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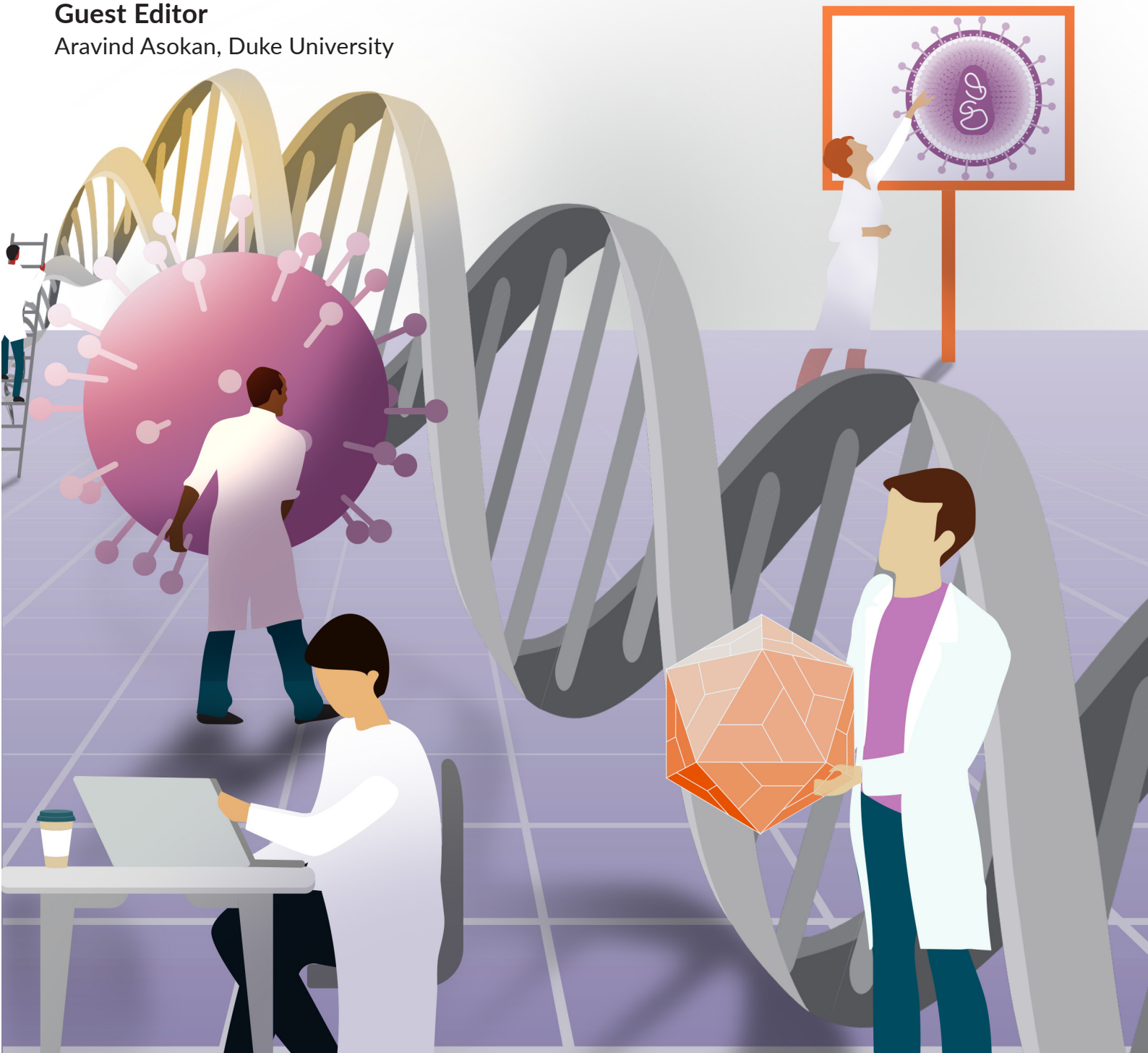
CELL & GENE THERAPY INSIGHTS

SPOTLIGHT ON
Gene delivery platform evolution part 1: viral

Guest Editor
Aravind Asokan, Duke University



CELL & GENE THERAPY INSIGHTS

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SPOTLIGHT

INTERVIEW

Assessing recent milestones in AAV capsid discovery & development for CNS applications

The CNS presents a formidable challenge to gene therapy developers, but the rapidly growing sophistication of viral gene delivery systems offer new hope to the field. **David McCall,** Senior Editor, BioInsights, speaks to (pictured) Voyager Therapeutics' **Mathieu Nonnenmacher,** Vice President, Novel Capsid Discovery about his team's work in discovering and developing novel AAV capsids with enhanced properties including tissue tropism.

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Q What are your working on right now?

MN: I am working on several approaches to discover AAV capsids with enhanced tropism for various tissues, mostly the central nervous system (CNS) and muscle. Our group is focused on evolving AAV vectors with improved transduction from intravenous dosing, so we are particularly interested in the transcytosis/extravasation process that allows transport of AAV vectors across endothelial cells. Our approaches are based on iterations of the proprietary TRACER™ RNA-driven evolution platform that we described in 2020 with

multiple AAV serotypes, capsid surface loops and screening methods (in cultured cells, rodents or non-human primates). We also perform extensive investigation into the mechanisms used by novel capsids to reach their target.

Voyager's TRACER™ AAV capsid discovery platform has generated novel capsids with high target delivery and blood–brain barrier (BBB) penetration at low doses, potentially addressing the narrow therapeutic window associated with conventional gene therapy delivery vectors. This platform is fueling alliances with Pfizer Inc., Novartis and Neurocrine Biosciences as well as multiple programs in Voyager's own pipeline. Voyager's pipeline includes wholly owned and collaborative preclinical programs in Alzheimer's disease, amyotrophic lateral sclerosis (ALS), Parkinson's disease, and Friedreich's Ataxia, with a focus on validated targets and biomarkers to enable a path to rapid potential proof-of-biology.

Q The CNS is an increasingly active and important therapeutic area for the *in vivo* gene therapy industry – what for you is driving this surge/resurgence in the field?

MN: I think the CNS offers both a formidable delivery challenge and a vast landscape of unmet medical need. Multiple hereditary or idiopathic neurological disorders are caused by well-defined mechanisms with known genetic targets, but they are currently out of reach due to the challenge of delivering genetic payload across the BBB. The current biologics or gene delivery vectors are not crossing the BBB efficiently and require either high doses or invasive local delivery, both of which present high risks of toxicity. This narrow therapeutic window is a major limitation for successful gene therapy of neurological disorders, and this is precisely what we are trying to solve by engineering viral vectors with improved BBB penetration and broad CNS targeting.

Q Systemic dosing to CNS may result in high viral load in the brain – how does this compare to viral load upon direct CNS dosing?

MN: A potential issue with direct CNS dosing is the steep gradient of vector accumulation between the injection site and the distal tissues. Because of limited diffusion of viral vectors through brain tissue, we and others have observed that large amounts of AAV vector are detected in the immediate vicinity of the injection site, but rapidly taper off in more distal regions. This could lead to a 'too much or too little' scenario where the biodistribution of the vector would go from one extreme to another, making it difficult to find the adequate balance between toxicity and efficacy. By contrast, delivery from the bloodstream typically allows a more homogenous and broad distribution through the brain and spinal cord. The CNS is a highly vascularized tissue, with a very dense network

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"Secondly, we and the field at large are constantly searching for capsid variants showing reduced liver accumulation relative to natural AAV capsids. This can be accomplished both by empirical evolution..."

of arteries, veins and capillaries, and it is commonly admitted that most brain cells lie within 10 or 20 microns of the nearest blood vessel. As a result, delivering a BBB-penetrant capsid from the vasculature allows a more homogenous and controllable distribution, as observed in rodent and primate studies.

Q High viral load in the liver has resulted in toxicity – what precautions can/should the field be taking when dosing CNS tropic viruses systemically?

MN: I believe there are two answers to this challenge. Firstly, a basic principle of toxicology is that the dose makes the poison. Our goal is to generate vectors capable of reaching their intended target at a dose that minimizes exposure to the liver or other off-target tissues. We have presented data indicating that some of Voyager's evolved capsids were capable of broad brain and spinal cord delivery without measurable liver toxicity and with minimal liver transduction. Secondly, we and the field at large are constantly searching for capsid variants showing reduced liver accumulation relative to natural AAV capsids. This can be accomplished both by empirical evolution, relying on chance to identify capsid variants with spontaneous de-targeting from the liver, or by rational capsid modification, using mutations known to affect hepatic distribution.

Q What are the chief measures being taken to mitigate the immune response to AAV capsids, and what is your interim analysis of progress to date in this area?

MN: Full disclosure: I am not an immunologist. That being said, I think most of my peers would agree that we must accept that AAV capsids, like most viral particles, will elicit an immune response. Before going further, we should draw a distinction between the various types of immune response against gene therapy vectors, and the rationale for their mitigation. Systemic injection of AAV vectors typically elicits both humoral and cell-mediated

immunity against the capsid, and in some instances, a response against the transgene product. The antibody response against the capsid is generally not considered problematic unless a re-dosing is necessary - for example, in the case of Pompe disease. Recent work from Barry Byrne, notably, has shown that formation of de novo antibodies against AAV vectors can be successfully blocked by a combination of steroids, mTOR inhibitor, and B cell depletion.

Perhaps more problematic is the induction of a cell-mediated immune response following high-dose AAV delivery. This T cell response can result in liver toxicity, as observed in various animal studies and clinical trials. This response is typically managed by prophylactic or therapeutic administration of immunosuppressants, and is thought to be initially triggered by the activation of the Toll-like receptor TLR9 by unmethylated CpG present in the AAV genome. This has led to multiple research efforts aiming at engineering CpG-depleted or 'cloaked' genomes containing TLR9 antagonist sequences, and is still a very active area of investigation. A third aspect of AAV immune response relates to the high prevalence of anti-capsid antibodies in the human population, as a result of natural infections in early childhood. This is problematic because even low levels of circulating antibodies can dramatically reduce the efficiency of AAV vectors. The strategies aimed at solving this issue include either the removal of circulating antibodies by biochemical or enzymatic methods, the use of capsids with non-primate hosts such as AAV5 that have been shown to largely evade neutralization, or the engineering of capsids by systematic mutation of dominant epitopes.

Q Tell us about the state-of-the-art in AAV capsid engineering tools as you experience it today – what for you have been the most significant advances over recent times, and equally, where would you like to see efforts around further innovation and improvement focused?

MN: I believe (and this is obviously very subjective) that there were three major advances in AAV capsid evolution: 1) the seminal studies from Martin Trepel's and Michael Hallek's groups in 2003 that first established a successful platform for AAV capsid evolution, 2) the first use of AAV barcoding and next-generation sequencing allowing high-throughput capsid analysis from Hiroyuki Nakai's lab in 2014, and 3) the work by Ben Deverman in 2016 showing that cell-specific functional screens provided a significant advantage over passive recovery. Most current efforts in AAV capsid evolution are using some combination of these three elements, with an impressive record of success over the past few years.

The addition of machine learning to the arsenal of tools used for capsid engineering is also very promising. Lastly, the field has largely pivoted away from rodent studies towards non-human primates (NHP), which hopefully will provide more predictive data for the translation of engineered vectors in humans.

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"The focus is now moving to the characterization of capsid tropism in human tissues, using multiple methods (in vitro assays, organoids, whole organ explants, cross-species equivalence, receptor identification)."

Q Voyager has enjoyed notable licensing and partnering success with its novel AAV capsids – what are the key characteristics and differentiators of the Voyager approach that enable this?

MN: The clinical potential of engineered AAV capsids is now widely recognized, and this is especially true in the CNS where preclinical studies have repeatedly demonstrated that evolved AAV variants could be a game-changer. Voyager benefits from having been an early proponent of RNA-driven evolution of AAV libraries, which combines the dual advantages of function-driven screening and in-primate evolution. This allowed us to discover some of the first capsids with dramatic improvements in primate CNS transduction. In addition, Voyager has built a fully integrated pipeline of capsid discovery, manufacturing and characterization, using state-of-the-art technology in all three areas to ensure rapid and accurate analysis of capsid tropism. The TRACER™ platform is generating a large number of capsid candidates, far more than we could develop internally, so we believe there is great potential for additional partnerships. Our capsid licenses are structured around the target, not the capsid, so multiple partners may select the same capsid, and we may also select that capsid for some of our own internal programs.

Q Looking to the future, what will be some key directions or next steps for discovery in the novel AAV capsid space?

MN: Given the breakneck pace of new discoveries and technical upgrades in the AAV capsid field, it would be presumptuous to predict the directions it will take, but we certainly hope that clinically meaningful next-generation capsids are right around the corner. The goal posts have progressively shifted from mouse capsids to primate capsids, and now to NHP-human translation. The focus is now moving to the characterization of capsid tropism in human tissues, using multiple methods (*in vitro* assays, organoids, whole organ explants, cross-species equivalence, receptor identification). Another key development may be the further refinement of capsids towards more cell or organ specificity via iterative cycles of empirical or structure-guided engineering. This, in conjunction

with the constant progress in transgene optimization, will hopefully allow the assembly of customized gene transfer vectors restricting expression exclusively to the target cell population, with minimal exposure of other tissues.

Another key progress in capsid engineering will be the systematic identification of the attachment receptors used by engineered capsids. The last years, or even months, have seen very rapid progress in this area thanks to the work performed in rodents by the groups of James Wilson, Ben Deverman, Viviana Gradinaru, and Aravind Asokan. This has a crucial importance in the understanding of capsid properties and for designing innovative receptor-first engineering strategies.

Another emerging avenue of research with very high potential resides in the vast untapped resource of autonomous parvoviral vectors. Research from the laboratories of John Engelhardt and Robert Kotin, notably, have shown that these naturally-occurring viruses come with unique tissue tropisms and – for some – a significantly larger packaging capacity than AAV-derived vectors. It will be exciting to see these vectors being developed and added to the list of candidates for a variety of clinical indications.

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

MN: My team is determined to generate capsids with the highest possible performance and the highest confidence for translation into human patients. We are pursuing all of the possible approaches to achieve that goal using the full potential of the TRACER™ platform. We are in the process of generating, improving and designating the best possible capsid candidates to meet the specific profiles that will be the best fit for our most advanced gene therapy programs. My group has also been able to identify the cell surface receptors used by some of our engineered capsids to cross the BBB in primates, which is an area of research that we are particularly excited about. To my knowledge, these are the first in-primate models of capsid-receptor interaction capable of ferrying viral vectors across the BBB, and this has very broad implications for the design of both viral and non-viral brain therapeutics.

BIOGRAPHY

MATHIEU NONNENMACHER is currently the Vice President of the Novel Capsid Discovery group at Voyager Therapeutics, a Cambridge-based gene therapy company using AAV for treatment of severe neurological diseases including Huntington's disease, Friedreich's Ataxia and amyotrophic lateral sclerosis. Mathieu obtained his PhD at the Pasteur Institute Paris, where he worked on human papillomavirus under the direction of Professor Gerard Orth. After this, he joined the group of Thomas Weber and Roger Hajjar in Mount Sinai School of Medicine, New York, to study the basic biology of Adeno-Associated Virus with a strong focus on cell

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biology and viral particle trafficking. Later during his postdoctoral studies, he identified a natural mechanism of AAV life cycle allowing an unexpectedly high capsid-genome correlation in the production of mixed viral populations. This discovery had important implications for the development of complex AAV libraries for Directed Evolution of AAV capsids. Mathieu Joined Voyager Therapeutics in December 2014 as a Senior Scientist in charge of capsid engineering. There, he developed the TRACER™ functional biopanning platform for functional and cell-specific screening of capsids with enhanced transduction properties in specific tissues. The platform was validated in mouse in 2020 and has since been successfully applied to non-human primates, leading to the identification of numerous AAV variants with improved tropism in primates. The TRACER™ platform has led to several partnerships between Voyager Therapeutics and major actors in the biopharmaceutical industry (Pfizer, Novartis, Neurocrine Biosciences), and several variants are currently under evaluation for multiple clinical programs. Currently, Mathieu's group at Voyager Therapeutics is pursuing TRACER™ capsid development using multiple approaches and is simultaneously investigating the molecular mechanisms leading to the improvement of capsid properties.

AFFILIATION

Mathieu Nonnenmacher, PhD
Vice President,
Novel Capsid Discovery

AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

GENE DELIVERY PLATFORM EVOLUTION
PART 1: VIRAL

SPOTLIGHT

INTERVIEW

Utilizing AI to drive AAV-based gene delivery to the CNS

Artificial intelligence and machine learning are entering an era of practical application in the cell and gene therapy space—with exciting early results. **David McCall,** Senior Editor, BioInsights, speaks to **David Huss,** Chief Scientific Officer, Shape Therapeutics, about ShapeTX's application of AI across the organization, from novel AAV capsid discovery and development to honing core business practices.

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Q What are you working on right now?

DH: At Shape Therapeutics, we focus on building an end-to-end drug discovery and development pipeline, combining payload, delivery, and manufacturing technologies. We assembled a team of people who are excited about technology development and creating new drugs, combined with people who have experience in the machine learning/AI space and want to apply it to biological problems. At our core, we have a team of people who like to solve hard problems. We have focused our problem-solving in a field that we call programmable RNA medicine. Within this field, we work on creating effective medicines quickly, targeting both diseases with large patient populations and individuals who have rare mutations.

We have created a suite of platform technologies, which cover everything from our RNA-editing payloads to engineering AAV vectors to have precise targeting so the payloads are delivered to the right cells in the body. We have also done a lot of work on the manufacturing side, which is vital because charging millions of dollars for gene therapy is not tractable when you are talking about large indications like Alzheimer's or Parkinson's disease. We therefore work on innovations to improve quality and drive down the cost of manufacturing and subsequently, of the therapies themselves.

Q How does ShapeTX harness AI to inform and guide its drug discovery and development?

DH: ShapeTX is at the forefront of a new era in drug discovery and development, where recent advances in generative machine learning are being harnessed to solve critical biological challenges. In the tech space, we have seen the development and implementation of cutting-edge AI—tools like DALL·E 2 or ChatGPT. We are applying similar approaches to biological data. For example, an RNA molecule is a combination of four nucleotides, four letters, positioned one after another, which means that you can use language processing algorithms to learn about RNA characteristics that yield a specific biological outcome. Additionally, RNA folds in a dynamic way and creates structures that are akin to images, enabling the application of diffusion models to create an RNA molecule for a specific biological outcome. Importantly, with any type of AI or machine learning, you have to consider the quality of the foundational data sets that build the models. That is why we spent the first couple of years of ShapeTX's existence doing very intensive high-throughput screening. We generated billions of data points on aspects ranging from creating RNA molecules and novel AAV capsids to manufacturing technology. Our sole focus was on generating foundational data sets to which we could apply different AI principles.

Ultimately, this allowed us to create generalizable models, so that if we see a new mutation or a new target, we no longer need to perform extensive high-throughput screening on that target. Instead, we can go to the AI model that has been trained on these billions of data points and ask it to create for us a new solution for this new target. That is the level that we have reached in terms of using AI to interpret biological data and generate novel solutions, and it allows us to find a solution much more quickly than if we had to go back to the screening phase.

Q What are the chief considerations for the AAV vectors ShapeTX utilizes—for example, relating tropism and the RNA payloads they carry—and can you talk us through the key related choices you have made?

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"An optimal gene therapy delivery solution to the brain for example, would likely include the ability to give an IV-administered dose that allows very good brain penetration into deep brain structures."

DH: Those in the gene therapy field recognize that when you give a wild-type AAV such as AAV5 or AAV9 through an IV administration, most of it goes to your liver. That has important consequences. Firstly, liver toxicity has long been one of the key concerns in the gene therapy space. Secondly, if your drug product is primarily going to the liver, unless you are treating a liver disease, it is not getting to where you want it to go. An optimal gene therapy delivery solution to the brain for example, would likely include the ability to give an IV-administered dose that allows very good brain penetration into deep brain structures. If this could be achieved, it would allow for a reduced dose with the safety benefits that go with it (including a reduction in the risk of liver toxicity).

At ShapeTX, because so many of the diseases that we are working on are central nervous system (CNS) disorders, it was a natural synergy to also apply high-throughput screening with AI technology to go after the engineering of viruses. The platform that we developed, which we call AAVid™, was built on the concept of screening massive diversity AAV libraries. We screened over a billion unique capsid variants in non-human primates (NHPs) to try to understand the properties of capsids that get into the brain and not the liver. Then, using that biological data, we built AI models that allow us to predict capsids that would be even better than those tested in that first screen. Of course, just predicting the capsid is not good enough—we actually need to go back and put those into NHPs to validate the models. Indeed, we now have capsids that give us one hundred times more brain penetration than a wild-type AAV9, and one hundred times less penetration in the liver.

While CNS is the area in which we have placed a lot of our initial focus, we are also working on diseases where we want to target cardiac or skeletal muscle, or photoreceptors in the eye. There, we took the same principles and applied them to some of these other capsid profiles. In terms of making choices when designing our vectors, we start by considering the disease, tissue, and cell type that we are targeting. We create a target product profile from which we then start to build and make choices regarding which one of our novel capsids best fits that profile. When we are thinking about our RNA payloads, we also do a lot of work in designing not just the guide RNA that recruits the ADAR enzyme inside the cell, but also methods to express enough of that guide RNA. We have done a lot of work on engineering synthetic promoters to drive robust, stable expression of RNA molecules. That is another

"we can go to the AI model that has been trained on these billions of data points and ask it to create for us a new solution for this new target. That is the level that we have reached in terms of using AI to interpret biological data and generate novel solutions..."

area where we think a lot about how much editing we want, and what expression level is appropriate. Overall, we take a fit-for-purpose, tailored approach.

Tropism is certainly one of the big challenges for gene therapy, but immunogenicity in vectors is equally as important. Once you have viral entry into the cell, you must drive a high level of functional transduction. We have conducted a lot of our work using AAV5 as our starting point, because it has the lowest level of pre-existing immunity in the general population. There is a lot of work being done by others on creating a window into the immune system (for example, using different types of immuno-suppressant drugs) so that you could enable your virus to deliver the payload before allowing the immune system to recover. There are a lot of novel approaches in the field aimed at either allowing re-dosing, or dosing in patients who have pre-existing immunity.

Q In addition to a lack of tissue-specific delivery systems, ShapeTX has cited limitations of traditional gene therapy approaches including the risk of introducing harmful genetic alterations and high manufacturing costs—can you expand on these challenges and how ShapeTX's approach addresses each of them?

DH: In terms of genetic alteration, much depends on the payload that you are delivering. With a DNA editing payload, there is the risk of making a permanent, heritable change to the cell. Any type of off-target consequence could be quite deleterious and would be passed on to daughter generations of that cell. In contrast, the beauty of our core payload technology, which is RNA editing, is that RNA is a transient molecule—therefore, an off-target would be captured only for the life of that RNA. Furthermore, RNA editing does not cause permanent changes to the genome in that cell. We also harness a protein called adenosine deaminase (ADAR), which acts on RNA and is present in every cell of the human body. By doing so, we are able to avoid the requirement of delivering a foreign bacterial enzyme, which is necessary with many of the CRISPR-Cas systems. By relying on a natural, fully-human protein, we reduce the risk of immunogenicity.

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There are a number of additional advantages to using an approach that targets RNA instead of DNA. With DNA, it is a bit of an 'all or nothing' proposition: if I make an edit in the DNA, one hundred percent of the RNA transcripts will be impacted. Sometimes, that is a desirable feature, but at other times, it could be a disadvantage. By targeting RNA, we are able to dial the efficiency of our payloads either up or down, so that we can make the appropriate impact. Furthermore, if I am thinking about altering RNA splicing or inhibiting a protein-protein interaction, or even knocking down a specific transcript, these are all things that we can do with an RNA editing technology.

Turning to manufacturing costs, most people in the AAV field use a triple transient transfection-based approach. Every time they make a batch of virus, they grow a producer cell line, and then add in three separate plasmids. This leads to a heterogeneity in the product from lot to lot, but even within a production run, some of the cells will take up all three of the plasmids needed to produce virus while others may only take up two. This is costly and inefficient. It is now possible to scale this type of process up to 2,000 L or even 4,000 L bioreactors, but in reality, you are just scaling up an inefficient process.

At ShapeTX, we took a step back and decided that there had to be a better way to produce higher yields with greater consistency. We decided to take a look at producer cell lines, starting in the antibody production field. Antibody processing also began by using a transient-based system, but the field then switched over to stable producer cell lines. Instead of transiently transfecting in the plasmids, you can stably integrate the instructions into the genome of the producer cell line, which allows you to grow much larger amounts. In addition, every single time you make that batch, it becomes much more consistent.

The problem in the virus production world is that two of the three plasmids required to produce virus are toxic to the cells—when you add them to the cells, the cells start dying. So, the key question was: could we stably integrate each of these components in the host cell genome, but in a silent manner? Similar to the antibody world, this would allow us to have master cell banks. We can grow them at a much greater density and can control when virus is produced from those cells, which leads to a much more homogenous product and lot-to-lot consistency that you simply do not get with transient transfection systems.

The results have been pretty remarkable. We are seeing much higher yields on a per cell basis, much greater infectivity, and much higher packaging for our cell line that we call TruStable™, because each of the components is truly stably integrated. Tying this back to cost, what it means is that instead of doing 2,000 L bioreactor runs and obtaining a certain amount of material, we can do things on a much smaller scale but produce more material. Ultimately, that allows us to drive down the cost.

Q You have touched on the measures to mitigate the immune response to AAV capsids—can you expand on the steps ShapeTX is taking in this regard?

DH: At ShapeTX, wild-type AAV5 was our starting point for capsid engineering because it has the lowest level of pre-existing immunity in the population. And as I mentioned, others in the field are exploring approaches to create a window in the immune system that enables treatment of individuals with pre-existing immunity. An alternative approach taken by some is trying to create AAV capsids that are 'immune stealth', that is to say, unrecognized by the immune system. If successful, that would mean you could re-dose with AAV over and over again in a patient.

While immune stealth viruses would have incredible value to the gene therapy field, as an immunologist, my personal opinion is that the immune system is extremely good, particularly upon re-exposure to a virus that has not undergone further mutation. Therefore, a truly immune stealth virus will be a hard proposition. The first time the immune system encounters a virus, there is a time delay in its response. This is what allows AAV to deliver its payload effectively the first time around. But the second encounter is a different story and immunological memory kicks in and quickly clears the virus. That is why I think that creating something that is truly immune stealth will be very challenging. What this means is that the way we think about the immunogenicity challenge, whether it relates to re-dosing or simply getting an initial payload into a patient who has a pre-existing immune response to the vector, needs to be concentrated more on targeting the immune system directly rather than on engineering a virus to do that job. It also means that at ShapeTX, we are creating multiple AAV variants with a similar biodistribution profile that could be seen by the immune system as a 'first encounter' and allow delivery of the gene therapy payload.

Q Finally, can you sum up one or two key priorities that you have for ShapeTX as a whole over the coming 12–24 months?

DH: We spent the first few years of the company focused heavily on building our foundational platform technologies to ensure they were industrialized, scalable, and could enable a robust drug discovery pipeline for ourselves and our partners. A lot of that was done with the tools I have mentioned, like high-throughput screening to create massive data sets and applying cutting-edge AI to make sense of it all. We have now progressed into a new phase of the company, where we are moving beyond building technology pipelines to moving therapies towards the clinic in specific disease areas. This is our main focus for the next one to two years. This involves building out pharmacology data sets that allow us to selectively develop candidates and advance them towards the clinic.

A second priority will be continuing to improve our AI processes and implementing them into all aspects of the company. We do not use AI solely in our scientific endeavors—we also use it, as an example, in our human resources department to make sure that we have pay equity, and to ensure that we are not creating unseen biases in our hiring practices. It has been impressive to see how one can use AI throughout the organization, including for

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aspects that are more on the core business side. For us, continuing to be at that forefront of applying AI to all sorts of different problems, and coming up with novel solutions across the company, is vital.

BIOGRAPHY

DAVID HUSS obtained his PhD in Molecular, Cellular and Developmental Biology from The Ohio State University where he established himself as a card-carrying T cell immunologist. During his PhD, exposure to both basic research and clinical and translational medicine ignited David's interest to pursue a career in the biotech industry. In his professional career, David has held roles along the entire drug development lifecycle from discovery research to post-approval sales and marketing. David is currently Chief Scientific Officer at ShapeTX and has driven all aspects of the business, including financings, scientific strategy, and business development. His team of >60 scientists is developing programmable RNA medicines. Their innovations have caught the attention of pharma and venture capitalists alike. Under David's leadership, ShapeTX raised $ 150 million and secured a $3B research collaboration with Roche. Prior to ShapeTX, David led a T cell engineering team at Juno Therapeutics. Before Juno, he led preclinical research programs at Biogen and contributed important scientific insights into the Daclizumab and Tecfidera mechanisms of action, resulting in four peer-reviewed publications.

AFFILIATION

David Huss
Chief Scientific Officer,
Shape Therapeutics

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CELL & GENE THERAPY INSIGHTS

SPOTLIGHT

INTERVIEW

Leveraging derisked AAV vector development & delivery approaches to enhance speed to market for rare disease gene therapies

Speed to clinic and to market reign supreme in today's gene therapy biotech sector, so why reinvent the wheel? **David McCall,** Senior Editor, BioInsights, speaks to (pictured) **Adrien Lemoine,** Co-Founder and Chief Executive Officer, Bloomsbury Genetic Therapies about the efficiencies and derisking benefits to be derived from building on established know-how and regulatory precedents in the AAV-driven gene therapy space.

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Q What are you working on right now?

AL: We have been busy working on four programs since Bloomsbury Genetic Therapies began operations in August 2022. One of the main things that we are working on alongside our colleagues at University College London (UCL) is bringing our

first program to the clinic for ornithine transcarbamylase deficiency (OTCD) as a Phase 1/2 clinical trial. The clinical trial application (CTA – the UK's Investigational New Drug [IND] equivalent) was filed in December 2022 and we expect that the study will start this summer.

Q Tell us more about Bloomsbury's approach and platform

AL: I co-founded Bloomsbury with four academics at University College London (UCL) – Professors Paul Gissen, Manju Kurian, Ahad Rahim, and Simon Waddington. We were becoming frustrated with the time it was taking to get gene therapies for rare diseases approved in the field. To me, the five or so gene therapies approved to date did not seem to be a productive outcome for thirty years of research. Gene therapy development should be relatively simple and fast in rare diseases because most are monogenic disorders. Alongside my co-founders at UCL, we wanted to figure out how to develop drugs that can move quickly from the bench to clinic, with approvals being the endgame, since the only way to provide access to as many patients as possible is to gain regulatory approvals.

The beauty of gene therapy is its conceptual simplicity, but it has been challenging to translate this simple concept into a flurry of approvals. Now that we finally have a critical mass of approved products or programs which have shown success in the clinic, we thought a smart way to go about developing new programs was to try and leverage the successes of these pioneering programs.

My co-founders and I collaborated and we brought into Bloomsbury a few programs from their labs, which all had the same unified approach to development of using well-known capsids that have been used in other programs that have either gained regulatory approval or demonstrated clinical proof of concept. The reason we use well characterized capsids is to derisk safety, distribution, and manufacturability. If you work with capsids that have already jumped through the hoops and are potentially being utilized in an approved product, you know about the safety profile, you know where they distribute, and you know they can be made with the right quality, titer, and other important manufacturing metrics such as empty/full capsid ratio. All of those features have already been derisked by other companies and their manufacturing partners. However, this approach does require finding an indication that lends itself to using the same capsids and the same route of administration as used in more advanced therapies for other indications.

So, the common thread between our programs and our approach to development is learning from first-generation companies, particularly in terms of navigating regulations. This has been made possible by the fact that the goalposts of regulatory approval are finally stabilizing.

Beyond this, we have chosen to work with a Contract Development and Manufacturing Organization (CDMO) with commercial experience from the get-go to make near-commercial grade product for our first-in-human trials. This is beneficial because it enables us to deploy the strategy of conducting a single, Phase 1/2/3 trial to approval. This approach can be more expensive to begin with, but it can ultimately accelerate development considerably.

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"The need to perform additional trials in rare diseases can kill development timelines and therefore projects, because the patient populations are limited."

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We are driven by the urgency of bringing potentially curative treatments to patients who often have few other treatment options. The people in my small team are highly experienced in gene therapy work, with individuals who have gained experience with companies such as Orchard Therapeutics, GenSight Biologics or Freeline Therapeutics.

Q What does Bloomsbury's specific pipeline look like?

AL: The lead program is for OTCD, which is an X-linked liver disorder condition. In its most severe form, it presents as a complete lack of the ornithine transcarbamylase (OTC) enzyme, which is an essential component of the urea cycle. Without it, patients accumulate ammonia, which often can be lethal in the first days of life if not rapidly managed. Currently the only way to cure the condition is with a liver transplant. Patients who survive in the early weeks of life will usually be on a restrictive diet and symptomatic treatment for life, and they still risk brain damage or death from spikes in ammonia levels. A gene therapy for this disease could be curative. We are going with an approach using a capsid with enhanced liver tropism, adeno-associated virus (AAV)-LK03, which has been tested in humans already by Spark Therapeutics in hemophilia A and shown to be safe and effective.

Our other three programs are all for central nervous system (CNS) disorders. One is for a disease called dopamine transporter deficiency syndrome (DTDS). This disease was discovered ten years ago by one of my co-founders, Professor Manju Kurian. It presents in the early weeks/months of life with motor symptoms such as dyskinesia, and eventually evolves into full-blown parkinsonism and neurodegeneration when patients reach the approximate age of ten. The disease usually leads to death in the teenage years or early adulthood, following the progressive loss of motor function.

Our approach is to inject a vector coding for that missing dopamine transporter protein directly into the part of the brain where it needs to be restored. We have very encouraging preclinical data showing complete prevention of disease in a mouse model, including preservation of normal survival and motor function. This program was inspired by the seminal work of Professor Krystof Bankiewicz at the Nationwide Children's Hospital, who has his own program for a similar condition (aromatic L-amino acid decarboxylase deficiency, or

AADC – the same indication that the gene therapy for intra-brain administration Upstaza is approved for in Europe). Professor Bankiewicz has been a long-term advisor to the team at UCL and now, to Bloomsbury Genetic Therapies. We have borrowed heavily from his approach: we inject in the same part of the brain and use the same capsid (AAV2) – again, it is about leveraging what has already worked to derisk as much as we can in our own target indication. When we met with the Medicines & Healthcare products Regulatory Agency (MHRA) a few weeks ago to present our approach, we received vindication through the confirmation that we do not need to do any further non-clinical work to progress to filing for a CTA for a Phase 1/2/3 clinical trial.

Our other two programs are for whole-brain disorders – Niemann-Pick disease type C (NPC) and infantile neuroaxonal dystrophy (INAD). Both of these diseases affect every neuron in the brains of the children born with the conditions. The idea here is to transduce as many neurons as we can to restore function in every neuron of either the NPC1 gene for the NPC disease, or the PLA2G6 gene for INAD. We are using AAV9 here in tandem with intracerebro-ventricular (ICV) injection to maximize the diffusion of the vector in the brain. This is an approach that has been pioneered by others. We have completed our short-term preclinical efficacy studies in mice and are currently completing longer-term preclinical efficacy studies, which will be complete by the end of the year. We met with the MHRA at the end of April regarding both programs, and will be able to say more once we have feedback from them. I am confident that we will be able to move faster than many other gene therapy programs thanks to leveraging the AAV9 ICV approach which is already well-studied.

Q What are the key current obstacles to the rapid and cost-efficient translation of AAV-driven gene therapies into and through the clinic?

AL: Historically, the two main challenges have been safety and manufacturability, and capsids link both aspects. By using well known capsids like we do, you can derisk much of the process. Another element that is relevant is translatability, because what works in mice obviously does not necessarily work in non-human primates or humans. This is particularly true for AAV capsids – there are many that work very well in mice, but are poor at transducing the equivalent organ or cell type in humans. For our OTCD program for instance, the preclinical efficacy work was based on an interesting chimeric model – the FRG mouse model, which is basically a mouse grafted with the liver of an OTCD patient. The thinking was that if it works in that model, there is a very high conviction that it will work in humans.

Q Can you expand on the measures that you are taking to enhance tropism and mitigate the immune response to AAV capsids?

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"Route of CNS administration is an exciting and still-nascent field. There is a wide range of interesting approaches including intraparenchymal, ICV, intra-cisterna magna, intrathecal, or intrathalamic injections."

AL: It revolves around the choice of capsid, promoter, dose and route of administration. For instance, our OTCD program uses the AAV-LK03 capsid, which was designed at Stanford University as a second-generation capsid to improve the liver transduction of some first-generation capsids, such as AAV8.

The route of administration is also important. The location of the injection will naturally affect where the vector goes. As I mentioned, for the CNS disorders in our portfolio (DTDS, NPC, and INAD), we believe that direct brain administration is the right way forward to maximize efficiency and safety.

Injecting in a localized way allows lower dosages, which means better safety (and lower cost of goods) – and with direct administration in the brain, we have the additional benefit of it being a relatively immuno-privileged organ. This means that you see fewer neutralizing antibodies and less T cell reaction to brain administration compared to intravenous delivery, where you elicit certain responses that could either neutralize some of the therapeutic effects or create toxicities.

Route of CNS administration is an exciting and still-nascent field. There is a wide range of interesting approaches including intraparenchymal, ICV, intra-cisterna magna, intrathecal, or intrathalamic injections. Your choice of these options depends on where you want to go, which will depend on the indication. The capsid itself can either have a magnifying role or reduce distribution. Therefore, the combination of the region of interest and capsid must be tailored to the indication of interest.

Using IV administration to get into the brain is a challenge because, firstly, you may need a higher dose than when injecting directly in the brain and you expose the body to the potential toxicity of your vector (particularly the liver). Secondly, you need to cross the blood-brain barrier, which can be challenging. Then, either directing the vector to a particular part of the brain where you want therapeutic effect or ensuring diffusion in the whole brain (depending on the disease of interest) poses further technical challenges.

Q You worked on some trailblazing advanced therapy products at the likes of GlaxoSmithKline and then Orchard Therapeutics – can you share any particular reflections or lessons learned from those early

'pathway to commercialization' experiences that you seek to apply today?

AL: I was lucky enough to be part of the teams that worked on Strimvelis and Libmeldy at GlaxoSmithKline and Orchard Therapeutics, which were the first two *ex vivo* hematopoietic stem cell (HSC) gene therapies approved. One of the issues that I witnessed first-hand at those companies was facing the differing expectations of the regulators. To give you an example, in rare diseases, it is often challenging to get comparator data and it is not always ethical to run a placebo-controlled trial, so you need to rely on natural history studies. One option is to run a retrospective natural history study with data already collected by academic centers. This means that data was not designed to be leveraged for registrational purposes. So, such academic database has to be converted into being more like the kind of database an industry developer would collect during a registrational clinical trial, which can be very challenging when data points are missing, for instance. The European Medicines Agency has been sympathetic to the plight of the developers in this regard – I would say that the US Food and Drug Administration (FDA) has been somewhat more stringent, often wanting prospective studies instead, which represent a significant investment of time, effort, and cost for developers, particularly when multiple years of follow-up is required. The fact that the FDA has a higher bar in place than is the case in Europe was a challenge that caught most first-generation developers off guard, but now the field knows to be vigilant for this. Hopefully, at the FDA, the pendulum may now be swinging back into a more European stance. I am particularly optimistic about the work of current Center for Biologics Evaluation and Research Director, Dr Peter Marks. The COVID-19 pandemic led to a reduction in the available workforce with gene therapy expertise at the FDA in particular, as many people were deployed on COVID vaccine work: this is changing now, too.

Gene therapy is an interesting field in that we do not typically see academia going all the way to clinical development on their own. In the early 2000s, there was no money from industry to fund gene therapy in the aftermath of the high-profile adverse events in the *ex vivo* HSC gene therapy field. Those academics who took matters into their own hands at that time and sponsored trials deserve kudos. This did, however, create the challenge of having amazing data, but data that was generated in an academic setting, and which was not meant to be for registration purposes. Those studies did not use commercial-grade material, for example. It has been a challenge to figure out how to leverage this great data, but also to manage the expectations of investors. Just because you have amazing data, it does not mean you can file for approval next month – there is still a lot of work to be done.

One area of growing significance is the importance of newborn screening. For many conditions affecting the CNS, the window for treatment is narrow and we cannot reverse the loss of neurons caused by the disease. We often need to treat before the damage happens and the window can sometimes be only a matter of a few years. Oftentimes, patients are only diagnosed when symptomatic, and it is already too late.

The only way to detect these diseases in time for treatment is through newborn screening, which needs to be more systematic. There are many campaigns led by patient associations and

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industry developers, to expand the use of newborn screening to new indications and to ensure newborn screening occurs more consistently across US states and EU countries. In particular, they are leveraging the lower cost of genome sequencing. The UK is doing well in this area with a couple of pilots and initiatives, which should pave the way for a more general adoption of modern techniques for sequencing newborns and identifying disease early. This will hopefully lead to more patients receiving timely treatment for those diseases that currently have an approved treatment option, which is sadly still a minority.

Q What are your key goals and priorities for Bloomsbury Genetic Therapies over the next few years?

AL: On the business front, my main priority today is fundraising in this challenging environment. On the operations front, we are very excited that our collaborators at UCL and Great Ormond Street Hospital are on course to bringing our OTCD program to the clinic this summer. We will be able to see first clinical data soon after trial patients are treated, because this disease allows for quick readouts.

We will also leverage the great feedback we have received from the MHRA and we intend to move forward with our clinical development plans for BGT-DTDS – we aim to submit a clinical trial application to the MHRA in 2024. This will begin with making GMP vector at our CDMO of choice, which was carefully chosen following an extensive selection process. We are also looking forward to implementing the remaining IND-enabling studies for our NPC and INAD programs that will pave the way for translation into clinical trials.

BIOGRAPHY

ADRIEN LEMOINE is the CEO and Co-Founder of Bloomsbury Genetic Therapies, to which he brings his 20 years of experience in pharma and biotech. Following roles of increasing responsibility in commercial analysis, strategy, operations, business & corporate development at GSK and AstraZeneca, Adrien's previous appointment was Chief Business Officer of Orchard Therapeutics. Adrien holds an Engineering Degree from CentraleSupélec (Paris, France) and a MSc in Engineering and Physical Science in Medicine from Imperial College (London, UK).

AFFILIATION

Adrien Lemoine
Co-Founder and Chief Executive Officer,
Bloomsbury Genetic Therapies

AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

SPOTLIGHT

INTERVIEW

At the cutting edge of AAV capsid engineering

Having emerged from the Harvard Lab of George Church, Dyno Therapeutics has set about tackling the key challenges and opportunities for the AAV gene therapy field through an AI-enabled approach to capsid engineering. **David McCall,** Senior Editor, BioInsights speaks to (pictured left to right) Dyno's **Eric Kelsic,** CEO & co-founder and **Adrian Veres,** CSO & co-founder, about their vision and future plans to extend the reach of gene therapy.

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Q What are you working on right now?

EK: We have been hard at work on Dyno Therapeutics for the past five years and now we are excited to share updates on all the progress we have made solving *in vivo* delivery by engineering improved adeno-associated virus (AAV) capsids. In particular, we have made capsids that represent a more than 100-fold improvement over the

best natural serotypes, delivering to the retina after intravitreal injection, and also delivering pan-brain having crossed the blood–brain barrier following IV injection. In both eye and central nervous system (CNS), these capsids produce just as well as natural serotypes, and the CNS capsids also de-target the liver 10-fold. Based on everything we have seen to date, we believe that these are the best capsids available today for delivery to retina or CNS.

AV: It's been a long journey getting here with a lot of scientific problem-solving and company-building along the way – for example, we are now more than 100 employees strong. We have established great partners in eye, muscle, liver, and CNS, and thanks to this progress, we are now excited to partner even more broadly across these areas and beyond. Characterizing these optimized capsids in detail and preparing to share these updates has been keeping us very busy!

Q Dyno is pioneering the use of artificial intelligence and machine learning to improve AAV capsid engineering—can you go deeper on the discoveries that led to this approach, and what those entail in technological terms?

EK: Prior to Dyno, I led a team at Harvard in George Church's lab working on two technologies that catalyzed the formation of Dyno in 2018 to carry the vision forward. First was the ability to measure the properties of AAV capsids *in vivo* in high-throughput—in particular, using DNA synthesis, DNA barcoding, and DNA sequencing. We showed we could measure the tropism of AAV capsid libraries in mice at the scale of hundreds of thousands of different sequences, and then later at Dyno, we optimized those methods to do the same for non-human primates in order to generate the most relevant data for predicting translation to humans. Secondly, we showed that we could input all of this data to machine-learning models, and predict the performance of capsid sequences which the models had never seen. That enables us to ask these models about all of sequence space to identify the most promising capsids with improved delivery properties. We learned how to automate this workflow (what most people are now calling generative artificial intelligence, or generative AI), published on that in *Science*, *Nature Biotechnology*, and several other preprints, and then began operationalizing these workflows to make better capsids so that we can make more effective and safer gene therapies to help more patients. There is a lot of information available on Dyno's website about the improvements we have made since, which I will just summarize by saying that the scale and breakthroughs built into Dyno platform methods are equally impressive compared to the improved capsids mentioned earlier. Right now, for us, it is probably the most exciting time to be working at the intersection of technology and biology, due to how powerful these technologies are becoming and how much impact they can have.

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Q Dyno has highlighted the value of multiple trait engineering – which feature(s) do you consider to be particularly critical/most pressing for the clinic right now?

AV: Successful delivery is only valuable if it can be done safely. That starts with highly efficient on-target tissue delivery. For example, if you are developing a therapeutic product for a CNS disorder, you must achieve efficient crossing of the blood–brain barrier and broad AAV transduction of neurons across brain regions. An important consideration then becomes reducing the amount of AAV delivery to off-target tissues. If you are aiming for CNS delivery, you want to minimize delivery to tissues such as the liver, dorsal-root ganglia, or spleen, which could cause toxicity. Finally, an AAV capsid with great on-target and off-target profiles will only truly be useful if it can be produced efficiently. Manufacturing efficiency impacts both the pace and cost of discovery work by making preclinical and clinical development slower and harder. Manufacturability will also be a key consideration that impacts cost of goods when bringing a new gene therapy to market—an important consideration when imagining a world where gene therapies are used broadly. Altogether, this set of properties:

1. Maximizing on-target delivery;
2. Minimizing off-target delivery; and;
3. Ensuring manufacturability, are the most important on which to focus.

We optimize for these properties across all of our AAV capsid engineering programs, and we've made progress designing capsids that are improved across all these dimensions simultaneously.

Q Can you expand on the measures being taken to mitigate the immune response to AAV capsids—both by Dyno and the field at large—and their relative prospects for success, from your viewpoints?

AV: When thinking about how AAV capsids interact with the immune system, it is important to consider two types of encounters. The first is pre-existing immunity, the second is inflammation in response to treatment. In both cases, one of the challenges capsid engineers face is thinking about not just a new capsid sequence, but also how it interacts with an individual's unique immune system. In the first case, that individual has been infected by a circulating, natural AAV serotype and developed a neutralizing immune response. This is a big issue for gene therapy development, as this can exclude—on the basis of their immune status—large (20–80%) fractions of the population as ineligible. Beyond

"To verify production efficiency, we always experimentally validate our best capsids with a production and purification workflow that is very similar to what gene therapy drug developers will use themselves..."

– **Eric Kelsic**

the core properties I mentioned before (on-target delivery, off-target delivery, and production), the ability to evade pre-existing immunity is another key property. It's a lot harder to measure at scale, but the good news is we have made interesting progress on this front that we hope to share soon. Since neutralizing antibodies bind to specific capsid locations, engineering these to differ from natural serotypes and thus evade pre-existing immunity becomes another property to optimize via sequence design, which is Dyno's core strength.

We think about the second topic, inflammation and immune response to the gene delivery itself, differently. The causes of the inflammatory response are not yet fully mapped out: teasing out exactly what is causing it is difficult, when it could be any one of: therapeutic dosage, the AAV capsid, the payload, or even other contaminants that might have been co-purified with the capsid. Steroids and other immuno-modulating agents clearly help and will play a role. From a capsid engineering perspective, our strategy is to follow the heuristic that the lower the viral dose that can achieve the same outcome, the lower the risk of inflammation in general. Now that we have capsids that deliver to a meaningful number of cells in the brain (~10–25% at a modest 1×10^{13} vg/kg intravenously-administered dose) the goal becomes: how do we achieve the same absolute rate delivery with an even lower dose? This also means there is enormous long-term value in continuously improving the efficiency of delivery: with a lower dose, gene therapies become both safer and easier to manufacture.

Q What are the considerations for process and analytical development that come with Dyno's approach, and how are you addressing them?

EK: As Adrian mentioned, at present we already optimize for efficiency of capsid assembly and genome packaging as part of our screening platform. And actually, this is one property that our machine learning models are very good at predicting—they are correct eight or nine times out of ten. This predictive accuracy is much better than we had imagined it would be, since from the work I did before starting Dyno, we knew that the vast majority of randomly mutated capsids are non-functional. To verify production efficiency, we always experimentally validate our best capsids with a production and purification workflow that is very similar to what gene therapy drug developers will use themselves, and are also in many

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cases working directly with contract development and manufacturing organizations (CDMOs) to produce Dyno's capsids during our validation workflows.

One new direction that I'm very excited about is Dyno's potential to directly partner with CDMOs, since we both support gene therapy developers. When we setup Dyno's capsid products within a CDMO partner's production system, this shortens the time needed to develop a gene therapy product, helping Dyno's therapeutic partners, and it brings more business to our CDMO partners, so it's a win for all parties, most especially for patients.

Q What will enable patients to benefit most from these advances in AAV capsid engineering?

EK: Last year, I had the opportunity to join a working group organized by the American Society for Gene and Cell Therapy (ASGCT) and present recommendations to the US FDA on sponsor applications using novel capsids, as part of ASGCT's annual FDA liaison meetings. I was glad that we were able to find agreement among capsid engineering experts from both industry and academia on how to demonstrate the therapeutic potential for using engineered capsids with optimized properties when drug sponsors are sharing data with the FDA to gain approval for starting human clinical trials. There are a number of different scenarios [1] that we imagined for how to demonstrate safety, vector efficacy, and payload efficacy across different organ targets and animal models. I am looking forward to seeing how the FDA will integrate these recommendations into their guidance to sponsors, and I am happy that the ASGCT chose to prioritize this topic for their annual meeting. As we mentioned earlier, it is an exciting time for the field, since the improvements we are seeing from engineered capsids hold the promise to make gene therapies safer and also applicable to a broader range of diseases, many of which lack good treatment options currently.

Q Looking to the future, what might be some key R&D directions or next steps for innovation in the novel AAV capsid space?

AV: Universal capsids capable of treating all patients. Because we know that naturally occurring AAV serotypes most often differ in about one third of their ~735 amino acids, it stands to reason that we could create completely novel serotypes once we are changing ~200 or more amino acids. A fully synthetic capsid, where most amino acids have been modified and designed to ensure both dramatically improved properties and to be different from what is seen in nature, should enable treatment of all patients who may be in need of a gene therapy. Given the complexity of the functions carried-out by an AAV capsid, creating a universal AAV capsid is a bold scientific undertaking, even in this era of rapid progress in

protein engineering. We are also in a prime position to take this challenge on, given our expertise in both machine learning and the experimental aspects of AAV engineering.

> "...continued progress will mean making therapies safer, more targeted, and more affordable, enabling gene therapy to become more applicable to more widespread conditions."
>
> – **Adrian Veres**

Q Lastly, can you each sum up one or two key goals and priorities that you have for your own work over the foreseeable future?

AV: Our first priority is to continue advancing the frontiers of gene delivery, because every step we take on this journey enables gene therapies to reach more patients. We have really only just started. With our platform up and running and a strong team behind us, we are expecting to see a lot of progress in the next two years. It is now possible to imagine reaching 90–100% of cells in a target tissue. From there, continued progress will mean making therapies safer, more targeted, and more affordable, making gene therapy applicable to more widespread conditions.. We are tremendously excited about that!

EK: Dyno's business is partnership-centric: we partner with gene therapy developers, providing them with the best capsids so that they can invest their efforts at the leading edge of genetic medicine. Because we are not concurrently developing our own gene therapy products, that makes us different from every other major player doing capsid engineering. We chose this path because we are confident that these new technologies will yield multiple generations of improved capsids that will be transformative in terms of what is possible for gene therapy, and we want to get there as soon as possible. Our business model also comes from being highly committed to maximizing our impact on gene therapy patients by ensuring these technologies are broadly distributed across the industry. I am excited about the progress we have made with capsid engineering and also how we have built our business to support partner success—now it is time to deliver on our promises to partners and form new relationships that will enable us as a field to more fully realize the potential of genetic medicines to help patients in need.

BIOGRAPHIES

UNDER ERIC KELSIC'S leadership, Dyno signed partnership agreements with Novartis, Sarepta, Roche, Spark and Astellas, and raised $109M in financing, including a 2021 Series A led by a16z. Dyno was named Xconomy's 2020 Startup of the Year and Eric was recognized as one of Endpoint's 20 under 40 next- gen biotech leaders in 2021. Prior to founding Dyno, Eric

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co-discovered the AAV MAAP gene and led a team in George Church's lab at the Wyss Institute of Harvard Medical School to measure a comprehensive fitness landscape of the adeno-associated virus (AAV) capsid protein and develop the technology underlying Dyno's artificial intelligence powered capsid engineering platform. He earned a PhD in Systems Biology from Harvard University and a BSc in Physics from Caltech.

ADRIAN VERES is a scientist who is most satisfied interfacing advanced experimental and computational approaches to solve complex systems. Prior to founding Dyno, Adrian was focused on applying next-generation sequencing-based assays and high-throughput screening techniques to advance cell therapies for Type 1 Diabetes through directed differentiation of stem-cell derived human beta cells in collaboration with Semma Therapeutics. He received his MD with Honors from Harvard Medical School and his PhD in Systems Biology from Harvard University.

AFFILIATIONS

Eric Kelsic, PhD
CEO & co-founder,
Dyno Therapeutics, Inc.

Adrian Veres , MD & PhD
CSO & co-founder,
Dyno Therapeutics, Inc.

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CELL & GENE THERAPY INSIGHTS

GENE DELIVERY PLATFORM EVOLUTION
PART 1: VIRAL

SPOTLIGHT

Harnessing the untapped potential of commensal viruses in genetic medicine

Joseph M Cabral
Ring Therapeutics

"Tissue specificity, immune evasion, re-dosabiity, and lack of integration are all unique attributes that make vectorizing anelloviruses so appealing and compelling..."

VIEWPOINT

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The emergence of gene therapy as an effective tool to treat once intractable indications has opened a door of tremendous opportunity to reach countless patients in the clinic and give hope to their families and loved ones. With only a handful of approved gene therapies in the clinic, we are still in the early days of gene therapies reaching patients. Despite the

tremendous potential, hurdles remain, especially in delivery. The limited toolbox for delivery of gene therapies is a result of the largely stagnant development landscape of new delivery modalities, which has existed for decades.

Viruses are biological entities that have evolved elegant, specific, and highly effective mechanisms for delivering genetic material into host cells. This core function of viruses has made them one of the most attractive and effective tools for delivering gene therapy payloads to patient cells. While viruses such as herpes simplex virus (HSV), adenovirus, and retroviruses (including lentiviruses) have been used in various gene therapy applications, for the treatment of most monogenic disorders, adeno-associated virus (AAV) has been the primary vector of choice. Originally discovered as a contaminant of an adenoviral prep (hence the name) [1], AAV is a small, single-stranded DNA virus of the genus *Dependoparvovirus* within the *Parvoviridae* family. The 'dependo' in the genus name comes from the fact that AAV lacks all the machinery needed to fully highjack a cell and initiate its own replication. AAV relies on superinfection of a helper virus, such as adenovirus, to highjack the cellular machinery required for AAV replication.

In the absence of a helper virus, AAV is thought to be non-pathogenic, which has made it a potent tool for gene therapy payload delivery; however, like any tool, it has limitations. Neutralizing antibodies against AAV strains used in gene therapy have been found in patient populations [2] which can often preclude many patients from being a candidate for therapy. And high doses of AAV can elicit a strong immune response that may prevent re-dosing without the aid of immunosuppressive agents. Natural tropism for tissues of interest has also been a barrier for AAV. Great efforts have been made to engineer AAV capsids to have specific tropism, and recent breakthroughs have been reported for some tissues. However, the engineering and screening for AAV variants with specific tropism can be both costly and time consuming.

But what if there were viruses that were weakly immunogenic and could be found in almost every tissue in the human body? The field of virology has largely focused on characterizing viruses that are associated with disease, but the sequencing of the human virome has revealed the presence of viruses that have seemingly evolved to be ubiquitous, apathogenic commensal human viruses, existing in concert with us rather than in conflict. Such viruses have tremendous potential for expanding the gene delivery tool kit to reach even more patients in the clinic. At Ring Therapeutics, our mission is to turn the most abundant family of commensal viruses, anelloviruses, into a leading gene therapy delivery tool that could lead to transformative therapeutic opportunities.

Anelloviruses have now been shown to be a major component of the human commensal virome, establishing themselves in nearly every human by 12 months of age [3]. Anelloviruses that infect humans come in three genera: alpha-, beta-, and gammatorqueviruses. These viruses have negative-sense, single-stranded, circular DNA genomes and display incredible diversity in human populations [4]. By sampling and sequencing a variety of human tissues, our genomics efforts have found scores of anelloviruses in every tissue we have sequenced. By hunting for anelloviruses in specific tissues of interest, we can quickly compile a list of lead vector candidates with potential specificity for their tissue of origin. Rapidly unlocking tissue tropism through these means represents a unique advantage for these commensal viruses as a gene delivery tool.

Because they are ubiquitous human commensal viruses, it is unsurprising to find that they appear to be weakly immunogenic [5]. We recently reported that anelloviruses transmitted through blood transfusion from a sample of donors to recipients did not illicit a detectable antibody response in three out of five recipients. For the other two recipients, the response was significantly delayed

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(~100–150 days post-transfusion) when compared to typical seroconversion after viral infection (~14 days). The lack of a strong antibody response to anelloviruses in conjunction with high viral diversity suggests pre-existing neutralizing antibodies may not be as significant an issue for anellovirus-mediated gene therapy. It also suggests that there is a strong potential for re-dosing patients if therapeutic durability were to wane. This is a unique advantage anelloviruses have as a gene delivery tool, and it may have significant implications for its future utility in the clinic.

Another advantage of anelloviruses as vectors is that they do not integrate into the human genome like retroviral and lentiviral vectors do. While integration provides excellent durability for transgene expression, it carries with it a risk of oncogenesis as a result. Anelloviruses remain in the nucleus as an extrachromosomal episome, alleviating concerns over potentially oncogenic integrations. However, if durability of transgene expression were to wane from cellular proliferation, anellovirus-based vectors could be readministered to maintain clinically relevant gene dosage due to their weak immunogenicity.

As the gene therapy field continues to mature, the need for expanding our gene delivery tool kit will become increasingly apparent. The untapped potential of the human commensal viruses represents a rich and diverse reservoir of novel vectors-in-waiting that can complement the viral vectors already in the tool kit, and deliver unique characteristics that are sorely lacking today. Tissue specificity, immune evasion, re-dosabiity, and lack of integration are all unique attributes that make vectorizing anelloviruses so appealing and compelling – but there will never be one tool that is right for every task. Sometimes you need a hammer. Sometimes you need a wrench. I expect that over the next few years, we will see more efforts in building unique and novel delivery tools. By diversifying the viral vectors available for use in gene therapy, we can reach more patients, treat more indications, and transform many more lives.

BIOGRAPHY

JOSEPH M CABRAL has bachelor's degrees in Cinema Studies and Molecular, Cellular, and Developmental Biology from San Francisco State University and the University of Colorado Boulder, respectively. He received a PhD in Virology from Harvard University where he studied in the lab of Prof. David Knipe. His graduate work focused on early events in chromatin formation on HSV DNA and epigenetic silencing of viral gene expression by host restriction factor ATRX. He worked on AAV novel capsid engineering at Voyager Therapeutics and then at Biogen. Dr. Cabral is currently a Senior Scientist at Ring Therapeutics where he leads the Vector Engineering team that conceives, designs, builds, and tests revolutionary anellovirus-based vector systems for gene therapy payload delivery.

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AFFILIATION

Joseph M Cabral
Senior Scientist, Vector Engineering,
Ring Therapeutics

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CELL & GENE THERAPY INSIGHTS

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CHANNEL CONTENT

EXPERT ROUNDTABLE

Cell culture in immune cell therapies: do more with less



In this expert roundtable, **David McCall,** Senior Editor, BioInsights, speaks to (pictured left to right) **Anastasiya Smith,** Senior Director R&D, CellReady **Josh Ludwig,** Global Director, Commercial Operations, ScaleReady, & **David Hermanson, Senior Manager of R&D Applications, Cell and Gene Therapy, Bio-Techne.** The panel discusses the importance of cytokine levels and the effects on phenotype, in addition to how to reduce complexity and cost from early research through commercial scale-up in a closed system.

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Q How would you rank the chief obstacles facing T cell therapy upstream processing currently?

JL: The biggest obstacles are simply standardizing the way we receive, collect, and initiate our processes from the raw material standpoint. At ScaleReady, we are

focusing on some of our newer instrumentation, including the Cue Cell Processing System from Fresenius Kabi, to begin standardizing apheresis collection and ensure that we have healthy, consistent cells going into these workflows. Ultimately, we are focused on driving the field towards a simplified and standardized approach that starts from upstream processing.

AS: Not having a robust or reproducible process will only lead to failure. Having too many open steps and operator interventions will pose many challenges going into clinical development Phases 2 and 3 and commercialization. Another obstacle is having a process that is not scalable. Using the wrong type of vessel that is either a prototype or one that does not scale well in the future is a problem.

DH: Certainly, the apheresis collection and starting material are key areas for standardization, but standardization is needed across everything. We are in a field where currently, the process is the product and the product is the process. In other words, we have as many processes as we do products in this field. Without standardization, those processes can be extremely challenging for reagent manufacturers or therapeutic companies in ensuring supply and therapeutic results. The more we can standardize certain things that we all agree on, the better off we will be.

JL: Standardizing does not mean 100% the same. We are talking about the 80:20 rule. The whole industry moves a lot faster if it does not continue to reinvent the wheel and spend the same money at each institute or company over and over again. If we focus on what we know through already commercialized products and the work happening now on developing protocols, there are a lot of consistencies and the same processes taking place. We need to share enough of that information, whilst knowing that there are plenty of aspects in terms of constructs, patient dosing, and targeting that make these therapeutics unique. We need to drive the field forward with more information sharing, starting with upstream processing and continuing throughout the whole manufacturing workflow. Whether you are manufacturing reagents or products to service the therapeutic developers in this field, or you are a therapeutic developer yourself, trying to piece together a process from hundreds of different options is not a sustainable model for the field.

Q What are the key lessons to learn in order to achieve better T cell phenotypes?

AS: To achieve better phenotypes, although every T cell product is unique and has its own kinetics, generally decreasing *in vitro* culture time is going to be favorable. Taking our MultiTAA cell therapy at Marker as an example, we initially licensed our process from the Baylor College of Medicine as a 36 day process. After optimizing the process, we scaled it down to 9 days. This decrease in manufacturing time resulted in younger cells, a

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"To achieve better phenotypes, although every T cell product is unique and has its own kinetics, generally decreasing *in vitro* culture time is going to be favorable."

– Anastasiya Smith

greater proportion of naive and central memory cells, fewer differentiated cells, better viability, and better potency. Extensive culture time leads to a greater proportion of CD8+ cells (especially when you are using IL-15) and also more differentiated cells. There is a loss of naive cells and central memory cells, and we now know that more differentiated cells have limited *in vivo* persistence. The key is to aim for a manufacturing process that is long enough to achieve the cell numbers required for clinical treatment, but short enough to maintain a favorable phenotype. The culture time is highly dependent on cell doublings. The variable impacting cell numbers is the relationship between cell doubling and cell death. If you have cells that divide but a large portion of your culture is dying, then those live cells have to work twice as hard and keep dividing to achieve your cell yield, which leads to longer culture time and a more unfavorable phenotype. The driving force for cell aging is the cell doubling.

DH: Just about everything has a trade-off. Is phenotype more important? Is cell number more important? If you are editing the cell therapy, the mode chosen will impact the phenotype. Transposon-based non-viral gene editing methods have been shown to have more central memory or stem cell memory phenotype than using viruses, which themselves do a better job of editing effector memory or effector function cells. Determine the critical parameters that you truly care about, and if a phenotype is one of those, then you need to start optimizing for phenotype early on in your process. The shorter the culture time, the better the phenotype you will have. You also need to optimize and evaluate your activation reagents, cytokine usage, media, culture vessel, and any small molecule modulators from the beginning. You must actively go after the phenotype you want. Most cell therapies are looking for the central memory and stem cell memory phenotype.

JL: One lesson that we have learned with the G-Rex is that once you feel that you are driving towards the optimal phenotype and have confidence in the reagents and process steps required to generate the cells that you want, start scaling that process and remove as many interventions and touchpoints as possible. It can be hard to generate a consistent final material when dealing with T cells and NK cells from different donors. Can we eliminate all those interventions and steps to make it as simple as possible? This allows the process to be more repeatable. Over the last decade, I have seen several groups make mistakes in small-scale studies, which then become written in stone. As you scale the process,

it then becomes apparent that the phenotypes are not the same. Process simplification needs to be the focus from the beginning, once you have defined what your reagents and components are in your process. Otherwise, you risk problems in terms of having a consistent phenotype.

DH: Complexity is not the answer – simplicity is always going to win when it comes to cell culture processes. Starting with that end in mind is key. If the phenotype is what is important, which is becoming increasingly apparent, then that needs to be the goal from the beginning. Ten years ago, the focus was getting enough cells to treat the patient. We are now over that hurdle, as we have learned that we do not need as many cells as we think. Now, the focus is identifying where you want to head early and ensuring it will scale. Do not base processes off only a couple of donors, especially since most processes are optimized on healthy donors and do not truly reflect what will be seen in patient donors. In the autologous field, if you do not have excellent phenotypes with healthy donors, do not expect that to get better when you move to sick patients.

JL: To begin any process development effort, there are so many unknowns and decisions to be made. You need to figure out that funnel of complexity, define your phenotypes, and then quickly move to commit to a process that has its eyes on simplicity. Consider what the field has aligned on that works consistently, and that can become a starting point rather than starting from scratch.

Q What tips do you have for those selecting a cytokines supplier?

DH: There are some obvious factors to this, such as quality, activity, lot-to-lot variability, supply capabilities, and price. We could group all of those things into 'pay-to-play'-type requirements. Most cytokine manufacturers will cover those things, not to say that they are not immensely important. If you have a supplier lacking in any one of those areas, you should consider looking into finding a new supplier. I pay the closest attention to supply capabilities. The last thing you want to do is to be scaling up and then realize that something went on backorder. cGMP is well understood now and the quality is easy to find, but those supply levels need to be there, too.

Moving beyond the pay-to-play requirements, there are other considerations such as how easy your vendor is to work with. How responsive are they? How open to customization are they? You should look for a company that is willing to partner with you and does not simply treat the relationship as transactional. How much do you trust that vendor is going to be able to overcome any bumps in the road?

AS: From my perspective, I suggest that people investigate a vendor that has good manufacturing practice (GMP)-grade cytokines to enable an easy transition. This means you will not have to look for an additional vendor at a later stage. In addition,

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the reagents should be vetted by your quality team to ensure that all required paperwork is provided by the vendor and satisfies GMP requirements. At Marker, we have run into suppliers that list their reagents as GMP, but when they are audited by the quality team, it is determined that they are not quite GMP.

"Defining variables early and ensuring that your cytokine vendor and supplier can help you to do more with less or move faster is key."

– Josh Ludwig

JL: At ScaleReady, we are partnered with Bio-Techne which has the longest track record of manufacturing cytokines in the field, and has made a massive commitment to GMP cytokines in terms of both capacity and innovation. Defining variables early and ensuring that your cytokine vendor and supplier can help you to do more with less or move faster is key. With Bio-Techne, we have access to Research Use Only (RUO) animal-free versions of the cytokines produced in the same large-capacity dedicated facility. Those same proteins, with a little more quality control (QC) testing and a more robust certificate, are fully GMP-validated products. There are no question marks or concerns about taking an early research process and scaling that linearly. Trusting your supplier is crucial. Are they willing to provide information on capacity? Will they take you on a tour of the facility so you can see where your cytokines are manufactured? You cannot have a hiccup on any single component within your process, or it ends up being the one thing that breaks tight timelines at your organization.

Q What upcoming tools or innovations will further enable researchers to achieve optimal cytokine concentrations?

DH: When it comes to determining optimal cytokine concentrations, it boils down to doing the work and titrating. As a cytokine supplier, we can certainly do some of that titration, but it will be specific to your process. One of the great things about working with the G-Rex is that because you are not perfusing media, you can upload those cytokines and they are good for the entire time. This helps save on costs and the number of cytokines needed, whereas some of the perfusion-type systems require a constant influx of cytokines.

In terms of innovations, we are going to be moving towards closed cytokine systems. Cytokines tend to be an open step in the processes. As we move to closed cytokines, you will no longer be reconstituting and aliquoting. Having a supplier who is willing to work with you to custom-fill closed cytokines so that you get the amount that you want is an important consideration here. When closed cytokines become more widely available, it will emphasize how important that supply partnership is. At Bio-Techne, we have put a lot of work into

determining the correct doses up front so that we have fill sizes that hopefully will meet the therapeutic company's needs.

JL: I am excited about the work that David and the applications lab have been doing on titrating. In today's climate of tough financial headwinds, as an industry, we need to continue to focus on pushing the cost of goods down. We need that right amount instead of the historical mentality of overdosing cytokines. If you are overdosing cytokines, not only are you wasting precious dollars as you drive towards commercialization, but you are potentially driving to a more differentiated, less desirable phenotype. Closed cytokines will be a challenge, but partnering with a group that can custom-fill and work with you as your process evolves will help overcome that challenge.

AS: From a customer perspective, I cannot wait to see an exact quantity of cytokine in a vial that you can pop into a G-Rex and not have to worry about aliquoting or overdosing. Many people are following an inherited protocol that is not optimized, and they do not realize how many cytokines they are wasting and how potentially detrimental it could be for the cells.

DH: Cytokine analysis needs to go deeper than just looking at the traditional memory markers like CD45RA, CD62L, and CCR7. You need to look at the additional functional markers that you have determined are important. One of those is the IL-7Rα, a crucial receptor in those cells for IL-7 signaling for homeostasis of T cells in the body. With too-high doses of IL-15, we saw a drop off in IL-7Rα expression.

Q What are the key manufacturing process components to successfully transition your immune cell therapy research from discovery to the clinic?

AS: A key component is to start from the end. This means thinking ahead and determining what will be required for GMP manufacturing and what will be compatible for future commercialization. There are proof-of-concept technologies and research-grade reagents that cannot move into GMP and are going to be hard to scale. This mindset still needs to be incorporated early in the academic environment. Because G-Rex devices are not only scalable but available as open and closed, and GMP and research grade, they are an excellent choice to adopt early on in an academic setting.

We have treated 170+ patients with the MultiTAA therapy we licensed from Baylor. The initial open process we licensed was complex and was not appropriate for commercialization. We spent several months trying to simplify and close the process. Now, the process is more sophisticated and the product has a better phenotype and potency. This took a great deal of work that could have been easily avoided if the process had a foundation of GMP readiness

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"The regulatory bodies are paying more attention to whether these processes have a chance to reach commercialization, so you need to be thinking about that from the beginning."

– David Hermanson

in mind. There needs to be a collaborative effort between industry and academia to prevent this type of scenario in the future.

DH: As a therapeutic company, you are always trying to balance speed to clinic with spending the appropriate amount of time in process development and planning for manufacturing success. There is a temptation to rush toward first-in-human trials, but this does not fly anymore. The regulatory bodies are paying more attention to whether these processes have a chance to reach commercialization, so you need to be thinking about that from the beginning.

There are many things to be considered before you get to first-in-human trials. Is delivery going to be viral or non-viral? Are cytokine and media supply stable? Can the process be scaled up/down/out? Can you treat the required number of patients?

JL: One area that needs to be considered in academic institutes is how they can sustain and drive value for the research that they are doing. As cell therapy becomes more mainstream in the sense that there are many more indications showing promise, institutes need to consider how to do more with less space. They need to lower the burden of the operator doing the process due to skilled operator shortages across the board. The real solution is starting from the early processes being developed at academic centers. Processes need to be developed with limited space and numbers of people in mind. At the point of partnering and licensing things out to industry, those challenges will be heightened, so simplifying early means you will not need to go back and redo everything. There are suppliers today who are making decisions with the long game in mind.

Q The industry seems to have pushed back on standardization of process development. However, the data (see Box 1) shows that simplification is possible. Do you agree or disagree with the latter statement, and why?

JL: I agree. Ultimately, the regulatory bodies including the FDA have placed a focus on standardization. The California Institute for Regenerative Medicine (CIRM) has

BOX 1

CASE STUDY: Titration of IL-7 & IL-15 for T cell expansion: David Hermanson.

Memory T cells are the desired population of cells when manufacturing T cell immunotherapies. IL-7 and IL-15 are two key cytokines that are used in the *ex vivo* expansion of T cells, replacing the single cytokine use of IL-2. IL-7 binds CD127 (IL-7Rα) and promotes the survival of memory T cells. IL-15 is known to drive antigen-independent cell growth through STAT5 signaling. Titration of these two cytokines can improve the overall T cell phenotype, and potentially save on reagent costs.

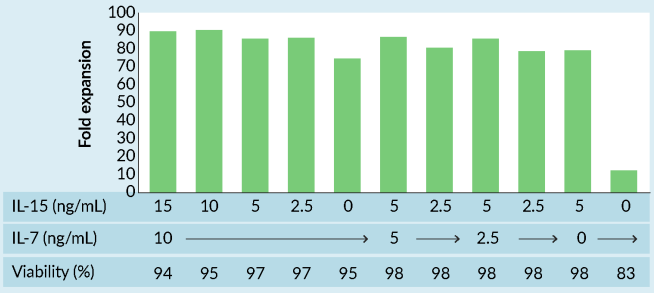
T cell phenotype refers to the naïve or stem-cell memory population, which is defined here by CD45RA, CCR7, CD62L, and CD127 positivity. The central memory population is CD62L$^+$, CCR7$^+$, CD127$^+$, and CD45RA$^-$. T effector memory is defined by CD62L$^-$, CCR7$^-$, CD127$^-$, and CD45RA$^-$.

The workflow used begins with thawing cells and activating them in a G-rex 6M plate on day zero.

On day two, the media undergoes QS up to 100 mL. From day 13 to day 14, there is a no-touch expansion protocol, and collection and phenotyping on day 14.

During T cell expansion, in all conditions except no cytokine control, acceptable levels of fold expansion were seen (Figure 1).

Figure 1: T cell expansion in the presence of IL-7 & IL-15



IL-15 was necessary to achieve the fold expansion potential of T cells with doses as low as 2.5ng/mL achieving complete growth. IL-7 alone enhances cell growth above no cytokine controls, but not as well as IL-15.

Characterizing cells by CD45RA and CCR7 phenotypes revealed minimal differences in T cell memory except when 0 ng/mL of IL-7 was utilized. This revealed that IL-7 is key to maintaining optimal T cell memory phenotype.

Further analysis of the cell phenotypes revealed that the CD127 expression is inversely proportional to IL-15 concentration in both CD4$^+$ and CD8$^+$ T cells (Figure 2). CD127 expression was found to be stable independent of IL-7 titration (Figure 3). This indicates that the IL-15 concentration is critical in the amount of CD127 expression found in cell culture. Overdosing of IL-15 can decrease the expression of the key memory marker IL-7Rα, especially in the CD8$^+$ subset of T cells.

This experiment demonstrates the importance of titrating the level of cytokines and observing the impact on the final T cell memory phenotype. In this case, IL-7Rα expression levels were the most impacted. Dosing of IL-7 and IL-15 in *ex vivo* T cell expansions should be considered during process development. This study concluded that 5 ng/mL of IL-7, and 2.5 ng/mL of IL-15 is a good starting point for titration experiments.

Figure 2: IL-7Rα expression is inversely proportional to IL-15 concentration in CD4+ and CD8+ T cells

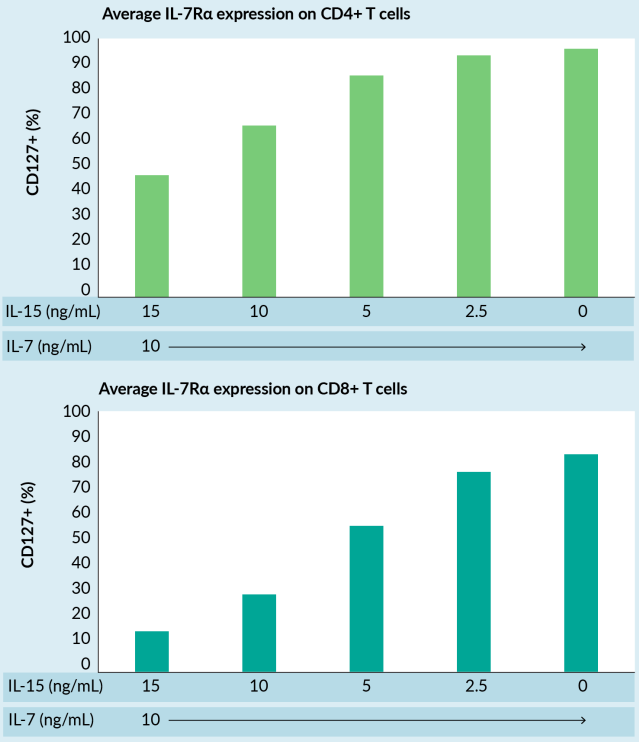
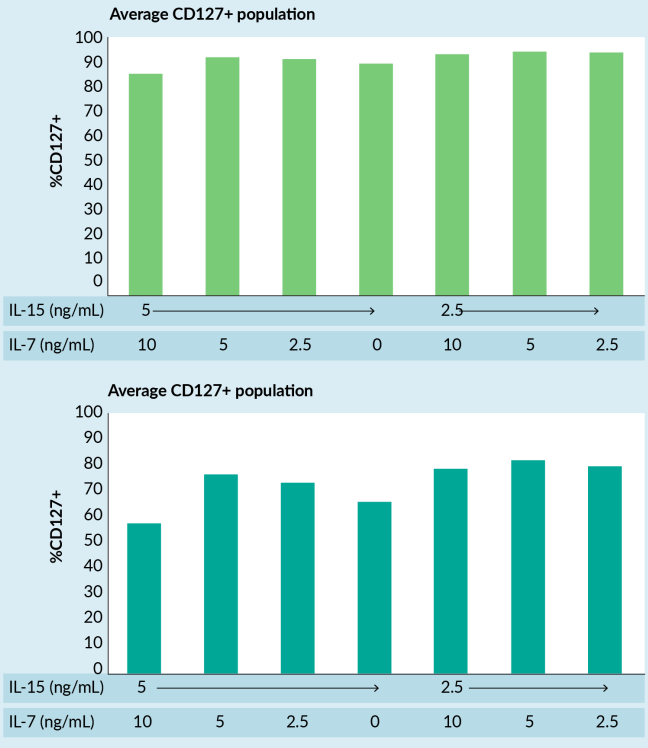


Figure 3: IL-7Rα expression as a function of IL-7 cytokine dosing



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placed a focus on grant money for people who are standardizing and sharing their best practices. Everything is pointing to the need to think of the greater good for the industry, and no longer think that everything you are doing needs to be kept secret. In terms of investments in this space, people are no longer willing to fund an expensive science experiment for 3–4 years, so we need to move faster to get meaningful clinical data. We can do this by sharing best practices and sharing what works more openly. The momentum for standardization is finally here, and I am excited about what is to come in the next months and years.

AS: Everybody has their secrets, but those secrets are such a small component of the work we are doing, and we all share the same basic components. There is no need for a hundred companies to waste money and resources to optimize things like the isolation of peripheral blood mononuclear cells, seeding density, or cytokine concentration. There are great companies out there that have the answers to what works. Simplification is going to be necessary for scalability in commercialization to avoid failures.

DH: All three of us have talked about standardization and simplification, and not only is it possible, but it is also prudent and necessary. I have a process development background and incredibly often, we see processes that are too complex when they happen for real – i.e. with patient samples in a clean room. If your process is not simple outside of that setting, you will struggle when you start to move into either working with patient material or in clean rooms.

JL: Hopefully, we are at the end of the 'black box manufacturing' era. Doing things in the proper order is another key. Simplifying leads to standardizing, which leads to repeatability and scaling. Then, focus on automation where it matters. Automation is impressive and powerful and has changed many industries. It has not been changing our industry as fast as it should because people are trying to automate a complex non-standardized process. You cannot build 1,000 different automated systems and workflows; you need to build the automation into workflows applicable across various cell types and strategies.

Q What do you see as the next big thing for the future of immune cell cultures in terms of driving improvements in cellular immunotherapeutic potential?

DH: Shortening the overall *ex vivo* or *in vitro* expansion timeframe is key for the autologous space. We have seen a few different trials come out with 2–3 day manufacturing processes. Early on, people were worried about QC, but avenues do exist for QC to be completed in a very short timeframe. Shortened expansion supports the use of something like the G-Rex, where you can park the cells overnight in the presence of cytokines to allow them to recover from either a transduction or an electroporation reaction, before infusing

BOX 2

CASE STUDY: Achieving rapid & robust cell expansion with G-Rex: Josh Ludwig.

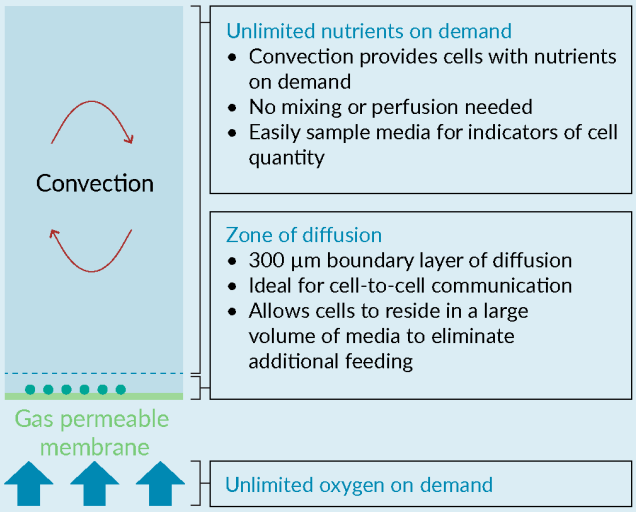
The G-Rex bioreactor plays an integral part in successfully achieving an efficient dosing regimen of cytokines for maximum proliferation and importantly, ideal cell phenotypes. The G-Rex technology is uniquely able to produce consistent, repeatable, and standardized results through its simplicity.

G-Rex is a gas permeable rapid expansion system, and its mode of action is outlined in Figure 4. G-Rex was developed to break the rules of traditional static culture and act as a game changer for T cell and natural killer (NK) cell companies growing cells simply and effectively. It begins with the gas-permeable membrane at the bottom of the vessel which allows unlimited access to oxygen on demand, allowing fluid to be stacked so the cells have unlimited nutrients on demand via convection. The 300 µm boundary layer of diffusion allows cells to reside in a large volume of media to eliminate additional feeding. The large waste sink means no media change is required and lactate build-up at the cellular level is not an issue. This creates a system free from media changes, perfusion, and technician intervention.

A maximum cell density can be achieved at a high rate, and 30–40 million+ cells/cm^2 generate a good linear expansion throughout our system. Paired with Bio-Techne's research use only (RUO) animal-free and GMP reagents, the system is easily scalable depending on need.

Small-scale experiments easily scale up from benchtop to clinical engineering runs. Using a scale-down model for process development and culture optimization is preferable to save time and money. G-Rex allows linear scale-up and scale-down as needed. Dosing at the correct levels can enable incredibly efficient use of space, time, resources, and reductions in the need for skilled labor. This benefits speed to market and the opportunity to deliver life-saving therapies to patients faster and in more geographies. .

Figure 4: Outline of G-Rex: how it works



them into a patient. You can contrast that against bioreactors that are designed to maintain the cells for longer periods.

In the allogeneic space, maximal expansion is key. How do we get more doses out of less starting material? The future of that lies in identifying new pathways to allow that process intensification while maintaining cell health and phenotype. The ratio of live cells versus apoptotic cells matters. If you have a bunch of cells dying, living cells must work hard to overcome that deficit. We must keep cells happy and healthy, whether that is through knocking out a potential pathway or silencing a pathway for a period, or through over expression allowing the cells to proliferate longer and stay healthier. I think those answers are coming.

AS: There is something simple that everyone can do to improve their processes – remove unnecessary elements from the process, such as unnecessarily counting cells or changing the media, or overfeeding your cells with unnecessary quantities of cytokines. These simple things can improve your yield and make your process the bare minimum reproducible process.

JL: You should move a process towards its most simple and elegant form. Whittling it down to its core components can be hard. Certainly, there are reasons during your early research to measure everything for characterization and understanding purposes. However, once you start manufacturing, you need to cut anything that is not adding value.

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We have so much good technology available today that works. We need a mindset shift to crowdsourcing and sharing the building blocks of what works in these settings. There is a lot of good happening out there and we should be sharing that to give people the best chance to succeed.

BIOGRAPHIES

ANASTASIYA SMITH has over 10 years of immunotherapy experience both in academic and industry settings. Dr Smith is currently the Senior Director of R&D at CellReady. She served as the Director of Research and Development at Marker Therapeutics, Inc. Dr Smith has a broad research experience and has made contributions to the fields of infectious diseases, cell biology and adoptive T cell therapy. Over the last several years at Marker and in previous industry positions, Dr Smith has focused her efforts on optimizing manufacturing processes for engineered and non-engineered adoptive T cell therapies. Dr Smith received her PhD in Immunology and Infectious Diseases from the Department of Biomedical Sciences, a program co-administered by the University at Albany's School of Public Health and the New York State Department of Health's Wadsworth Center. She also completed a post-doctoral fellowship at the Baylor College of Medicine investigating NK cell antibody-dependent cell-mediated cytotoxicity against Cytomegalovirus infected and tumorigenic target cells.

JOSH LUDWIG leads commercial operations as the global director for ScaleReady. He closely collaborates with the EMEA commercial and leadership teams to ensure ScaleReady addresses the longstanding industry need for a truly scalable and practical solution. Josh has more than a decade of experience in commercial leadership and business development in the medical device industry with a specific focus on engaging directly with customers and achieving customer and company goals. Before ScaleReady, Josh spent more than seven years supporting technical and business development at Wilson Wolf, a founding partner of ScaleReady. He successfully established protocols and executed all aspects of global business development, customer service, and marketing efforts. Josh's career in Life Sciences began by obtaining a Bachelor of Science in biology and a minor in chemistry from the University of St Thomas.

DAVID HERMANSON is the Senior Manager of R&D Applications in Cell and Gene Therapy at Bio-Techne. He works with Bio-Techne's suite of cell and gene therapy products to establish appropriate protocols and workflows for the modification of immune cells include CAR T and CAR NK cell workflows. His career has focused on non-viral gene editing of T and NK cells using transposon systems and continues to drive the adoption of TcBuster™ for cell therapy.

AFFILIATIONS

Anastasiya Smith, PhD
Senior Director R&D
CellReady

Josh Ludwig,
Global Director, Commercial Operations,
ScaleReady

David Hermanson
Senior Manager of R&D Applications,
Cell and Gene Therapy,
Bio-Techne.

AUTHORSHIP & CONFLICT OF INTEREST

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DOI: 10.18609/cgti.2023.070

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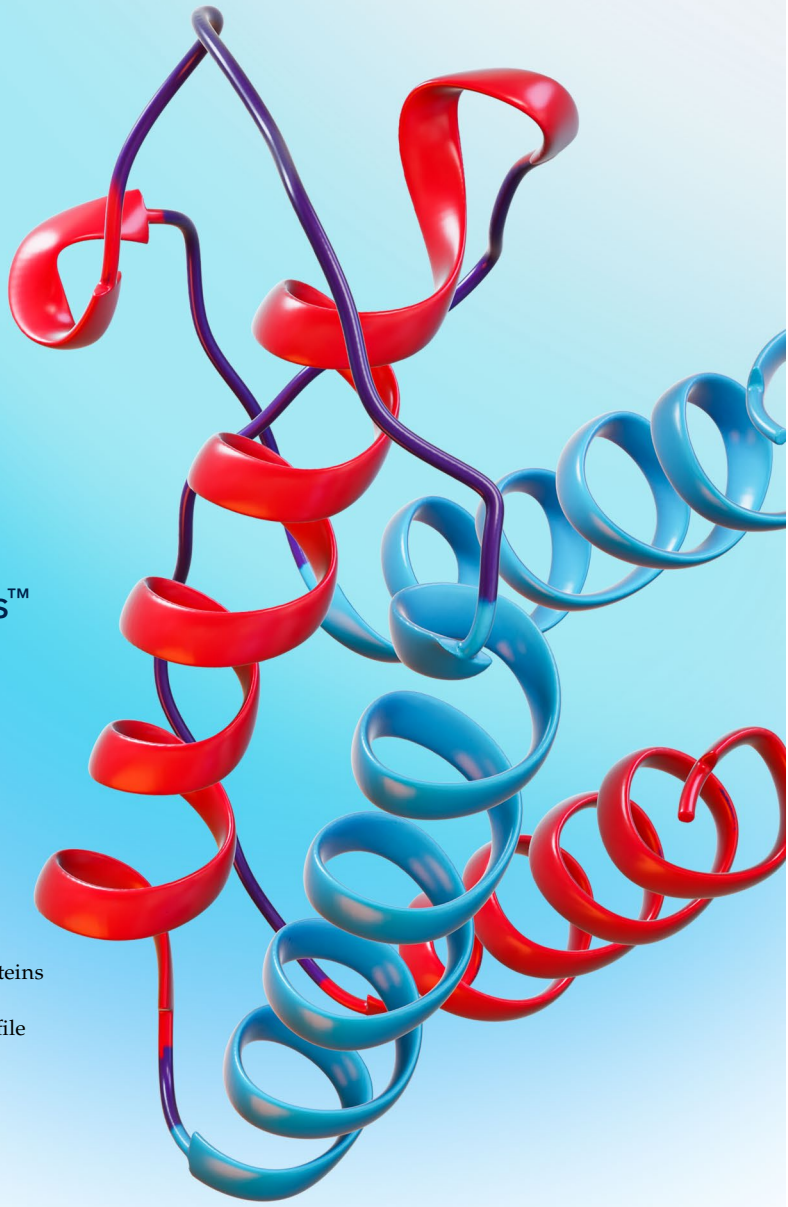

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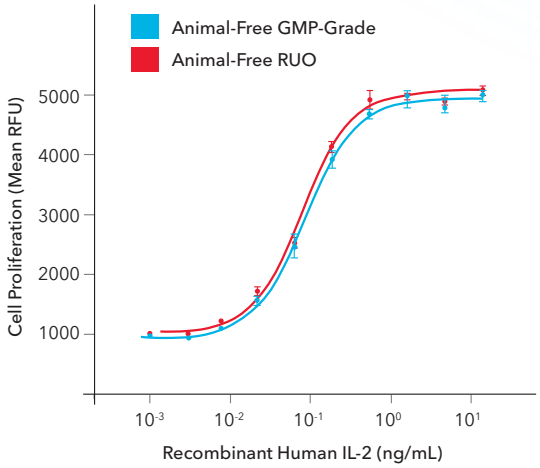

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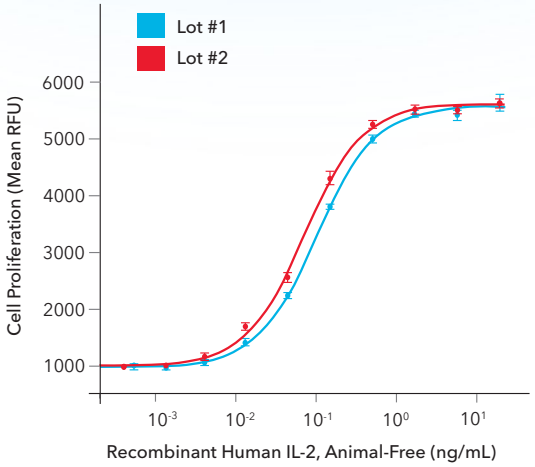

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FASTFACTS

Navigating the gene & cell therapy regulatory landscape: when, why and how to deploy GMP gene editing materials

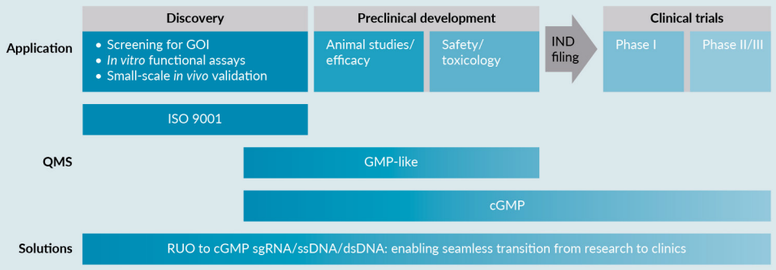
Michael Lau PhD MBA, Head of GMP Business Development, GenScript

The evolving regulatory landscape around the use of cGMP material for therapeutic gene editing can lead to costly delays in development pipelines. Recent FDA guidelines recommend cGMP reagents be used prior to IND enabling studies and clinical trials of novel advanced therapies, including for master cell bank generation. To prevent regulatory issues, researchers must source phase-appropriate cGMP reagents and submit detailed production documentation with their IND filing. This FastFacts poster will demonstrate how GenScript is supporting gene and cell therapy development from research to clinic with their recently expanded cGMP manufacturing capacity.

FROM DISCOVERY TO CLINICAL TRIALS

GenScript provides CRISPR proteins, single guide (sg)RNA, and non-viral payloads (including single-stranded DNA, double-stranded DNA, and GenCircle miniaturized plasmid) from discovery level at the research grade, to preclinical development with animal and safety and toxicology studies, to clinical trials and beyond. They offer all appropriate quality documentation in support of IND and BLA filings, with phase-appropriate quality management systems from ISO9001-controlled environments to Class A clean rooms and GMP manufacturing. They provide seamless solutions for the transition from research use only (RUO) to cGMP to help researchers achieve their goals efficiently, quickly, and adequately **(Figure 1)**.

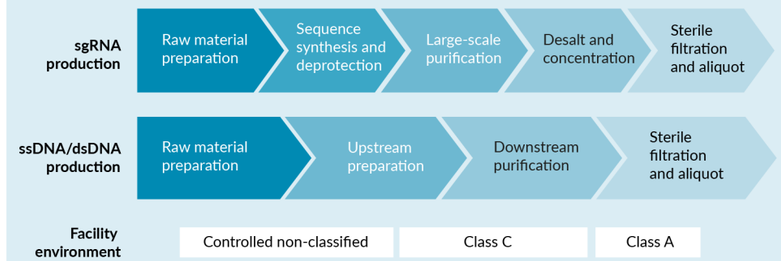
Figure 1. Phase-appropriate materials and comprehensive QA/QC documentation for successful IND filing.



MANUFACTURING & QC STRATEGY

Genscript's sgRNA and non-viral payload production process is well-established and robust. Following ICH and USP guidance, CRISPR oligos are manufactured from a Controlled environment to class C isolators for purification, and class A clean rooms for fill and finish. GenScript can manufacture products suitable for IND-enabling studies, clinical trials, and commercial launches **(Figure 2)**.

Figure 2. Manufacturing workflow strategy.



cGMP CAPABILITIES

Limited production capacity has led to a global bottleneck in cGMP gene editing raw materials supply. However, GenScript is supporting gene and cell therapy development with a new state-of-the-art cGMP facility **(Figure 3)** that will increase production capacity.

In 2020, GenScript established a 21,500 sq. ft. cGMP plant in Nanjing, comprising five cGMP production lines for synthetic CRISPR sgRNA and non-viral DNA payloads. As of April 2023, GenScript has delivered 56 batches of cGMP quality gene editing products to global biotechnology and pharmaceutical companies in the gene and cell therapy field, supporting 18 project applications, five of which have received IND approvals.

The recent expansion adds 400,000 sq.ft. of cGMP manufacturing space, bringing onstream four additional production lines equipped to deliver up to one gram of sgRNA or 10 milligrams of ssDNA or dsDNA per batch, respectively, with superior purity. With this expansion in manufacturing capacity, GenScript clients can reliably source CRISPR gene editing materials at the required quantity and purity, and with the necessary documentation for successful IND submission and clinical trials.

Figure 3. Zhenjiang cGMP facility.

As science advances, regulations must advance with the industry to ensure patient safety. While the evolving regulatory landscape around the use of cGMP material for therapeutic gene editing can lead to costly delays, working with a single source supplier for all phases of gene and cell therapy development can help save time and money.

CELL & GENE THERAPY INSIGHTS

Cell and Gene Therapy Insights 2023; 9(4), 521; DOI:10.18609/cgti.2023.076

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CELL & GENE THERAPY INSIGHTS

OPTIMIZING MATERIALS & CONSUMABLES SOURCING STRATEGY THROUGH SCALE-UP/-OUT

CHANNEL CONTENT

INTERVIEW

Quantifying manufacturing uncertainty and developing digital tools for supply chain optimization

Abi Pinchbeck, Assistant Editor, Cell and Gene Therapy Insights, speaks to **Maria Papathanasiou,** Assistant Professor, Department of Chemical Engineering, Imperial College London, about her work on developing tools for optimizing the advanced therapy medicinal product supply chain, and how we learned to prepare for future uncertainty from the COVID-19 pandemic.

Cell & Gene Therapy Insights 2023; 9(4), 441–446

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Q What are you working on right now?

MP: In my lab, we are currently developing supply chain optimization tools for the manufacture and distribution of advanced therapeutic medicinal products (ATMPs). We particularly focus on viral vectors and CAR T cells. Another activity we have launched is the quantification of uncertainty, especially with respect to manufacturing. This is

complementary to the supply chain and informs our supply chain models. In that space, we are looking at how novel products such as viral vectors or T cells may be subject to uncertainty when it comes to manufacturing, for example in terms of titers or batch sizes, and how that may impact supply.

Q Where do the key challenges or bottlenecks lie in terms of supply chain scaling and modeling for ATMPs?

MP: For ATMPs in general, we see different production distribution models. For example, viral vectors are batch distributed in larger quantities than CAR T cells, which are currently more bespoke. One great challenge in the field is how to orchestrate the overall supply chain network globally to integrate vectors and T cells as well as vaccines that use, for example, vectors in a resilient global supply chain network. One of the biggest challenges within this is the different business models.

CAR T cells are produced in a bespoke, one-to-one process, beginning with extracting the leukapheresis sample from the patient and then sending the therapy to that same patient. Everything happens in a 'black box' production unit, which can be challenging to plan in terms of volumes. In terms of timing and orchestration of activities, CAR T cells are subject to the patient's state and health condition and the manufacturing must be scheduled around the patient. Manufacturers need to understand and plan for a fine balance between different manufacturing models and scales. In addition, cost can be a significant challenge in scaling those therapies up and offering them to a wider patient cohort. Although CAR T therapies are expensive, there are ways to potentially bring down the cost, for example by decentralizing the manufacturing to be closer to the patient and more reactive to their schedule and reduce the cost of logistics.

Q How can digital twins be useful tools for optimizing cell and gene therapy supply chain strategy?

MP: The digital twins we are developing are mainly looking at investment planning decisions and how we can accelerate those. We are also looking into integrating the level of uncertainty we may have for products that are new or currently under development. If you compare CAR T cells, for example, to other established products like antibodies, there are a lot of things we do not know.

Digital twins give us the ability to run simulations on a computer to allow us to perform low-cost experiments that give us an idea of how the system may perform under certain conditions. In the space of supply chain optimization, this is powerful as manufacturers can assess different network structures and identify those that are more resilient to unforeseen circumstances. This means we will always be in a position to supply at the requested demand, and we also have the ability to assess the financial profile of all those networks. Another component that we have been integrating into our tools is the patient experience of the performance of the

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proposed network. We are able to monitor the return times of therapy to patients, which is a strong component of the social responsibility and sustainability of the network. Digital twins are a great help to expedite and accelerate the assessment of different modalities and different network structures, especially when it comes to novel products or products under development.

"Competitive supply chains are supply chains or products that use the same resources for materials, transportation, or storage."

Q What lessons have been learned from the COVID-19 pandemic and the response to it in terms of supply chain optimization?

MP: COVID showed us that if everything can go wrong at the same time, it probably will. We must be resilient to unforeseen events and circumstances, in addition to disrupted or competitive supply chains. Competitive supply chains are supply chains or products that use the same resources for materials, transportation, or storage. During COVID, much of the workforce and ATMP resources were redirected to ramp up vaccine manufacturing and distribution, and maintaining other activities became a great challenge.

One key message that we can distill from the pandemic is that the synergies between academia, industry, and stakeholders can accelerate the adoption and application of cutting-edge research to the benefit of society. This is what happened during COVID and is why vaccines were established so quickly. Of course, there were years of knowledge and work on the vaccine formulations previously, but the acceleration to get those vaccines to people came from the synergies that fell into place, in addition to the funding support from stakeholders, industry, and government.

Another thing that we can take away is that we are not as prepared as we would hope to deal with any kind of unforeseen circumstance, pandemic, or extreme situation. All other operations have not remained unchanged, and COVID has created a backlog in many different industries, some of which are still suffering. We need to investigate how we can optimize our supply chains to be agile and diversify for the benefit of society. This will ensure that when the next unforeseen event occurs, everything else will remain in operation, ideally as normal.

Q Could you tell me more about your lab's work on the development of tools and strategies to ramp up the manufacturing and supply chain distribution capacity of COVID-19 vaccines?

MP: In the early days of the COVID-19 pandemic, I had the chance to collaborate with colleagues from the Departments of Chemical Engineering at Imperial and UCL through a UK Research and Innovation (UKRI) COVID responsive mode grant

"The question we are trying to answer is: when is a good time for manufacturers to invest in a new expansion or facility?"

that we received to help ramp up the manufacturing and supply chain of COVID-19 vaccines. This yielded interesting results on how the different vaccine products, based on their requirements for transportation and storage, should be distributed. We achieved good insights into the manufacturing and supply chain bottlenecks in a short period of time. For example, the AstraZeneca and the Pfizer vaccines were distributed at different temperatures, which translates into different logistical requirements. This was only feasible because there was a significant amount of research done previously through the Vaccine Manufacturing Hubs, looking at platform technologies and how those could help in pandemic situations. That research was accelerated and put to good use.

We saw the real-life advantages of platform technologies. When manufacturers and logistical centers gain knowledge of how platform technologies and vaccines work, it can accelerate the development of a new vaccine in the event of a different type of virus or product.

Q What do you see as the crucial next steps of the advanced therapy field in terms of preparing future supply chains for any uncertainty that may happen?

MP: Currently, my lab is looking at quantifying the uncertainty that comes from manufacturing. We have been developing digital tools to be able to first identify and then quantify the uncertainty that comes with scaling up. We link the scale-up to the different phases of clinical trials. At every phase, we look at the differences in terms of batch size or the number of doses. The question we are trying to answer is: when is a good time for manufacturers to invest in a new expansion or facility?

In parallel, we are looking into mapping good candidate global supply chain networks. The immediate goal for us is to bring the two together. We are currently looking into integrated models that can do both at the same time and provide manufacturers with a tool that will be able to design supply chain networks based on the level of uncertainty that exists. This links well with the existence or absence of prior knowledge with respect to the product under development. This exists at an investment planning level, but if one wanted to look at the scheduling, how the unit operations will take place, and the day-to-day factors, model-based control would have a strong role to play. We are learning from other classical chemical engineering applications of model-based control. We aim to propagate this knowledge and bring it into supply chains. We want to be able to use smart controllers to change our decision-making

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portfolio based on what is happening. It will be especially interesting to see how well this could perform in a pandemic situation with many unexpectedly changing factors. We are exploring the open research question of whether controlling settings can yield useful tools, especially for manufacturers for online decision-making.

Q What are the key goals for your lab over the next few years?

MP: One angle of my lab is the quantification of uncertainty and supply chain optimization in the space of ATMPs, but we also do a lot of work on accelerating process development. We have been developing a framework whereby manufacturers can study, design, and operate decisions at the same time. For example, if looking at a chromatographic column, a manufacturer may want to consider the type of resin to choose whilst also looking at operational flexibility. Integrating the accelerated process development and accelerated uncertainty analysis under one larger umbrella is of great interest to us. That would enable manufacturers to accelerate development and assist them in filing for approval, due to our data-rich approaches and tools.

From a personal perspective and research curiosity, we have now started looking at threefold sustainability in pharma: economic, environmental, and social sustainability. Social sustainability describes our duty to deliver high-quality therapeutics to patients. Economic sustainability is whether the models we are producing are financially viable. Importantly, environmental sustainability is now being considered more within pharma and biotech. We want to do things in an environmentally sustainable way without jeopardizing either the supply or the quality of the therapeutics. The sustainability theme will be central to my lab's activities over the next few years.

BIOGRAPHY

MARIA PAPATHANASIOU is a Lecturer (Assistant Professor) at the Department of Chemical Engineering, Imperial College London. She holds a PhD degree in Process Systems Engineering from the Department of Chemical Engineering, Imperial College London. Dr Papathanasiou leads the Life Science and Process Systems Engineering lab, focusing on the development of digital twins and control methodologies for with application to personalized therapies, (bio-) pharmaceuticals, food and energy systems engineering. She has been recognized as an emerging leader in process systems engineering, receiving the Junior Sargent Medal (2023), the most prestigious award for early career academics in the field.

AFFILIATION

Maria Papathanasiou
Department of Chemical Engineering, Imperial College London, UK

AUTHORSHIP & CONFLICT OF INTEREST

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MAY 2023 Volume 9, Issue 4

CELL & GENE THERAPY INSIGHTS

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LIVE30 TRANSCRIPT

Advancing the purification of VSV-G pseudotyped lentiviral vectors by using affinity chromatography

Pim Hermans & Frank Detmers

Cell and gene therapy vectors derived from lentivirus (LV) offer unique advantages over more conventional retroviral gene delivery systems. Considering the ability to integrate the host cell genome, LV vectors have become effective tools to transduce both dividing and non-dividing cells, thereby providing long-term stable gene expression. With a growing pipeline of LV particle-based therapies comes a prominent need for more efficient manufacturing processes that are meeting the demand of functional LVs required for clinical trials. Despite the manufacturing process improvements achieved over recent years, current unit operations are still unable to reverse the significant loss of biological LV particles during the downstream process. One of the major challenges has been the development of a truly selective affinity chromatography resin that can bind the viral envelope and simultaneously allow the preservation of its biological activity during elution. This article describes a new affinity resin, suitable for the purification of VSV-G pseudotyped lentivirus particles.

Cell & Gene Therapy Insights 2023; 9(4), 389–397

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LENTIVIRUS PURIFICATION CHALLENGES

With the elevated use of lentivirus (LV) vector-based therapies in clinical trials, there is an increasing demand for good quality, highly pure vectors. Nevertheless, there is a plethora of purification challenges to overcome in order to reach the desired purity levels needed for clinical use.

LV is an enveloped virus that is produced using mammalian cell lines, such as HEK293 cells. One of the major challenges process developers face is separating LV vectors from

the large variety of closely-related product forms in the feedstock – for instance, exosomes. LV particles and extracellular vesicles such as exosomes follow a similar expression route in the cell. Consequently, the production of LV vectors yields a number of variations on both the vector and the exosomes, which is illustrated in Figure 1. These product-related impurities need to be removed from the final product.

A further challenge is the separation of particles with and without a genetic payload. Besides a very complex feedstock, LV vectors are relatively unstable. Therefore, sheer stress, high salt concentrations, and high osmolarity should all be avoided. In addition, only a narrow range in pH and temperature can be used when handling these particles.

The combination of these factors makes finding a suitable and efficient purification strategy challenging. Current processes report total recoveries of approximately 30% or less.

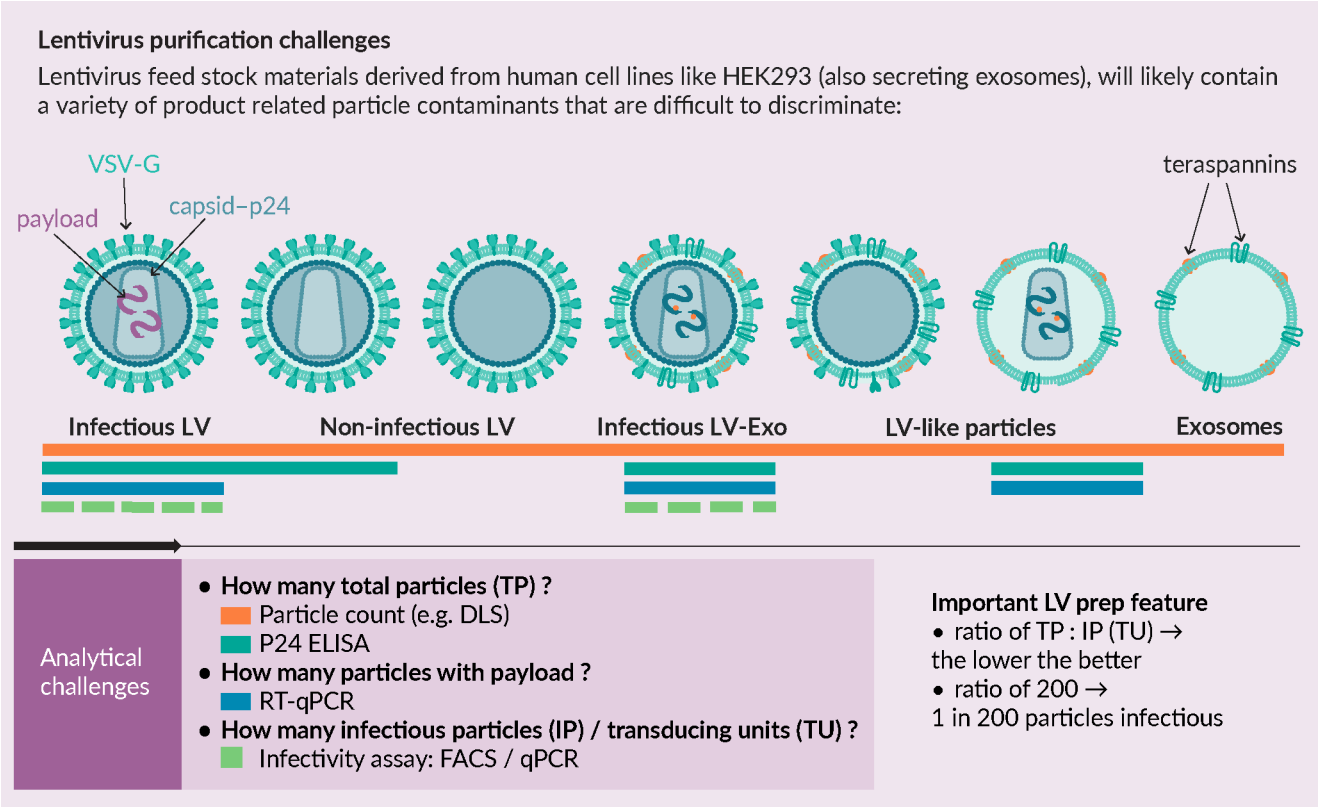
To determine both the quality of the feedstock and required steps of the purification process, it is essential to have the correct analytics in place. Important factors are the total number of particles (TP), the amount of particles with an effective payload (IP), and the ratio between these two groups (TP:IP). Figure 1 shows an overview of the various analytical assays and how they can discriminate between the different particles present in the cell culture feed or purification samples.

AFFINITY RESIN DEVELOPMENT USING CAPTURESELECT™ TECHNOLOGY

To overcome the challenges in LV purification, an affinity resin targeting the VSV-G membrane protein was developed using

FIGURE 1

Overview of lentivirus purification challenges and the analytics associated with process development.



DOI: 10.18609/cgti.2023.059

the CaptureSelect technology and resin development process. CaptureSelect ligands are based on single-domain antibody technology. The ligands are developed using an extensive screening technology where final process conditions are already implemented during screening. Ligands are tested for specificity, mild elution conditions, and stability to allow use in chromatography processes. The final ligand is recombinantly expressed in a yeast production process, which is free of animal components. CaptureSelect products are used in late clinical-stage and commercial processes. Resins are developed in a variety of drug development areas such as antibodies, biosimilars, plasma proteins, and viral vectors. The preferred resin features for the Lenti VSVG resin are shown in Box 1.

Firstly, a library was created to identify binders to the VSV-G target protein. Secondly, ligands capable of binding the target were screened using a Surface Plasmon Resonance (SPR) array-based system to monitor the selectivity and the ability to release under mild elution conditions. Three ligands demonstrated good binding in the SPR assay and selectivity was confirmed using a

BOX 1

Preferred design features for the design of the CaptureSelect VSVG affinity matrix.

High purity and yield in a single capture step

- Good HCP and DNA clearance
- Reducing the number of purification steps
- Suitable for cell clarified harvest (no concentration)

Target release under mild elution conditions to retain LV infectivity

- Good recoveries of active LV particles
- Improved TP:IP ratios

Scalable

FIGURE 2

Ligand evaluation experiments showing SPR binding curves at different LV doses (left) and release efficiency using a mixed set of buffers and elution conditions (right).

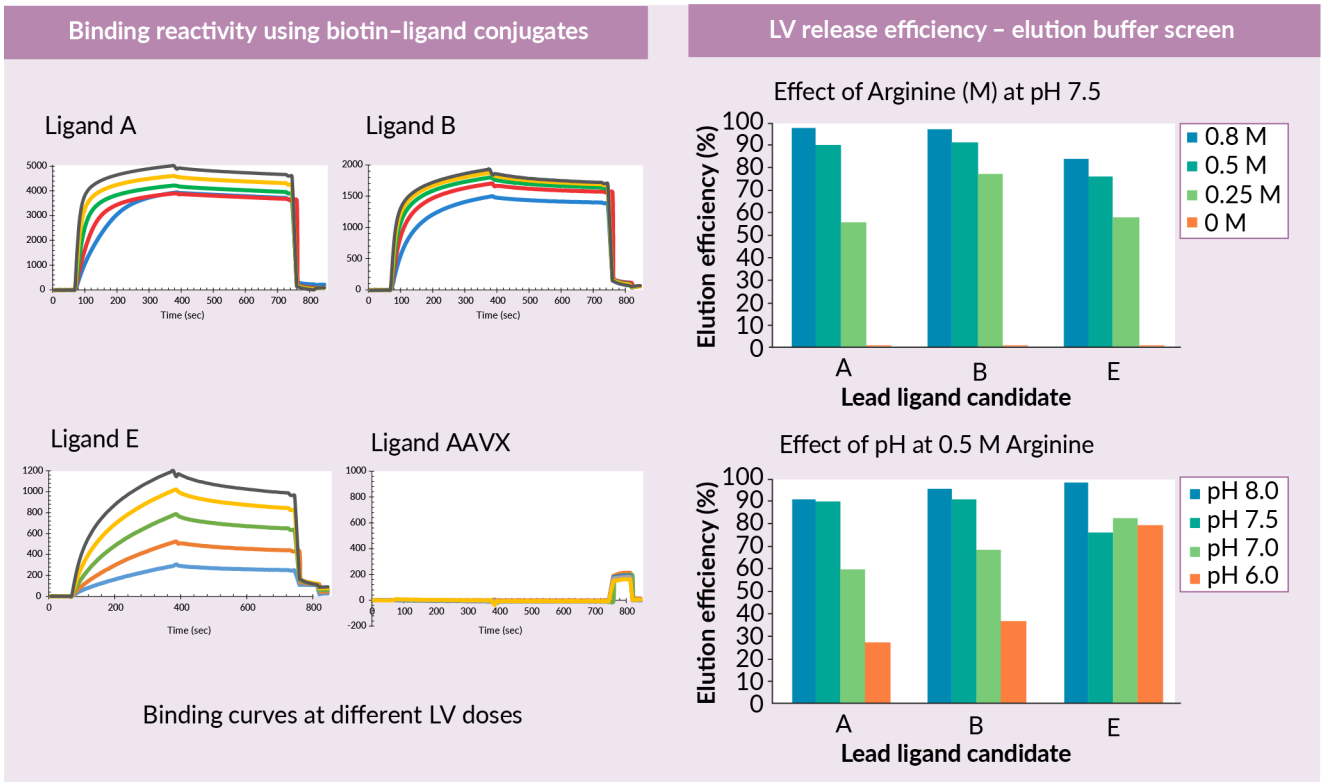
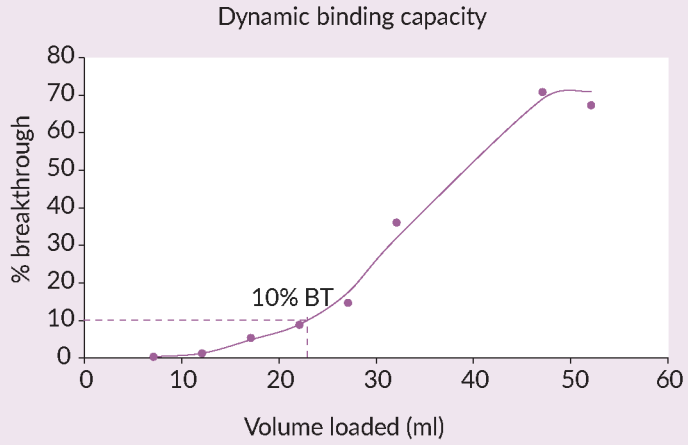


FIGURE 3

Graph showing DBC of the resin. 10% breakthrough is estimated at 1×10^{11} particles/mL.



DBC is determined by P24 total particle ELISA

Fraction	Volume (mL)	TP/mL	C/C_0
Start		3.98×10^{9}	
1	7.1	1.55×10^{7}	0.39
2	12.1	5.24×10^{7}	1.32
3	17.1	2.14×10^{8}	5.39
4	22.1	3.53×10^{8}	8.88
5	27.1	5.85×10^{8}	14.7
6	32.1	1.44×10^{9}	36.09
7	47.1	2.82×10^{9}	70.84
8	52.1	2.68×10^{9}	67.33

24.6 mL

- 10% breakthrough (C/C_0= 10%) estimated from the curve at 24.6 ml loading
- This relates to 9.78E10 total particles/ml resin (= 1×10^{11})

non-related ligand binding to AAV (Figure 2, left). In addition, a concentration of 0.8 M Arginine at neutral pH was identified as a compatible elution buffer for VSV-G pseudotyped LV vectors (Figure 2, right).

After screening, three ligand candidates were expressed in a yeast production system and developed into resin prototypes, using different backbones. Resin prototypes were tested extensively in a small-scale chromatography set-up in order to determine a small selection of lead candidates for final resin development.

LENTI-VSVG RESIN CHARACTERISTICS

Dynamic binding capacity (DBC) of the resin was determined using the p24 total particle ELISA. Results are shown in Figure 3. A feed containing 4×10^{9} total particles/mL was loaded onto a 1 mL column and flow-through fractions were analyzed. Based on the results, a binding curve was plotted and the 10% breakthrough point was determined. These results show that the DBC of the resin is 1×10^{11} total particles/mL resin.

Next, purification conditions were determined in two consecutive runs on a 10 mL chromatography column, using 200 mL load material, a flowrate of 150 cm/h, and 2 min contact time. The feed was endonuclease treated, followed by a clarification on a 0.4 µm filter and direct loading on the column. Column equilibration was performed using a 50 mM HEPES buffer at pH 7.5, containing 150 mM NaCl. Elution was performed using the same HEPES buffer containing 0.8 M Arginine. After the run, a strip of the column was performed using 50 mM sodium phosphate pH12. The chromatographic profile and a close-up of the elution peak are presented in Figure 4.

Fractions of the two chromatography runs were further analyzed to determine the ratio of total particles versus infectious particles.

TABLE 1

Overview of total particles and infectious particles, and their ratio (TP:IP).

Sample	TP/mL	IP/mL	TP/IP ratio
1. Feed	1.10×10^{10}	7.98×10^{7}	138
1. Flow through	3.25×10^{8}	8.30×10^{5}	392
1. Elution	4.44×10^{10}	4.42×10^{8}	100
2. Feed	1.11×10^{10}	9.00×10^{7}	123
2. Flow through	1.28×10^{9}	5.45×10^{6}	235
2. Elution	2.6×10^{10}	4.66×10^{8}	56

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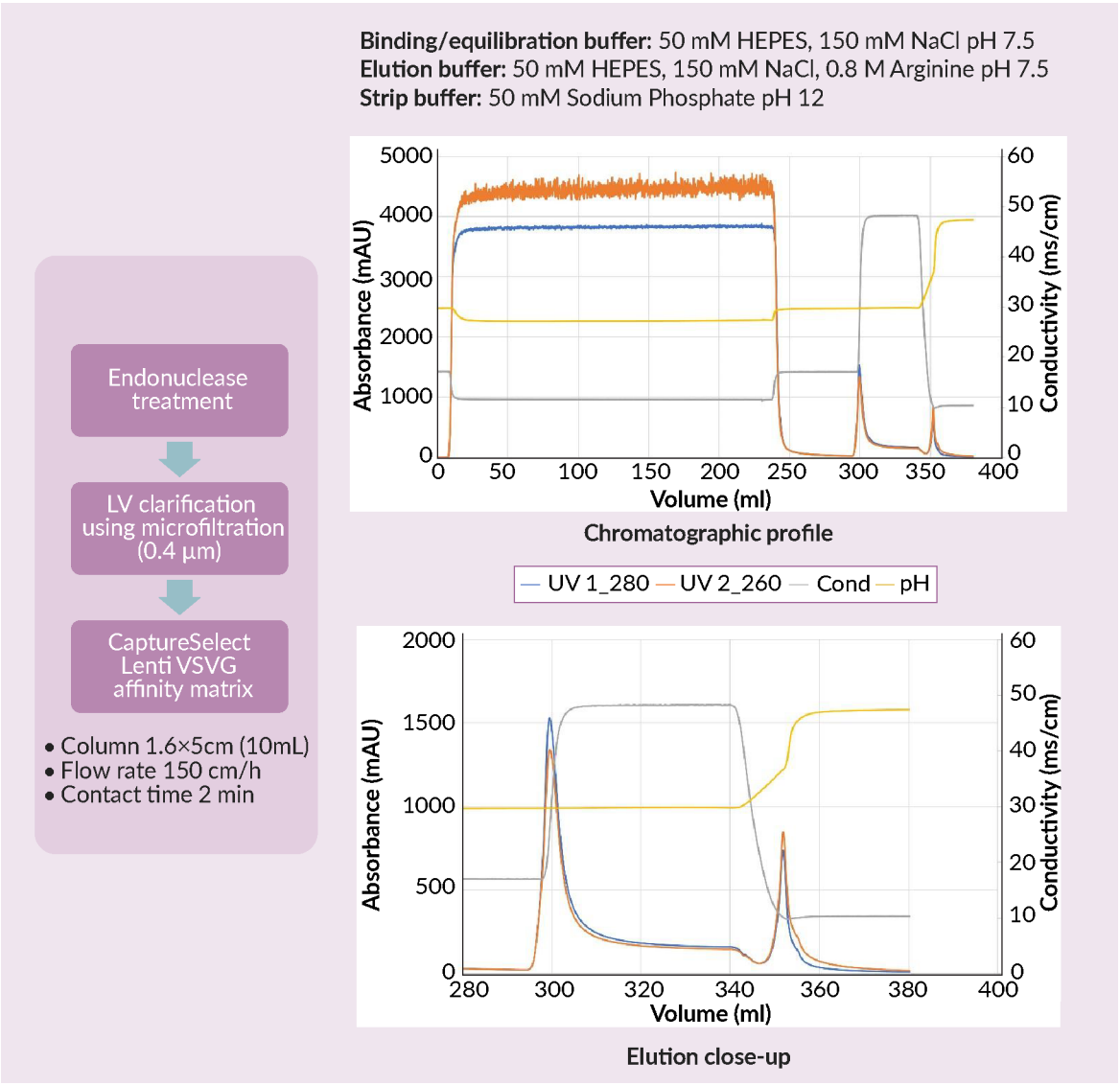
FIGURE 4

The chromatography process using the Lenti VSVG affinity matrix (left) and the chromatographic profile (right).



The results, demonstrated in Table 1, reveal a five-fold enrichment of the infectious particles in the final elution fraction and a decreasing TP:IP ratio. In addition, host cell protein (HCP) and DNA removal, along with total recovery of the elution fractions, was determined. Total recovery of the LV particles was between 50–60% and HCP and DNA impurity removal was considered to be highly efficient; between 80–99% (Table 2).

CONCLUSION

The CaptureSelect Lenti VSVG affinity matrix is designed to help increase productivity and efficiency in the downstream process of VSV-G pseudotyped lentiviral vectors from suspension culture. It provides gentle elution conditions, based on Arginine, to retain infectivity of the LV particles. Furthermore, the resin is a scalable affinity purification method without animal-derived components.

TABLE 2

Final recovery, HCP, and total DNA removal from the elution fractions of two chromatography runs.

Sample	Volume (mL)	IP/mL	TU (Transduction units)	Recovery	HCP removal	Total DNA removal
1. Feed	250	7.98×10^{7}	1.99×10^{10}			
1. Flow through	258	8.30×10^{5}	2.14×10^{8}			
1. Elution	22.5	4.42×10^{8}	9.95×10^{9}	49.9%	98.7%	80.2%
2. Feed	230	9.00×10^{7}	2.07×10^{10}			
2. Flow through	240	5.45×10^{6}	1.31×10^{9}			
2. Elution	25.6	4.66×10^{8}	1.19×10^{10}	57.7%	97.1%	96.5%

ASK THE EXPERTS

Abgail Pinchbeck, Assistant Editor, BioInsights speaks to (pictured left to right) **Pim Hermans,** Director of Ligand Discovery for BioProduction Group, Thermo Fisher Scientific and **Frank Detmers,** Director of Ligand Application for CaptureSelect, Thermo Fisher Scientific

Q Can you expect performance differences between suspension and adherent cultured feedstocks?

PH: Performance differences can be expected. It depends on the quality of the material. In suspension cell feeds, the ratio of total particles versus infectious particles is quite low. In adherent cell feed stocks, where the ratio can be approximately 1000:1, the composition of the material is quite different and the number of actual infectious particles is relatively low compared to the suspension cell feeds. Purification will therefore be more challenging, even for affinity solutions.

Q Can the purity level of the elution fraction regarding host cell proteins and residual DNA be further optimized?

FD: We have seen in ongoing customer evaluations that increasing the NaCl concentration between 300–450 mM for an intermediate wash buffer before eluting can help in further reducing these types of impurities. When you implement an affinity resin, the wash conditions and elution conditions are the steps that often need some optimization.

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Q Does the resin also work for non-VSV-G pseudotyped lentiviruses?

PH: For the development of the resin, we focused on a specific protein that is expressed by lentivirus. In this case, the VSV-G protein was chosen, which means the resin only binds to VSV-G pseudotyped lentivirus particles.

Q Are there any plans to make this research-use-only resin suitable for bioprocessing?

FD: There are plans for upscaling of the resin, making it suitable for bioprocessing. It is scheduled to be available by the end of this year. It will come together with all the support packages needed such as a ligand-leakage ELISA and a regulatory support file. In addition, we are planning to generate supplementary data.

BIOGRAPHIES

PIM HERMANS leads the ligand discovery team at Thermo Fisher Scientific. Antibodies and affinity research have been a common theme throughout his whole career. After receiving his Bachelors degree in Biochemistry he proceeded to work at Holland Biotechnology where he carried out research on the production and purification of recombinant cytokines and monoclonal antibodies. By joining Unilever-Bestfoods, Pim was one of the first scientists involved in the early development and exploration of camelid derived single domain antibodies (VHHs). He joined the Bio Affinity Company (BAC, now part of Thermo Fisher Scientific) in 2003. As head of the Ligand Discovery Department Pim is responsible for the development of VHH based affinity ligands for applications in process – and analytical affinity chromatography. Through the introduction of new selection and screening methodologies he and his team enabled rapid development of affinity purification - and detection tools serving a broad variety of targets.

FRANK DETMERS received his PhD at the department of Molecular Microbiology of the University of Groningen (The Netherlands). From 2001 until 2004 he worked as a post-doctoral researcher at the Department of Cell Physiology at the Nijmegen Center of Molecular Life Sciences (NCMLS, Nijmegen, The Netherlands). He joined BAC BV (Leiden, The Netherlands) in 2004 and the focus of his work is immobilization of affinity ligands on solid supports and the development of new applications of the CaptureSelect ligands. Currently, Frank is director of ligand application for CaptureSelect at ThermoFisher. His work is focusing on the development of new purification tools in the field of antibodies, therapeutic proteins, and gene and cell therapy.

AFFILIATIONS

Pim Hermans
Director of Ligand Discovery for BioProduction Group,
Thermo Fisher Scientific

Frank Detmers, PhD
Director of Ligand Application for CaptureSelect,
Thermo Fisher Scientific

AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

EXPERT ROUNDTABLE

Navigating evolving regulatory CMC guidance in the AAV gene therapy field

Elisa Manzotti, CEO of BioInsights, speaks to (pictured left to right) **Michael Brewer,** Director, Global Principal Consultant, Regulatory, Bioproduction Group, Thermo Fisher Scientific, **Alexis Cockroft,** Director & Regulatory Consultant, Lex Regulatory Ltd, **Christina Fuentes,** Senior Consultant, Dark Horse Consulting Group, **Christine Le Bec,** Head of CMC Gene Therapy, Sensorion Pharma & **Yan Zhi,** Director and Cell and Gene Therapy Product Owner, Process Engineering, CSL Behring





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Q What would you pick out as the key areas of regulatory guidance that have evolved and impacted the AAV gene therapy field in the past 2–3 years?

> "...we are seeing greater clarity from regulators (and particularly [FDA]) in terms of their expectations."
>
> – Christina Fuentes

CF: We are now seeing accelerated growth in the number of clinical programs utilizing adeno-associated virus (AAV), so we have a large amount of clinical data. This is coupled with new and improved analytical methods offering more refined information from raw materials to the product itself. As a result, we are seeing greater clarity from regulators (and particularly the [FDA]) in terms of their expectations. There is now expanded access to current thinking at CBER's Office of Therapeutic Products (OTP) through the initiation of Office of Tissues and Advanced Therapies (OTAT) 'townhalls', as well as advisory committee meetings.

Over the past couple of years, it has become exceedingly clear that total capsid dose is correlated with safety profiles in clinical programs. The greater the viral load, the greater the immune response. For the product, that means wanting to ensure you remove empty capsids because they contribute to the total capsid dose without contributing to product efficacy. This led to the proposed draft guidance [1] we submitted.

We are also seeing additional clarity on various aspects such as the potency assay. Early on in development, a quantitative measure is generally accepted to support first-in-human studies. However, as you advance your program, you want to move towards assays that look at biological effects, and it is never too early to have engagement with regulatory agencies. We are seeing feedback coming from the FDA as early as the pre-IND meeting.

AC: I will give a few specific examples of guidance available to help AAV developers. First, in the EU, there is the European Medicine Agency's Q&A on the principles of good manufacturing practice (GMP) for the manufacture of starting materials of biological origin used to transfer genetic material for the manufacturing of ATMPs [2]. This was well received because it gives clarity on the expectations for plasmids used in AAV production – the expectation is that you consider which principles of EU GMP to apply when you are sourcing plasmids.

In terms of viral safety, it is good to see that ICH is revising its Guideline Q5A. There will now be an annex dedicated to viral vectors like AAVs, which are small enough to have a clearance capability. The British Pharmacopeia (BP) has been doing good work in this area, too – for example, there was a consultation on the characterization of AAV particles at the end of January 2023 with a view to developing future guidance in this area. There is also guidance on vector copy number and flow cytometry as they relate to AAV.

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"...the FDA's Cellular, Tissue and Gene Therapies Advisory Committee convened to discuss some of the challenges around AAV-related toxicities and adverse events. Following that meeting, some guidance came out in October 2022 to provide more advice on the characterization of recombinant AAV."

– Michael Brewer

Finally, a particular thorn in our side in the EU and UK is genetically modified organism (GMO) legislation. We were glad to see that updated in January 2022 with the introduction of a common application form that you can use for GMO applications in the EU related to AAV products [3].

CLB: The balance between full and empty capsids is an important one. If you want to enrich full particles, you need more time for development. It also depends on the type of administration – for example, with systemic administration, you inject a huge amount of vector genome copies into the patient. It is about the balance between safety and risk. There are also discussions to be had surrounding starting materials, and whether they need to be GMP from the beginning of development.

YZ: From the developer side of things, it is always nice to see more regulatory guidance available to help people fact-check. Regulatory requirements have been evolving in the past few years. People developing products in the field must keep in mind that what is permitted now may not be applicable in the next 3–5 years. There are certain things for which there is still room for definition, such as in residual and host cell DNA. However, in general, regulatory agencies seem to be more open-minded and are looking at justifications on a case-by-case basis.

Full versus empty particles is always a big debate in the field. On the one hand, we want to have a clear guidance document to outline the cut-off in this respect; on the other hand, it may be appropriate to continue assessing this aspect case-by-case, although that does create uncertainty. In this new field, everything is quickly evolving and developing, which makes it an exciting place to be.

MB: On the regulatory front, one of the most significant happenings was in September 2021, when a meeting of the FDA's Cellular, Tissue and Gene Therapies Advisory Committee convened to discuss some of the challenges around AAV-related toxicities and adverse events. Following that meeting, some guidance came out in October 2022 to provide more advice on the characterization of recombinant AAV. This

guidance is focused on neurodegenerative diseases, but it can still form the basis of general guidance around host cell-related process impurities, and it can also apply to other applications of AAV-based gene therapy.

Q What are the most significant ways in which the AAV analytical toolkit has developed, particularly in response to current regulatory opinion?

CLB: The greatest evolution has been in product characterization, particularly surrounding full and empty particles. We need further tools for process characterization. The process for purification of AAV takes 2-3 days, and there are not currently many tools available for following the process and product in real-time. We have the ability to characterize drug substance, but we need further process characterizations: we need to know the integrity of the particles and of the genome, and the quantity of plasmid DNA inside the particles. We have quantitative PCR (qPCR) or digital PCR (dPCR) for copy number, but we need to develop more tools for full and empty particle analysis. Ideally, we would have different orthogonal methods to understand the product.

MB: As guidance has evolved, it has transitioned from only quantifying the amount of host cell DNA to also assessing the size of the DNA in the final product, in addition to the content of vector DNA. If you are using cell lines that contain oncogenic genes, you need to characterize and potentially measure the size of those oncogenic fragments of DNA that are part of the host cell genome. We need specific and sensitive assays to potential oncogenes, with an approach to allow size assessment of specific genes, to provide the assurance that if they are present, they are not full length.

This speaks to the importance of choosing an appropriate residual host cell DNA assay. Typically, people now use qPCR, dPCR or array-based digital PCR. It is important to target a high-copy genetic element that is spread across the genome in order to get a full picture of what the host cell DNA is carrying through in the purification process. Then, using highly sensitive and specific assays for vector components as part of the AAV process can enable the demonstration of high levels of clearance of those components.

We also need advanced tools for contaminant testing, such as specific qPCR assays for viral contaminants. Keep in mind that these cell culture harvests need to be tested for mycoplasma. Many factors such as this are often overlooked when manufacturing a viral vector as opposed to a recombinant protein or monoclonal antibody.

YZ: As this is an actively developing field, there are many tools and platform technologies popping up. As a scientist, I am always excited about these new instruments or platforms, as they offer a different viewpoint and provide different data. As my career has progressed, I have spent more time in the chemistry manufacturing organization (CMC) space,

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particularly in late-stage development/commercial. New technology requires you to take a pause and consider how to handle the new data that you are being offered. Depending on the stage of product development and the application, there is a fine balance to consider regarding technology and the usefulness of the data it offers.

CF: Emerging technology can be used if you demonstrate that the method is fit for purpose through characterization of aspects such as accuracy, precision, linearity, and limit of quantification. It is true that you need to establish a specific purpose for their application early on, but these technologies can certainly be very powerful tools in product development for understanding what is in your product so long as the method is reliable.

We are seeing improvements in next-generation sequencing (NGS) that can help characterize the heterogeneity and impurities in AAV products. For example, if you have many partially-filled capsids, you can assess if they contribute to product efficacy in terms of having a therapeutic benefit. NGS data may also be useful in leveraging for comparability studies after making a process change.

Tying these pieces together is important. From the development perspective, sequencing is powerful for characterization. Release tests are moving towards assays that are more sensitive, accurate, and precise. For example, for dose-determining assays for vector genome titer, groups are moving away from qPCR in favor of dPCR.

Q As you have mentioned, the US FDA in particular has been clear in relaying the importance of full/empty/partially-full capsid ratio to the safety and efficacy of the final drug product – can you expand on the tools and methods that stand out for you in terms of maximizing the quality of the final vector product in this specific area, both in terms of processing and analytics?

MB: As we have discussed, there has been an evolution towards dPCR and array-based digital PCR platforms, which have improved accuracy. This is particularly critical for counting vector.

The method chosen for the quantitation of viral capsid and correlating the ratio of full/empty is also critical. People frequently use ELISAs for this purpose, but there have been developments in analytical ultra performance liquid chromatography (UPLC) methods that can distinguish between empty, full, and partially-full capsids. This method can even determine a percentage of overly-full capsids that have fragments of host cell DNA incorporated into the vector.

NGS-based techniques are certainly evolving. Applying these methods in combination is critical, as is optimizing your manufacturing process and lot-release testing package.

"To improve full/empty/partially-full capsid ratio, another consideration is if there is anything that can be done to maximize full capsids via vector design regarding optimization of genome size and consideration of hybrid capsids."

– Alexis Cockroft

YZ: Full/empty/partially-full AAV particle ratio has become a critical quality attribute (CQA) for gene therapy products, despite no clear release specification requirement by regulatory agencies as yet. It is necessary to have a solid method to assess the full/empty capsid ratio at a minimum, and ideally, also an idea of your partially-full capsid population. There are many emerging technology platforms that have been developed to address this particular issue. However, because many are still so new, they are not yet GMP-ready. Data traceability and integrity as per 21 CFR part 11 compliance needs to be considered before an assay is chosen for release testing.

So far, sedimentation velocity analytical ultracentrifugation (SV-AUC) and transmission electron cryomicroscopy (CryoTEM) have been adapted by different companies for use in product release testing, following assay validation as per ICH guidance. Typically, sponsors must provide at least 100% empty particles, and as high a percentage of full particles as they can afford to achieve (in terms of the requisite purification steps and analytics), to support full scope assay validation before release testing. Even so, despite these efforts, it is still possible that for partially-full particles, you may not have full confidence in the validated result. In those cases, you must communicate with regulatory agencies on a case-by-case basis.

It is understandable that there are safety concerns surrounding AAV, due to the well-documented clinical incidents in which high doses of AAV have caused the deaths of patients. Total particle analysis is certainly required in response. However, the question of whether analyzing the distribution of the different types of particle is entirely necessary is still open to debate. Current publications surrounding whether empty particles are beneficial to some degree still engender varying opinions in the public domain.

Personally, I am more inclined to highlight the importance of batch-to-batch consistency over the absolute full/empty/partially-full value for each batch, particularly if you have solid clinical data for an early-stage asset. The minimum requirement you need is consistency. If consistency is guaranteed, the likelihood of safety events is kept to a minimum.

AC: To improve full/empty/partially-full capsid ratio, another consideration is if there is anything that can be done to maximize full capsids via vector design regarding optimization of genome size and consideration of hybrid capsids.

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"We must bear in mind the definitions of full and empty particles. We must fully characterize these and know what is inside. NGS is being used to understand the quantity and the size of the host cell DNA, the plasmid DNA, and the capsid itself."

– Christine Le Bec

CF: There is no magic number. Many people postulate numbers such as 70% or 80% full to guarantee a safe product, but as my colleague, Don Fink, likes to say, everything is based on the totality of data. Both preclinical and clinical data will be used to assess product safety. It is clear that lot consistency is important, as is early characterization of full/ empty/partials. If you make a manufacturing change, for example, it can significantly impact your full/empty/partial profile. It is key to have consistency and well-characterized attributes, so that if and when you make a manufacturing change, you can demonstrate comparability.

In terms of the available analytical toolkit, there are a few methods out there. AUC is commonly used for release testing, as it allows the resolution of empty, full, and intermediates in an accurate and precise way. However, when you think about your manufacturing process, you may also want to characterize full enrichment. This can be where other methods come into play because AUC requires a lot of purified material. Example methods for this include charge detection mass spectrometry (CDMS), mass photometry, and many others. The method you chose should be based on your understanding of the specific context.

CLB: People are mostly trying to enrich capsids at the beginning of the process. Many people are working more on vector design and using two plasmids instead of three to enrich capsids and achieve a high yield. During the purification, it is difficult to achieve 100% full particles. You can achieve 90–95% if you pre-refine with cesium chloride gradients, although this type of process can pose challenges for clinical applications. For clinical applications, ion chromatography approaches such as anion exchange chromatography (IEX) are the most commonly used methods.

We must bear in mind the definitions of full and empty particles. We must fully characterize these and know what is inside. NGS is being used to understand the quantity and the size of the host cell DNA, the plasmid DNA, and the capsid itself.

Q How would you go about establishing partially-full capsid percentage as a CQA, and then establishing a release assay and release specifications?

YZ: Right now, I think that even SV AUC is not at the stage where you can use it to easily quantify partially-full percentage accurately – particularly if you have multiple intermediate populations. My sense is that this is very much on a case-by-case basis. What I mean by that is it will be highly beneficial if you can have supporting data to show whether there is any biological function for those intermediate species. Then you can have a different conversation with the regulators.

Everyone knows an empty particle is an impurity – there is no question about that. However, if you have data to show the intermediate species actually has a biological effect, then the conversation changes: it is no longer an impurity, it's just a different form of your product. This might open the door to consider full and partially-full particles together.

Again, though, I think this is very much case-by-case at the moment, and it depends heavily on how much information you have and what kind of story you want to tell.

MB: Setting the acceptance criteria for partially-full capsid population is really dependent on the capabilities of the analytical methods you are using to differentiate or discriminate between populations.

Q Vector characterization and CMC considerations are often given relatively little consideration in the early stages of development due to the obvious need to prioritize investment in progressing as swiftly as possible to the clinic. What would you say are the 'must-do's' in terms of early product development?

AC: For any direct injection vector, first-in-human trials is not your goal; if you want to give patients access to your product for a prolonged period of time, your goal must be the marketing application. Consequently, it is important to make every bit of data count. I strongly advise an integrated development plan from an early stage, and applying quality by design (QbD) principles. A quality target product profile should be created early and CQAs should be considered. An analytical development plan is also needed early on.

When considering what is needed for a clinical trial application, as a minimum, one should know both the FDA and the EMA guidances for clinical trial applications off-by-heart. Specifically, these are the FDA Gene Therapy Guideline for Investigational New Drug Applications [4], and the draft EMA guidance on investigational advanced therapy medicinal products [5]. These will outline the requirements for the CMC section of your clinical trial application. As early as is feasible, critique your vector design, and question every single component for its benefit and safety profile. Ideally, keep a vector design file so that you have all that information to hand if a regulator asks these questions.

You need at least one batch manufactured in accordance with your proposed manufacturing process for the clinical trial. If you have manufactured non-clinical studies using a

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different manufacturing process, then consider the changes that have been made and whether they would impact the interpretation of the non-clinical studies. In short, are the non-clinical batches representative of what you are going to put into patients?

Having a shelf-life strategy means that at early stages, think about which batch is leading. It is almost impossible to set a shelf-life on the batch you are going to use clinically. Design your production so that you can have an initial batch assigned to stability, in order to have stability data, and indicate which (potential) CQAs will be tested. Once you have established a stability profile, you can propose a meaningful, practicable shelf-life.

Ensure you have completed compatibility studies in addition to a potency assay (which may not need to be a full biological activity assay). Qualify the assay that will be used for dose determination, otherwise you risk wasting clinical data if those results are not robust.

Process-related impurities, such as any raw materials being used that could have a potential toxicological or pharmacological action, should be risk assessed. If you suspect they could have an impact on patient safety, develop a test to control them on each batch.

Qualify your starting materials and consider whether each material you are using is the best for the job. Ask yourself: what do these materials mean in terms of their potential impact on both product and patient safety?

MB: Adding to what Alexis said, I think it's critically important to plan for success. Incorporate and qualify your analytical methods early, ensuring they provide the performance to give you a high degree of confidence that they can be validated and then accepted later on, when you are filing your biologics license application (BLA).

I really can't emphasize it enough: plan for success. Don't go down the road of using characterization assays that regulators won't accept later on.

CLB: For me, I think it is really important to qualify your method early, but as Alexis said, don't forget to design your product with clinical application in mind. Also, don't forget also to retain some material - from some tox batches, for example – for when you come to develop the potency assay, and to go back to when you are at the BLA stage. At that point, you will need to have the ability to make a comparability assessment with all of the different batches over the course of your development.

YZ: I think Alexis hit all of the main points. I would probably just emphasize one small point, which is that even though your assay may not be fully validated from the product standpoint at an early stage, you should nonetheless have some understanding of the CQAs. For each CQA (with the possible exception of dosing) you should ideally have a prototype assay ready

CF: I think some great points have been raised. If there is one thing I would want readers to take away, it would be to take retains, as Christine mentioned – especially if you are starting this work early. You may not have all your methods developed yet, so having those retains will be really important, particularly once you have your dose determining assay.

"As we all know, for regulatory approval of an AAV-based gene therapy product, either a potency assay or a potency assay matrix is an absolute requirement. The potency assay or assay matrix needs to be fully validated as per ICH guidance..."

– Yan Zhi

The other thing to consider is something I have seen often, whereby clients start early with a process – iodixanol density gradient purification, for example – in order to get a few batches of material that they can start testing. But eventually, for clinical manufacture, they will want to move towards chromatography-based purification. As my colleague Kevin Whittlesey and I often recommend, the earlier you can make the transition to a representative manufacturing process, the more of your preclinical data you can leverage later on.

Finally, we talked about the need for a qualified vector genome titer assay for dose determination. You need to save your preclinical lots because ideally, you will test your titer assay on those retains. Once you have your qualified method in place, you can again leverage the data to determine your starting dose range for clinical use.

Q No discussion of product development/characterization in the AAV gene therapy field can be complete without some discussion of potency assay development – what is your advice to those facing this particularly thorny challenge?

YZ: As we all know, for regulatory approval of an AAV-based gene therapy product, either a potency assay or a potency assay matrix is an absolute requirement. The potency assay or assay matrix needs to be fully validated as per ICH guidance in order to provide a quantitative result to meet the preset specifications or acceptance criteria. It also needs to adequately reflect the mechanism of action. Ultimately, the potency result should be well correlated to your clinical efficacy – the potency assay is required not only for lot release, but also for the comparability and stability studies.

Potency assays tend to be highly product-specific. However, for a typical AAV-based gene therapy product, at least two levels of biological activity will be required to demonstrate potency. One is the ability for the vector to transduce a cell in the target organ or tissue. The second level is the ability to express the therapeutic protein after transduction in order to provide the desired biological effect. As a result, *in vitro* cell-based potency assays that

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correlate to a well-characterized and defined reference standard, have been widely used by industry for AAV-based gene therapy products.

In order to develop a brand-new *in vitro* cell-based potency assay, the totality of product knowledge becomes critical. For example, you need to consider whether an established cell line, an engineered cell line, or even primary cells (even though not preferred, you may have to go down that route) is optimal. This decision may be driven by your understanding of which option is most compatible with your specific AAV serotype, or by any specific need relating to your gene of interest being driven by a tissue-specific promoter, or by any enzymes or proteins that may be required in order to achieve the desired biological effect. All of these things must be considered.

At an early stage of product development, it may be beneficial to explore different types of potency assay rather than just one, so that you may obtain different readouts for biological effect. But as a minimum, you should have an expression-based assay (ELISA) to look for therapeutic protein expression, or even mRNA expression (real-time PCR or RT digital PCR).

Then, once the product has matured and you have accumulated more knowledge, you can select the assay that best fits the bill in order to start validation towards it becoming a release test. Certainly, it will be a lot easier to remove an assay rather than add a new one at the later stages of product development.

The other thing you need to consider early is that your potency assay must ultimately be a key part of GMP-compliant release testing. To support the GMP compliance of a potency assay, the qualification of any critical reagent in that assay needs to be in place. (Ideally, you would have supply chain redundancy in place as well, so that you don't have to rely on a single source for any of the critical reagents). On top of this, you need to consider the technical staff training as well as any specialized instruments you may need to implement in a QC environment. Sometimes a complex potency assay may work very well in your analytical development department, but when you transfer it to QC, you find that it is not so robust.

The majority of people working in the AAV gene therapy field today will probably be using a relative potency (RP) assay. As you can imagine, for any RP assay, the quantifications, characterization, and stability of the reference material become extremely important.

Last but certainly not least, I would always recommend communicating early and often with the regulatory agencies to discuss your potency assay design and strategy.

AC: Just to reiterate what Yan said, it can be incredibly challenging to have enough batch data from patients who had a positive clinical outcome to justify the acceptance criteria for your commercial product. I therefore agree that it is important to develop a suite of assays early on, so that by the time you get to your pivotal study, you know which assay or assays you are going to implement because you are confident in their performance. Ensure the dataset for setting the acceptance criteria, which is based on a positive clinical outcome, is sufficient.

A potency assay can be a marketing application killer.

Q Lastly, could you comment on the usefulness (or lack thereof) of infectious titer assays?

CLB: I wouldn't say infectious titer assays are useless. I think they can be interesting to use at the beginning of development, when it can sometimes be hard to have a potency assay but you need to demonstrate firstly that your vector can infect your cells. Even if you have some variability with the infectious titer assay method, it still gives you a value you can work with, and some form of comparability from one batch to another.

So, while it is an old method, I would say it is good to have for your early-stage development work. Then, once you have developed a potency assay, you can remove the infectious titer assay and go on to something that is more qualified.

CF: Infectious titer assays are a tricky one. It is dependent on the serotype you are using.

As Christine noted, infectious titer can be used as a surrogate for potency very early on, while you are still exploring methods and defining your potency assay. It tells you whether the vector particle gets into the cell or not. It doesn't tell you if there is a biological effect or if anything is being expressed, but it is a piece of the puzzle nevertheless.

I think it again comes back to the question of is the assay fit for purpose, because you may have serotypes that are very difficult to transduce *in vitro* and in those cases, you will get quite a high degree of variability in your results. The extent to which you can then leverage or rely on the data then becomes a bit more limited.

BIOGRAPHIES

MICHAEL BREWER is the Director, Global Principal Consultant, Regulatory for the BioProduction Group (BPG) at Thermo Fisher Scientific. In this role, Michael is responsible for providing global support to BioProduction customers and serving as the regulatory thought leader and expert across all technology areas within BPD. Prior to moving to this role, he led the Pharma Analytics business, a team responsible for development and commercialization of testing applications for Microbiology, Analytical Sciences and Quality control. The products are fully integrated, solutions for Glycan profiling, Bacterial and Fungal identification, Mycoplasma and Viral detection and host cell DNA and protein quantitation. Michael has over 30 years' experience in the Biopharma industry, including, Scios, Synergen and Amgen in a variety of roles including Discovery Research, Analytical Sciences and Quality Control. Prior to joining Thermo Fisher Scientific, he led a group at Amgen that developed qualified, validated and implemented molecular methods for host cell DNA quantitation, contaminant (Mycoplasma, Virus and Bacteria) detection, contaminant identification, strain typing and genotypic verification of production cell lines. Additionally, his group supported regulatory submissions including IND, NDA, and CMC updates, Regulatory inspections, NC/CAPA investigations, contamination investigations and remediation and developed regulatory strategy for implementation of new methods.

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ALEXIS COCKROFT has worked in CMC regulatory affairs for more than 17 years and focused solely on gene/cell therapy products for the last 11+ years. She is currently a freelance regulatory consultant as Lex Regulatory Ltd and works with a number of organizations developing advanced therapies. A large proportion of her experience is with gene therapy products. She has accumulated knowledge from more than 25 years in science-related roles and finds the complexities and challenges of advanced therapies very rewarding.

CHRISTINA FUENTES is a Senior Consultant at Dark Horse Consulting Group where she provides technical expertise in Cell and Gene Therapy to help clients address unique challenges in the field. Her specialties include supporting clients utilizing engineered nucleases for gene modification and AAV for *in vivo* therapies. Prior to joining Dark Horse Consulting, Christina completed her PhD at University of California, Berkeley in David Schaffer's lab where she engineered systems to enhance cell and gene therapies, including the development of a self-inactivating AAV mediated CRISPR-Cas system for therapeutic genome editing *in vivo*. Dr. Fuentes co-authored the first externally prepared proposed draft guidance on "Testing of Adeno-Associated Viral (AAV) Vector-Based Human Gene Therapy Products for Empty Capsid During Product Manufacture" which was formally submitted to the FDA for consideration on May 15, 2022. Most recently, Christina co-authored a white paper titled "Beyond Empty and Full: Understanding Heterogeneity in rAAV Products and Impurities." This document details the importance of product characterization and heterogeneity in product-related impurities to improve product understanding and inform manufacturing strategy.

CHRISTINE LE BEC, PhD, joined Sensorion Pharma, a small biotech company, in early 2020 as Head of CMC Gene Therapy. She is responsible for CMC development (process & analytical development, product characterization) including non-clinical and clinical manufacturing, CMC transfer to CDMO/CRO and CMC regulatory issues. Prior to joining Sensorion Pharma, she worked for more than 20 years at Genethon, a French non-profit organization, in the field of Gene Therapy vectors (AAV, lentivirus, baculovirus) for rare diseases. She has a strong expertise in the development, qualification, validation of analytical methods for product characterization, release testing of gene therapy products and in stability studies. She also has a solid knowledge of international regulations and reviewing CMC documents for clinical applications.

YAN ZHI completed postdoctoral research training with Dr James M. Wilson at Institute of Human Gene Therapy, University of Pennsylvania, after which Yan spent over 15 years in global contract testing organizations (WuXi AppTec and Charles River Laboratories) to provide scientific leadership to Biologics testing services with the strong focus on cell and gene therapy industry as well as a global contract development and manufacturing organization (Fujifilm Diosynth Biotechnologies) to design cell and gene therapy product development programs from clinical to commercial manufacturing. In 2020, Yan joined Spirovant Sciences, Inc. to lead the analytical development and develop CMC strategy of a novel serotype AAV based in vivo gene therapy product for IND submission. Since 2022, Yan has been working with CMC, regulatory, quality, supply, and commercial teams at CSL Behring to support commercial launch of an AAV gene therapy

product as well as manufacturing process and analytical methods transfer to a second site for future registration. Yan received a PhD in Microbiology and Molecular Genetics from University of California, Irvine, and a BA in Molecular Biology from University of Science & Technology of China. She is a leading author and co-author of numerous publications in peer-reviewed scientific journals and a patent holder.

AFFILIATIONS

Michael Brewer
Director, Global Principal Consultant,
Regulatory, Bioproduction Group,
Thermo Fisher Scientific

Alexis Cockroft
Director & Regulatory Consultant,
Lex Regulatory Ltd

Christina Fuentes
Senior Consultant,
Dark Horse Consulting Group

Christine Le Bec
Head of CMC Gene Therapy,
Sensorion Pharma

Yan Zhi
Director and Cell and Gene Therapy Product Owner,
Process Engineering,
CSL Behring

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AUTHORSHIP & CONFLICT OF INTEREST

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Designing viral vector clearance studies for CGT products according to ICH Q5A (R2): part 1

Tareq Jaber PhD, Associate Director, Process Evaluation

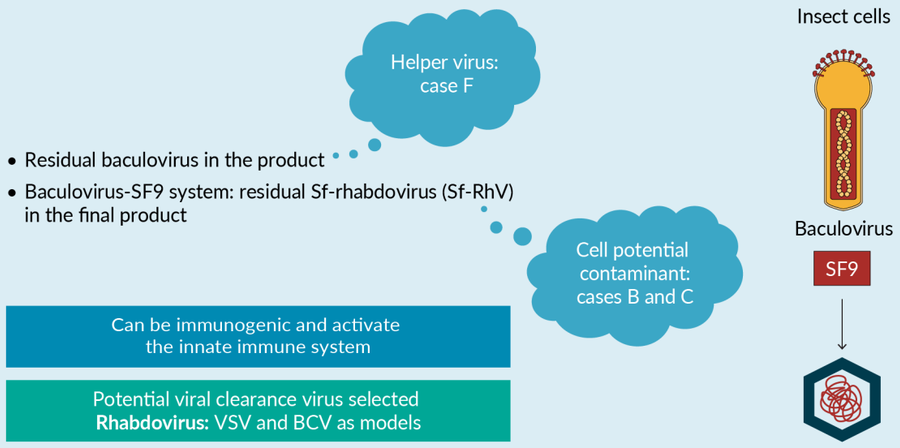
In October 2022, a new draft of ICH Q5A (R2) was released. This draft revision was necessary to reflect current scientific knowledge and biotechnology advances such as new product types that are amenable to viral clearance and alternative virus clearance validation strategies. In Part 1 of this FastFacts series, sources of contamination and viral clearance processes will be explored.

Some limitations exist when it comes to ensuring viral clearance for gene therapy medicinal products (GTMPs), as many utilize viruses as their mechanism of action. The revised guidance covers some of these issues and allows for alternative test methods.

STARTING MATERIAL CONSIDERATIONS FOR GTMPS

Starting material for gene therapy product manufacture is a key consideration for the assessment of viral safety testing and viral clearance. When considering potential sources of contamination, the general principles of the ICH Q5A (R2) guideline should be followed. This states that contamination could arise from the cell line, such as from the use of contaminated cell substrate, latent/persistent viruses, or endogenous contaminants. Adventitious viral contamination could also occur through the use of contaminated raw materials/reagents. Other sources of contamination include those caused by manufacturing operators or by using a virus or viral vector to induce the expression of specific genes.

Figure 1. Case study 1: residual baculovirus as a potential source of contamination in gene therapy.



REVIEW OF CASE STUDIES IN ICH Q5A (R2)

One potential source of contamination where viral clearance is recommended is residual baculovirus **(Figure 1)**. The addition of Case F to the document focuses on helper viruses used in gene therapy products.

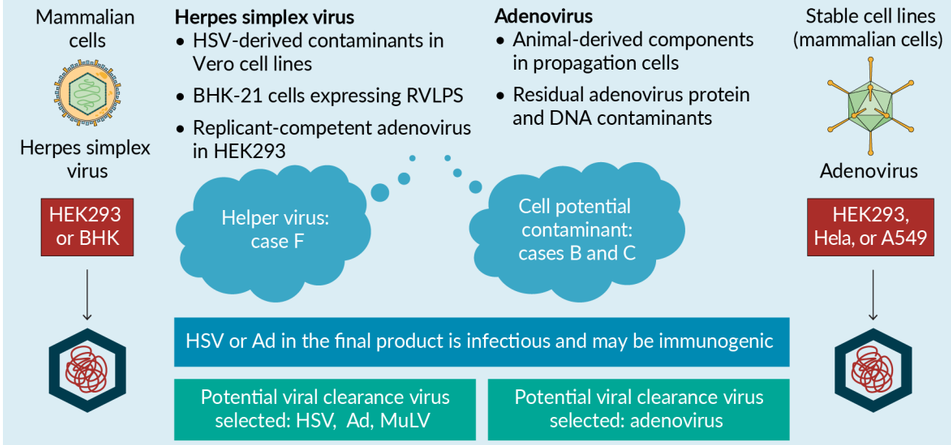
The second example within the document covers the contamination potential posed by human viruses, namely herpes simplex virus (HSV) and adenovirus (Ad) **(Figure 2)**.

LIMITATIONS OF VIRAL CLEARANCE PROCESSES

Since the possibilities for applying viral clearance steps during production are limited for many types of GTMPs the viral safety of these products should be ensured by applying a combination of measures. The document shows an understanding of these limitations.

Virus safety should focus on testing and control of the raw materials and reagents and the manufacturing process. The use of well-characterized cell banks and virus seeds can reduce the risk of virus contamination. Manufacturers should avoid using human- and animal-derived raw materials in their manufacturing processes when possible. Cell culture media or media supplement treatments such as gamma irradiation or virus filtration can be used as additional virus risk mitigation measures. Closed processing, testing, and other preventative controls can be used.

Figure 2. Case study 2: HSV and Ad as potential sources of contamination in gene therapy.



DETERMINING THE APPROPRIATE LOG REDUCTION FACTOR

When appropriate, viral clearance studies should be performed to determine virus reduction factors for the relevant step(s) of the production process. The virus clearance should be validated using representative and qualified scale-down systems. Virus clearance validation should include model viruses representative of adventitious, endogenous, and, if possible, the relevant helper virus. Acceptable log-reduction factors can be based on risk assessment, although no particular value is given in the document. This can be dependent on factors such as the cell type used, whether a helper virus is used, the potential of contamination, and the control level over downstream steps.

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FASTFACTS

Designing viral clearance studies for CGT products according to ICH Q5A (R2): part 2

Tareq Jaber PhD, Associate Director, Process Evaluation

In October 2022, a new draft of ICH Q5A (R2) was released. This draft revision was necessary to reflect current scientific knowledge and biotechnology advances such as new product types that are amenable to viral clearance and alternative virus clearance validation strategies. In Part 2 of this FastFacts series, the evaluation and characterization of viral clearance procedures will be detailed.

A FOCUS ON CHROMATOGRAPHY

ICH Q5A (R2) placed a specific focus on chromatography, specifically the function and regeneration of columns for protein A affinity chromatography.

Process parameters for chromatography have not changed. In general, when scaling down a production system, factors such as column bed height, linear flow rate, buffer and gel types, and concentration of protein must still be considered.

The new addition to this document surrounds protein A affinity. Prior knowledge indicates that virus removal is not impacted or slightly increases for used chromatography media/resin. Therefore product-specific studies with used resin are not expected.

Prior knowledge might also apply to other chromatography types involved in viral clearance (e.g., anion exchange or cation exchange). To support repeated resin use for other chromatography types, equivalent prior knowledge including in-house experience and a detailed justification should be provided instead of product-specific viral clearance studies with end-of-life-time resin.

APPLICATION OF PRIOR KNOWLEDGE FOR EVALUATION OF VIRAL CLEARANCE

The decision on the acceptability of virus clearance data without product-specific experiments is made on a case-by-case basis, considering the whole viral safety concept for a medicinal product.

When using prior knowledge, the process steps must be well understood. The representativeness of the prior knowledge for the specific process step should be clearly justified.

The use of external prior knowledge can enable a demonstration of comparability of the processes across the manufacture of different products involved, in addition to comparability of the product intermediates. External knowledge can be supportive in nature, providing insights into the mechanisms involved and defining critical process parameters; however, this must be carefully assessed and supplemented with in-house experience.

A STEP-BY-STEP OVERVIEW OF PLATFORM VALIDATION

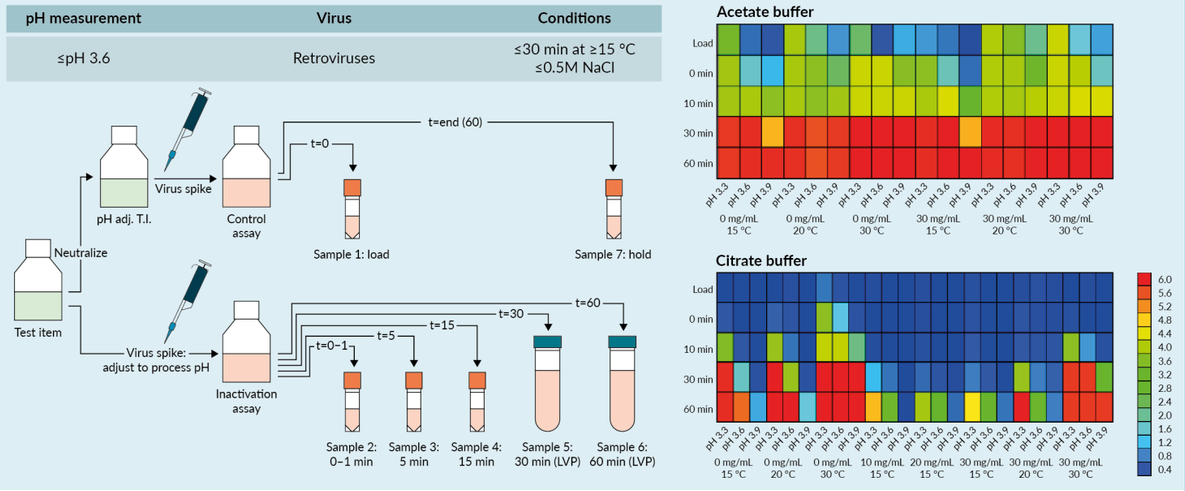
Examples to illustrate the steps suitable for a platform validation approach for gene therapy viral clearance are included in the document, namely detergent treatment, low pH treatment, and viral filtration. An outline of a step-by-step case study for low pH treatment suitable for a platform validation approach can be found below.

Process parameter impacts for low pH treatment are shown in **Table 1**. An overview of how to apply a platform approach to low pH treatment is shown in **Figure 1**.

Table 1. Low pH treatment: process parameter impacts.

Process parameter	Potential impact	Rationale
pH	High	Inactivating agent
Incubation time	High	Mechanism of inactivation is time-dependent
Temperature	High	Impact on inactivation kinetics
Buffer matrix	High	Available data show that inactivation robustness depends on buffer matrix
Product concentration	Low	No impact on inactivation observed
Type of product	Low	No impact on inactivation observed for MAb, half antibody, bispecific antibody, fusion protein or recombinant protein
NaCl concentration	Low	No impact if ≤500 mmol/L NaCl
Potential interaction between virus particle and product	Low	No impact on inactivation observed

Figure 1. Low pH treatment: how to apply a platform validation approach.



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FASTFACTS

Efficient, scalable purification of VSV-G lentivirus by novel affinity chromatography

Frank Detmers, Director of Ligand Application for CaptureSelect, Thermo Fisher Scientific

Lentiviral vectors have emerged as a long-term stable gene expression tool for cell and gene therapies. However, large-scale production of purified clinical-grade lentiviral vectors remains a challenge because of the complex feedstock and its sensitivity to changes in temperature, ionic strength, pH, and other environmental factors. This poster presents the chromatography conditions and performance of a recently developed affinity chromatography resin for the purification of lentivirus particles.

LENTIVIRUS PURIFICATION CHALLENGES

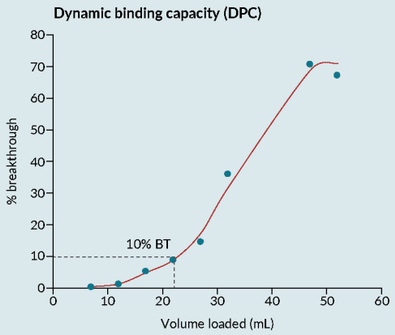
Lentiviral vectors (LVV) have limited stability, requiring a narrow range of pH, temperature, shear stress, salt concentration, and osmolarity. Because of this, traditional methods of purification suffer from difficulties relating to yield, purity, and scalability. With these methods, general recoveries in the field are not higher than 25–30% for the overall process, with a significant part of the losses being in the final filtration step utilizing a sterilizing-grade filter. Thermo Fisher Scientific recently developed an affinity chromatography resin, CaptureSelect™ Lenti VSVG Affinity Matrix, as a solution to these challenges.

DYNAMIC BINDING CAPACITY OF CAPTURESELECT™ LENTI VESICULAR STOMATITIS VIRUS G (VSV-G) AFFINITY MATRIX

Based on CaptureSelect™ technology, the immobilized ligand is developed to specifically bind to the VSV-G envelope protein present in the vast majority of recombinant lentiviral pseudotypes. Lentivirus produced in HEK-293 cells in suspension is loaded on 0.66 x 3 cm column containing 1 mL of CaptureSelect™ Lenti VSV-G resin, equilibrated in 50 mM HEPES buffer solution, 150 mM NaCl pH 7.5.

As shown in **Figure 1**, 10% breakthrough of the lentivirus particles is reached after loading 24.6 mL of the feed material, resulting in a dynamic binding capacity of the resin of $1x10^{11}$ total particles/ml of resin. C_0 is the titer of the feedstock (3.89×10^9 particles/mL), and C is the titer measured in the flow through fractions. The 10% breakthrough point is interpolated from the breakthrough curve.

Figure 1. Dynamic binding capacity (1 mL column).



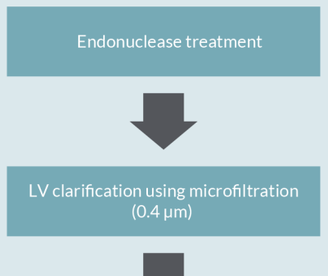
DBC is determined by P24 total particle ELISA

Fraction	Volume (mL)	TP/mL	C/C_0	
Start		3.98E9		
1	7.1	1.55E7	0.39	
2	12.1	5.24E7	1.32	
3	17.1	2.14E8	5.39	
4	22.1	3.53E8	8.88	24.6
5	27.1	5.85E8	14.7	
6	32.1	1.44E9	36.09	
7	47.1	2.82E9	70.84	
8	52.1	2.68E9	67.33	

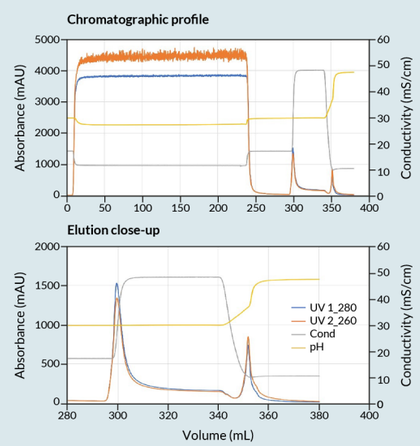
- 10% breakthrough (C/C_0=10%) estimated from the curve at 24.6 mL loading
- This relates to 9.78E10 total particles/mL resin (=1E11)

Dynamic binding capacity at 10% breakthrough is 1E11 total particles/mL resin

Figure 2. Chromatography conditions of the CaptureSelect™ Lenti VSVG affinity matrix.



Binding/equilibration buffer: 50 mM HEPES, 150 mM NaCL pH 7.5
Elution buffer: 50 mM HEPES, 150 mM NaCL, 0.8 M arginine pH 7.5
Strip buffer: 50 mM sodium phosphate pH 12



The CaptureSelect Lenti VSVG affinity matrix demonstrates an efficient elution profile

CHROMATOGRAPHY CONDITIONS

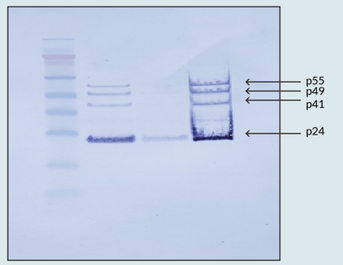
Figure 2 illustrates that the elution with 50 mM HEPES, 150 mM NaCl, 0.8 M arginine pH 7.5 is efficient and has good compatibility with the enveloped virus particles, resulting in high concentrations of infectious particles in the elution fraction. Depending on the feed and application, optimization of the elution buffer might be needed with adjustments of the arginine concentration, pH, or combinations thereof.

COMPARISON OF TOTAL PARTICLES TO INFECTIOUS PARTICLE RATIOS

The total concentration of infectious particles increases after purification **(Figure 3)**. Total particles are determined by p24 ELISA and infectious particles are determined through a cell infectivity assay. In the first run, 1 in every 100 particles is infectious in the elution fraction, while in the feedstock it is 1 in every 138 particles. In the second run, this ratio becomes 1 in 165 particles in the feed to 1 in 70 particles in the elution fraction.

Figure 3. Concentration of infectious particles in the elution fraction.

P24–WB pattern



Total particle (TP) and infectious particle (IP) ratio

Sample	TP/mL	IP/mL	TP/IP ratio
1. Feed	1.10E10	7.98E7	138
1. Flow through	3.25E8	8.30E5	392
1. Elution	4.44E10	4.42E8	100
2. Feed	1.11E10	9.00E7	165
2. Flow through	1.28E9	5.45E6	245
2. Elution	2.6E10	4.66E8	71

(n=2)

- The eluted fractions show a more than 5-fold increase of the infectious particle concentration compared to the load

The concentration of infectious particles in the elution fraction has been enriched through purificaton using the Lenti-VSVG resin

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