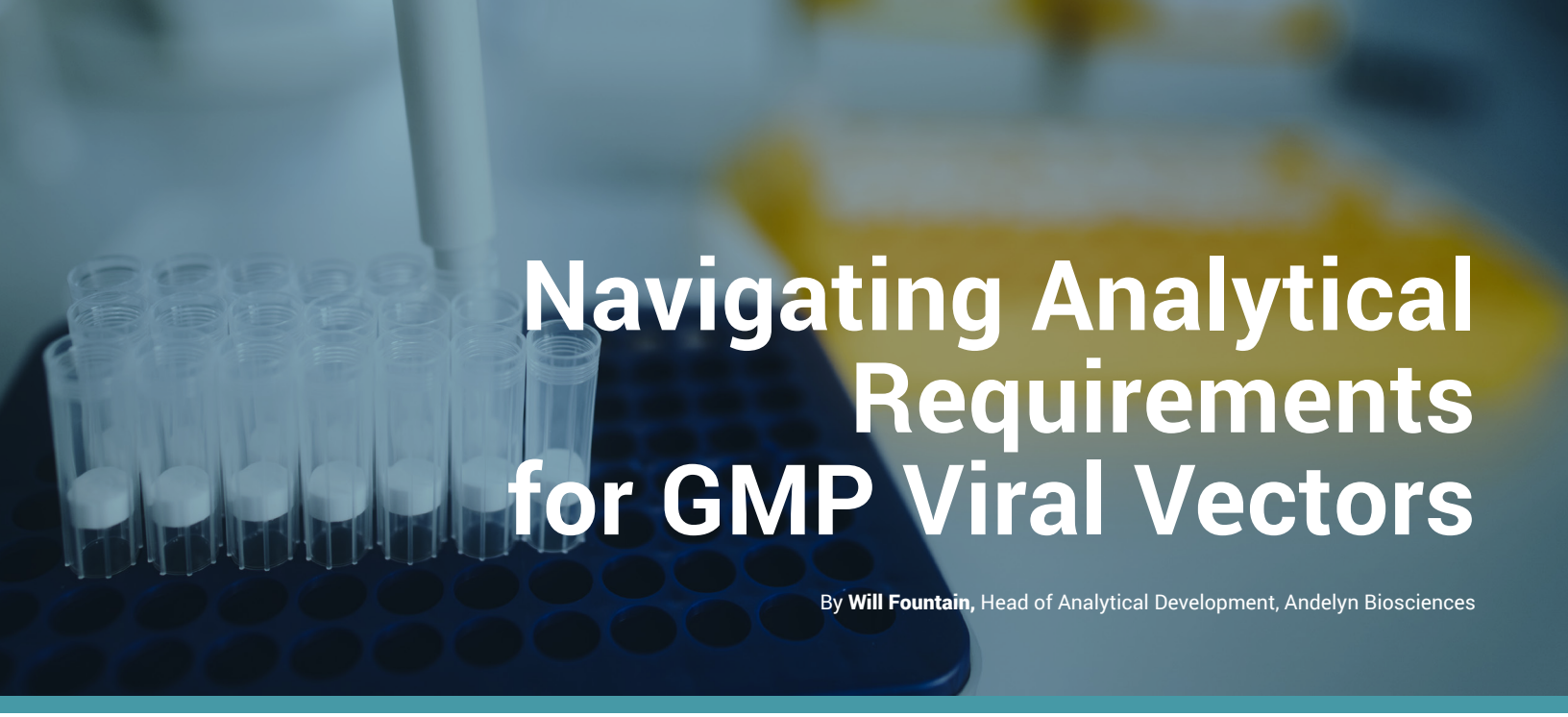

Navigating Analytical Requirements for GMP Viral Vectors

By **Will Fountain,** Head of Analytical Development, Andelyn Biosciences

The analytical requirements for viral vectors used to deliver gene therapies are complex and constantly evolving. The keys to success, in addition to the necessary extensive experience in analytical development for viral vectors, is establishing appropriate strategies beginning with fundamental testing, expecting additional regulatory requests, being prepared for assay variability, recognizing the limits of current understanding, and ensuring that comparability studies will be possible by retaining material from preclinical batch runs and stability studies.

Many Analytical Development Challenges

The cell and gene therapy field is evolving rapidly, and, as the understanding of optimal approaches to cell and gene therapy expands and viral vector engineering advances, the supporting analytics must be modified and, in some cases, new techniques and methods must be developed.

The nature of viral vectors adds further complexities. There are multiple serotypes of adeno-associated virus (AAV) vectors, and each one can behave differently and require slightly different production processes and associated analytical methods. The same is true for vectors based on different viruses, such as AAV and lentiviral (LV) vectors.

In addition, each manufacturer has its own strategies for performing key analyses. They may even have their own proprietary analytical reagents or report results using different units. Even within companies, different sites may use different approaches. Furthermore, there are no monographs that establish limits for quality and purity, so defining release specifications for viral vector products can be somewhat challenging, and specifications can thus differ widely.

Comparability can be a real challenge, both for assays performed at different sites or by different companies and specifications for products that are transferred from one contract manufacturing partner to another, typically when going from early- to late-phase development.

This need to demonstrate comparability is a key driver for pursuing analytical development early in the development process. The generic platform-based assays used in R&D are not the same as the product-specific assays required for GMP production, but switching from one assay to another requires a demonstration of comparability, which can be complicated. Developing some GMP assays earlier in the process can shorten timelines and eliminate some of this complexity. However, it is also important to not develop product-specific assays too early, because if the product evolves many times before it reaches initial GMP production, those assays may no longer be relevant.

There has been significant discussion in the industry about establishing standardized approaches to analytics for cell and gene therapy, but little progress has been made for the methods necessary to determine most critical quality attributes (CQAs).

A NOTE ABOUT PCR TECHNOLOGIES

Until recently, quantitative polymerase change reaction (qPCR) technology was the gold standard for many different assays used to evaluate gene therapy products. That began to change in 2014 with application of digital PCR (dPCR) to gene therapy analysis. Today, there is tremendous focus on dPCR, because it is much more precise than traditional qPCR technologies and utilizes absolute and not relative quantification.

Although it is not yet a mature technology, dPCR is helping to reduce the variability and increase the accuracy of many gene therapy assays. Currently, one equipment vendor has the majority of the market share, with multiple companies introducing their own versions. However, none of the platforms are equivalent, and it is not possible to develop an assay and run it on any one of these instruments. Sample preparation is not yet well defined, either; different groups using different processes get different results. Given the rapid expansion of the dPCR field, these issues should be resolved sooner rather than later.

Some assays, such as those for sterility and endotoxins, can be readily standardized, but, because each serotype and transgene is different, each viral vector presents a different analytical target. In addition, once a group settles on a specific analytical method, it is typically difficult to get them to switch to another.

While standardization is attractive from a business perspective, because it helps eliminate the risks associated with using proprietary tests conducted by only at specific CROs or at certain testing facilities, the scientific hurdles are significant. There is hope, though, that, as viral vector science continues to evolve and researchers rapidly expand their knowledge and understanding, opportunities for standardization will be identified.

Start with the Fundamentals

The solution to overcoming these challenges is to begin with the fundamentals. On the basis of our many years of experience in viral vector development and manufacturing, Andelyn Biosciences has identified a standard testing package for GMP viral vector production comprising a couple dozen different analyses. The package consists of the minimum set of tests that the U.S. Food and Drug Administration (FDA) and other regulatory agencies expect for viral vectors intended for use as delivery vehicles for phase I gene therapies.

Expect Regulatory Agency Requests

With the cell and gene therapy field evolving so rapidly and knowledge expanding at a tremendous pace, it is not unusual, even when results from our standard set of tests are included when filing an Investigational New Drug (or similar) application, that the FDA and other regulatory authorities request additional testing for later-phase studies.

There are two possible strategies that can be adopted to meet these requirements:

1. **DEVELOP AND PERFORM THE ADDITIONAL TESTS** before phase I to minimize work later on and reduce the time needed to get to the later trial stage or
2. **PUSH THE WORK OFF** to facilitate quick entry into the clinic and avoid unnecessary testing if the product is unsuccessful.

Get Clarity on Variability

Unlike biologics like monoclonal antibodies or traditional small molecule pharmaceuticals, for which most analytical results are constant, there is significant variability in the assays for viral vectors and formulated gene therapy products. Even physical titer assays can be more variable than those who work with traditional pharmaceuticals may be used to. Analytical experts in the field are working to address this issue, but, at present, the variability in viral vector assays is simply a limitation of the technology that must be managed.

No Such Thing as Too Much Retains

While a few large biopharmaceutical manufacturers and even some biotech startups have elected to build their own viral vector manufacturing capacity due to the limited number of contract manufacturers with this capability, much of viral vector development and manufacturing is outsourced. Projects may even move from a small R&D lab to a phase I producer and then to a manufacturer capable of producing GMP material for late-stage trails and commercial launch.

Transfer of AAV production from one site to another, as mentioned above, requires demonstration of comparability of the analytical assays that were initially used. Bridging studies involving the analysis of retains from previous productions and material produced at the new site are necessary to demonstrate equivalence of the method or to identify the need for a correction factor.

Therefore, it is essential to keep retains from all preclinical batches and material used for toxicity studies. Contract manufacturers like Andelyn cannot develop custom assays for each client; it is simply time- and cost-prohibitive. We use platform technologies that may not be fully optimized for each application but are sufficient to provide the basic information needed to get a project started. If the product shows promise and moves into human clinical trials, we eventually create those product-specific assays and conduct studies using retains to compare and correlate the results.

The problem arises when a client comes to a new contract manufacturing partner having already produced preclinical batches, either themselves or at another service provider. Often, the results of assays performed at the new CDMO do not match those of the historical assays. Comparability studies are essential, but often no retains are available, and trying to figure out how the methods compare can be challenging. The key message: retaining as much product as possible early in the process can avoid considerable headaches down the road.

More Understanding Needed

Despite constant advances in viral vector manufacturing and analytics, there are still several areas where greater understanding of assay results and their impacts is needed.

For instance, the limit for residual host-cell DNA in a vaccine dose is 10 ng or less according to WHO guidance. Should this limit also apply to cell and gene gene therapy products? A vaccine dose is typically 0.5–1 mL, while a subretinal gene therapy dose is typically <100 µL and a systemic intravenous dose may be tens or hundreds of mL. In addition, there can be host-cell DNA inside and outside of the viral particles. While manufacturing steps can degrade the non-encapsulated host-cell DNA, there are no methods for removing the material packaged inside the particles. That level could be much greater than 10 ng, because AAV is known to promiscuously package genomic DNA. Clearly, there can be a large disconnect between the actual results and the expectations in this area if the vaccine guidance is assumed to apply to gene therapies as well.

Aggregation of AAV particles is another example. While there are many methods for measuring aggregation (e.g., dynamic light scattering, size-exclusion chromatography), there is little knowledge or understanding

FOCUS ON NEXT-GEN SEQUENCING

Next-generation sequencing of viral DNA is very complex. It provides large quantities of data that must be parsed into a reportable result.

A few different platforms exist today. Illumina-based short-read sequencing, PacBio long-read sequencing, and Oxford Nanopore sequencing, which is the newest, are three of the leaders.

The Illumina technology is most widely used and excellent for understanding variant levels, with million-fold coverage of viral DNA. It provides accurate assessment of a particular position in the theoretical sequence, allowing developers to determine how many DNA strands contain nuclear types other than what is expected. However, it does not provide a good picture of what the entire DNA molecule looks like. Viral DNA can be full-length in the viral particles or it can be truncated to varying degrees, with near-full-length DNA more biologically efficacious than a DNA strand that is only half the intended length. The latter will be missing a huge chunk of the transgene and could either be nonfunctional or even potentially create undesirable reactions *in vivo*.

That is where the PacBio platform shines, because it sequences entire DNA strands, not just sets of chopped up pieces. This technology enables cell and gene therapy developers to gain a better understanding of the population of DNA molecules by sequencing many different individual DNA strands.

The reality is that no one platform is superior in all cases. Both short-read and long-read technologies have roles to play, and there are clear advantages and disadvantages to both. The real challenge remains making sense of the data that are generated in order to present those data as a quantifiable result.

For the Illumina platform, for instance, a result in one region might be that 99% of the reads match the expected sequence, while in another 90% match, which might have resulted simply because it is a GC-rich region or has a difficult secondary structure that decreases the fidelity of the sequencing data. This is just one example to demonstrate how very challenging it is to obtain an unambiguous, quantifiable, and reportable result using any of these technologies.

Traditional Sanger sequencing technology provides a single output sequence that can be compared to a theoretical sequence to determine whether there is or is not a match. But Sanger sequencing really struggles with secondary structure in DNA, and it is often impossible to get sequencing all the way through inverted terminal repeats (ITRs) or GC-rich regions. As a result, the data may match the theoretical sequence for the areas it covers, but they do not cover 100% of the theoretical sequence. In addition, Sanger sequencing cannot determine whether the primers are annealing to the left or right halves of self-complementary viral DNA, resulting in an incomplete picture.

THERE HAS BEEN SIGNIFICANT DISCUSSION IN THE INDUSTRY ABOUT ESTABLISHING STANDARDIZED APPROACHES TO ANALYTICS FOR CELL AND GENE THERAPY, BUT LITTLE PROGRESS HAS BEEN MADE FOR THE METHODS NECESSARY TO DETERMINE MOST CRITICAL QUALITY ATTRIBUTES (CQAS).

regarding the potential negative impacts of aggregation in general, the level of aggregation, or the types of aggregates (dimers or trimers vs. aggregates comprising hundreds of viral particles). Due to this lack of understanding, no limits have yet been established for aggregation in cell and gene therapy products. The FDA is more frequently asking to inspect aggregation data, but it is not specifically required at this point. The agency wants researchers to start studying the issue in order to gain that understanding, but it seems inevitable that this will be a requirement down the road.

DNA contamination is a third area that requires more development and understanding. We do not yet know the impacts of various types of DNA contamination or different degrees of contamination or which methods are best for assessing contamination. Most AAV production platforms today use a two- or three-plasmid transfection process. Each plasmid possesses its own contaminants, including residual *E. coli* DNA and trace DNA contaminants originating from raw materials.

With next-generation sequencing, it is possible to detect DNA contaminants present at one part per billion, but DNA is everywhere, from the lab to the sequencing technology itself. That raises the question of the meaning of trace DNA detection. In addition, the risk of a very small amount of contaminating DNA that carries a toxic gene or a pathogenic gene would be greater than the risk presented by a larger amount of non-problematic human DNA.

While there are no defined limits at this time, in discussions Andelyn has heard the agency indicate that no amount of DNA contamination in plasmids is acceptable. But the issue is not a black or white one.

Comprehensive Support from Andelyn

Andelyn Biosciences was recently spun out from Nationwide Children's Hospital (NCH) as a for-profit entity providing contract development and manufacturing services to cell and gene therapy companies. We began as a small research-grade vector producer and expanded to GMP vector production, initially supporting NCH's clinical programs but ultimately taking on projects from external clients, including foundations, biotech startups, and Big Pharma companies. So, while Andelyn is a new name, we have experience that dates back to the 2006 founding of the NCH GMP vector production facility and R&D vector production since the late 1990s.

During this journey, we scaled up our manufacturing processes and converted our existing R&D assays to assays appropriate for large-scale GMP manufacturing of AAV vectors and developed new, additional assays as needed. Through that process, we have learned countless lessons about productivity and cost-efficiency. We also have worked at the R&D scale with approximately 15+ different AAV serotypes on a semi-regular basis, a significant number of which have migrated to GMP production, so we have knowledge about which serotypes are best for different applications.

Our early R&D experience also sets us apart from other viral vector CDMOs. A lot of these other firms touch early-phase R&D production, but it is truly the foundation from which Andelyn grew, and that activity remains an important part of the business. The majority of products we manufacture for clinical use originate in that research production facility, whether they come from an academic client or Big Pharma.

When those projects move on to the clinic, the same analytical technologies and scientific methods used at the research scale are employed through clinical manufacturing and commercial production. Scientifically, they are fundamentally the same and share the same workflows, reagents, and other factors, which helps to demonstrate comparability over the life span of those products.

In addition, we can leverage not only the viral vector core research experience through GMP production, but also plasmid R&D and GMP manufacturing capabilities. We have had the privilege to watch patients benefit from the gene therapies we make, as shown by our name, which is a portmanteau of two NCH patients named Andrew and Evelyn. This gives our employees an invaluable perspective on the impacts these novel treatments can have.

On the analytical side, we recently split our quality control group into complementary QC and analytical development groups. Quality Control will continue to focus on the GMP analytics required to support our clinical-phase productions. The new Analytical Development group will focus on three different areas. The first is true assay creation, development, and implementation using new technologies and instruments. The second is routine R&D quality control and testing of research samples from the various R&D groups within Andelyn (viral vector, plasmid, process development), serving those internal clients on a daily basis. The third is assay qualification and validation. The development team will create assays, and the R&D QC team will use those assays routinely in an R&D capacity. When they are ready for prime time, they will go through qualification and validation as part of that transfer to the GMP QC team.

ABOUT THE AUTHORS

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Will Fountain has lead the analytical testing at Andelyn since the company's initial founding in 2006 as part of Nationwide Children's Hospital. He has been in the pharma and biotech industry for more than 20 years gaining experience from the University of Kentucky College of Pharmacy, Ventaira Pharmaceuticals, and Battelle Memorial Institute. His area of expertise is in the development of assays specific to adeno-associated viruses.

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