these analytical assays. This poster will summarize some of the most recent regulations concerning analytical methods to characterize the properties of a biological product. A range of regulatory-validated solutions to overcome these obstacles will also be introduced.

RESIDUAL HOST CELL DNA TESTING GUIDANCE

In order to minimize the biological activity of any residual host cell DNA associated with viral preparation and maintain the safety of the final product, these guidelines must be followed:

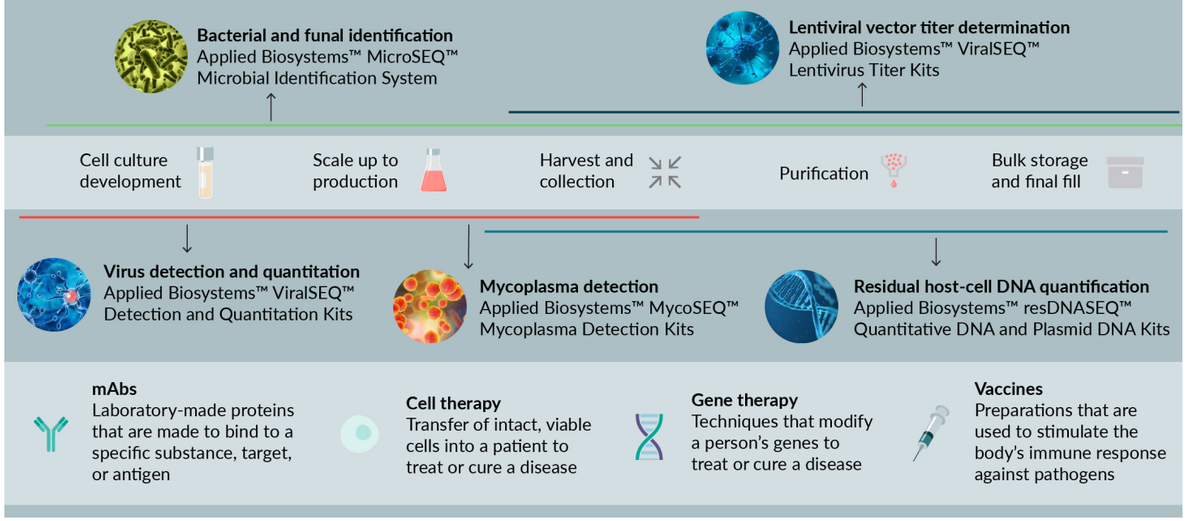
- Reduce the size of the DNA to below the size of a functional gene
- Decrease the amount of residual to 10 ng per dose
- Consider the characteristics of cell lines used in the manufacture of viral vectors, including the presence of tumorigenic sequences
- Optimize the manufacturing process to reduce non-vector DNA contamination

GENE TESTING FOR HEK293 GUIDANCE

In addition to controlling the host cell DNA content and size, the level of relevant transforming sequences should also be controlled in order to limit patient exposure. This can be accomplished by adhering to the following guidelines:

- Products made in 293T cells should be tested for adenovirus E1 SV40 Large T antigen sequences
- Products made in Hela cells should be tested for E6/E7 genes
- Tests should be appropriately controlled and of sufficient sensitivity and specificity to determine the level of the aforementioned sequences in your product

Figure 1. Integrated solutions across bioprocessing workflows from preclinical through commercial manufacturing.



MYCOPLASMA TESTING GUIDANCE

Vector safety testing should include sterility, mycoplasma, endotoxin, and adventitious agent microbiological testing. Mycoplasma testing specifically occurs in these scenarios:

- Mycoplasma testing is required at cell culture harvest for viruses used in gene therapy and for transduction of T cells in cell therapy
- In the manufacturing process of recombinant AAV, mycoplasma testing is typically done at the stage of bulk harvest from the bioreactor producing the recombinant virus
- Downstream, residual DNA impurities such as plasmid host cell DNA and E1A are measured

ASSAY VALIDATION GUIDANCE SUMMARY

The reliability of assays or tests used to evaluate a cell substrate in the context of intended use must be demonstrated. Assays related to assurance of safety should be scientifically valid prior to initiation of clinical trials. Guidance regarding validation of analytical assays may be obtained from the ICH Q2(R1) documents.

REGULATORY-VALIDATED SOLUTIONS

The constantly evolving regulatory environment for HEK293 cells and AAV-based therapeutics manufacturing necessitates integrated solutions. Thermo Fisher Scientific has established a multitude of real-time qPCR and dPCR assays in order to enable the manufacture of HEK293 and AAV-based therapeutics whilst meeting all regulatory requirements **(Figure 1)**. Each of these assays has been validated by multiple end users globally.

Watch the webinar here

Read the full article here

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