Key Considerations in Gene Therapy Manufacturing for Commercialization

An important discussion about gene therapy manufacturing, including the latest in manufacturing methods, analytical analysis and key regulatory considerations. Topics to be discussed include:

- Planning for scale up, technology transfer and regulatory considerations on the way to commercialization.
- In-house vs. Outsourcing manufacturing.
- Ensuring scalability and efficient timelines in manufacturing while still maintaining reasonable cost.
- Incorporating the right analytics in gene therapy manufacturing.
- Where are we at as an industry and where is there room for improvement.

Cell Culture DISH Inc.

The Future of Medicine: Gene Therapy Makes a Compelling Case

There are many reasons to be excited and optimistic about the future of gene therapy. Just the thought that we can use precise gene-modifying technology to repair or correct genetic disorders is remarkable. On the tumultuous journey from the first approved gene therapy study in 1989 to today, there have been successes and stumbles. However, last year represented the most promising year thus far with three new gene therapies approved by the FDA. For the purposes of this article, we will use the term gene therapy to mean the use of a gene-modifying technology to treat or prevent disease. The tremendous potential of gene therapy is the result of decades of research, implementation of select best practices from the field of biologics development, and recent impressive clinical results that collectively have led to establishing a pathway to regulatory approval.

So what makes the case for gene therapy so compelling? I'll start with the potential to revolutionize difficult to treat cancers. The American Society of Clinical Oncology's (ASCO) Clinical Cancer Advances 2018 report recently named adoptive immunotherapy with chimeric antigen receptor T cells (CAR T as the most important clinical cancer advance of the year.

CAR T-cell therapies are both gene therapies and immunotherapies. In brief, CAR-T takes immune cells, called T cells, from a patient then genetically engineers them to express chimeric antigen receptors (CARs) that recognize the patient's cancer cells. Cells are then infused back to the patient (this process is called adoptive cell transfer, or ACT). These engineered cells circulate in the bloodstream, becoming "living drugs" that target and kill the antigen-expressing cancer cells. With many early successes in clinical trials using CAR-T, there is great hope that it can be used to treat a wide variety of blood and solid tumor cancers.

In August 2017 the FDA approved two CAR T-cell therapies in 2017. In August 2017, Novartis' Kymriah® (tisangenlecleucel) was the first adoptive cell immunotherapy and gene therapy for cancer to be approved by the FDA for the treatment of recurrent pediatric acute lymphoblastic leukemia (ALL). ASCO's report stated that, "this [tisangenlecleucel] represents one of the most remarkable advances in the treatment of childhood cancer in the last decade." ALL is one of the most common cancers in children and will recur in about 600 children and young adults each year despite response with initial therapy. According to the report, "remission rates with current standard therapies in prior clinical trials have been only 20% with chemotherapy and 33% with targeted therapy. In a clinical trial of children and young adults with relapsed or refractory ALL, cancer went into remission within 3 months of receiving tisagenlecleucel in 52 (82%) of 63 patients, and 75% of patients remained relapse free at 6 months."

The ASCO report also highlights the effectiveness of CAR–T cell therapy against another hard to treat cancer, diffuse large B-cell lymphoma (DLBCL), which is the most common type of non-Hodgkin's lymphoma.

The report describes a clinical trial where patients with relapsed or refractory DLBCL, refractory primary mediastinal B-cell lymphoma or transformed follicular lymphoma were treated with Kite/Gilead's Yescarta® (axicabtagene ciloleucel). The response rate was 82% with complete remission in 54% of patients. At an 8.7 month follow up, 39% of patients were still in complete remission. In October 2017, Yescarta® was approved to treat adult patients with relapsed or refractory large B-cell lymphoma after two or more lines of systemic therapy, including diffuse large B-cell lymphoma (DLBCL) not otherwise specified, primary mediastinal large B-cell lymphoma, high grade B-cell lymphoma, and DLBCL arising from follicular lymphoma.

The American Society of Clinical Oncology recently named adoptive immunotherapy with chimeric antigen receptor T cells (CAR T)- as the most important clinical cancer advance of the year.

In another clinical trial for patients with relapsed DLBCL (after at least two prior therapies), 59% of patients responded to tisagenlecleucel and 43% went into remission. At 6 months, 79% of these patients had not had a recurrence of lymphoma. In May 2018, Kymriah® was also approved for the same indication.

Cancer is by far the largest group of diseases (65%) being investigated in gene therapy clinical trials. The second largest group (11.1%) is inherited monogenetic diseases. Gene therapy is unique in this area, as it has the potential to correct inherited

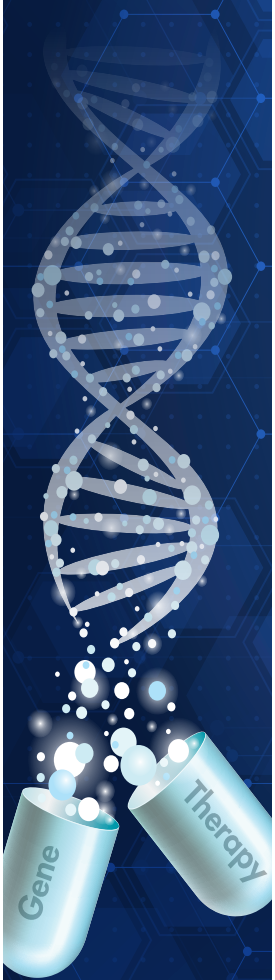


photo courtesy of WuXi

gene disorders by delivering the missing or damaged gene. In late 2017, Spark's Luxturna® was approved to treat an inherited retinal disease due to mutations in both copies of the RPE65 gene. A one-time gene therapy treatment in each eye, restores the visual cycle by delivering the corrected gene, thereby enabling retinal cells to produce the *RPE65* protein. Other major areas being explored include infectious diseases and cardiovascular diseases.

The recent clinical success and the subsequent increased investment from the market have permitted innovator companies to look toward manufacturing and commercialization of their gene therapy products. As gene therapy commercialization is relatively new, there are a host of issues that still need to be addressed. One major challenge is that gene therapies represent a new medical paradigm.

What makes gene therapies so different?

- They offer the possibility, in many instances, of an actual cure instead of chronic treatment.
- Frequently the treatment is a one-time only treatment.
- Gene therapies can be expensive to produce and administer which can present as a challenge in terms of reimbursement.
- In many instances gene therapies are highly customized, patient-specific therapies, which present unique challenges from a logistics and distribution perspective.
- The regulatory pathway across multiple markets, is still evolving.

Inspired by the incredible clinical results and the promise of more gene therapies to come, we at Cell Culture Dish began to consider the challenges facing gene therapy manufacturing and commercialization. We wanted to create a guide identifying key considerations in gene therapy manufacturing for late-stage clinical development and subsequent commercialization. We assembled a panel of experts in their respective fields as they relate to gene therapy manufacturing and commercialization. We were lucky to bring together such a talented group of authors from several different companies, who all saw the importance of coming together to create a piece that is educational and identifies the key challenges, new developments, recent successes, and areas for improvement.

Our goal was to address some key areas that we identified up front as being important to the successful commercialization of gene therapies. This included discussion about gene therapy manufacturing, the latest in manufacturing methods, analytical analysis and key regulatory considerations. The key areas we identified include:

- Planning for scale up, technology transfer, and regulatory considerations on the way to commercialization.
- In-house versus outsourcing manufacturing.
- Ensuring scalability and efficient timelines in manufacturing while still maintaining reasonable cost.
- Incorporating the right analytics in gene therapy manufacturing.
- Identifying where the industry is currently and areas in which advances are taking place.

The industry faces some formidable challenges ahead, but there are enabling technologies being developed, excellent resources and experts available, and importantly, a successful regulatory and manufacturing pathway has been paved for other companies to follow (thanks to the 2017 approvals). In this eBook, with the help of experts sharing their perspectives on various topics related to gene therapy manufacturing, we examine the current state of the industry and how we can continue to advance these life-changing medicines for the benefit of patients.

We sincerely hope this serves as a good resource for those wishing to learn more about gene therapy, its manufacture, and commercialization.

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Editor-in-chief
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Marketing Director
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PhD in Medical Science at Uppsala University followed by 2 years of post doc at the Max Planck Institute for Biochemistry in Munich. After that switching to industrial roles in product marketing first at Roche biochemicals and then to Amersham Biosciences involving definition of user requirements for development and launch of new ÄKTA protein purification systems used in academic research and biopharmaceutical industry. Before joining Vironova as marketing director in 2015 Nina Forsberg also has had several senior marketing roles at GE Healthcare involving the launch of complex solutions and platforms for bioprocessing.

Clive Glover, PhD

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Dr. Glover leads Pall's Biotech's cell and gene therapy business. Previously he was responsible for driving product development efforts around cell therapy at GE Healthcare and has also held positions in marketing and product management at STEMCELL Technologies. Clive holds a PhD in Genetics from the University of British Columbia.

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Dr. Joseph Hughes is the Chief Scientist and Vice President in charge of process and analytical development. He has over 30 years' experience in virology, molecular biology, product development in pharmaceutical and biopharmaceutical industries. His responsibilities include overseeing process development and QC/technical development groups that assess new technologies and develop new platforms as well as develops or tech transfers in new client programs primarily for Cell and Gene Therapies. In addition, Dr. Hughes is the Co-chair for the PDA Committee and serves on other industry committees on viral safety, and regularly presents at Cell and Gene Therapy conferences. He received his Ph.D. in Microbiology-Virology from Northwestern University.

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Pratik Jaluria is the Executive Director of the Process Development & Manufacturing group at Adverum Biotechnologies, Inc. In this role, he leads a department of scientists developing and transferring the manufacturing process for Adverum's AAV-based gene therapies. Prior to this position, he worked for several organizations focused on bringing biologics to market for a variety of serious and life-threatening diseases. Earlier in his career, Pratik worked in vaccine development focusing on novel therapies for infectious diseases. Dr. Jaluria received a Ph.D. in Biomolecular Engineering from Johns Hopkins University and B.S. in Biochemical Engineering from Rutgers University. In addition, Dr. Jaluria was awarded an Intramural Training Fellowship allowing him to conduct research at the National Institutes of Health, where he studied modulating cellular properties including growth, adhesion, and adaptation with the use of bioinformatics tools. Dr. Jaluria is the author of numerous peer-reviewed journal articles and other publications. In addition, he is a co-inventor on several patents relating to targeted gene expression and manufacturing processes.

Debbie King

Scientific Technical Writer

Debbie is a scientific technical writer and regular contributor to The Cell Culture Dish, Inc. specializing in editorial content in the cell culture and gene therapy space. Debbie previously worked at STEMCELL Technologies, Inc. where she initially gained experience in Quality Control for primary and stem cell culture media. Subsequently, her work on process development for the commercialization of human embryonic/induced pluripotent stem cell culture media – mTeSR1 – paved the way to her transition to Manufacturing Sciences Scientist where she dedicated her time to translating R&D methods to larger-scale manufacturing-friendly protocols. She also has experience as a scientific educator, having traveled within the US and abroad to lecture and teach other scientists how to use STEMCELL media products. Her practical laboratory experience has been an asset to her as a technical writer.

John Madsen, PhD

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Dr. Madsen obtained his Ph.D. in Biomedical Science from the University of Texas Health Science Center, MD Anderson Cancer Center. He obtained his post-doctoral training at the Albert Einstein College of Medicine and then at the Walter Reed Army Institute of Research. There he developed liposome-base vaccine technology in collaboration with Medimmune, Inc. He then joined EntreMed, Inc., focusing on the development of anti-angiogenic/anti-cancer small molecules and biologics. Subsequently, at SAIC Frederick, Inc., he was Director of both Outsourcing and Manufacturing for the Vaccine Clinical Materials Program (VCMP) Vaccine Pilot Plant, where plasmid DNA- and adenoviral vector-based vaccines were manufactured under cGMPs for the NIAID/Vaccine Research Center. He then joined Novavax, Inc., a biotech focusing on the development of vaccines against Influenza and Respiratory Syncytial Virus (RSV), based on Virus-Like Particle (VLP) platform vaccine technology. There he was responsible for process development, clinical manufacturing, and manufacturing collaborations. He was a key member of the team that secured a contract award valued at up to $179 million by BARDA for the advanced clinical and manufacturing development of recombinant vaccines for the prevention of seasonal and pandemic influenza. He is an inventor on ten patents.

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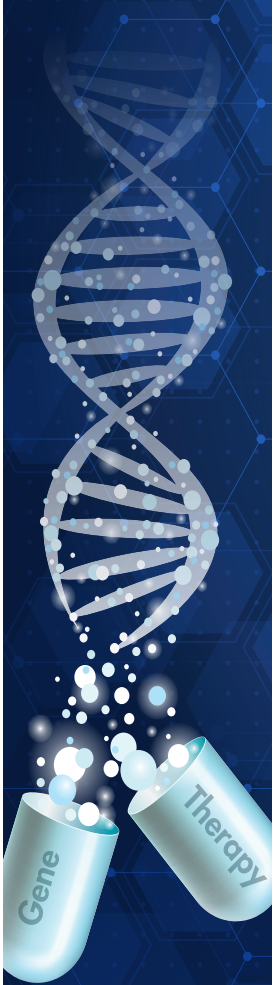
Alan Moore is responsible for commercial activities supporting biopharmaceutical products and advanced therapies such as cell and genetic therapies. Previously he oversaw cGMP-compliant contract manufacturing of innovative cell and gene therapies for WuXi AppTec, Inc., as well as supported strategic biopharmaceutical clients. He held positions of increasing responsibility at BioReliance Corp. (Microbiological Associates) leading to Director of Business Development. He served as President of Genzyme Transgenics Washington Laboratories, and Senior Regulatory Coordinator for Genzyme Corporation supporting products such as Carticel®, Epicel®, Cerezyme®, and later as Vice President of Biopharmaceutical Development Services at Genzyme Corporation. Prior to joining WuXi AppTec, Mr. Moore served as Executive Vice President and Chief Business Officer at Althea Technologies, a leading provider of innovative technologies along with manufacturing and aseptic fill and finish services. Mr. Moore has presented on biopharmaceutical, cell and gene therapy development and safety to the U.S. FDA Center for Biologics Evaluation and Research, various U.S. FDA Advisory Committees, Health Protection Branch, Drugs Directorate (Canada), Therapeutic Goods Administration (Australia), Japan Health Science Council (Ministry of Health), and the U.K. Xenotransplantation Interim Regulatory Authority (UKXIRA).



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Dr. Nilsson has her PhD in Biophysics and Structural Biology from the Karolinska Institute from where she has gained specialized expertise in structural analysis of viruses and nanoparticles, electron microscopy and its industrial applications. Since 2009, she has been responsible for Vironova's EM services offering electron microscopy and image analysis services specialized for biopharmaceuticals. Josefina has served to shape the Vironova services and solutions related to characterization of nanoparticles with particular focus on virus, virus like particles and gene therapy vectors. Vironova offers beside services also innovative hardware and software solutions for advanced electron microscopy image analysis.

Steve Pettit, PhD

Scientific Technical Writer

Steve Pettit is a scientific technical writer for cellcutluredish.com. Steve specializes in editorial content on viral manufacturing and upstream monoclonal antibody bioprocessing. He received his Ph.D in Biochemistry/Virology from the University of Alabama at Birmingham and continued to work for 15 years in virology at the University of North Carolina at Chapel Hill. Dr. Pettit also served for 10 years as the Director of the cell culture program at InVitria.

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Brandy Sargent

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Brandy Sargent is the Editor in-chief and frequent author of The Cell Culture Dish and The Downstream Column, She has worked in the biotechnology industry for over eighteen years, first in corporate communications and public relations, then in technical sales and marketing, and most recently as a writer and publisher. She strives to introduce topics that are interesting, thought provoking, and possible starting points for discussion by the biomanufacturing community. She has been fascinated by the different applications of biotechnology since she first started working in the industry and continues to be fascinated as her experience as her exposure has grown. Brandy shares her enthusiasm by authoring scientific articles and she enjoys watching the industry evolve and thrive.

Chad Schwartz, PhD

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Chad Schwartz, Ph.D. is currently the Global Product Manager for the Analytical Ultracentrifugation (AUC) product line at Beckman Coulter, Inc. He earned his Bachelor of Science in both Agricultural Biotechnology and Biology from the University of Kentucky in 2008. Upon graduation, he was accepted into the University of Cincinnati's Biomedical Engineering graduate program under the laboratory supervision of Professor Peixuan Guo. Following Guo to the University of Kentucky's Pharmaceutical Science program in 2011, Chad's dissertation studies involved the mechanistic and biochemical studies of bacteriophage phi29, often using AUC and other biophysical techniques. Upon graduation in 2013, he accepted a position as a postdoctoral scholar at the University of California, Davis studying prostate cancer under Professor Allen Gao. Following that, in 2014, Chad moved to industry, taking the role of Senior Application Scientist at Beckman Coulter. In January of 2016, he moved into his current position and has helped to bring the Optima AUC to market. Chad has 13 refereed publications and has provided countless seminars at a number of scientific conferences and events.

Tracy L. TreDenick

Head of Quality & Regulatory
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Tracy TreDenick is one of the founding partners of BioTechLogic, formed in 2004. She is the Head of Regulatory and Quality and has over 20 years experience in Pharmaceutical Quality, Manufacturing and Regulatory. Most recently she has supported the development of 7 Breakthrough therapy products including 4 gene and 3 cell therapies, including one that is approved. This responsibility included development of combination product CMC strategies, preparation of CMC sections for IND and BLA submissions, as well as development of strategies related to vendor qualification, risk management and cross-contamination controls. This experience enabled a clear understand of the expectations for the development and commercialization of Breakthrough therapy products. Prior to joining BioTechLogic, Ms. TreDenick directed the validation and pre-approval readiness programs for Biopharmaceutical products within Pfizer (formerly Pharmacia Corporation), including the process validation, registration and commercialization of Somavert®, a protein product. Ms. TreDenick received her B.A. in Biology/Pre-Med from Indiana Wesleyan University. She also completed graduate study courses in Business Management at Keller Graduate School of Management.

Key Considerations for Gene Therapy Commercialization

By Brandy Sargent, Clive Glover, Pratik Jaluria, John Madsen, Alan Moore and Tracy TreDenick

Gene Therapy Industry

Gene therapy is the use of a gene-modifying technology to repair, replace or correct damage in the body. The first approved gene therapy study was conducted by the National Institutes of Health (NIH) in 1989 and provided evidence for the first time that human cells could be genetically modified and returned to the patient without harm. To date, approximately 2,600 clinical trials and six gene therapy products have been approved in various countries.[1]

While the majority of current trials are still in Phase I or Phase II (combined ~ 77%), there are over 100 trials worldwide that are in Phase III or IV. This suggests there could very well be dozens of gene therapy product approvals in the coming years. Last year was a significant year for gene therapy with two pioneering Chimeric Antigen Receptor T Cell (CAR T) cell therapies for cancer approved in the US: Novartis' Kymriah® and Kite/Gilead's Yescarta®. Additionally, in late 2017, Spark's Luxturna® became available to correct a retinal disease.

In light of the impressive remission rates and the recent regulatory approvals, it is no surprise that the gene therapy market has also increased significantly with $7.5 billion raised in 2017 by advanced cell and gene therapy companies.[2] Cell and gene therapies have been called the future of medicine and stand to revolutionize the current healthcare paradigm from patient treatment to drug supply logistics.

How Does Gene Therapy Work?

For gene therapies to work, genetic material must be introduced into cells to treat disease, which is most effectively achieved using a vector delivery system. Viruses make good vectors for delivering genetic material because they have evolved to do just that—deliver genes by infecting cells. Viral vectors for gene therapy are modified to ensure that they don't cause infectious disease in the patient. The most commonly used viral vectors for gene therapy include retrovirus, adenovirus, adeno-associated virus (AAV), and lentivirus. While non-viral approaches for delivering gene therapy are being explored, viral vectors are still the most popular approach with two-thirds of the clinical trials to date delivered via viral vector.[1]

Gene therapies can be delivered by injecting the vector directly into the patient (*in vivo*) or the vector can be delivered to specific cells harvested from a patient's blood or tissue collection (*ex vivo*). The vector is introduced into the patient's cells using a method called transduction. With *ex vivo* approaches, the modified cells are subsequently expanded in cell culture before they are injected back into the patient.

CAR T cell-based gene therapies have been able to provide unprecedented remission rates and have demonstrated success where other therapies have failed.

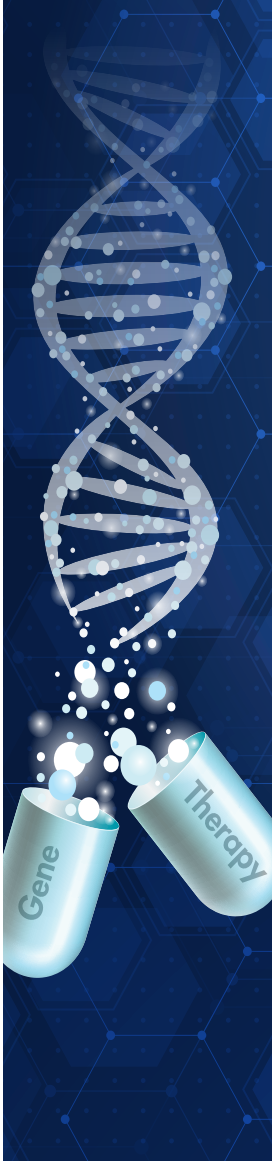
Non-viral approaches, offer some advantages including delivery of larger genes, simplified production, and reduced biosafety concerns. However, they have been shown to be less efficient at delivering the genetic material, and in some instances, the therapeutic benefits have been short term. Recent improvements in non-viral methodologies are increasing interest in this approach.

Indications

Perhaps the most talked about gene therapies have been the cell-based gene therapies for cancer immunotherapy called CAR-T. Novartis' Kymriah® and Kite/Gilead's Yescarta® are examples of these types of cell-based gene therapies. These cell-based gene therapies have been able to provide unprecedented remission rates and have demonstrated success where other therapies have failed. Cancer is by far the largest category of indications being investigated with 65% of the gene therapy clinical trials in this area. The second most popular category of indications is inherited monogenetic disease with 11.1%, followed by infectious diseases (7%) and cardiovascular diseases (6.9%) rounding out the top four indications.[1]

Keys to Successful Gene Therapy Commercialization

With clinical success and increased investment from the market, many gene therapy companies are looking toward manufacturing and commercialization of their lead therapies. As gene therapy commercialization is relatively new—only



six approved products worldwide—there are still several challenges that must be considered when looking at how these products will be manufactured consistently, at an appropriate scale, and delivered to patients. Complicating this task is the fact that until recently there hasn't been much in the way of enabling manufacturing technologies designed solely with gene therapy manufacturing in mind. Additionally, there is no "one size fits all" approach as gene therapy products are complex and can be manufactured in a variety of ways, using a variety of vectors and cell lines. Lastly, in the case of *ex vivo* therapies where genetic modifications are made to harvested patient cells, they can also include a cell culture component. These autologous therapies using the patient's own cells, adds an additional layer of complexity to manufacturing. The logistics of collection and delivery of cells to and from patients can be challenging and often requires a tight turnaround time. And of course, all this must be achieved at the lowest possible cost while still maintaining stringent quality standards to ensure patient safety and comply with regulatory guidelines.


photo courtesy of Fujifilm

Gene therapy companies should also evaluate whether their current Intellectual Property (IP) portfolio offers them sufficient freedom to operate in terms of commercial manufacturing. The IP landscape around the critical components of the process, including unit operations, needs to be understood and evaluated as soon as possible. Developers can then identify barriers and find alternatives early on in the development process. This will save time and money for the developer.

It sounds daunting, but there are excellent resources available and thanks to the 2017 approvals, a recent and successful regulatory and manufacturing pathway has been paved for other companies to follow. In this article, we have pooled many years of experience with experts in various areas of gene therapy manufacturing to create a list of key considerations and best practices.

Product Efficacy and Safety

For therapeutic products to be made available for the general public, products must demonstrate efficacy and safety. Considering the biology of gene therapies, durable efficacy and long-term safety are required by regulatory agencies. Data demonstrating durable efficacy will help to persuade payers and providers that these potentially higher priced therapies are better options for patients compared to traditional therapies. Payers will likely want to see data beyond clinical trials to justify long-term product coverage.

For gene therapies that provide a modest improvement over standard of care, payers will expect the price to be commensurate to the level of improvement. For gene therapies that provide transformative improvement over standard of care, for example a cure, payers will want to look at long-term data with a larger group of patients to justify the higher upfront cost and overall economic benefit of these medicines.

Gene Therapy Manufacturing

Gene therapy manufacturing is a critical part of whether a gene therapy will be successfully commercialized or not. Can the product be manufactured in the quantity and of the quality needed to meet demand? Can it be manufactured at a cost that makes it accessible for patients? These are just some of the issues that need to be addressed.

It is important for stakeholders to include process development and manufacturing programs as early as possible in development to keep pace with the rapid movement of gene-based products through the clinical landscape. There is a key balance to be struck between investing too early in manufacturing technology before the product has been fully characterized and running the risk that the manufacturing process doesn't produce the correct product. On the opposite end, investing too late means attempting to scale up a process that may not meet needs, which could become very expensive and risky.

Gene therapy companies also need to understand what regulatory pathways are available to them based on whether their product fills an unmet need or addresses a serious, life-threatening condition. As such, there are several expedited pathways that may be available for gene therapy developers. If the product is designated under an expedited timeline then this will impact manufacturing timelines and thus needs to be considered during process design, scale-up, qualification, and continuous verification.

Viral vector systems are by far the most widely used methods to delivery therapeutic gene products because of their infectious nature and ability to introduce specific genes into a cell. Most often, the

therapeutic DNA is delivered using viral vector systems based on adeno associated viruses (AAV), lentiviruses (LV), and adenoviruses (Ad). Of these, AAV-based vectors are the most widely used vehicles for delivery in gene therapy indications. According to market experts, AAVs are used in nearly 50% of the 483 currently ongoing gene therapy trials.[3] However, a major hurdle for translation of promising research through clinical evaluation to market approval of these therapeutics is meeting the demand for large quantities of viral vectors that this requires. The vector production gap is one of the main challenges facing the industry today. Key elements to the development of large-scale, optimized production are harvesting and purification strategies and the analytical tools to monitor quality attributes ensuring a safe and efficacious product. For the purposes of this article we will focus on viral vector manufacturing.

Key Factors to be Considered in Designing the Manufacturing Process

Evaluating the Existing Process

Many pre-clinical processes for making viruses are currently based on academic protocols where scale and quality are not of the highest importance. It is important to determine whether the current process is scalable to clinical and commercial manufacturing and whether quality demands can be met. Gene therapy developers can have a wide range of perceptions as to how mature their manufacturing processes actually are. The Good Manufacturing Practices (GMP) requirements for raw materials, cell substrates and process consumables are much more demanding and the potential long lead times for obtaining those critical items can be challenging. Therefore, evaluating existing processes will help design process development for clinical and commercial manufacturing. It will also help to develop accurate time lines as academic processes that need more changes will take longer to move to a clinical/commercially compatible process.

Scalability

Scale is one of the biggest considerations when designing the manufacturing process. This can be a challenge to achieve, particularly if a product's approval timeline is accelerated. Determining scale means identifying how much material is needed for clinical trials and ultimately commercial manufacturing. Projected manufacturing scale is calculated by taking the number of patients to be treated per year x dose / desired number of batches per year.

So for example, if you plan to treat 200 patients per year at a dose of 10^{14} viral genomes (vg) per patient, then one needs to understand how much a process and a facility can make in a single batch. For example, if a facility has a process that generates 2 x 10^{15} vg per batch, then the program will need upwards of 10 GMP batches produced, tested and released per year. Based on assumptions about upstream and downstream yields, the scale of the process can be determined. This calculation gets refined through the course of process development and different clinical phases as real numbers get substituted for the assumptions that are initially made. For instance, a 100% success rate is highly unlikely and so a more appropriate level of 70-85% should be used to adequately plan for meeting supply needs.

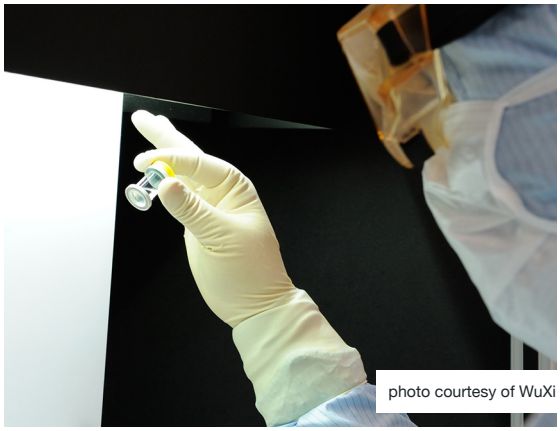
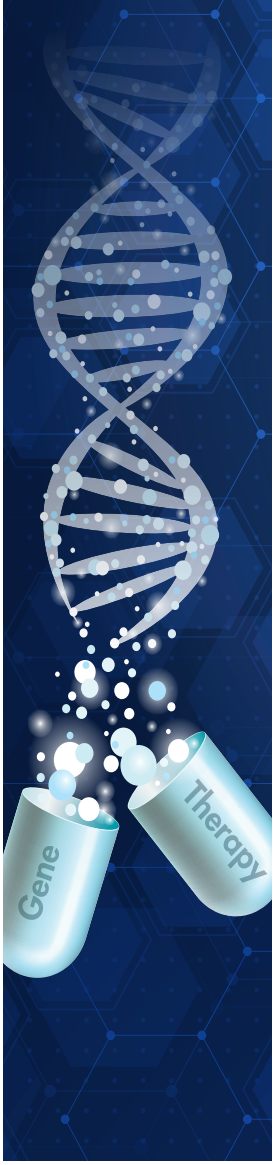


photo courtesy of WuXi

Because product demand and the number of patients treated will usually increase as a product progresses through clinical trial phases and on to commercialization, it is important that the designed process is scalable and that critical quality attributes are well characterized. Therefore, investing in process development and optimization must be appropriately timed, particularly if the product has been granted an accelerated approval process by regulators.

It is best to use consistent, scalable technologies throughout the development process when possible. For example, using a bioreactor system that is available in a range of sizes from the beginning allows for more predictable movement between each phase compared to a sudden switch in technology where significant process development may be necessary. Another example is the downstream purification of viral vectors. Many small-scale processes use cesium chloride ultracentrifugation, however, chromatographic methods are preferred for their scalability and better GMP control strategies. Attempting to switch to more scalable chromatographic methods half way through therapy development could significantly delay timelines and could potentially lead to a product that differs in purity from the original methodologies. Scalable processes also make it possible for cell therapy manufacturing to be produced at multiple GMP facilities rather than being restricted to a single site on a single piece of equipment.



It is also possible that a gene therapy will not need to be scaled-up, for instance in rare monogenic diseases with very low patient populations. In these cases it may be possible to meet all product demands without the need to scale-up. This is why understanding the scale of a disease indication is so critical in manufacturing process design.

In-house or Outsourced Manufacturing

The patient population being treated is a key consideration in deciding between in-house manufacturing versus outsourced manufacturing to a Contract Development and Manufacturing Organization (CDMO). In conventional medicine, the ratio of the incidence to prevalence of patients for a given disease is expected to be high so that capital expenditures, such as those required to build a manufacturing facility, can be amortized over a number of years. In contrast, for genetic diseases, the ratio of incidence to prevalence population can be quite low. This means that the prevalence population gets treated in the first few years after launch and then a manufacturing facility is only required to treat the incidence population which can be very small for rare and ultra-rare diseases. This means the capital associated with building a manufacturing facility for a single gene therapy has to amortized over just a couple of years after product launch. This can make the business case for building your own facility quite high.


photo courtesy of Fujifilm

Beyond the expense and dedication of capital to build manufacturing capacity, time and resources required of gene therapy companies and their management must be considered. Companies must be able to establish the design and scale requirements for the facility, often with limited certainty as to the scale ultimately required, the potential launch quantities, and market penetration to guide planning. Outsourcing can serve as an effective and economical bridge until greater certainty is gained as to product demand.

However, for gene therapy companies who either have difficulty finding a CDMO partner or securing production slots in the timeframe they need, building a small, early phase GMP facility may make sense. There are also unique partnership agreements that can be established in lieu of full outsourcing, for example, a monoplant or condo arrangement.

Ultimately gene therapy companies will have to weigh several factors before deciding whether to outsource manufacturing or manufacture in-house and the appropriate time to implement each option.

Working with CDMOs and Suppliers to Ensure Technology Transfer Best Practices

A well-documented process, detailed bill of materials, and qualified analytical methods contribute to efficient technology transfer, as does the availability of subject matter experts with process knowledge at the targeted scale of manufacture.

When seeking to outsource production, contract negotiation and production slot reservation are first steps. Once technology transfer begins, developers of gene therapies need to consider that timeline from initiation of an order to the batch release of GMP vectors is the aggregate sum of time required for acquisition of plasmid DNA for transfection, of qualified raw materials and components, availability of manufacturing capacity and quality testing and final release of the vector. Allowing adequate time for each of these components requires effective planning, as does care in selection of available, GMP grade raw materials. Product specific analytical assays must also be ready for transfer as the lack of these tools can significantly hamper the progress toward GMP manufacturing.

Early interaction with a CDMO can be valuable and can establish realistic timelines for development and GMP manufacturing. The timeline from contract negotiation to released product can take up to 12 months. Often gaps that present hurdles to manufacturing can be identified and addressed in these early interactions. Moreover, adaption of the CDMO's manufacturing platform can be leveraged to reduce the overall time and expenditures in creating their own process. For example, platforms established at CDMOs for upstream and downstream processing have already addressed availability of materials and components of appropriate quality for use in GMP manufacturing, so these common gaps are eliminated. Early interactions are also important in creating the custom and semi-custom assays used for in-process and release testing.

It is also important to engage with equipment providers early during the process. The experience of equipment providers for similar processes can be leveraged to facilitate design development.This can be done either onsite or as a contract service provided by the supplier if there are particular specialty pieces of equipment that you want to adopt into your process. In other words, aligning the equipment used in process development and analytical development with the equipment to be used in GMP manufacturing is an often overlooked, but critical aspect of having a successful technology transfer.

Logistics and Distribution

This is particularly important for cell-based gene therapies, which are personalized, autologous treatments. In these therapies, the coordination of collecting material from patients and shipping this material to and from the manufacturing area in a timely manner is critical. For instance Novartis has stated that their target manufacturing turnaround time from receipt of patient material to return of product is only 22 days. Gilead has stated that the median turnaround time for Yescarta® is 17 days. This kind of expedited turnaround on personalized therapies requires extensive data and temperature tracking systems to ensure a patient receives their own cells and also to ensure that the product is kept at the correct temperature.

Regulatory Approval

The challenge and benefit of the Breakthrough Therapy designation, often granted to gene therapy products, is the difference in the program development timeline compared to traditional biologics. Authors from the Friends of Cancer Research organization have published an article stating that median approval times for drugs with a breakthrough therapy designation is 2.9 months faster than traditional products. It is estimated that non-breakthrough therapy products are approved within 7.4 years from submission of the IND compared to 5.2 years for breakthrough therapy products.[4]

A successful gene therapy regulatory strategy requires: new product and process validation strategies to accommodate accelerated development timelines, active communication with the FDA and the dedication to pursuing and adopting new technologies to tackle vexing scientific challenges, particularly in the area of product characterization.

Typical gene therapy information requests that arise during reviews include providing more information on manufacturing methods and reagents used for master cell banks, working cell banks and the vector, information on plasmid manufacturing and release testing, freezing rates of the drug product, labeling methods for the frozen product, and method of assigning the product's date of manufacture (see 21 CFR 610.50). As would be anticipated, there are many questions regarding analytical methods (e.g. potency), specifications and method validation. Specifications are typically based on a limited number of early phase lots, which leads to questions regarding comparability with the proposed commercial process. Similar to PEGylated biologics and the amount of free-PEG (Polyethylene glycol) in the product, regulators are also concerned with empty capsids controls.

As is often the case with emerging therapies, there are more questions than answers. However, gene therapies are unquestionably moving toward fulfilling their tremendous promise. Industry and regulators alike are working in an increasingly focused manner to determine how the needed regulatory constructs can be practically applied with product characterization technologies and better understanding of mechanics of action at the heart of the matter.

Reimbursement and Market Access

There are several factors to establishing a reimbursement strategy for gene therapies. One of the first is to keep the cost as reasonable as possible, recognizing that payers have limits, even if the therapies are one-time administration, life saving medications. Payers will be evaluating not only the efficacy and safety of a product but also its cost-effectiveness. Gene therapy companies need to engage early in the process with payers to ensure that their product will be covered.

Another important aspect is working with payers in advance to determine what documentation and data will be required to obtain reimbursement and broad coverage for patients. If the gene therapy has received an accelerated approval designation, this will reduce the time available to work with payers, so this will need to be done as quickly as possible.

In addition, coding needs to be considered. In order to receive payment in the United States, the gene therapy needs to have a code for reimbursement, which takes a significant amount of time to apply for and be granted. In fact, some novel products end up going to commercial launch without a billing code, which can delay patient access and payment. In cell-based gene therapies there are several components to treatment that all need coding, for instance cell collection, cell processing, and infusion back into the patient.

Because gene therapies are novel, payers will need education around the benefits of this type of therapy both clinically and from a long-term cost benefit perspective. Education is also critical in the area of cell-based gene therapy coding so that payers understand how the different components fit together and how they should be coded.

One gene therapy product that struggled with reimbursement and ultimately failed was Glybera®. Glybera® was granted EMA market authorization in 2012 to UniQure as an AAV gene therapy for lipoprotein lipase deficiency disease for which the patient population is very small. Treatment was a one-time series of injections of the viral vector carrying the intact copy of the lipoprotein lipase gene and the cost was around $1 million based on patient weight. Since approval in 2012, it has only been used and reimbursed once. Because Glybera® was never able to get approval from the national regulators in Europe, who decide which drugs get

reimbursed, this left insurers to make decisions about whether treatment would be covered on a case-by-case basis and the burden of getting approval on the physician and patient.[3,5] Due to the lack of use, UniQure will not submit renewal for marketing authorization of Glybera® from the European authorities when it expires in October.

In contrast, Spark Therapeutics has recognized that reimbursement is a major component of a gene therapies' success. For their new therapy, Luxturna®, which corrects a rare inherited retinal disease, they have developed a novel threefold plan for ensuring coverage for their patients. The current cost of Luxturna®, is $850,000 per patient. In order to ensure reimbursement from payers, they are working to create contracts with individual payers or their specialty pharmacies who will then negotiate payment to the treatment centers to reduce the risk of non payment to the centers who decide to administer the therapy. They have also announced a rebate program based on treatment effectiveness at 30 to 90 days and then again at 30 months. If efficacy isn't proven over those timeframes rebates will be provided to payers. Lastly, they are in discussions with the Centers for Medicare and Medicade Services to create a plan that would allow payers to reimburse the cost of the therapy in installments over several years.[6]

These innovative approaches for reimbursement of the more expensive gene therapies are a great step in the right direction of ensuring access to treatment for patients and the long term success of the therapy.

Provider and Patient Education and Access

It is important that providers and patients have access to the product. This access can be in the form of logistics, for example with cell-based gene therapies, making sure patients can get to a site to collect their cells or tissue and provide infusion of the treatment. It is important that providers understand proper collection and infusion methods. Cost is also a part of access, so securing reimbursement coverage is important. In addition, patients are increasingly becoming advocates of their own treatment and connection with thought leaders and patient advocacy groups can be invaluable in helping to educate the community on the product, as well as working with payers to ensure coverage. Having a commercial group involved early in a company's evolution can be very beneficial. These professionals can make commercial projections, identify and interact with patient advocacy groups, and establish the framework for a suitable Target Product Profile. Initial and ongoing stakeholder education is a critical part of any commercialization plan.

In Closing

The past decade has seen a surge in the development of cell and gene therapies and recent successes have begun a paradigm shift in how we treat disease. The approval of these products will drive other similar products through the pipeline at an even faster pace because now there is a regulatory and manufacturing blueprint that industry stakeholders will be able to follow. However, it is important to remember that cell and gene therapies are still relatively new and there are still challenges regarding manufacturing and commercialization that need to be addressed.

The industry has a great appreciation for the challenges associated with manufacturing scale up and are actively looking to develop processes that are suitable for large-scale production. Previously, most manufacturing tools for cell and gene therapy were borrowed from either academic or from monoclonal antibody manufacturing and were often not fit for purpose. While this is still true of many tools, there are increasing numbers of tools that are designed specifically for viral vector manufacture and tools companies are now starting to invest in this. This is all good news in terms of creating manufacturing that is more efficient and cost-effective. These manufacturing improvements will enable more therapies that previously weren't able to be manufactured efficiently or at a reasonable cost and will increase access and reimbursement of gene therapies as a whole.

References: Referenceses

1 Ginn, S.L., et al., *Gene therapy clinical trials worldwide to 2017: An update.* J Gene Med, 2018. **20**(5): p. 3015.

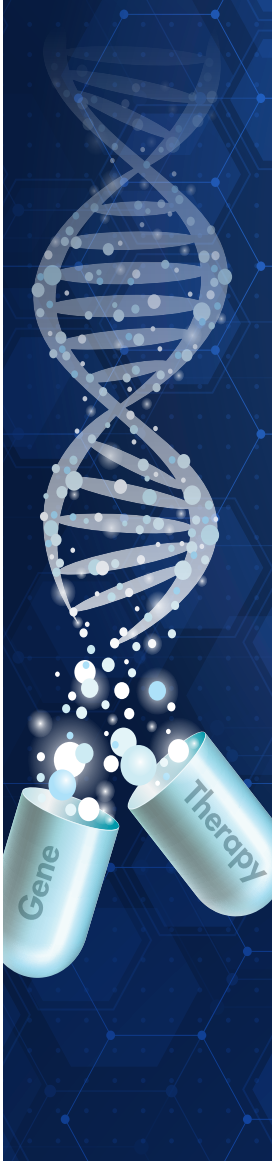
2 Source: Alliance for Regenerative Medicine. State of the industry, 2018.

3 Roots Analysis Report: Gene therapy market 2015-2025, 2015.

4 raps.org. *Analysis: breakthrough therapies cut development timeline by two years.* 2016; Available from: www.raps.org/regulatory-focus%E2%84%A2/news-articles/2016/3/analysis-breakthrough-therapies-cut-development-timeline-by-two-years.

5 Regalado, A. T*he world's most expensive medicine is a bust.* 2016; Available from: www.technologyreview.com/s/601165/the-worlds-most-expensive-medicine-is-a-bust/.

6 *Spark therapeutics announcesfirst-of-their-kind programs to improve patient access to LUXTURNA™ (voretigene neparvovec-rzyl), a one-time gene therapy treatment*; Available from: ir.sparktx.com/news-releases/news-release-details/spark-therapeutics-announces-first-their-kind-programs-improve.



The State of Gene Therapy Regulation

Overview of the Regulatory Path to Commercializing a Gene Therapy

By Tracy TreDenick

After decades of research, gene therapies now comprise much of the R&D and manufacturing pipelines in the United States and around the world. Huge strides in biological understanding and the development of key enabling technologies are at long last delivering on the promises of precision and personalized medicines. While there is still much more to learn and develop, regulatory constructs keeping pace with the speed of innovation remains a sizeable challenge.

The guiding principle of successful regulation is to ensure patient safety. However, as novel therapeutics have emerged, meaningful challenges have manifested as regulators seek to define key attributes leading to efficacy and patient safety. Adding to the challenge is the accelerated speed in which many of these products are being brought to market as nearly all gene therapies under development have been assigned one of the FDA's expedited approval designations.

Overview of FDA's Expedited-Approval Designations

The FDA's and U.S. Congress' objective of speeding the path to market for therapeutics to address unmet medical needs started with the Accelerated Approval Program introduced by the FDA in 1992. Additional legislation including the FDA Modernization Act of 1997, the FDA Safety and Innovation Act of 2012 and the 21st Century Cures Act of 2016 provided further expedited approval pathways. Currently, there are five expedited approval designations:

Fast Track designation – This designation is for therapies that treat serious conditions where there is an unmet medical need and they receive the benefit of more frequent meetings and communication with the FDA, a rolling review of the Biologic License Application (BLA) or New Drug Application (NDA), and qualify for Accelerated Approval.

Breakthrough Therapy – This designation is assigned to drugs that treat a serious condition when preliminary clinical data show significantly improved outcomes compared to treatments currently on the market. Breakthrough therapies are eligible for: Fast Track designation benefits, and extensive FDA guidance early in the development process, as well as organizational commitment, including access to FDA senior managers.

Accelerated Approval – This can be given to drugs that meet a serious unmet medical need, and approval is based on a surrogate endpoint. However, in 2012 Congress passed the FDA Safety Innovations Act (FDASIA), which allowed approval to be based on either a surrogate endpoint or an intermediate clinical endpoint.

Priority Review – For this designation, the drug must treat a serious condition and offer a significant improvement in safety or effectiveness over drugs currently on the market. Designation is assigned only at the time of the original NDA or efficacy filing and means that the FDA's goal is to act on an application within six months.

Regenerative Medicine Advanced Therapy Designation (RMAT) – This designation requires that the drug is a regenerative medicine therapy, which is defined as a cell therapy, therapeutic tissue engineering product, human cell and tissue product, or any combination product using such therapies or products. The therapeutic must be intended to treat, modify, reverse, or cure a serious or life-threatening disease or condition, and preliminary clinical evidence indicates that the drug has the potential to address unmet medical needs. In November 2017, the FDA clarified that gene therapies are eligible for RMAT designation. While not guaranteed as part of the designation, products may be eligible for Priority Review and Accelerated Approval.

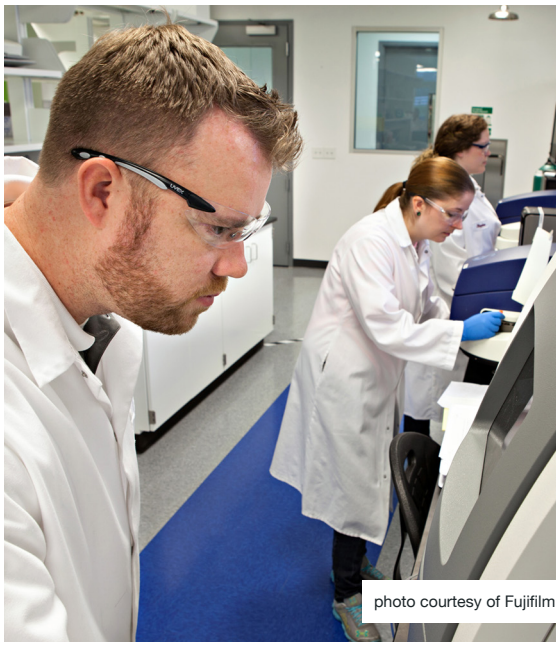
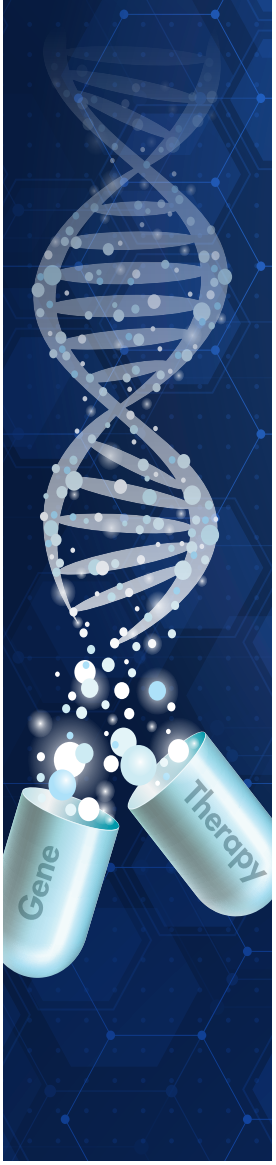


photo courtesy of Fujifilm



Expedited Designation versus Traditional Development Program Timelines

To illustrate the benefit, yet the challenge of an expedited approval classification, the chart below compares the CMC program timeline for a drug with Breakthrough Therapy designation with the traditional timeline. As you will see, the Breakthrough Therapy designation accelerates a CMC program by almost two years.

However, despite sharply reduced timeframes, manufacturers cannot compromise patient safety or product supply. Therefore, characterization of critical product and process attributes is typically required much earlier in the process than under the traditional timeline.

Common Gene Therapy Regulatory Challenges

While gene therapies present challenges in every aspect of development, arguably the most challenging is the product testing and characterization that leads to safe and robust manufacturing processes. As is the case with any GMP-manufactured product, a complete and detailed description of all steps in manufacturing needs to be designed, documented, and understood. For a viral vector, this description would include cell growth, transfection, viral harvest, and additional steps such as purification, formulation, filing, and storage. For the final formulation, all components must be described in detail.

Safety tests are common to all biologics, with the basic requirements for this testing including sterility, mycoplasma, and endotoxin. In addition, given that gene therapy vectors are manufactured using materials of biological origin, developers must perform adventitious agents safety testing.

In final product testing for complex biologics, such as gene therapies, the active ingredient is often not well defined, and significant variability both in the cell source and the manufacturing process may exist. The goal should be to produce a safe and consistent product that is characterized to an appropriate extent—but the phrase "to an appropriate extent" is open to considerable interpretation.

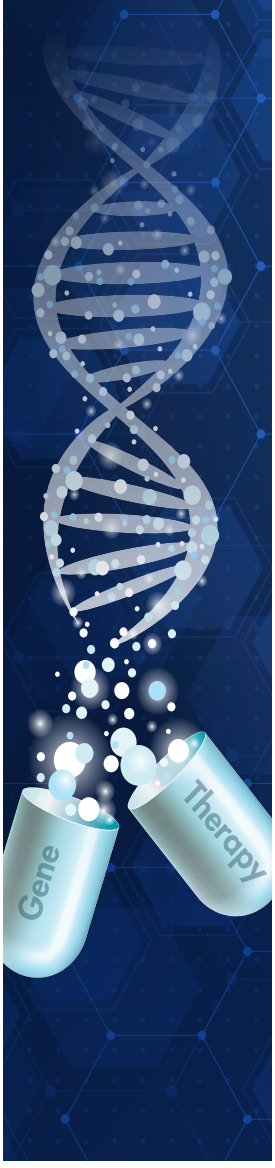
To what degree must critical quality attributes (CQAs) and mechanisms of action be understood in order to project likely clinical outcomes? What levels of understanding do developers and regulators need in order to gain confidence in the product? These are not easy questions to answer. Although gene therapy development has advanced markedly in recent years, our understanding of structural-function is still evolving. In short, there are still gaps in our knowledge as to why a gene therapy works or does not work or more specifically why one batch has a higher titer than the next. Despite the observation of promising clinical outcomes, the mechanism of action is not always well understood, such that the processes can be replicated or improved for additional or improved versions of a product.

In order to achieve a sufficient level of understanding, developers must perform assays unique to their own gene therapy platform that are specific to genomic and infectious titer, purity (e.g. total impurities and % empty capsid), vector genome identity, *in-vivo* potency, process related impurities (e.g. endotoxin and residual agents), and appearance/infectious. For many of these assays, regulators require the use of accompanying reference standards according to the two-tiered strategy outlined in ICH (Q6A), which may be challenging to qualify and establish. Additionally, product characterization must continue to become more robust as product development advances.

Table 1. Expedited Designation versus Traditional Development Program Timelines

	Breakthrough Therapy Program	Traditional BLA /NDA Program[2]
Phase 1 Clinical Study	18 months	Several months[2]
Decision on BT Application		N/A
EOP 1 Meeting Confirms Breakthrough Therapy Status	6 months	N/A
Phase 2/3 Clinical Study	24 months	Phase 2: Several Mo. to 2 years[2] Phase 3: 1 to 4 years[2]
EOP 3/Pre-NDA/Pre-BLA Submission Meeting		
NDA/BLA Preparation	6 months	
NDA/BLA Review	6 months	10 months
Development Timeline	~ 5.2 years[1]	~7.4 years[1]

[1] https://www.raps.org/news-articles/news-articles/2016/3/analysis-breakthrough-therapies-cut-development-timeline-by-two-years
[2] https://www.fda.gov/ForPatients/Approvals/Drugs/ucm405622.htm



Successful Gene Therapy Regulatory Strategies

A successful gene therapy regulatory strategy requires: new product and process validation strategies to accommodate accelerated development timelines, active communication with the FDA and the dedication to pursuing and adopting new technologies to tackle vexing scientific challenges, particularly in the area of product characterization.

Developers must proactively set the stage for successful later-phase development and this often means working well beyond IND requirements. Determination of stability-indicating assays is often one of the most vexing challenges and one way to address this challenge is by performing forced- and accelerated-stress studies early in the development process.

Additional elements of gene therapy regulatory success include:

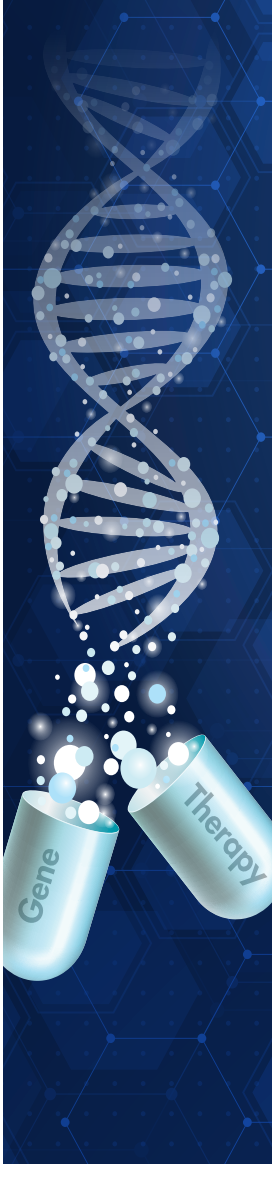
- Transparent communication with the FDA throughout the entire approval and post-market process
- Additional data submissions via amendments during the review cycle, and in some cases, post-market
- Novel statistical models and approaches
- Freely acknowledge where data is limited, demonstrate that the missing data pose no risk to patient safety or product supply and outline post-market strategy for acquiring the missing data
- Focus on patient safety and reliable supply of quality product at launch, not process optimization
- Enhance analytical methods and understanding to offset more limited process understanding and to support future comparability work
- A post-approval product lifecycle management plan is necessary, and it needs to be included in the filing to support deferred CMC activities

Conclusion

As is often the case with emerging therapies, there are more questions than answers. However, gene therapies are unquestionably moving toward fulfilling their tremendous promise. Industry and regulators alike are working in an increasingly focused manner to determine how the needed regulatory constructs can be practically applied with product characterization technologies and better understanding of mechanics of action at the heart of the matter.

References:

1. Bauer, S. *Development of strategies to improve cell therapy product characterization.* Available from: www.fda.gov/BiologicsBloodVaccines/ScienceResearch/BiologicsResearchAreas/ucm127182.htm.
2. Gadlock, P. HCT/P regulation - 351 vs 361 *products,* Arent Fox, LLP (Pharmaconference presentation). Feb 15, 2017; Available from: www.pharmaconference.com/Attendee_Files-PDF/HCTP2_2017/13 Gadiock - HCTP 351 vs. 361 Products.pdf.
3. *FDA announces comprehensive regenerative medicine policy framework.* Nov 16, 2017; Available from: www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm585345.htm.
4. Byrnes, A. *The chemistry, manufacturing and controls CMC section of a gene therapy IND* (presentation). Apr 6, 2016; Available from: www.fda.gov/BiologicsBloodVaccines/NewsEvents/ucm241305.htm.
5. Preti, R. *Process improvement and accelerated CMC development workshop, Cell and Gene Meeting on the Mesa* (video recording). Oct 2016; Available from: www.youtube.com/watch?v=t05tD1UizHk.
6. Naso, M.F., et al., *Adeno-Associated Virus (AAV) as a Vector for Gene Therapy.* BioDrugs, 2017. **31**(4): p. 317-334.
7. Ayuso, E., et al., Manufacturing and characterization of a recombinant adeno-associated virus type 8 reference standard material. Hum Gene Ther, 2014. **25**(11): p. 977-87.



Upstream Manufacturing of Gene Therapy Viral Vectors

By Steve Pettit, Clive Glover, Joseph Hughes, John Madsen, and Pratik Jaluria

Overview

The majority of gene therapy applications in development utilize viral vectors to carry the therapeutic gene into the target cells. Cells may be genetically modified either *in vivo* or *ex vivo*. In the *ex vivo* applications cells are modified in culture, which also allows for cell expansion and analytical characterization prior to re-infusion of the treated cells. Typically, cells are examined pretreatment and post treatment for viability, density, expression level, etc. The *ex vivo* process can sometimes result in more efficient transduction.

There are several possible viral vectors systems available and the decision of which to use depends on many factors that include tissue tropism, desire for integrating or non-integrating modification, *in vivo* or *ex vivo* process, prior immune exposure, whether the target cell is replicating or non-replicating, and safety. High efficiency transduction and robust levels of transgene expression are desired outcomes in applications with viral vectors.

One of the greatest safety concerns with viral vectors is generation of wild type infectious virus from vector components. For this reason, viral vectors are typically manufactured from 2, 3 or even several separate expressible units to significantly reduce the possibility of forming a wild type particle via recombination. The expressible units are typically separate plasmids introduced to producer cells either by transfection or through "helper" transducing viruses. The expression units are usually further engineered with mutations to disable the function of the wild type virus should they be formed by a recombination event. For example, newer HIV lentiviral vectors delete genes for virulence factors *tat*, *vpr*, *vpu*, *nef* and/or have *gag* and *pol* on separate plasmids from *rev* and *env*[1] and/or contain other mutations such as deletions in the 3' LTR.[2]

The overall goal in viral vectors design is to efficiently package the therapeutic gene or nucleotides into infectious viral particles and avoid the generation of wild type particles or empty particles. Thus, many vector systems are manufactured by transient co-transfection of multiple plasmids for either safety, for convenience, or by necessity in order to avoid toxicity of a vector component in producer cells (Figure 1).

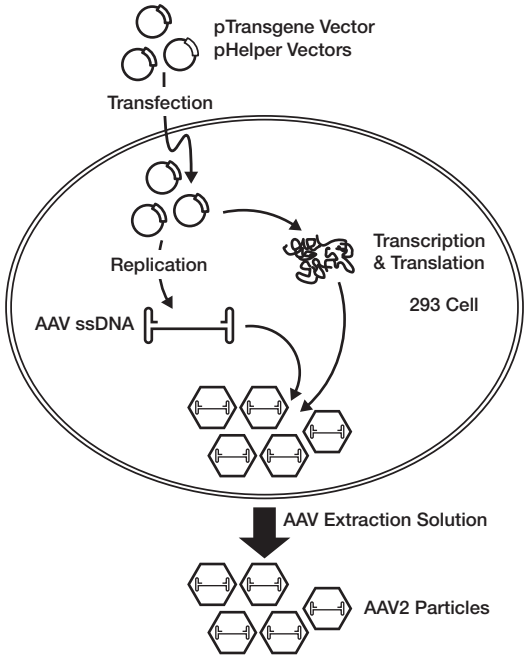
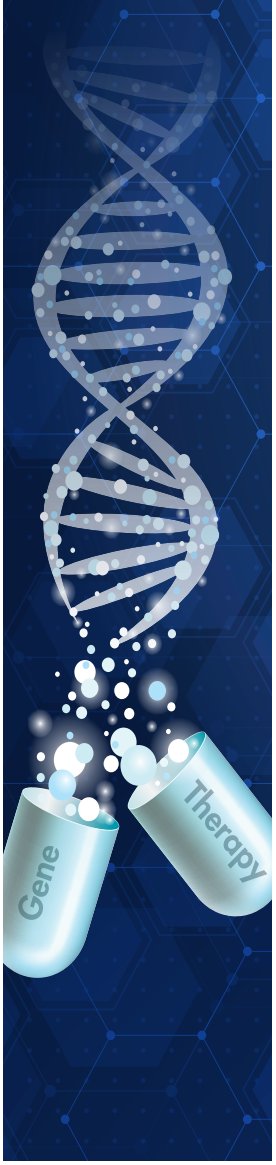


Figure 1. Schematic of the production of AAV vector via transfection.
AAV vector components and therapeutic gene are transfected, usually as separate expression cassettes from different plasmids. Expression cassettes are expressed within the cell resulting in viral proteins and a genomic ssDNA containing the expression cassette for the therapeutic gene(s). In the AAV system, viral particles containing the transgene assemble in the cytoplasm. Particles are released to the media via cellular lysis prior to further purification and characterization.

Major Viral Vectors in Use

Historically, there has been more focus on retroviral and adenoviral vectors, which have now generated a 30-year history.[3] However, because of enhanced safety and improved target tissue expression profiles, vectors derived from adeno-associated virus (AAV) and lentiviral vectors have advanced in development. Herpes simplex virus and pox/vaccinia vectors also show promise for their effectiveness as oncolytic vaccines.

AAV is the most commonly used vector for *in vivo* genetic modification, and different serotypes (naturally occurring or recombinant) can be used to target different tissues within the body.[4] AAV is not known to be pathogenic and is thought to be well tolerated, resulting in lower inflammatory response.[5,6] Moreover, unlike other viral vectors such as lentivirus that integrate into the host cell genome, AAV is thought to primarily remain episomal.[3,7]



One downside to AAV is its low transgene packaging capacity relative to other viruses[5] but this is not currently a major problem with the diseases being targeted or investigators have designed mini-genes (that fit within AAV) for the disease targets. AAV also has a propensity to package any and all DNA in the vicinity including host cell DNA and plasmid backbone DNA.[8, 9]

For *ex vivo* genetic modification, lentiviral vectors are now the most commonly used. Lentiviral vectors have advantage over classical gamma retroviral vectors because they can transduce both dividing and non-dividing cells. Lentiviral transduction of slowly dividing $CD34^+$ HSCs has been applied to several genetic diseases, including β-thalassemia[10], X-linked adrenoleukodystrophy[11], and metachromatic leukodystrophy.[12] There are also fewer challenges associated with insertional oncogenesis compared to gamma retroviral vectors (MuLV) like those that hindered early clinical progress with gene therapies retroviral vectors.[1,13]

The table below summarizes some physical properties of the various viruses that are used for gene therapy:

Overview of Viral Vector Manufacturing

The manufacture of viral vectors may require several manufacturing phases or platforms. Initially, the materials needed to manufacture the therapeutic viral vector must be generated. These include plasmids encoding helper-virus functions and the therapeutic gene, cell lines used to manufacture the vector, and other materials (See Figure 2, plasmid manufacturing). In some cases, helper transducing viruses may be substituted for plasmids. Alternatively, stable producer cell lines may be created in order to reduce or eliminate the transfection and/or transduction steps and simplify the production process.

The next step in vector manufacturing involves the generation of infectious viral vector (Figure 2, viral manufacturing). Cells are transfected with plasmids to generate the viral vector, which is harvested. AAV viral particles accumulate in the cytoplasm and the media, so total yield can be enhanced by lysing the cells. Harvested vector is then concentrated, purified, titrated, characterized, and stored for later *ex vivo* or *in vivo* used.

In *ex vivo* transduction (Figure 2, cell processing), target cells are collected and modified by the viral vector. Following modification, the cells are harvested, characterized, and formulated prior to transplantation. In some processes, transduced cells may be expanded in cell culture prior to the re-infusion into the patients.

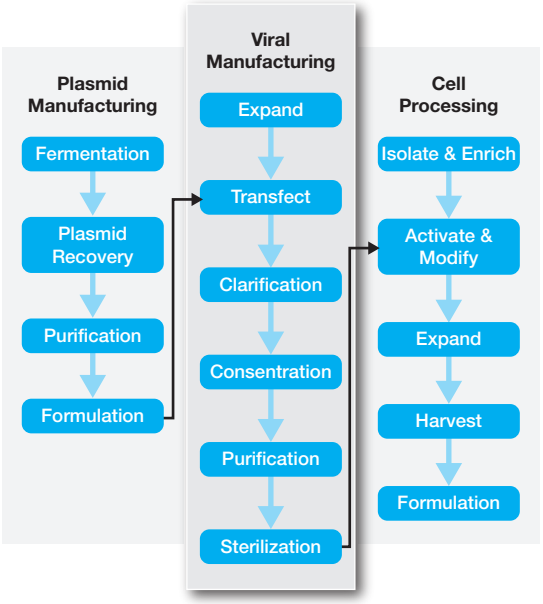
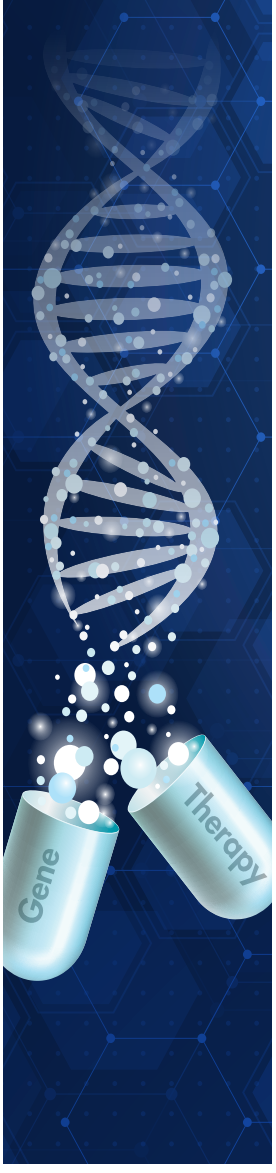


Figure 2. Example of 3 manufacturing platforms for the generation of modified cells for *ex vivo* gene therapy via viral vectors produced by transfection.

Table 1. Overview of common viruses used for generating gene therapy viral vectors.

Parameter	Retrovirus	Lentivirus	AAV	Adenovirus
Coat	Enveloped	Enveloped	Non-enveloped	Non-enveloped
Packaging capacity (Kb)	8	8	~4.5	7.5
Tropism/infection	Dividing cells	Broad	Broad excluding hematopoietic stem cells	Broad
Inflammatory potential	Reduced	Reduced	Reduced	High
Host genome interaction	Integrating	Integrating	Integrating/ non-integrating	Non-integrating
Transgene expression	Long lasting	Long lasting	Potentially long-lasting	Transient or long-lasting depending on immunogenicity



Generation of Viral Vectors by Transfection, Infection, or by Stable Producer Cells

In general, there are two modes of vector production: transient production systems or producer cell lines, which may be mammalian or insect cells in the case of baculovirus vectors. Transient production systems involve either transfection of one or several plasmids encoding the helper virus functions or alternatively transient systems may use other viruses to provide helper function.[14] At the present time, many vectors are currently generated by transient transfection either due to convenience, expense, or because of the lack of a stable producer cell line.

Manufacturing viral vectors by transfection offers advantages and disadvantages. It is flexible and efficient, does not require time-consuming development of stable cell lines, and allows for successful vector generation should any viral component produce cellular toxicity upon expression.[15] However, transfection-based methods have disadvantages due to the requirement for costly GMP grade plasmids for each transfection lot and the possible requirement of additional purification steps to remove plasmid and cellular DNA. Moreover, transfection of suspension cells at large scale can prove difficult and can result in relatively low yields.

Among the transfection methods, polyethylenimine (PEI) transfection has advantages over calcium phosphate since it is less toxic and may eliminate the need for a media exchange. PEI transfection is also less dependent on pH, the presence of serum, and is effective for both adherent and suspension cultures.[16] Drawbacks are the amount of costly plasmid required for large suspension culture and the absence of an analytical method to quantify PEI in the purified vector preparation.[17] Lipid-based transfection reagents are also effective; however, expense may make them impractical for use at larger scales.[18] Non-chemical methods such as electroporation show promise, however, the need to concentrate cells can also make its use impractical at large scales.[17] Transfection-based methods, while fast and efficient, can pose significant challenges, particularly at larger scale manufacture.

Use of Baculoviral Vectors for Therapy or to Produce Other Viral Vectors

Low toxicity and inability of baculoviruses to replicate in mammalian cells make them potential candidates for therapeutic gene delivery.[19] Baculovirus-mediated gene delivery into dividing and non-dividing mammalian cells has demonstrated therapeutic efficacy in both *ex vivo* and *in vivo* gene therapy studies.[20, 21, 22]

A suitable expression cassette and/or a pseudotyped envelope such as VSV-G can be used to transduce various cell types.[23, 24] Baculoviral vectors can mediate high-level transient expression of transgenes in many stem cells[25] and can be modified to permit stable transgene expression.[26] The high-level of transgene expression from baculoviral vectors is well suited for cancer gene therapy[22, 27] and the lack of pre-existing antibodies to baculovirus in humans is an advantage for *in vivo* therapy.

Baculoviral vectors have advantages and disadvantages. Baculoviral vectors offer an attractive alternative to transfection for vector generation and have been used in the production of other vectors such as AAV from insect cells[28] and lentiviral vectors from suspension 293T cells.[29] Baculovirus and other infection-based systems for the generation of vector are typically easier to work with at large scale than transfection but require additional manufacturing steps in order to produce the baculoviral vector. There are some drawbacks to the use of baculoviral systems. Some cell culture media formulations can result in reduced transduction efficiency.[20] Another challenge with this approach is that health authorities are increasingly interested in ensuring minimal levels of residual baculovirus (infectious or not) remain in the final product.

Overall, there is much promise for the use of baculoviral vectors in the future.

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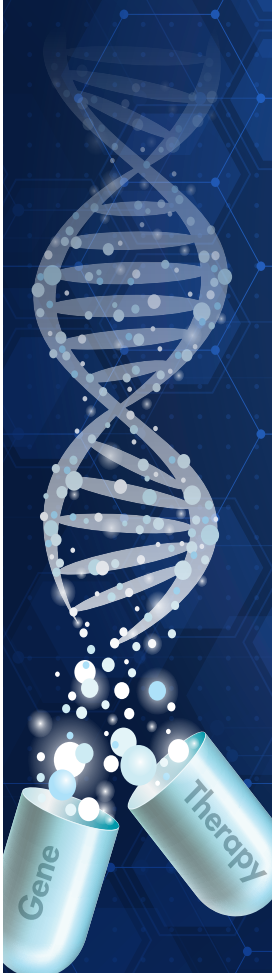
Stable Producer Cell Lines

Cellular toxicity from the expression of a required vector component has complicated the establishment of stable producer cell lines for many vector systems. Some stable producer lines utilize inducible promoters to minimize toxicity, and most stable MuLV retroviral cell lines are inducible.[14] One example of cellular toxicity produced from a vector component is the pseudotyped retroviral envelope VSV-G.[1]

Increasingly, the field is thinking that stable cell lines with inducible systems to express the viral component is the way to go. Toxicity is one aspect. Another difficulty is the prospect of making infectious virus without any nucleotides packaged. That effectively becomes an impurity that must be cleared via the purification process.



Similarity, toxicity from lentiviral components (*pol*, *rev*) has thwarted the development of stable lentiviral producer cell lines. Only recently are methods being established for generating clinical-grade cell lines that continuously produce lentiviral vector.[30]

The development of stable cell lines is expected to significantly improve the current challenges associated with producing viral vector at scales needed for therapies with large therapeutic demand or those requiring high titers of vector. The use of a stable cell lines simplifies manufacturing steps, lessens regulatory oversight, and lowers cost. This, in turn, could significantly enable the development of new vector-based gene therapies.

Choice of Producer Cell Line

Many factors influence the choice of cell line for vector generation. A key factor is production titer. Increased production titers of therapeutic vector can greatly reduce both production scale and lower costs. For vectors produced by transfection, transfection efficiency is a major factor. HEK 293 and HT1080 cells can be transfected at efficiency of near 90% or they also produce high titers of vector.[14] Among the many HEK lines, HEK 293T cells reportedly produces increased titer because of the presence of SV40 T-Ag.[1] Recently, "Fast" HEK 293FT cells, which reportedly have shorter doubling times and greater production are showing promise.[31] Many companies are actively expanding their repertoire of available cell lines by exploring unconventional sources such as engineered human cell lines with minimal prior viral infection.

Scaling of Adherent Cells for Manufacturing Vector

The majority of the cell lines used to generate viral vectors are naturally adherent, with the exception of some tumor cell or blood lines which can grow in suspension.[14] Adherent cells are limited by the surface area of the device. Hence, production scaling is accomplished either by increasing the number of identical culture systems units (flasks, roller bottle, cubes, HYPERStacks®) (scale out) or using successively larger devices (scale up).[32]

Much work to date with viral vector manufacturing has used adherent based processes generally in multilayer cell factories (CF) and HYPERFlasks/Stacks (HY) (Corning). Cell factories have been used for the production of preclinical and clinical vector batches of γ-retroviral, lentiviral[33] or AAV vectors.[34] CFs can provide up to 2.5 m^2 a significant increase over a roller bottle with area up to 0.17 m^2.

Cell factories, HY, and roller bottles are unit production systems. Any significant increase in production capacity requires additional culture units (scale out).[35] Roller bottle expansion can be assisted by automation, which is commonly used in the vaccine industry. Cell factories are more difficult to scale and limitations in gas exchange have been shown to decrease viral titer for AAV production.[36] However, some cell factory systems are available in semi-closed loop providing some advantage.[37]

Newer, HYPERFlasks® and HyperStacks® have a membrane for gas exchange which has been shown to increase lentiviral production.[38] These are more flexible than cell factories in media/surface area volumes and they offer areas of up to 1.8 m^2/unit. However, these also require the addition of more units for large scale production. The Corning CellCube® system can scale up to 34 m^2. However, the CellCube® system is only partly single-use.[14]

The use of bioreactors enable scale up options and they also have additional advantages in that it is easier to monitor and control processes, reduced record keeping, fewer unit operations, lower contamination risks, and lower operation costs.[14,39,40] The improved control of culture condition in bioreactors may also result in improved productivity. Furthermore, many bioreactor systems can also be used in a perfusion mode which is an attractive option for retroviral and lentiviral production.[14]

Hollow fiber bioreactors (e.g., the Quantum® bioreactor, Terumo BCT)[41] and fixed-bed bioreactors look promising for attachment-dependent cells as they offer increased scale[42] and sometimes scalability options within a unit. For example, the iCELLis® fixed-bed bioreactor (Pall) has two models. The iCELLis® Nano system is a smaller bench top unit and the larger 500 model is a stand-alone unit (Figure 3).

Figure 3. The iCELLis® 500 bioreactor system.

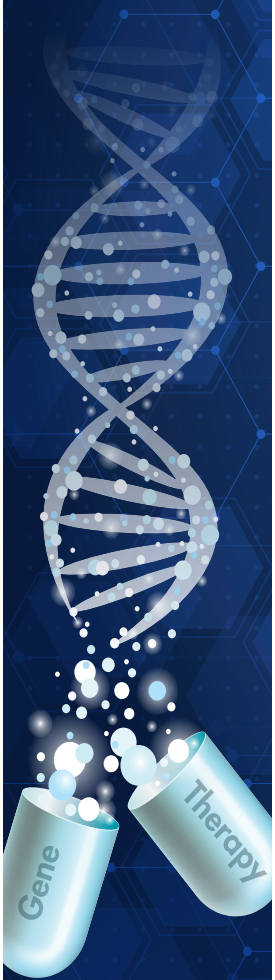


Table 2. Scalability options for the iCELLis® Nano and iCELLis® 500 bioreactors.

	Bed Size		Surface Area/m²	Equivalent Units		
IBioreactor	**Bed Diameter (cm)**	**Bed Height (cm)**	**Carrier Compaction Low-High**	**850-cm² Roller Bottles**	**10-Layer CellStacks**	**HYPERStack 36**
iCELLis Nano	11	2	0.53 - 0.8	6.2-9.4	0.04-0.08	0.3-0.4
iCELLis Nano	11	4	1.06 - 1.6	12.4-18.8	0.2-0.5	0.6-0.9
iCELLis Nano	11	10	2.65 - 4.0	31.8-47.1	10-25	1.5-2.2
iCELLis 500/100	86	2	66 - 100	776-1116	104-157	37-56
iCELLis 500/200	86	4	133 - 200	1565-2353	209-314	74-111
iCELLis 500/500	86	10	333 - 500	3918-5882	524-786	185-278

The fixed-bed consists of polyester (PET) macrocarriers (13.9 cm^2 each) fixed inside a housing where media flows from the bottom to the top. The height of the bed can vary as can the compaction density of carrier which enables a wide range of scalability options up to 500 m^2 (Table 2). The maximum area of 500 m^2 offers greatly increased scale which is equivalent to roughly 5900 roller bottles, 780 10-layer stacks, or 280 HYPERStacks (Table 2). In addition, the iCELLis® bioreactor enables high cell density growth of 10^8 cells/mL carrier and produces similar cell-specific titers compared to adherent culture.[43]

While hollow fiber and fixed-bed bioreactor systems offer significant increases in scale, the scale is currently limited. Another option for adherent cells is the use of microcarriers which can provides a large surface area for cell attachment in rocking bioreactors or stirred tank bioreactors. Rocking bioreactors with microcarrier or single cell suspension culture are an option to produce smaller quantities of vector for research or clinical studies, as their currently capacity is limited 500L at low cell density. Rocking bioreactors are disposable and can also be used to provide cell seed for larger bioreactors.

Virus production in the vaccine industry has been successfully scaled up to 2000+L using microcarriers in stirred tank bioreactors.[44] A single 2000L bioreactor operating at 10 cm^2/mL microcarrier density offers 2,000 m^2 of surface area. Drawbacks to microcarriers are developmental considerations of optimizing cell attachment, growth, and viability. Moreover, the ability to transfect/transduce cells growing on a microcarrier needs to be understood early in development.

Stirred-tank bioreactors are by far the most prevalent bioreactor used for the commercial manufacturing of mAbs and recombinant proteins. As a result, the technology is very well characterized and both industry and regulatory authorities are very familiar with their operation. They are the most efficient means of scaling up to large volumes. While these bioreactors can be used to grow adherent cells with the use of microcarriers, they are best adapted to the growth of suspension cells. Stirred tank bioreactors come in a variety of size and configuration options. For example, the Allegro™ STR (Pall) is a single-use bioreactor that is available in 50, 200, 1000 and 2000 L scales (Figure 4). In addition, these bioreactors have a compact footprint due to their atypical cubical design.

Figure 4. Allego™ STR jacketed 50 L, 200 L, 1000 L and 2000 L bioreactors.

Scaling of Suspension Cells for Manufacturing Vector

Single-cell suspension culture has significant advantage over adherent cells in that they can easily be scaled out from spinner, to laboratory scale bioreactor, to production bioreactor without cell detachment. Several 293 cell lines used for vector production (293T, 293FT) are prone to adaptation to suspension culture in chemically defined media[45] and suspension 293 cell lines preadapted to commercial media are available on the market. The use of animal-free media is a desirable feature for manufacturing due to the decreased risk of adventitious agents and the reduced purification burden. However, adapting custom cells to suspension culture can be time consuming and prolong process development time.

Currently, there are challenges associated with transfection of cells at scales >200L which is a significant limiting factor to widespread adoption of suspension culture for processes dependent on transfection.[17] More recently, published reports have proven that high titers of lentiviral vector can be achieved using a suspension adapted helper cell line in small scale bioreactors.[46] Although promising, methods using suspension cells at larger scale have not yet been widely adopted for routine vector production.

Cost Considerations

There are several factors that can influence the cost of viral based gene therapy manufacturing. These include production titer, the ability to upscale production processes, regulatory burden, and other factors.

Production titer is a key factor as higher titer results in a smaller, more cost-effective production process. High titer production of vector can lower reagent demand, labor, and facility requirement. One possible route to increase titer is by control of cell environment and production conditions. In this regard, controlled bioreactor vessels can present an advantage over flasks, roller bottles, or CF's in productivity.

In addition, use of bioreactors can simplify scale up of a process as previously described. From a capex, point of view, scaling up multilayer stacks, requires considerable space. Since clean room space is expensive, this can be a very significant cost. In contrast, implementation of a bioreactor system can be done in the same space required for a modestly size multilayer stack system.

Implementation of a bioreactor system can decrease labor and consumables costs 3-fold and there is considerably less plastic waste generated with a bioreactor system resulting in reduced disposal costs. Most bioreactor systems come with some degree of automation which manifests itself as a 3-fold decrease in labor compared to more manual systems like multilayer stacks. Overall a >50% reduction in costs can be achieved by implementing a bioreactor system compared to a multilayer stack of similar scale.[47] Scaling out a process that uses adherent cells in multilayer CF requires many connections and incubators, so contamination risk increases along with capex costs. Finally, disposable systems like the iCELLis® bioreactor, or other single use systems can provide savings as cleaning, sterilization as well as extensive validations are reduced or eliminated.

Suspension bioreactors also have lower risk due to the reduction of production units, especially compared to a scale-out, multi-flask configuration. With very small clinical trial material requirements, adherent cell technology may suffice with some cost savings on capex.

Process Design and Regulatory Burden as a Cost Determinant

Production design can have a large effect on cost. While quick, easy, and flexible, vector production by transfection poses both increased cost and challenges as scale increases. The use of helper viruses (for direct infection) rather than plasmids may reduce transfection needs and provide a path for scaling up production. In this regard, the use of a stable producer cell line would provide the greatest benefit through the reduction of manufacturing steps. The use of a stable producer cell line over transfection methods may also facilitate process consistency, which is an attribute for GMP vector production.[48] However, the use of inducible cell lines may require additional steps to remove inducing agents.

Since all components of vector production intended for therapy require GMP manufacture, optimization or elimination of costly GMP steps, such as the manufacture of plasmid, provides large benefit.[49] Design optimization of the overall process in light of regulatory burden for purity and safely could significantly lower production costs.[50] These include the presence of potentially toxic or immunogenic impurities, contaminants such as HCP proteins and DNA, plasmid DNA, reagents from transfection, potential transduction inhibitors, induction agents, antibiotics, and others.[51] Other areas include screening for producer cells and vector batches for replication-competent viruses.[48] Consideration of vector sterilization may also reduce costs given that large viruses, such as HSV, may not be filter sterilizable, thus requiring aseptic processing, and validation that goes with that. As some gene therapy products are relatively new for regulatory authorities, it is highly likely that regulatory guidance will increase as more products are taken to market.[50]

Process Development Timelines

Adherent based bioreactor systems such as the iCELLis® have advantages over suspension systems in terms of process development timelines. Adaptation of a process from cell factories to adherent based bioreactors does take some effort but timelines can be relatively short. By comparison, converting an adherent system to suspension can be a very lengthy process (more than 1 year). Other possible complications are variation in product quality that would requiring extensive comparability testing because of biological differences between adherent and suspension systems.

Transfection based approaches are quick and flexible. However, they are expensive and more difficult to upscale. Transfection approaches may have benefit in that vector can be produced quickly to enter clinical evaluation. However, this approach may provide technical and cost difficulties for greater upscaling for vectors destined for wide therapeutic distribution.

Adapting an adherent system to a fixed-bed bioreactor does take some time but this is generally much shorter than adapting adherent systems to suspension, which can be very difficult and may result in decreased productivity.

Analytical Considerations

The final vector product intended for gene therapy and vaccine applications needs to be well characterized and of proper quantity, purity and potency. This can be achieved only if adequate characterization methods are in place and the viral production process is well established, understood and reproducible. Sample control and product characterization are not only important for the final product, but also during different steps within the production process. In depth knowledge of both up- and downstream processing is crucial since the upstream production greatly affects the downstream process. Sometimes even slight changes in the upstream process can result in a less efficient downstream process.

The well-established framework for traditional biologics (i.e. recombinant proteins, enzyme replacement therapies, monoclonal antibodies) is increasingly being applied to the field of gene therapies. As such, more investment in real-time, high-throughput technologies to characterizer both process and product intermediates is needed. The days of process = product are over and a new paradigm is needed to bring the potential of gene therapies to patients in need.

Impact on Downstream Purification

Many vectors are secreted into the media. In this case, virus can be collected from the media which will substantially reduce downstream burden. In some cases, a portion of the virus remains in the cells and so cell lysis with either detergent or physical means may be critical to improve yield. There are a wide variety of processes that can be used for purification, including clarification by filtration or centrifugation; chromatography steps for affinity, ion exchange, sizing, multi-modal and other resins; concentration steps with TFF or ultracentrifugation; and enzymatic treatments to reduce nucleic acids. When the cells must be lysed to release the vector products, multiple steps may be needed to optimize the purification.

The development of single-cell stable producer cell lines may further increase production scale.

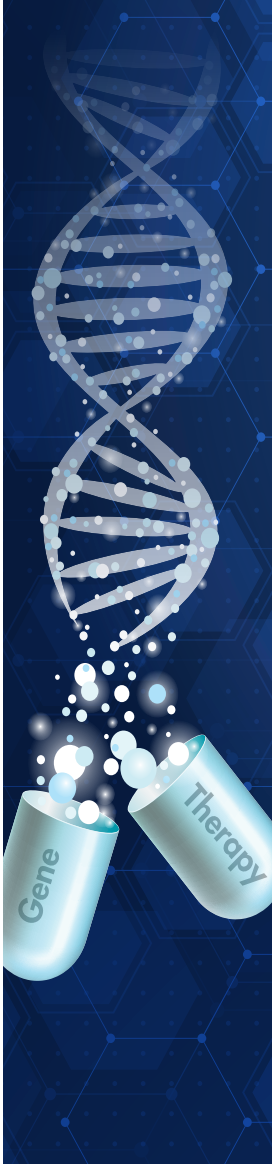
The choice of upstream production process can affect downstream purification burden. For example, shear forces on adhered cells on microcarrier systems or other systems with fluid motion can increase cell debris. Another example is that a fixed-bed bioreactor can sometimes offer some benefit some impurities can be trapped into the bed.

Traditional purification steps have relied on ultracentrifugation, flocculation, and other difficult-to-scale technologies. Recent advances have enabled the same level of impurity removal to be obtained using highly scale-able chromatography and tangential flow filtration.

Room for Improvement

There are several areas of improvements for viral vectors for gene therapy. As mentioned earlier, transfection can be both problematic and expensive at larger scales required for wide clinical demand. As such, advancement in the use of stable producer lines, particularly in the HIV lentiviral system is needed. The cost of vector and vector-derived therapies continues to present a challenge towards advancement. Methods need to be developed which result in lower costs.

The development of single-cell stable producer cell lines may further increase production scale, and in particular if the cell can be adapted to growth in suspension, by enabling the use of large tank bioreactors. These, in turn, can be used in closed systems with less risk of contamination. Moreover, the integration of serum-free media will lessen purification burden and animal-free media will improve regulatory safety profiles.



Current methods for lysing cells (example AAV) are also crude and there is room for improvement. Indeed, Triton™-X, which is traditionally one of the most widely used lysing agents, has restrictions on use in the EU.[52] In addition, there are often shortages of commonly used items, such as tissue culture plastic ware used for expanding adherent cells due to the very rapid increase in the number of gene therapy projects.

As more serotypes of certain vectors are being designed for more tissue specific uses there is an increased need for more generic and specific affinity reagents and new techniques for vector purification.

Despite the ongoing need for improvements in therapeutic vector production, there is much optimism for the future of viral based gene therapy. In the future, vector based gene therapy may not only offer gene replacement, but could advance to offer gene editing functions as well.

References:

1 Milone, M.C. and U. O'Doherty, *Clinical use of lentiviral vectors. Leukemia*, 2018. **32**(7): p. 1529-1541.

2 Dull, T., et al., *A third-generation lentivirus vector with a conditional packaging system*. J Virol, 1998. **72**(11): p. 8463-7

3 Hastie, E. and R.J. Samulski, *Adeno-associated virus at 50: a golden anniversary of discovery, research, and gene therapy success--a personal perspective. Hum Gene Ther*, 2015. **26**(5): p. 257-65.

4 Balakrishnan, B. and G.R. Jayandharan, *Basic biology of adeno-associated virus (AAV) vectors used in gene therapy.* Curr Gene Ther, 2014. **14**(2): p. 86-100.

5 Colella, P., G. Ronzitti, and F. Mingozzi, *Emerging issues in AAV-mediated in vivo gene therapy.* Mol Ther Methods Clin Dev, 2018. **8**: p. 87-104.

6 Merten, O.W., *AAV vector production: state of the art developments and remaining challenges.* Cell GeneTherapy Insights, 2016. **2**(5): p. 521-551.

7 Srivastava, A., *Advances and challenges in the use of recombinant adeno-associated virus vectors for human gene therapy.* Cell GeneTherapy Insights, 2016. **2**(5): p. 553-575.

8 Chadeuf, G., et al., *Evidence for encapsidation of prokaryotic sequences during recombinant adeno-associated virus production and their in vivo persistence after vector delivery.* Mol Ther, 2005. **12**(4): p. 744-53.

9 Schnodt, M., et al., *DNA minicircle technology improves purity of adeno-associated viral vector preparations.* Mol Ther Nucleic Acids, 2016. **5**: p. e355.

10 Cavazzana-Calvo, M., et al., *Transfusion independence and HMGA2 activation after gene therapy of human beta-thalassaemia.* Nature, 2010. **467**(7313): p. 318-22.

11 Cartier, N., et al., *Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy.* Science, 2009. **326**(5954): p. 818-23.

12 Biffi, A., et al., *Lentiviral hematopoietic stem cell gene therapy benefits metachromatic leukodystrophy.* Science, 2013. **341**(6148): p. 1233158.

13 Hematti, P., et al., *Distinct genomic integration of MLV and SIV vectors in primate hematopoietic stem and progenitor cells.* PLoS Biol, 2004. **2**(12): p. e423.

14 Merten, O.W., et al., *Manufacturing of viral vectors for gene therapy: part I. Upstream processing.* Pharm. Bioprocess., 2014. **2**(2): p. 182-203.

15 Krieg, A.M., *Direct immunologic activities of CpG DNA and implications for gene therapy.* J Gene Med, 1999. **1**(1): p. 56-63.

16 Segura, M.M., et al., *New protocol for lentiviral vector mass production.* Methods Mol Biol, 2010. **614**: p. 39-52.

17 Merten, O.W., M. Hebben, and C. *Bovolenta, Production of lentiviral vectors.* Mol Ther Methods Clin Dev, 2016. **3**: p. 16017

18 Cribbs, A.P., et al., *Simplified production and concentration of lentiviral vectors to achieve high transduction in primary human T cells.* BMC Biotechnol, 2013. **13**: p. 98.

19 Kost, T.A., J.P. Condreay, and D.L. Jarvis, B*aculovirus as versatile vectors for protein expression in insect and mammalian cells.* Nat Biotechnol, 2005. **23**(5): p. 567-75.

20 Mahonen, A.J., et al., *Culture medium induced vimentin reorganization associates with enhanced baculovirus-mediated gene delivery.* J Biotechnol, 2010. **145**(2): p. 111-9.

21 Madhan, S., M. Prabakaran, and J. Kwang, *Baculovirus as vaccine vectors.* Curr Gene Ther, 2010. **10**(3): p. 201-13.

22 Wang, S. and G. Balasundaram, Potential cancer gene therapy by baculoviral transduction. Curr Gene Ther, 2010. **10**(3): p. 214-25.

23 Volkman, L.E. and P.A. *Goldsmith, In vitro survey of Autographa californica nuclear polyhedrosis virus interaction with nontarget vertebrate host cells.* Appl Environ Microbiol, 1983. **45**(3): p. 1085-93.

24 Tani, H., et al., *In vitro and in vivo in vivo in vivo in vivo gene delivery by recombinant baculoviruses.* J Virol, 2003. **77**(18): p. 9799-808.

25 Ho, Y.C., et al., *Transgene expression and differentiation of baculovirus-transduced human mesenchymal stem cells.* J Gene Med, 2005. **7**(7): p. 860-8.

26 Ramachandra, C.J., et al., *Efficient recombinase-mediated cassette exchange at the AAVS1 locus in human embryonic stem cells using baculoviral vectors.* Nucleic Acids Res, 2011. **39**(16): p. e107.

27 Luo, W.Y., et al., *Development of the hybrid Sleeping Beauty: baculovirus vector for sustained gene expression and cancer therapy.* Gene Ther, 2012. **19**(8): p. 844-51.

28 Urabe, M., C. Ding, and R.M. Kotin, *Insect cells as a factory to produce adeno-associated virus type 2 vectors.* Hum Gene Ther, 2002. **13**(16): p. 1935-43.

29 Lesch, H.P., et al., *Production and purification of lentiviral vectors generated in 293T suspension cells with baculoviral vectors.* Gene Ther, 2011. **18**(6): p. 531-8.

30 Sanber, K.S., et al., *Construction of stable packaging cell lines for clinical lentiviral vector production.* Sci Rep, 2015. **5**: p. 9021.

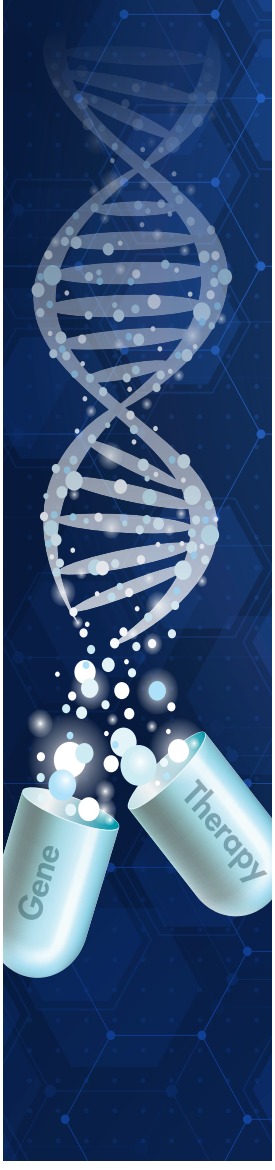
31 Fisher, T. 2018; Available from: Thermofisher.com/order/catalog/product/R70007.

32 Segura, M.M., et al., *New developments in lentiviral vector design, production and purification.* Expert Opin Biol Ther, 2013. **13**(7): p. 987-1011.

33 Merten, O.W., et al., *Large-scale manufacture and characterization of a lentiviral vector produced for clinical ex vivo gene therapy application.* Hum Gene Ther, 2011. **22**(3): p. 343-56.

34 Allay, J.A., et al., *Good manufacturing practice production of self-complementary serotype 8 adeno-associated viral vector for a hemophilia B clinical trial.* Hum Gene Ther, 2011. **22**(5): p. 595-604.

35 Spier, R., *Monolayer growth systems: heterogeneous unit processes., in Animal Cell Biotechnology,*, R. Spier and J. Griffiths, Editors. 1985, Academic Press: London.



References continued

36 Okada, T., et al., *Large-scale production of recombinant viruses by use of a large culture vessel with active gassing.* Hum Gene Ther, 2005. **16**(10): p. 1212-8.

37 Pakos, V. and A. Johansson, *Large scale production of human fibroblast interferon in multitray battery systems.* Dev Biol Stand, 1985. **60**: p. 317-20.

38 Kutner, R.H., et al., *Simplified production and concentration of HIV-1-based lentiviral vectors using HYPERFlask vessels and anion exchange membrane chromatography.* BMC Biotechnol, 2009. **9**: p. 10.

39 Ghani, K., et al., *Generation of a high-titer packaging cell line for the production of retroviral vectors in suspension and serum-free media.* Gene Ther, 2007. **14**(24): p. 1705-11.

40 Merten, O.W., *State-of-the-art of the production of retroviral vectors.* J Gene Med, 2004. **6 Suppl 1**: p. S105-24.

41 Sheu, J., et al., *Large-scale production of lentiviral vector in a closed system hollow fiber bioreactor.* Mol Ther Methods Clin Dev, 2015. **2**: p. 15020.

42 Lennaertz, A., et al., *Viral vector production in the integrity® iCELLis® single-use fixed-bed bioreactor, from bench-scale to industrial scale.* BMC Proc., 2013. **7**(S6): p. 59.

43 Lennaertz, A., et al., *Viral vector production in the integrity® iCELLis® single-use fixed-bed bioreactor, from bench-scale to industrial scale.* BMC Proc., 2013. **7**(Suppl 6): p. 59.

44 Barrett, P.N., et al., *Vero cell platform in vaccine production: moving towards cell culture-based viral vaccines.* Expert Rev Vaccines, 2009. **8**(5): p. 607-18.

45 Dormond, E., et al., *An efficient and scalable process for helper-dependent adenoviral vector production using polyethylenimine-adenofection.* Biotechnol Bioeng, 2009. **102**(3): p. 800-10.

46 Manceur, A.P., et al., *Scalable lentiviral vector production using stable HEK293SF producer cell lines.* Hum Gene Ther Methods, 2017. **28**(6): p. 330-339.

47 Science, P.L., *Cost modeling of upstream production process of lentiviral vectors in HEK-293 cells comparing multi-tray 10 stacks and fixed-bed bioreactor.* Poster ESACT 2007, 2017.

48 McCarron, A., M. Donnelley, and D. Parsons, *Scale-up of lentiviral vectors for gene therapy: advances and challenges.* Cell GeneTherapy Insights, 2017. **3**(9): p. 719-29.

49 Ausubel, L.J., et al., *Production of CGMP-Grade Lentiviral Vectors.* Bioprocess Int, 2012. **10**(2): p. 32-43.

50 White, M., et al., *A guide to approaching regulatory considerations for lentiviral-mediated gene therapies.* Hum Gene Ther Methods, 2017. **28**(4): p. 163-176.

51 Rodrigues, T., et al., *Purification of retroviral vectors for clinical application: biological implications and technological challenges.* J Biotechnol, 2007. **127**(3): p. 520-41.

52 CRS. *CRS-Chemical inspection and regulation service -REACH Restriction.* 2018; Available from: cirs-reach.com/REACH/REACH_Restriction.html.

Downstream Manufacturing of Gene Therapy Vectors

By Steve Pettit, Clive Glover, Joseph Hughes, John Madsen, and Pratik Jaluria

Introduction

The goal of downstream processing is to separate the viral vector from the various impurities produced during upstream processing and to get the virus into the appropriate state for formulation and administration to patients. Viral vector batches destined for clinical use must comply to increasing regulatory standards for impurities and contaminants as these can affect product safety and potency. In addition to providing purity, purification processes need to meet production scale, which can be quite large for clinical trials and commercial applications.

Approaches to vector purification exploit the physical characteristics of the viral particle such as size, surface charge, and hydrophobicity. Viral vectors vary greatly in physical characteristics depending on the type of virus, the serotype of the same virus, and can sometimes vary with different transgene inserts within the same vector (AAV). Table 1 shows some similarities and differences of the 4 commonly used viruses used as vectors: adenovirus (AdV), adeno-associated virus (AAV), retrovirus (RV), and lentivirus (LV).

Table 1. Differences and similarities in 4 common viruses used as viral vectors

Virus	Size (nm)	Envelope	Stability	Buoyant Density
Adenovirus	~90	No	High	1.34 (CsCl)
AAV	~ 20	No	High	1.41 (CsCl)
Retrovirus/ Lentivirus	~90-120	Yes	Low[a]	1.16 (Sucrose)

a. Pseudotyping with VSV-g is reported to improve stability to some extent[1]

The differences in physical characteristics between various vectors presents a diverse set of purification challenges that result in a multiplicity of needed techniques and solutions for the industry. For example, AAV also accumulated in the media and is partly released from cells via a lytic process, AAV viruses are generally harvested by cell lysis to improve yield. Cell lysis can generate significant quantities of host cell contaminants including DNA and protein that increase purification burden. AAV vectors can produce a significant portion of "empty" particles devoid of transgene at up to 95% to total particles.[2] Despite the ongoing debate over a possible benefit from the presence of empty particles in AAV therapies, these are still considered a major contaminant[3,4] and health authorities encourage sponsors to set limits on the amount of empty or partially full vectors.

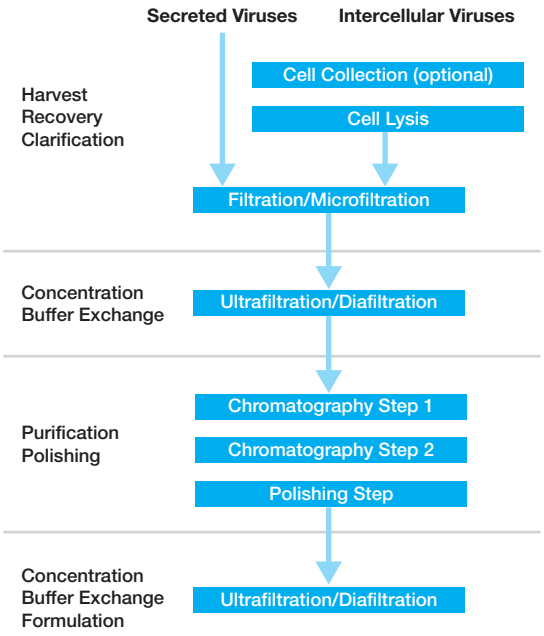
The downstream processing of RV/LV vectors presents its own unique challenges. The presence of a lipid envelope makes these vectors less stable. The instability of these particles can lead to a loss of yield - potentially up to 70% during the whole downstream purification process.[5] Pseudotyping can also influence RV/LV stability, which may be improved or made worse depending on the vector and pseudotype component.[1,6] Thus, each vector can present unique challenges during downstream processing.

There are other unique challenges for the purification of vectors that are generated because of the chosen upstream production process. For example, vector supernatants that are produced by transfection contain large amounts of contaminating plasmid DNA. Large viruses, such as herpes, may not be filter sterilizable, and may requiring aseptic processing, and the validation that goes with that.[7, 8, 9] The use of serum-supplemented cell culture media can result in a greater purification burden. Moreover, increased viscosity from the use of sera can affect downstream process efficiency.[10] In this regard, upstream production systems using serum-free media, such as common with cells grown in bioreactors and with baculovirus/insect cell systems, can produce a significant advantage downstream.

photo courtesy of WuXi

Despite the differences in physical characteristics between vectors, current downstream protocols generally have a similar work flow which involve a clarification step (which may be proceeded by cell lysis), purification (centrifugation or ion exchange/ affinity chromatography), and a polishing step (additional chromatography step/size exclusion) (Figure 1). In addition to the above, filtration/ dialysis/centrifugations steps are typically utilized to concentrate, for buffer exchange, or for final formulation.

Figure 1. Example of a downstream process for secreted viruses (retrovirus/lentivirus) and viruses released by cell lysis (AAV/adenovirus).



Clarification, Cell Lysis, and Filtration

The clarification step includes elimination of large debris and macromolecular complexes from the initial crude suspension and may include cell lysis in order to improve viral yield (AdV, AAV). Physical methods such as microfluidizers or heat-shock treatments can be used to promote cell lysis. Lysis using detergents such Triton™ X-100 or other detergents has proven inexpensive and efficient for many applications such as for AdV and AAV preparations and can be utilized for insect cell preparations as well.[11] However, the inclusion of Triton™ X-100 on the REACH list of banned substances in the EU limits its adoption.[12] Alternative approaches are available using Tween™ detergents[13] and there are analytical methods for the detection of Tween™ 20 in vector preparations. However, polysorbate 20/80 are thought to adversely impact chromatographic steps, reducing the dynamic binding capacity and fouling the underlying chemistry of the resin/ligand interactions.

During or after the cell lysis step, nucleases (example benzonase) are typically added in order to degrade nucleic acids and disturb the macromolecular complexes. Any cell lysis method requires optimization as lysis is highly dependent on the cell concentration, incubation time, incubation temperature, cell type, and the virus being released.[15]

The initial removal of cell debris and impurities during clarification typically involve a combination of filters with decreasing pore size down to 0.2 µm, which act as an important part of an overall bioburden control strategy. Note that clarification of larger viruses, may be limited to larger pore sizes due to possible retention of virus or issues with excessive shear force. Diafiltration can also prove effective for clarification in addition to serving other functions such as concentration and buffer exchange. A key consideration for this unit operation is the inclusion of a pre-filter with relatively large nominal pore sizes to remove cellular debris and protect subsequent filters from clogging.

During the processing of RV/LV, reduced viral yield due to the low stability of particles is an upmost concern. Process strategies may be need to be developed to minimize shear such as limiting flow rates or employing the use of slower speed centrifugation. At smaller scale, slow speed centrifugation can provide some degree of clarification for RV/LV, while at larger scale direct flow setups have been used with some vectors using 1 µm > 0.8 µm > 0.45 µm filters.[14] Alternatively, tangential flow filtration (TFF) has proven an effective method for both clarification and concentration with reports of recoveries as high as 90–100%, although flow rate may need to be adjusted.[15,16] Exploration of single-pass TFF systems is an active area of development given the potential to improve processing yields while minimizing potential shear forces.

The Limitation of Classical Centrifuge Methods

Purification of small quantities of virus for research or for small clinical studies have classically relied heavily on ultracentrifugation methods. In these methods, viral particles are typically pelleted and concentrated through a matrix, which is then followed by resuspension and purification via buoyant density using gradient ultracentrifugation. Several rounds of gradient ultracentrifugation may be required to further purify particles (Table 1).

CsCl has been commonly used for AAV and AdV ultracentrifugation in the past. However, iodixanol is now preferred over CsCl because it is less detrimental, needs less dialysis, lessens aggregation, and because run times are shortened from 24 h to few hours.[17] Because of the decreased stability of

enveloped viruses such as RV/LV, sucrose is generally used for density purification of these. For example, classical retroviral techniques using ultracentrifugation typically involve concentration by "pelleting" particles through a cushion of 20% sucrose, resuspending, and then "banding" by sucrose gradient ultracentrifugation(s).

A major problem with density methods is scale up due to centrifuge volume limitations and cost. Since larger volume ultracentrifuges are now available on the market, it is now possible to at least consider ultracentrifugation at some scales. However, any benefit must be weighed against the disadvantages of labor, processing time, possible capex, and other restraints.[10] In addition, there are health and safety issues associated with density ultracentrifugation as recovery of the vector usually requires sticking a needle into the side of the ultracentrifugation tube, which certainly increases safety concerns for using sharps with highly concentrated vectors.

Despite these and other drawbacks, ultracentrifugation has been adopted by a number of companies and academic institutions to produce early-stage clinical trial material. Some companies have shown ultracentrifugation has application in the later stages of an overall purification scheme and can in some instances satisfy most of the purification needs at smaller scale.[10] Moreover, buoyant density separation of some viruses such as AAV has clear benefits as it is serotype independent and it is one of the few methods that can separate full AAV particles from empty particles. Moreover, density ultracentrifugation can separate helper AdV from full AAV particles.[8,18]

Chromatography

The use of chromatography for the purification of vectors has advanced and is becoming an established method that can purify vector by its physical property of net charge, hydrophobicity, affinity to ligand, size, or other property. Chromatography is considered much more scalable and cost effective than centrifugation.[19] Columns can be reused multiple times and columns can be run in parallel or in tandem in different strategies. In addition, chromatography is an effective means of removing potential adventitious agents, a key aspect of late-stage development. Chromatography has been widely used for purification of vaccines and gene therapy vectors and it may be applied in various downstream steps, including capture, concentration, purification, and polishing steps.[20]

Ion Exchange Chromatography

Ion exchange chromatography (IEC) is based on surface charge and is a simple and cost-effective technique that can be applied to multiple virus types. Anion or cation exchangers can be used to bind either positively or negatively charged viruses. However, IEC requires developmental efforts to match the column type to individual virus surface properties, such as found in varying serotypes. One IEC step may not be sufficient to purify some viruses such as AAV, which then requires additional processing steps.[21]

The use of IEC can have clear benefits. IEC is versatile. For example, with AAV vectors, IEC can work across multiple serotypes and can also be used to separate helper viruses such as AdV and baculovirus from AAV preparations.[22] IEC is one of the few methods that can separate full from empty AAV particles due to a difference in the charge of full particles from the presence of full-length DNA.[23] IEC can also be effective in this regard as protocols have shown >90% elimination of empty particles.[23,24,25] Examples include a 2-stage process where cation exchange (Mustang® S, Pall Corp.) was used to retain empty particles, which is followed by strong anion exchange (Mustang® Q) to retain full particles. This process resulted in only 0.8% empty particles.[23]

One of the major considerations of moving to clinical manufacturing is how to employ increased production scale.

IEC theoretically should allow the separation of empty particles of all AAV serotypes; however, resins and conditions would likely need to be optimized for each individual serotype.[21,26] One drawback to the separation of empty particles is that substantial development time (>2 months) may be required as the gradients necessary for separation can be very shallow. Furthermore, anything that affects the charge of a vector, such as serotype, vector design, or length of the transgene insert, may require redevelopment of the gradient conditions.

IEC is a promising purification method. Currently, at larger scale, the only efficient way to separate full from empty particles is by use of ion-exchange chromatography.[3] There are many configurations available that include resins, faster flow membranes, such as Mustang®, or other configurations. IEC is flexible, such as being used as a capture step, which then can be followed by a buoyant density ultracentrifugation. This method has been used for the generation of GMP AAV vector for multiple

clinical studies.[3,27,28] In addition, IEC can produce LV vectors of high purity, although the use of too high of ionic strength solutions during the elution step can reduce LV infectivity.[29]

Hydrophobic Interaction Chromatography

Hydrophobic interaction chromatography (HIC) been used for viral capture/clearance in the vector and recombinant protein industry for years. In purification schemes, HIC has been used for the purification both AdV and AAV2.[30, 31] However, its use with AAV has not been extensively documented with different serotypes. Similar, to IEC use of high ionic strength solutions during binding can affect the stability of some viruses.[30]

Affinity Chromatography

Affinity chromatography (AC) relies on the interaction of the viral particle with a ligand. Heparin affinity chromatography has been used for purification of LV and other viruses and is also reported to handle large volumes.[32] The use of mild salt solutions and can help preserve vector integrity.[33] However, there are disadvantages including non-specific binding of impurities that then require additional purification steps[32], animal sourcing, and selectivity of only some AAV serotypes such as 2, 3, 6, and 13. [34] Cellufine™ sulphated cellulose is an alternative with similar specificity to heparin affinity for purification of various viruses.[35]

For AAV, AVB affinity resin (BAC/GE Healthcare) has been shown to be effective strategy for the purification of AAV serotypes 1, 2, 3, and 5 as recommended by the manufacturer.[36] Others have reported its successful use with other serotypes, although efficiency may vary depending serotype.[37,38] Moreover, an analytical kit is available to check for leakage of the recombinant protein ligand.[39] AVB has successfully been used in combination with IEC to purify a variety of AAV serotypes.[37]

Newer serotype-specific affinity matrices are available for AAV serotypes 8 and 9 (Thermo Fisher, POROS CaptureSelect™). These have resulted in the development efficient vector-specific purification protocols that consist of clarification, affinity chromatography, tangential flow filtration, and a gel filtration step for formulation.[3,19] These affinity matrices reportedly also lead to an increase in the overall yield[3], and a lower cost of goods.[40] Genethon has reported purification of AAV9 using CaptureSelect™ AAV9 at 10L and 50L scale with >80% recovery.[19]

One issue with the use of affinity chromatography with AAV is that full particles cannot be separated from empty particles, which results in additional steps such as ion-exchange chromatography or ultracentrifugation for that purpose.[37] Another potential issue with affinity chromatography is the possible leakage of ligand into the vector preparation. Leakage of ligand may require analytical steps and possible additional purification steps.

Size Exclusion Chromatography

Size exclusion chromatography (SEC) separates virus particles from contaminants on the basis of size and mass.[41] SEC has benefits in that it is very gentle on particles since there is no binding or elution. However, SEC may not be suitable for large-scale processing as it has low throughput, requires low flow rates that increase processing time, and it may dilute the sample and result in additional concentration steps. Moreover, it is commonly thought that SEC does not scale well beyond 1000L. Nevertheless, SEC has found use as a final polishing for many viruses including AAV and LV.[8,41,42]

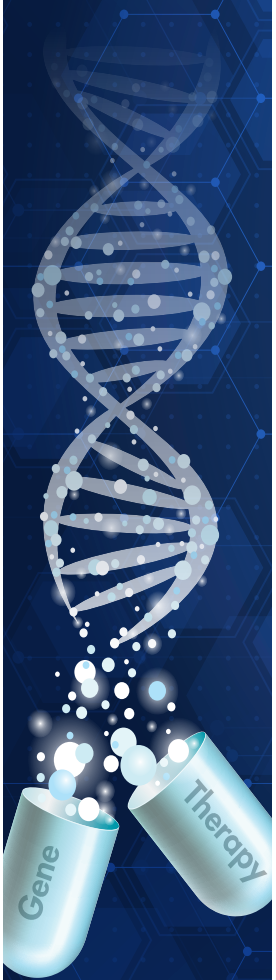
Concentration/Buffer Exchange

Pelleting virus by ultracentrifugation can provide effective concentrations and high vector yields for some vectors. However, pelleting has potential disadvantages such as losses of functional vector particles due to shear stress[43] and co-concentration of impurities.[42] The use of low-speed centrifugation for longer durations is a more gentle approach, which may prove valuable with less stable vectors to result in higher infectious particle recoveries.[44] However, as discussed above centrifugation methods are judged to have limited scalability.

TFF has advantages for concentration or buffer exchange in that it is typically scalable and allows for mild processing conditions for less stable vectors. Products are available in numerous configurations based on the molecular weight cutoff, the nature of the membranes, or the filter surface. Although the achievable concentration factors are often lower than for centrifugation methods there is an additional benefit in that some impurities may be removed during the process of concentration.[17,45]

Bioburden Control

Bioburden control is critical for products with a GMP regulatory requirement to offset the risk of microbial contamination of the final product. In most protocols, the final vector preparation is filtered by 0.2 µm filtration as component of control, although there may be variations in its placement in downstream protocols.[3] Some vector products are not filtered sterilizable because of the size of the vector. It is possible to skip sterile filtration provided that the process can be certified as being fully aseptic; however, this requires validation and operations must be performed in a clean room. Nevertheless, there are large-scale protocols without the sterile filtration step because



of the use of a semi-closed system.[7] TFF systems can provide increased throughput for sterilization and can have benefit in that regard.

Moving Downstream from Research Production to Clinical/Commercial Manufacturing

One of the major considerations of moving to clinical manufacturing is how to employ increased production scale. At larger scales, the purification methods need to accommodate large volumes with high efficiency and speed. One approach toward implementation of greater scale is to move away from poorly scalable centrifugation-based techniques to systems with greater throughput. Chromatographic approaches are considered to be the best technological method for manufacturing applications, due in part to increased scalability. Moreover, IEC or AC methods can be used as starting methods for the purification of many vectors.[10,23] When considering individual chromatography, efficiency is a huge factor that can lead to a smaller, simpler process and greater scale.

It has been shown that VSG-g LV vectors can be purified using membrane based Mustang® Q capsules (Pall Corp.) up to a volume of 1,500 L/day[46] and lead to vector preparations of high purity. However, LV recovery may not be as efficient as with ultracentrifugation due to the fragility of these vectors. In this case, there may a clear trade off between efficiency and yield.

Another major aspect is GMP compliance. Vectors for therapeutic use must meet stringent standards for purity and safety. In this regard, product quality is of upmost importance. Simplification of the process design and increase of efficiency can reduce the number of required costly GMP reagents and steps, which significantly lower costs. Adventitious agent control is an aspect of GMP that is only generally considered once a process has moved into clinical production. Adventitious virus control is a particular challenge as conventional virus control techniques developed for mAb production, such as the viral inactivation step or the viral removal step, may not be suitable when a virus is the product of interest. Closed processing can be helpful for agent control; however, many remain under development by the industry. Moreover, development of a closed system requires working closely with vendors to ensure that a fully closed flow path is designed and is suitable for the process.

Challenges in Downstream

There are always challenges with development of a downstream process. Yield is of paramount important. Loss of viral yield puts pressure on increased pressure on the upstream process for greater titer. Viral stability can be a huge problem for some vectors. Reducing the number of steps and time in process can support viral stability and improve overall yield. Enveloped vectors and some pseudotyped vectors can be especially unstable and conditions that reduce shear forces can help with yield. Storing of process intermediates and how infectivity is impacted by the hold conditions (temp, pH, etc.) is a major challenge.

Caution must taken be for the introduction of contaminations in the downstream process. The use of lysis buffers, affinity resins, and other methods can introduce contaminants, which would then require the need for analytical assays and the possibility of additional processing steps.

Aggregation can be issue during downstream processing particularly when viruses are released by lysis or when concentrated. Some methods are prone to producing aggregates, such as concentration by ultracentrifugation pelleting. Choosing alternate methods or conditions can help reduce aggregation.

Cost Considerations in Downstream Manufacturing

Cost is a combination of many factors such as, time, labor, operating expenses, and capital expenses. The technology used in downstream processing can impact the timeline of process development, product quality, ease of process scale-up, and cost of goods (COGs).[19] Many of the traditional methods of viral purification are time consuming, complex, have low productivity, are not efficient, and can add substantially to cost.[20] Replacing traditional methods with approaches that are more cost effective, such as chromatography, can lower costs.

photo courtesy of Pall

Implementing newer, more efficient strategies such as IEC or AC that have greater potential for throughput and upscaling can significantly simplify process strategy and increase efficiency. These simple and potentially platformable vaccine or vector purification schemes significantly shorten process development time and reduce COGs. In that regard, the newer affinity chromatography approaches, if applicable to the particular vector, can potentially simplify the process architecture to a single chromatography capture step accompanied only by a clarification step and a polishing step. The use of purification and polishing chromatographic systems can provide quality vectors of high purity as well.[10]

Development and use of lower costs structures such as (semi)-continuous processes systems can result in footprint reduction, increased productivity, automations, reduced inventory and storage, and fewer, unit operations, can reduce costs.[20,47] Reduction of the chromatographic unit operations, such as multi-column systems, and single-use systems can also reduce cost can cost.[20] An additional chromatographic approach to reduce costs is by employing column cycling with expensive resins. When implementing scalable solutions for downstream, such as depth filtration, chromatography etc. it is possible to reduce processing time multiple-fold as well as the process being scalable.

Enabling the use of tangential flow filtration (TFF), which can be scaled up during manufacturing can provide cost savings. In that regard, automated TFF systems are available to control both the transmembrane pressure as well as the level of concentration. Additionally, automation of TFF can provide an ergonomic and secure environment for the operator and facilitate the speed and the upscaling of the process. A 20–80x concentration factor can easily be achieved using these systems.[10]

Areas for Improvement

With respect with downstream there are several areas that need improvement or are in the process of being improved by the industry. Implementation of automation could be a big advancement. Automation is needed in several areas to reduce both labor and costs. Closed systems are needed for larger vectors that where sterilization by filtration is an issue. Closed systems also serve to protect both the product and the operators and will be of large benefit by several measures. In that regard, more development of processes similar to those that have benefited the recombinant protein industry is needed such as disposable technology, and continuous processing methods.

The continued movement away from older methods such as ultracentrifugation to more efficient processes such as chromatography will benefit the industry. Alternatives to traditional chromatography resins that incorporate improved designs for viruses such as nanofiber and macroporous filters show great promise to increase efficiency and scale. Moreover, the costs of some specific resins and filters are quite expensive and could be challenging during the launch of a clinical project[3] Some affinity resins are very expensive.

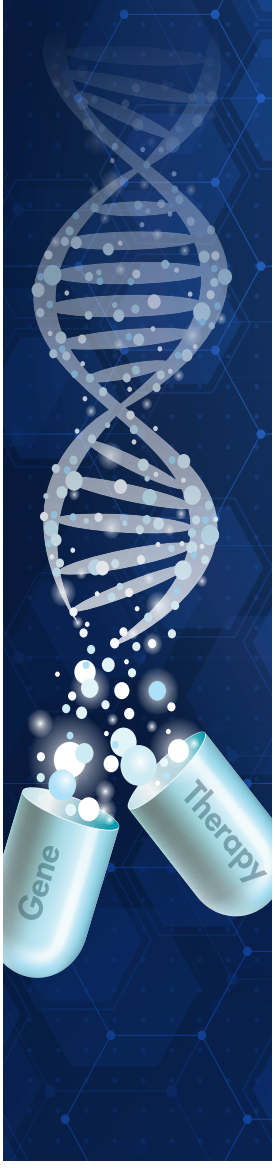
There continues to be a challenge with upscaling. Continued development of upscaling methods through the 2000L scale would benefit the industry greatly. There is some upscaling struggle for empty vs. full AAV separation and there are still several issues that need solutions. For example, volumes of buffers and fractionation could be an issue if the elution gradient is long.

There is an increasing range of options to process and obtain high titers and high-quality batches of viral vectors.

On the analytics side, implementation of at-line or inline analytics will benefit the industry by allowing more efficient time in process and lowering costs. Because the analytics can be quite imprecise and can take a long time to perform, downstream processing can take place “blind”. One of the results of this is that recovery from a downstream process can be very low with some vectors (between 5 – 30%). Another challenge with the analytics is achieving high throughput, which limits efficiency and can also extend development time.

Establishing efficient downstream protocols for LVs has proven challenging. Loss of yield due to virus stability is a crucial bottleneck with the manufacturing of many vectors. As a result, purification and concentration techniques have been often integrated together to create a step-wise downstream approach to minimize losses. Further improvements to enhance yield of less stable vectors, such as low shear systems, new handing methods, and stabilization methods will benefit the industry.

AC shows great promise as it could potentially enable a platform-like approach where a universal downstream scheme is suitable for purifying multiple products simply by switching AC columns. When chromatographic material is packed into highly efficient, single-use columns, process throughput can be further improved and substantial savings in infrastructures, operations and quality procedures can be realized.[48]



One drawback is that approaches that rely on particle charge or affinity to ligand, can be virus/serotype dependent, meaning that a major technological development must be undertaken for each AAV serotype. In addition, it is currently very difficult to separate the full and empty particles using these approaches.

Process Development Timeline

The timeline to develop a downstream process is dependent on many factors such as the type of vector, the intended use, the scale, cost considerations, and the technology available. Independent of the factors involved, reduced developmental timelines are a desirable goal. Moreover, reduced timelines lower cost. Timelines for scientific work on a downstream process for complex molecule can be extensive – several months - depending on the resources available. Technical transfer can add additional time.

One promising approach is that technology is advancing for some vectors that is enabling more "platform-like" or "plug and play" solutions for downstream. This is particularly being seen in IEC and AC chromatography. For example: most AdV 5 vectors should have similar surface properties. In that case, developmental timeline for various AdV 5 vectors could be reduced significantly since only tweaking of the process is needed. Similarly for AAV, the rise of vector-specific AC solutions could potently reduce developmental timelines in that columns can be switched for vectors of different serotype.[19]

Summary

In summary, there is an increasing range of options to process and obtain high titers and high-quality batches of viral vectors. These new developments, particularly in the chromatography approaches, have improved downstream process options, which have in turn enabled gene therapy. There are much needed improvements, especially in the areas of upscaling and lowering cost. Nevertheless, the industry is advancing, and the future is open to new innovations and capabilities.

References:

1 McCarron, A., M. Donnelley, and D. *Parsons, Scale-up of lentiviral vectors for gene therapy: advances and challenges.* Cell GeneTherapy Insights, 2017. **3**(9): p. 719-29.

2 Sommer, J.M., et al., *Quantification of adeno-associated virus particles and empty capsids by optical density measurement.* Mol Ther, 2003. **7**(1): p. 122-8.

3 Merten, O.W., *AAV vector production: state of the art developments and remaining challenges.* Cell GeneTherapy Insights, 2016. **2**(5): p. 521-551.

4 Gao, K., et al., *Empty Virions In AAV8 vector preparations reduce transduction efficiency and may cause total viral particle dose-limiting side-effects.* Mol Ther Methods Clin Dev, 2014. **1**(9): p. 20139.

5 Carmo, M., et al., *Relationship between retroviral vector membrane and vector stability.* J Gen Virol, 2006. **87**(Pt 5): p. 1349-56.

6 Tailor, C., *Challenges and advances in viral vectors.* Cell Gene Therapy Insights, 2016. **2**(5): p. 583-588.

7 Ausubel, L.J., et al., *Production of CGMP-Grade Lentiviral Vectors.* Bioprocess Int, 2012. **10**(2): p. 32-43.

8 Merten, O.W., M. Hebben, and C. Bovolenta, *Production of lentiviral vectors.* Mol Ther Methods Clin Dev, 2016. **3**: p. 16017.

9 White, M., et al., *A guide to approaching regulatory considerations for lentiviral-mediated gene therapies.* Hum Gene Ther Methods, 2017. **28**(4): p. 163-176.

10 Benchaouir, R., *Recent advances in the purfication of adeno-associated virus.* Cell GeneTherapy Insights, 2016. **2**(3): p. 287-297.

11 Dias Florencio, G., et al., *Simple downstream process based on detergent treatment improves yield and in vivo transduction efficacy of adeno-associated virus vectors.* Mol Ther Methods Clin Dev, 2015. **2**: p. 15024.

12 CRS. *CRS-Chemical inspection and regulation service -REACH Restriction.* 2018; Available from: cirs-reach.com/REACH/REACH_Restriction.html.

13 Healthcare, G., *Application Note: Optimization of midstream cell lysis and virus filtration steps in an adenovirus purification process.* 2018.

14 Merten, O.W., et al., *Large-scale manufacture and characterization of a lentiviral vector produced for clinical ex vivo gene therapy application.* Hum Gene Ther, 2011. **22**(3): p. 343-56.

15 Geraerts, M., et al., *Upscaling of lentiviral vector production by tangential flow filtration.* J Gene Med, 2005. **7**(10): p. 1299-310.

16 Cooper, A.R., et al., *Highly efficient large-scale lentiviral vector concentration by tandem tangential flow filtration.* J Virol Methods, 2011. **177**(1): p. 1-9.

17 Potter, M., et al., *Streamlined large-scale production of recombinant adeno-associated virus (rAAV) vectors.* Methods Enzymol, 2002. **346**: p. 413-30.

18 Zolotukhin, S., et al., *Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield.* Gene Ther, 1999. **6**(6): p. 973-85.

19 Zhao, M., et al., *Affinity chromatography for vaccines manufacturing: Finally ready for prime time?* Vaccine, 2018.

20 Silva, R., et al., *Improving the downstream processing of vaccine and gene therapy vectors. Pharm.* Bioprocess., 2015. **3**(8): p. 489-505.

21 Merten, O.W., et al., *Manufacturing of viral vectors for gene therapy: part I. Upstream processing.* Pharm. Bioprocess., 2014. **2**(2): p. 182-203.

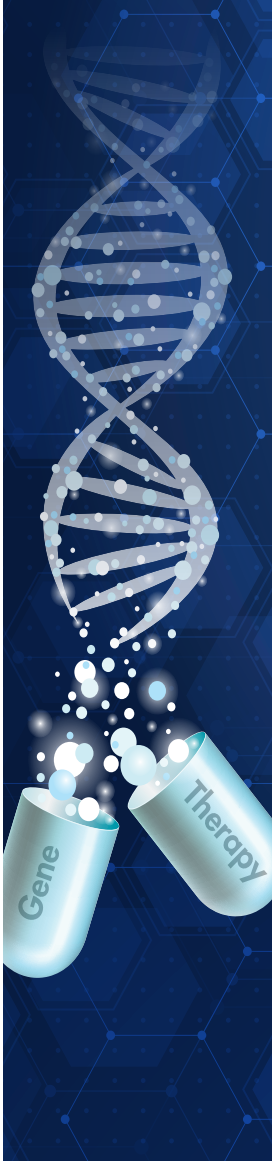
22 Khandelwal, P., *Practical guide: selecting theoptimal resins for adenovirus process purificatioin,* Bio-Rad, Editor. 2016.

23 Okada, T., et al., *Scalable purification of adeno-associated virus serotype 1 (AAV1) and AAV8 vectors, using dual ion-exchange adsorptive membranes.* Hum Gene Ther, 2009. **20**(9): p. 1013-21.

24 Urabe, M., et al., *Removal of empty capsids from type 1 adeno-associated virus vector stocks by anion-exchange chromatography potentiates transgene expression.* Mol Ther, 2006. **13**(4): p. 823-8.

25 Davidoff, A.M., et al., *Purification of recombinant adeno-associated virus type 8 vectors by ion exchange chromatography generates clinical grade vector stock.* J Virol Methods, 2004. **121**(2): p. 209-15.

26 Qu, G., et al., *Separation of adeno-associated virus type 2 empty particles from genome containing vectors by anion-exchange column chromatography.* J Virol Methods, 2007. **140**(1-2): p. 183-92.



References continued

27 Wright, J.F. and O. Zelenaia, *Vector characterization methods for quality control testing of recombinant adeno-associated viruses.* Methods Mol Biol, 2011. **737**: p. 247-78.

28 Manno, C.S., et al., *Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response.* Nat Med, 2006. **12**(3): p. 342-7.

29 Scherr, M., et al., *Efficient gene transfer into the CNS by lentiviral vectors purified by anion exchange chromatography.* Gene Ther, 2002. **9**(24): p. 1708-14.

30 Shambram, P., G. Vellekamp, and C. Scandella, *Purification of adenovirus*, in Adenoviral vectors for gene therapy 2016, Academic Press.

31 Chahal, P.S., M.G. Aucoin, and A. Kamen, *Primary recovery and chromatographic purification of adeno-associated virus type 2 produced by baculovirus/insect cell system.* J Virol Methods, 2007. **139**(1): p. 61-70.

32 Summerford, C. and R.J. Samulski, *Viral receptors and vector purification: new approaches for generating clinical-grade reagents.* Nat Med, 1999. **5**(5): p. 587-8.

33 Segura, M.M., et al., *A novel purification strategy for retrovirus gene therapy vectors using heparin affinity chromatography.* Biotechnol Bioeng, 2005. **90**(4): p. 391-404.

34 Mietzsch, M., et al., *Differential adeno-associated virus serotype-specific interaction patterns with synthetic heparins and other glycans.* J Virol, 2014. **88**(5): p. 2991-3003.

35 Kanlaya, R. and V. Thongboonkerd, *Cellufine sulfate column chromatography as a simple, rapid, and effective method to purify dengue virus.* J Virol Methods, 2016. **234**: p. 174-7.

36 Healthcare, G., AVB Sepharose™ *High Performance*. Data file 28-9207-54 AB.

37 Nass, S.A., et al., *Universal Method for the Purification of Recombinant AAV Vectors of Differing Serotypes.* Mol Ther Methods Clin Dev, 2018. **9**: p. 33-46.

38 Mietzsch, M., et al., *OneBac: platform for scalable and high-titer production of adeno-associated virus serotype 1-12 vectors for gene therapy.* Hum Gene Ther, 2014. **25**(3): p. 212-22.

39 Fisher, T., *CaptureSelect™ AVB Sepharose HP Ligand Leakage ELISA*.

40 Fisher, T. 2017; Available from: thermofisher.mediaroom.com/press-releases?item=122399.

41 Rodrigues, T., et al., *Purification of retroviral vectors for clinical application: biological implications and technological challenges.* J Biotechnol, 2007. **127**(3): p. 520-41.

42 Transfiguracion, J., et al., *Size-exclusion chromatography purification of high-titer vesicular stomatitis virus G glycoprotein-pseudotyped retrovectors for cell and gene therapy applications.* Hum Gene Ther, 2003. **14**(12): p. 1139-53.

43 Burns, J.C., et al., *Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells.* Proc Natl Acad Sci U S A, 1993. **90**(17): p. 8033-7.

44 Jiang, W., et al., *An optimized method for high-titer lentivirus preparations without ultracentrifugation*. Sci Rep, 2015. **5**: p. 13875.

45 Buclez, P.O., et al., *Rapid, scalable, and low-cost purification of recombinant adeno-associated virus produced by baculovirus expression vector system.* Mol Ther Methods Clin Dev, 2016. **3**: p. 16035.

46 Slepushkin, V., et al., *Large-scale purification of a lentiviral vector by size exclusion chromatography or Mustang Q ion exchange capsule.* Bioproc J, 2003. **2**(5): p. 89-95.

47 Hernandez, R., *Continuous manufacturing: A changing processing paradigm, in BioPharm International.* 2015.

48 Jacquemart, R., et al., *A Single-use Strategy to Enable Manufacturing of Affordable Biologics.* Comput Struct Biotechnol J, 2016. **14**: p. 309-18.

Viral Vector Characterization: A Look at Analytical Tools

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Introduction

As cell and gene therapy field continues to push products through the clinical landscape, the need to develop appropriate analytical tools has become increasingly evident. Because of their intended use in patients, gene therapy products must meet rigorous safety guidelines highlighting the importance of well-characterized analytics. The FDA introduced the Process Analytical Technology (PAT) initiative in 2004 for biopharmaceutical manufacturing, to emphasize the importance of in-depth monitoring/testing throughout the production process that goes beyond the traditional approach of process validation and extensive end product testing. The initiative has encouraged pharmaceutical companies to investigate the use of new analytical technologies to perform timely measurements of the critical quality attributes allowing for better understanding and control during the manufacturing process.[1]

For cell and gene therapy, one critical aspect of the supply chain is the vector used to introduce the gene of interest. Most commonly, viral vectors, derived from lentivirus (LV), adenovirus (AV) or adeno-associated virus (AVV) are used for these therapeutics. One of the challenges of characterization and quality control testing of viral vectors is their high degree of complexity. Even recombinant Adenovirus associated virus (rAAV), the smallest and least complex type of recombinant viral vectors, has a structure more complex than the most complex recombinant proteins. Key to the development of large-scale, optimized production, harvesting and purification strategies to meet the demand for viral vectors, is having accurate and reproducible analytical tools in place to monitor quality attributes ensuring a safe, high quality, consistent and efficacious product.

This article aims to provide an overview of analytical methods, with input from industry experts, used to characterize viral vectors to ensure their safety, potency, and purity to keep pace with the demands from the fast-growing field of cell and gene therapy.

A Brief Overview of Viral Vector Manufacturing

As the demand for gene therapy vectors in clinical therapeutics grows, robust, reproducible large-scale manufacturing platforms are needed (Figure 1). This may necessitate changes to:

- Components (i.e. changes in raw materials to improve traceability or compliance)
- Cell lines (i.e. adaptation to suspension cultures, use of microcarriers)
- Equipment (to increase scale of production)
- Manufacturing processes (i.e. optimize methodologies or to integrate automation)
- Manufacturing facilities

Changes in these parameters may require comparability studies to ensure that the safety, identity, purity and efficacy of the product remains unaffected. Scaling up from a research-scale protocol requires process development time to ensure all the critical quality attributes (CQAs) of the therapeutic product are maintained. The CQAs that are important to monitor during viral vector manufacturing include viral potency, identity, quantity, process residuals (i.e. Triton and deoxiribonuclease), aggregation, empty capsids, protein content and product safety. There are different characterization assays available and their applicability depends on the type of virus purified as well as the expression system used.

Gene therapy products must meet rigorous safety guidelines highlighting the importance of well-characterized analytics.

It is important to point out the upstream considerations prior to manufacturing that should be addressed, such as sourcing suitable raw materials (i.e. plasmids, vectors, reagents) and utilizing qualified cell and viral banks. The regulatory bodies mandate that that all components used during the manufacture are qualified and suitable for the intended purpose. After manufacturing, QC testing is performed to ensure the viral vector meets preset specifications for safety, identity, purity, potency, and stability and safety parameters. This information is combined into a release document (a Certificate of Analysis that lists the tests, specifications, and results) which is submitted to regulatory agencies, such as the FDA, for review.

During process development/optimization, the FDA mandates that overall product characterization under cGMP (current Good Manufacturing Practice) follows SISPQ (Safety, Identity, Strength/Potency, Purity and Quality):

- **Safety:** The product does not elicit unexpected side effects when used appropriately in the patient.
- **Identity:** The product is exactly what is described on the label.
- **Strength/Potency**: The product delivers the correct dosage over the shelf-life of the product.
- **Purity:** The product is free from physical, biological and chemical contamination.
- **Quality**: The product is manufactured using established quality systems to ensure product consistency and quality specifications are met.

There are some common quantification methods used by manufacturers to monitor key attributes to ensure specifications are met during the process development phase and beyond. These specifications should be established early on but should also be appropriate to the stage of product development, because release criteria will be refined and tightened as the product moves through clinical phases towards licensing. It is important to stress the need for high quality, properly characterized standards and controls for assay development, assay qualification, and product testing. Implementation of the appropriate standards for comparability studies that conform to cGMP is critical to overall success.

Analytical Assays

Because of the complexity of biomanufacturing of viral vectors, there can variability in the manufacturing that necessitates in-process product characterization to ensure lot-to-lot consistency. Gene therapy products must meet rigorous safety guidelines highlighting the importance of well-characterized analytics. Table 1 summarizes the assays that will be discussed in more detail in this section.

Identity and Potency

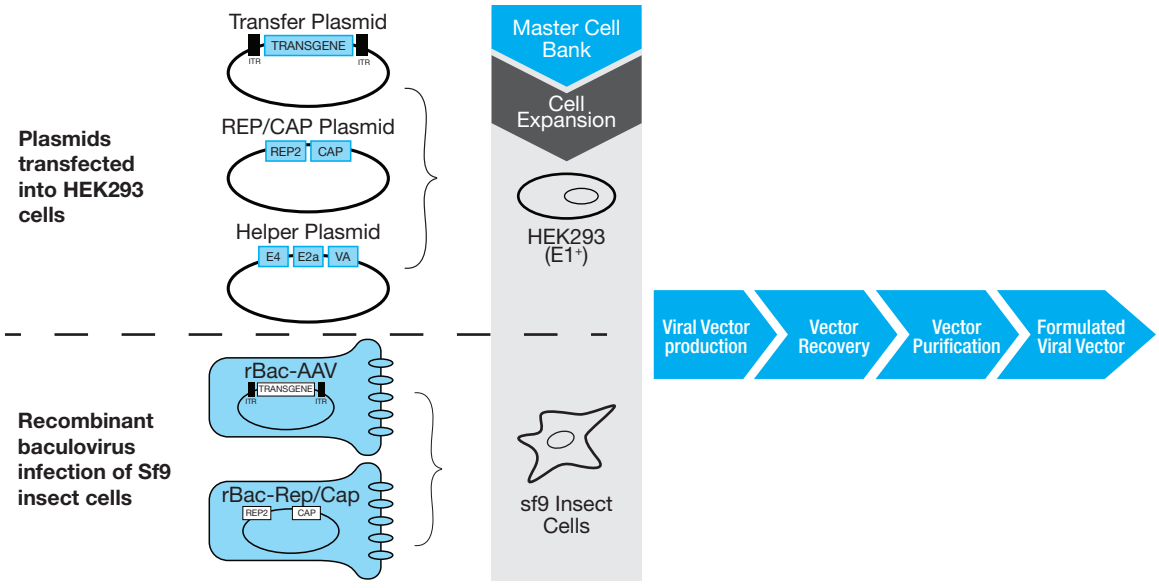
Identity

Tests can be run to ensure that the identity of the viral vector preparation. Assays such as SDS-PAGE, mass spectrometry, immunoblotting and ELISA can be used to look at viral protein expression. The proper number, molecular weight, and stoichiometry of the viral proteins can be used to positively identify the vector. Conversely, the vector genome can be evaluated using PCR or high throughput NGS (next generation genome sequencing) to ensure positive identity.

Physical Titer

Physical titer calculates the total number of alive and dead viral particles present and is expressed as the number of viral particles per mL (VP/mL), or for AAV as genome copies per mL (GC/mL). Clinical dosing of rAAV therapeutics is usually based on physical titer, thus having accurate quality control methods that determine the amount of vector being administered is important.[2] There are a variety of methods used to determine the physical titer of virus based on quantifying the concentration of viral genomes or viral proteins.

Figure 1.
Simplified workflow for the manufacturing of AAV vectors through transfection with vector plasmids of a mammalian production cell line (i.e. HEK293). As an alternative to the AAV production in mammalian cells, the baculovirus-Sf9 platform has been notably established as a GMP-compatible and scalable system. Recombinant baculovirus seeds are used to infect insect cells (Sf9) followed by vector production, purification and final vector processing.



Historically, DNA hybridization assays were used to quantify the number of viral genomes present in a preparation. Plasmid and unpackaged vector DNA from a sample are removed with nucleases so only viral genomes inside intact capsids will be titered. The vector DNA signal is compared to the signal from the plasmid DNA standard curve and extrapolated to determine a vector genome titer.

The Optical Density ($A_{260/280}$) Assay measures the concentration of viral DNA and protein. It is a physical assay measuring the concentration of viral particles (VP).

Several groups have investigated the NanoSight® device (Malvern Instruments Ltd) for viral particle quantification. This real-time nanoparticle visualization system is based on a laser-illuminated microscope technique detecting Brownian motion of virus particles in solution. While this method has utility, its technical limits are reached when host cell debris or other background particles are present.[3]

High-performance liquid chromatography (HPLC) is also a rapid method to quantify total viral particles since the column allows for the separation of intact virus particles from other cellular contaminants or virus particle fragments.[4]

The most widely used methods to quantify packaged AAV vector genomes are real-time PCR-based assays (i.e. qPCR, digital drop PCR) because they are robust, easy, fast, and convenient. The vector genome titer is determined by using appropriate primers and fluorescent probes. However, there can be substantial variability in the assay because PCR is affected by many experimental factors. This can affect the accuracy of the titer, which in turn affects the vector dosing in both preclinical and clinical settings.[5] Therefore, assay design and incorporation of appropriate standards needs to be well thought out.

Infectious or Functional Titer

Infectious or functional titer is always more accurate than physical titer because it measures how much virus can infect the target cell. It is always lower than physical titer, typically by a factor of 10 to 100-fold.

However, functional titer usually takes much longer to determine and is sometimes not practical during intermediate productions stages of the viral vector production.

Functional titer is expressed as transducing units per mL (TU/mL) for lentiviral or retroviral vectors. Adenoviral vector titer is generally expressed as plaque-forming units per mL (PFU/mL) or infectious units per mL (IFU/mL). The latter term is used for viral vectors that do not lyse cell membranes and therefore are not compatible with the plaque assay.

The viral plaque assay is one of the most widely used methods to determine infectious titer. Serial dilutions of the virus are used to transduce a permissive cell line. The cells are infected at very low multiplicity of infection (MOI) to ensure that most transduced cells are infected by a single viral particle for accurate quantitation. Viruses propagate in the infected cells eventually causing cell lysis, spreading to neighboring

Table 1. Overview of Analytical Methods used in Viral Vector Manufacturing

Quality	Attribute	Technique
Identity	Confirm presence and identity of viral vector	SDS-PAGE, Mass spectrometry (MS), Western blot (immunoblot), Genome sequencing (NGS), PCR
Potency	Physical viral titer	DNA hybridization, Real-time PCR (qPCR, ddPCR), Optical density ($A_{260/280}$), NanoSight, HPLC
	Functional viral titer	Plaque-forming assay, Fluorescence foci assay, $TCID_{50}$ (end point dilution assay)
Purity	Process-related impurities	MS, Chromatography, TEM
	Host cell-related Impurities	Host cell DNA/RNA: Picogreen, DNA Threshold assay, qPCR, Host cell proteins: ELISA,TEM
	Capsid content (empty: full capsids)	TEM, AUC
Safety	Sterility	Standard sterility tests (EP 2.6.1, USP71)
	Endotoxin	LAL method (EP 2.6.14, USP85), Rabbit pyrogen assay
	Mycoplasma	PCR, Cell cultured based-assays
	Replication Competent Virus (presence of *rep* or *cap* sequences)	Southern blotting, qPCR
	Adventitious Agents	I*n vivo* and *in vitro* assays
Stability	pH	Potentiometry
	Osmolality	Osmometry
	Aggregate formation	Light microscopy, DLS, SEC-MALS, TEM, AUC, FFF-MALS

cells eventually forming visible holes (or plaques) on the cell monolayer. Enumeration of the total plaques at particular dilution provides the number of plaque-forming units (PFU) per mL.

An Endpoint Dilution Assay ($TCID_{50}$) measures the infectious virus titer by quantifying the amount of virus required to kill or to produce a cytopathic effect in 50% host cells. Although both the plaque-forming Assay and Endpoint Dilution assay give the infectious titer, they are scored by the human eye and subject to human and procedural variations.

The Immunofluorescence Foci Assay (IFA) also known as fluorescent foci assay FFA, shows good correlation with the plaque assay and captures viruses that do not lyse cell membranes (which are not compatible with plaque assays) or do not exhibit detectable cytopathic effects (to perform $TCID_{50}$). IFA utilizes antibody-based staining methods to detect virally infected cells. The results of IFA are expressed in focus forming units (FFU) per ml, or IFU/mL. Overall, IFA is considered a more rapid and sensitive method to determine infectious titer than traditional plaque assays or $TCID_{50}$.[6]

Vectors carrying reporter genes such as the green fluorescent protein (GFP) can be easily titrated by flow cytometry in transduction units (TU/mL).[5]

Purity

Impurities can be derived from the host cell system within which the vector product is generated or from the downstream vector purification. Sources of host cell-related impurities are residual host cell proteins and nucleic acids derived from the production cells while other are process-related residuals from the cell culture medium (i.e. bovine serum albumin) and downstream purification processes (i.e. detergents and chromatography resin components). It is important to establish a good understanding of potential residuals early on in process development to establish acceptable tolerances in the final product.

Host Cell-Related Impurities

The quantification and removal of host-cell impurities is important since certain host cell molecules can have toxic effects in the final drug product or can act as an adjuvant to stimulate an anti-vector immune response.

Host cell nucleic acids (DNA/RNA) can be copurified with the viral vector product during production. PicoGreen and DNA Threshold Assays are two of the historical methods to determine residual DNA levels. More recently, quantifying host cell nucleic acids has been done using real-time or quantitative PCR (qPCR).

Detection of host cell-associated proteins is typically done with ELISA, where antibodies react with host proteins. One important point to consider is that certain host proteins are not recognized by commercial ELISA kits. For example, there are many variants of HEK293 cells used for virus production that can potentially express proteins not detectable by standard kits. Host cell protein impurities can vary in quantity and proportion from lot to lot during production.

These impurities can be also be successfully detected using transmission electron microscopy (TEM). MiniTEM™ by Vironova, is a novel, benchtop TEM platform that can inform on quality attributes during process development and be easily incorporated into existing QC workflows. The sample is prepared, mounted to a grid support with negative staining (to improve contrast) prior to microscopic analysis to detect impurities (i.e. proteasomes and host cell debris). The advantage over traditional TEM is that image acquisition and, more importantly image analysis, from the MiniTEM™ is automated allowing for the quantitative analysis of a greater total number of particles for more accurate and statistically significant results in shorter time.

Process Impurities

Process impurities are usually present in trace amounts but it is important that they meet pre-set safety guidelines. Mass Spectrometry (MS) and chromatography methods are widely used to identify detergents and organic solvents in the vector preparation.

The quality of vector preparation can also be analyzed through direct visualization with MiniTEM by Vironova. The system can assess viral particle morphology such as capsid integrity and detect particle aggregation.

Capsid Content

During the production of viral vectors, there exists a population of capsids that have failed to package the vector DNA. These empty capsids can represent up to 90% of the crude harvest for rAAV vector preparations.[5,7] In addition, incomplete encapsidation is also observed, leading to capsids containing truncated vector genomes or capsids that contain illegitimate, non-vector DNA (from plasmids, cells, or helper viruses). While their impact on the efficacy of gene therapy is not fully understood, these capsids are considered an undesirable quality attribute[5]. They are a source of unnecessary, potentially antigenic material, which could elicit an unwanted immune response in the patient. For example, in a clinical study for hemophilia B, capsid-specific cytotoxic T lymphocytes were implicated in an adverse immune response after successful expression of human coagulation factor IX using recombinant AAV2-mediated gene transfer.[7] Therefore, the capsid content (ratio of empty to full capsids) is an important

attribute to monitor in viral vector preparations. There are two main methods under investigation to evaluate capsid content: transmission electron microscopy (TEM) and analytical ultracentrifugation (AUC).

TEM (cryoTEM): Cryogenic TEM is a method where the biological specimens are vitrified by rapid freezing in liquid ethane thereby preserving their native structure. Analysis by cryoTEM combined with Vironova Analyzer Software (VAS), a stand-alone 21 CFR part 11 compliant software for TEM based image analysis of nanoparticles, is a GMP-validated method to identify and relatively quantify empty and fully packed particles. When imaged, there is a clear, morphological distinction between packed and empty viral particles where the empty particles appear as open circles with well-defined edges and no internal density and packed particles appear circular with a dense inner core. Currently, CryoTEM™ is performed as a service by Vironova.

AUC: Analytical ultracentrifugation (AUC) is a powerful tool to distinguish and quantify different AAV species by either mass (sedimentation velocity) or density (sedimentation equilibrium). Beckman Coulter introduced the first commercial AUC sample characterization instrument. Their newest platform, the Optima AUC is the most sensitive and robust to date, offering multi-wavelength absorbance capabilities to properly quantify both genomic DNA and viral capsid content in a single experiment.

AUC is a technique that monitors the sedimentation of particles over time under a centrifugal field, providing critical information on particle molecular weight, homogeneity, and interactions with other particles and itself. The empty capsids have a different density and/or mass than the correctly packaged, full particles and those partially filled, incomplete particles allowing baseline separation on the basis of hydrodynamics under centrifugal force. The main advantages are that it is highly reproducible, can quantify viral particles in the formulation buffer (no sample preparation required), works independent of the serotype or the size and type of transgene (single or double stranded DNA) and also requires no standard for comparison making it easy to incorporated into the manufacturing workflow with little to no development time.

Each technique differs in the physical measuring principle and has its own strengths and weaknesses, but it is valuable to obtain complementary data from orthogonal methods to increase confidence in the data particularly for a quality attribute where regulatory requirements have yet to be established.

Safety

Safety testing is conducted to ensure that process intermediates or final product is free of detectable contaminating agents that can pose risks to patients. The key parameters are sterility, endotoxin, mycoplasma, adventitious virus and the presence of replication-competent virus.

Sterility testing to assess bacterial or fungal burden of the product should be performed according to USP or EP recommendations.[5,8]

Endotoxin can be detected using the Limulus Amebocyte Lysate (LAL) Assay or the rabbit pyrogen test. The presence of mycoplasma DNA can be detected by PCR-based methods in Vero cells or cell culture-based methods where the viral vector preparation is inoculated into growth medium to detect any growth of mycoplasma.

Mycoplasma testing should be performed on the product at the manufacturing stage when the test is most likely to detect contamination; such as after pooling of cultures for harvest, but prior to cell washing to ensure that it is not present in the preparation prior to downstream purification steps that could filter it out.

photo courtesy of Vironova

The *in vivo* and *in vitro* assays to test for adventitious virus are designed to detect the presence of infectious viral agents of human or animal origin. The *in vitro* assay can be performed using a number of cell lines (e.g. human, murine, bovine, porcine, etc) while rodent and embryonated hen's eggs are commonly used for the *in vivo* assay.[9]

While viral vectors are engineered to be replication defective, generation of replication-competent virus can still occur during vector manufacturing by means of spontaneous recombination events within

the producer cells. This is obviously undesirable because many viral vectors are created from pathogenic viruses posing a significant safety concern for gene therapy. Therefore, it is important to test for the presence of potential replication-competent virus in the vector preparation prior to final product release.

For quantification of replication competent AAV (rcAAV), the vector sample is incubated on a permissive cell line in the presence of adenovirus or an alternative source for helper factors.[10] After three rounds of infection using cell lysate from the previous round, amplification of AAV rep or cap sequences is detectable by Southern blotting or qPCR. Vector production systems currently in use have been optimized to reduce rcAAV to levels ≤1 rcAAV per 10^8 vector genomes.[11]

Stability Testing

Stability testing of the purified viral vector at the proper storage temperature, formulation, and fill volume, and in the container used for patient doses (i.e. final product) should be performed to ensure that quality attributes are maintained and that the quantitative values (i.e. infectivity titer) are not adversely affected over the span of its shelf-life. This can include examining physical parameters such pH/ osmolality or the presence of protein aggregates. If these unwanted aggregates are not removed, they will become part of the finished biopharmaceutical drug product and may alter its bioactivity and/or cause adverse biological effects when administered to a patient (i.e. immunogenicity).

Aggregates can vary greatly in size from a few nm (subvisible) to a few mm (visible) in diameter and their formation during production, storage, shipment can be caused by numerous stressors.[11] Since no single technique is able to cover this large size range, a combination of several techniques is typically necessary to evaluate this parameter. These techniques rely on different separation and detection principles ranging from basic techniques such as visual or microscopic inspection (visible particles), to more sophisticated methods to detect subvisible particles, such as dynamic light scattering (DLS), size exclusion chromatography (SEC), TEM, AUC and field flow fractionation with multi-angle static light scattering (FFF-MALS).

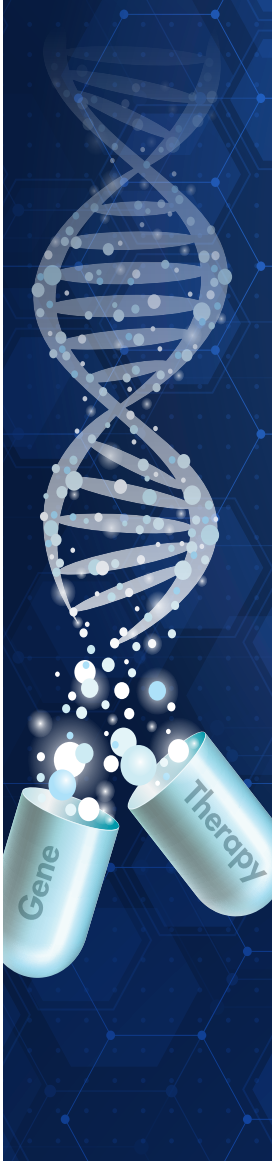
SEC is a long-standing industry standard as an inexpensive, high throughput method to quantify aggregates at various steps of protein product development. Ideally, SEC separates proteins and their aggregates based on their hydrodynamic volume. However, numerous factors have impacted the accuracy of analysis using SEC that typically arise from nonspecific interactions between the aggregates and the chromatographic material in the HPLC column or the column frits.[11]

More recently, hydrodynamic techniques such as FFF-MALS and AUC are being implemented as orthogonal methods to validate SEC aggregation data. SV (sedimentation velocity)-AUC is a robust tool to quantify aggregates because, unlike SEC, matrices are not involved in the analysis and the aggregate content often can be analyzed in the original protein formulation.[11] In addition to supporting efforts in the development of accurate SEC methods, SV-AUC can be used to generate extended biophysical characterization information on potential biopharmaceuticals to support formulation/process development and comparability studies.

Conclusion

This article has outlined an extensive list of analytical tools that can be applied to different stages of viral vector manufacturing. However, it should be emphasized here that not all of the analytical controls are suitable for every stage of product development and the extent/amount of testing should reflect the stage of development. A distinction can be made between techniques that would be routinely used in a QC testing for product release versus those for extended characterization during product development, whether it be due to the reproducibility or expertise/time differences required to execute the assays. For example, applying extended characterization assays during formulation and process development is necessary to gain product knowledge and to establish specifications early on, but often times, there is a limited quantity of the final, purified viral vector which may influence which assay and how many are needed to provide sufficient data on key quality parameters for an acceptable production lot.

As the cell and gene therapy field focuses on product commercialization, it is prudent for manufacturers and therapy owners to ensure all processes and procedures are adapted to meet the current regulatory requirements. This poses challenges since the parameters for what makes a cell and gene therapy product safe for patients are still evolving and the needs of the field are diverse. It is also vitally important to harmonize analytical methods to ensure quality attributes meet acceptance criteria for a specific application (this can vary based on the disease and method of delivery into the patient). Some of the analytical technologies described may still need fine-tuning but show that there is a vested interested to ensure the safest product possible can be manufactured for these therapies. Thus, improving viral vector production must be regarded as a collaborative effort of stakeholders and regulatory agencies.



References:

1 Kramberger, P., L. Urbas, and A. Strancar, *Downstream processing and chromatography based analytical methods for production of vaccines, gene therapy vectors, and bacteriophages.* Hum Vaccin Immunother, 2015. **11**(4): p. 1010-21.

2 D'Costa, S., et al., *Practical utilization of recombinant AAV vector reference standards: focus on vector genomes titration by free ITR qPCR.* Mol Ther Methods Clin Dev, 2016. **5**: p. 16019..

3 Heider, S. and C. Metzner, *Quantitative real-time single particle analysis of virions.* Virology, 2014. **462-463**: p. 199-206.

4 Kuhn, I., et al., *High-performance liquid chromatography method for rapid assessment of viral particle number in crude adenoviral lysates of mixed serotype.* Gene Ther, 2007. **14**(2): p. 180-4.

5 Dorange, F. and C. Le Bec, *Latest developments in vral & non-viral vector manufacturing.* Cell GeneTherapy Insights, 2018. **4**(2): p. 119-129.

6 Kumar, P., *Methods for rapid virus identification and quantification.* MATER METHODS, 2013. **3**: p. 207.

7 Wright, J.F., *Transient transfection methods for clinical adeno-associated viral vector production.* Hum Gene Ther, 2009. **20**(7): p. 698-706.

8 Husain, S.R., et al., *Gene therapy for cancer: regulatory considerations for approval.* Cancer Gene Ther, 2015. **22**(12): p. 554-63.

9 Mandel, R.J., C. Burger, and R.O. Snyder, *Viral vectors for in vivo gene transfer in Parkinson's disease: properties and clinical grade production.* Exp Neurol, 2008. **209**(1): p. 58-71.

10 Schnodt, M. and H. Buning, *Improving the quality of adeno-associated viral vector preparations: the challenge of product-related impurities.* Hum Gene Ther Methods, 2017. **28**(3): p. 101-108.

11 den Engelsman, J., et al., *Strategies for the assessment of protein aggregates in pharmaceutical biotech product development.* Pharm Res, 2011. **28**(4): p. 920-33