CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Breaking viral vector bioanalysis barriers with centrifugal force

Patricia Ahrweiler

Advances in viral vector gene delivery systems, particularly adeno-associated virus (AAV) and lentivirus (LV), have accelerated the development of new cell and gene therapies. Regulatory programs for accelerated review have added to the demand for the manufacture of viral vectors, exceeding capacity and creating backlogs. Improvements in analysis speed, accuracy, precision, and dynamic range are potential targets for accelerating production timelines. Plate-based enzyme-linked immunosorbent assays (ELISAs), commonly used in analysis of viral vector titer, purity, and potency, have laborious and time-consuming manual processing drawbacks as well as long processing times and poor precision. A novel automated microfluidic, compact disc (CD)-based immunoassay format that uses centrifugal force to precisely control the flow of sample and reagents has been notably effective in accelerating bioanalysis of antibody-based therapeutics with high-precision results. One-hour assay run times and wide dynamic ranges accelerate workflows, and 10 µL sample requirements minimizes consumption of limited production material. These dramatic immunoassay improvements are expected to alleviate the analytical delays in viral vector manufacturing.

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Cell and gene therapies have brought the promise of effective, even curative, therapies for acute or chronic genetic diseases where no treatment or only long-term symptomatic treatment exists. Over the past decade, advances in viral vector delivery systems, along with programs facilitating development of gene therapies and immunotherapies, have fanned the flames of an already active field [1,2]. Programs being actively promoted by regulatory bodies such as the Medicines and Healthcare Products Regulatory Agency (MHRA) and the United States Food and Drug Administration (FDA) include expedited development and review, fast-track designation, accelerated approval, and breakthrough therapy designation, all focused on accelerating therapies treating unmet medical

needs. This further availability of shortened development timelines and financial benefits has attracted pharma and biotech companies to invest in the development of genetic therapies with rigorously scheduled development plans.

These incentive programs have also had a noticeable effect on the expansion of the gene therapy pipeline – in just the last 2 years, the number of cell and gene therapies in clinical development has grown from 289 to 362, or a 25% increase [3]. In addition, the number of gene therapy clinical trials is expected to continue its meteoric rise from 775 in 2019 to >4,000 ongoing or completed in 2020 [4], projected to be nearly 11,000 by 2026 [5,6]. Lentivirus (LV) and adeno-associated virus (AAV) vectors are the most successful delivery systems for cell and gene therapies, and recent regulatory approvals utilizing these vectors for generation of chimeric antigen receptor (CAR) T cell cancer immunotherapies include: Tecartus™ (brexucabtagene autoleucel, Kite, a Gilead company) for treatment of mantle cell lymphoma, Yescarta® (axicabtagene ciloleucel, Gilead Sciences, Inc.) for treatment of relapsed or refractory large B-cell lymphoma, and Kymriah® (tisagenlecleucel, Novartis) for treatment of B-cell acute lymphoblastic leukemia.

AAV vectors have risen in popularity for systemic delivery of genetic therapies primarily because of their small size, tissue tropism, and low immunogenicity. Recent AAV approvals include Luxturna® (voretigene neparvovec-rzyl, Spark Therapeutics) for *RPE65* mutation-associated retinal dystrophy, and Zolgensma® (onasemnogene abeparvovec-xioi, AveXis, Inc) for the treatment of pediatric patients with spinal muscular atrophy (SMA).

BREAKING THE MANUFACTURING BOTTLENECKS

The popularity of AAV and LV vectors, along with the recent swell in the development pipeline for cell and gene therapies, has outstripped manufacturing capacity and created backlogs in the bioreactor production, along with compressed development and production timeline pressures [5,7].

Analytical characterization of viral vector identity, potency, purity, safety, and stability during manufacturing can contribute significantly to the overall timeline for vector therapeutic production [8]. Physical titer determination is an integral component of process monitoring from culture growth to downstream purification, and for quality control (QC) testing of product attributes of potency and purity. These assays may be included as critical quality attributes (CQAs) identified by the manufacturer as "a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality" as defined by the FDA [9].

THE NEED FOR IMPROVED ACCURACY, SPEED & LOW VOLUMES

Improvements in analytical techniques, specifically in analysis speed, accuracy, and sample volume consumption, have been identified as a target for helping to meet compressed timeline demands for the production of viral vectors [10–12].

Data quality

As the number of clinical studies for AAV and LV-based gene therapies grows, the FDA increasingly emphasizes the importance of vector titer assay reproducibility and the measurement of full:empty AAV capsid ratios to facilitate dose comparison between clinical programs. In a recent workshop, a target of ≤15% precision for measurement of empty AAV capsids was set as reasonable starting with early phase studies in order to compare clinical study efficacy and adverse events between studies. The discussion during the workshop indicated that improvements in

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the reliability of analytical methods for viral vector titer or a switch to newer technologies may be needed to reach this goal [10,13].

Speed

Compressed timelines for gene therapy manufacturing intensify the need for faster analytical approaches to characterize the therapeutic to verify quality and titer [7]. Many existing methods are time-consuming, producing results long after the time window for adjustment of growth or purification conditions has passed. Long assay times also add to the bottleneck in the development and production of new products [5,8].

Sample volumes

Regulatory demands have increased the number of analyses required for characterization, putting an even higher premium on analytical techniques requiring less sample. While suspension-adapted cultures of HEK293 or insect cell (Sf9) baculovirus expression system cultures facilitate process scale-up, the complex process of 3-plasmid transient transfection (AAV rep and cap genes, adenovirus helper genes, and therapeutic transgene) continues to hinder batch size, batch yields of under 100 mg of virus typical [14]. It was estimated by one CMC specialist that almost half of the viral vector production batch may be consumed during QC bioanalysis steps [10]. Any increases in viral vector production for preclinical or clinical studies will be a noticeable improvement.

MEETING IMMUNOASSAY CHALLENGES WITH (CENTRIFUGAL) FORCE

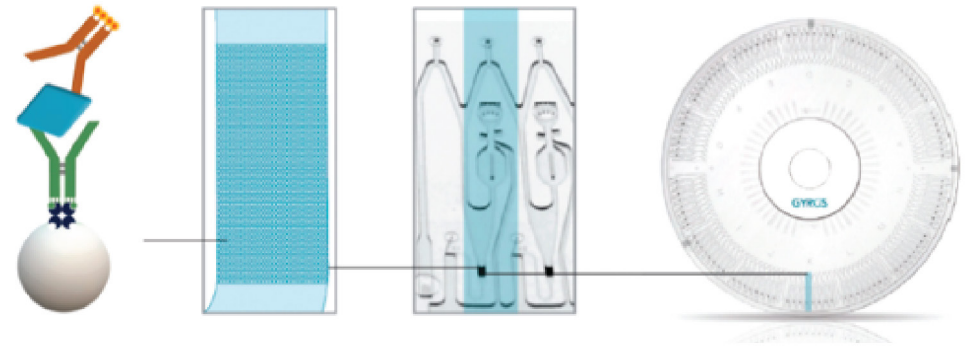
Immunoassays have long been an integral component of preclinical and clinical biotherapeutic development, as a well-established method for detection and quantitation of antibody-based therapies in pharmacokinetic (PK) and anti-drug antibody (ADA) immunogenicity. Immunoassays are also routinely used analytical assays during vector manufacturing and bioprocessing for titer, empty/full capsid ratio, and process impurity analysis [15]. Traditional plate-based immunoassay formats such as ELISAs are fraught with drawbacks of extensive manual manipulations, long incubation times, and high sample and reagent usage all stemming from the assay design – adhering the capture reagent to the bottom of flat well microplates and the addition or removal of assay reagents and wash buffers to or from the top of the well.

Immunoassay technologies that go beyond the microplate look to address these drawbacks. A microfluidic, CD-based immunoassay format that uses centrifugal force to precisely control the flow of sample and reagents to automate assay steps has been notably effective in accelerating bioanalysis of antibody-based therapeutics. The microfluidic technology integrated in Gyrolab® platform (Gyros Protein Technologies) with 96 or 112 flow through streptavidin-coated bead-based affinity columns for parallel assay automation, eliminates the need for lengthy incubation times (Figure 1). Laser-induced fluorescence (LIF) data is collected from each microcolumn during the run, to complete automate immunoassays with data production in about one hour. Control and analysis software designed for 21 CFR Part 11 compliance ensures that assays can be transferred to good laboratory or good manufacturing practice (GLP, GMP) environments.

This automated, microfluidic format with an affinity flow-through column format facilitates high binding capacity, resulting in a large dynamic range and shortens sample-contact time, minimizing assay susceptibility to matrix interference. These substantial improvements in immunoassay speed (4x faster), dynamic range (1-2 log expansion of dynamic range), and sample volume (20x less) are summarized in Table 1.

FIGURE 1

Gyrolab® Bioaffy™ CD-based microfluidic immunoassay design utilizing a 15 nL affinity capture column, streptavidin beads, and microstructures in a circular array for precise, automated liquid movements using centrifugal force.



Parallel processing of Gyrolab CD-based immunoassays on streptavidin beads within the affinity capture column uses centrifugal force and capillary action to precisely control the flow of reagents and samples over the column. On-column laser-induced fluorescence results are read automatically, and results are ready to analyze at the end of the run. The short contact times minimize matrix interference and dramatically shorten assay times.

VIRAL VECTOR TITER ANALYSIS: BREAKING BARRIERS

Total LV vector titer is typically monitored in-process during production and purification steps by quantitation of free p24 antigen, a component of the LV capsid (Figure 2). Advances in immunoassay p24 titer dynamic range, speed, and sample consumption have been made with the availability of Gyrolab p24 Titer Kit. Immunoassays measuring total LV vector titers using Gyrolab CD-based platform have been shown to cover a broad analytical range (0.2–1000 ng/mL), with 96 data points collected in 80 minutes, requiring less than 10 µL of sample. Intra- and inter-run precision run in duplicate in six runs on four instruments by three operators of ≤5.3 %CV [16] demonstrated an extremely robust assay as shown in Table 2.

Quantitation of AAV vectors during downstream processing can be complicated by the presence of empty and partially filled capsids and the need for serotype-specific assays [17]. Several analytical methods are routinely used for vector quantitation, all with distinctive drawbacks. The most widely used, quantitative real-time polymerase chain reaction (qPCR) is hampered by amplification

TABLE 1

Performance of Gyrolab® AAVX capsid titer immunoassay exceeds ELISA performance and suitability for bioprocessing development.

	ELISA	Gyrolab
Sample volume required	100-200 µL	8 µL
Number of hands-on steps	5	1
Total assay time	4 hours	1 hour
Dynamic range	1-2 logs	>3 logs

When compared to ELISA kits, Gyrolab microfluidic immunoassays greatly reduce the sample volumes, hands-on time required, and overall assay time, while expanding the assay dynamic range. These dramatic improvements in assay performance and sample consumption meet the demands for advances in vector titer bioanalysis required by gene therapy compressed production timelines and limitations on batch yields.

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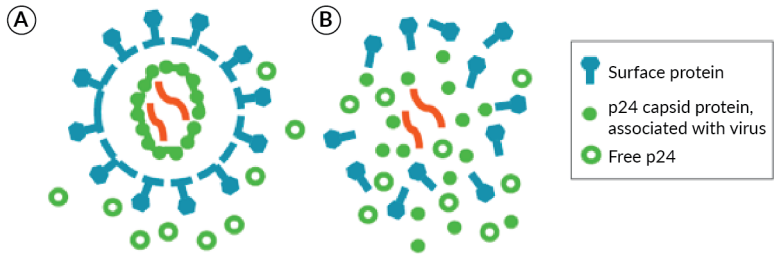
FIGURE 2

Lentiviral titer measurement using p24 immunoassays requires lentiviral particles A) to be disrupted with Triton X-100 detergent B) producing free p24 capsid proteins.



inefficiency, inhibitors, and a standard curve requirement [18] although the increasing adoption of droplet digital PCR (ddPCR) eliminates the standard curve requirement and is less subject to sample inhibition and amplification inefficiency. ELISAs detecting assembled AAV capsid proteins (of various serotypes) are commonly used for total AAV capsid quantitation but have the typical plate-based assay drawbacks of narrow dynamic range, long assay times, and involve many manual interventions.

AAV total capsid titer quantitation using the microfluidic format Gyrolab AAVX Titer Kit immunoassay has been shown to significantly improve data quality over plate-based methods by expanding the dynamic range, from 1.5 logs for a commercially available ELISA AAV2 kit to 3 logs for the Gyrolab AAVX Titer Kit (Figure 3) [19]. Functional aspects of the Gyrolab AAVX Titer Kit were also found to be highly beneficial to analysis workflows: assay time was shortened 4-fold, with one-hour assay times, and the volume required of limited AAV batch samples was shown to be reduced 10-fold to under 10 µL. Matrix-tolerant Gyrolab flow-through immunoassay formats have also demonstrated robustness towards bioprocess samples in host-cell protein characterization [20], AAV titer using commercially available ELISA kit reagents on Gyrolab platform [21], and using the Gyrolab AAVX Titer Kit (customer feedback, data not shown).

TABLE 2

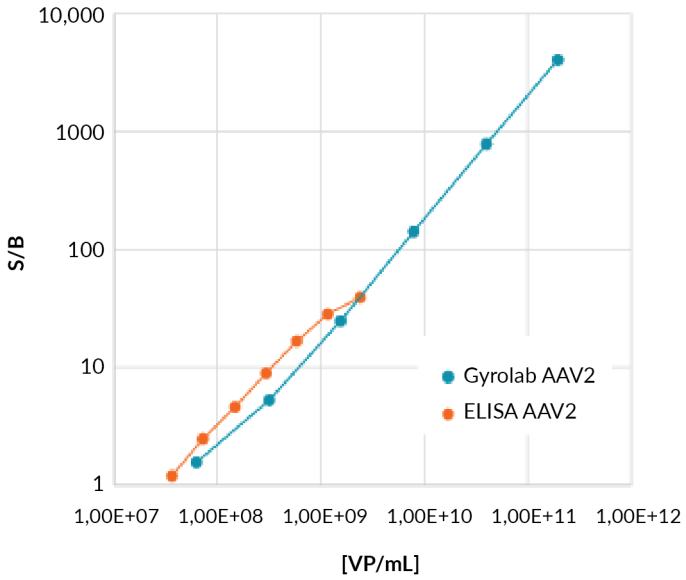
Intra- and inter-run precision for the Gyrolab® p24 Titer Kit standard curve samples.

	Expected conc (ng/mL)	Average measured conc (ng/mL)	Intra-run[1] CV (%)	Inter-run[2] CV (%)
Blank	0			
Standard 1[3]	1250	1250	3.6	3.1
Standard 2	250	251	2.3	1.9
Standard 3	50	50	2.8	2.7
Standard 4	10	10	2.9	2.8
Standard 5	2	2	1.7	2.1
Standard 6	0.4	0.4	2.0	1.8
Standard 7	0.08	0.08	5.0	5.3

Data for standard curve samples over the assay working range were run in duplicate in six runs on four instruments by three operators. (Six duplicate runs were performed on four different instruments, or N=12 per standard concentration). The intra- and inter-run precision was well under 10% (1.7–5.3%), demonstrating an extremely robust assay. The microfluidic design, flow-through affinity column, and automated assay all contribute to the reproducibility of assay results from run to run.
[1]Intra-run CV (%) = standard deviation of response divided by mean response from one run performed in duplicates.
[2]Inter-run CV (%) = standard deviation of means from six runs performed in duplicates divided by mean response for the six runs.
[3]Purified recombinant p24 standards diluted in assay buffer.

FIGURE 3

The dynamic range of AAV2 capsid titer immunoassay is expanded into higher concentration ranges with Gyrolab® AAVX Titer Kit compared to ELISA.



The broad dynamic range of Gyrolab AAV immunoassays reduces the frequency of sample re-runs and dilutions. Expansion of Gyrolab AAV immunoassay ranges to almost 2 logs higher concentrations is especially useful for high-titer AAV batch productions. Gyrolab AAV2 immunoassay was performed per the Gyrolab AAVX Titer Kit instructions. ELISA was performed per kit instructions (PROGEN). Duplicate data points were collected for AAV2 standards (Sirion Biotech GmbH) diluted 1:5 from 2.0E11 VP/mL (Gyrolab) and 1:2 dilutions from 2.4E09 for ELISA. (S/B, signal/background; VP/mL, viral particles per milliliter).

Gyrolab AAVX Titer Kits provide a single reagent set solution for most AAV serotypes, compared to the need for different detection reagents for each serotype in commercially available ELISA kits. Resolving the need to source multiple detection antibodies for different AAV serotypes, the Gyrolab AAVX Titer Kit incorporates Thermo Scientific™ CaptureSelect™ anti-AAVX (ThermoFisher Scientific) ligand that has binding selectivity and affinity for a range of AAV serotypes, with specificity towards assembled capsids. The biotinylated CaptureSelect anti-AAVX ligand as capture reagent and Alexa Fluor® 647-labeled CaptureSelect anti-AAVX as detection reagent was shown to be suitable for quantitation of AAV serotypes 1–8 and rh10 (Figure 4).

Combining qPCR or ddPCR techniques to measure vector genomes with ELISA for total capsid titer to determine AAV full-to-empty capsid ratios has been shown to be an effective and higher throughput method over transmission electron microscopy (TEM) and analytical ultracentrifugation [22] although ELISA has generally been considered to be an imprecise approach and limited to single AAV serotype assays. Gyrolab immunoassays for total capsid titer overcome these limitations can provide complete and rapid quantification of full-to-empty capsid ratios for accurate titer analysis, enabling data-driven decision-making relevant to production and bioprocess timelines.

IMPURITY ANALYSIS WHERE SPEED & DATA QUALITY COUNT

Process-related impurities present in biologics are highly regulated because of their immunogenic potential and associated risks to product safety, efficacy, and quality. Assays for measuring complex heterogeneous mixtures of culture-related host-cell proteins (HCPs) need to be robust and reproducible to meet regulatory

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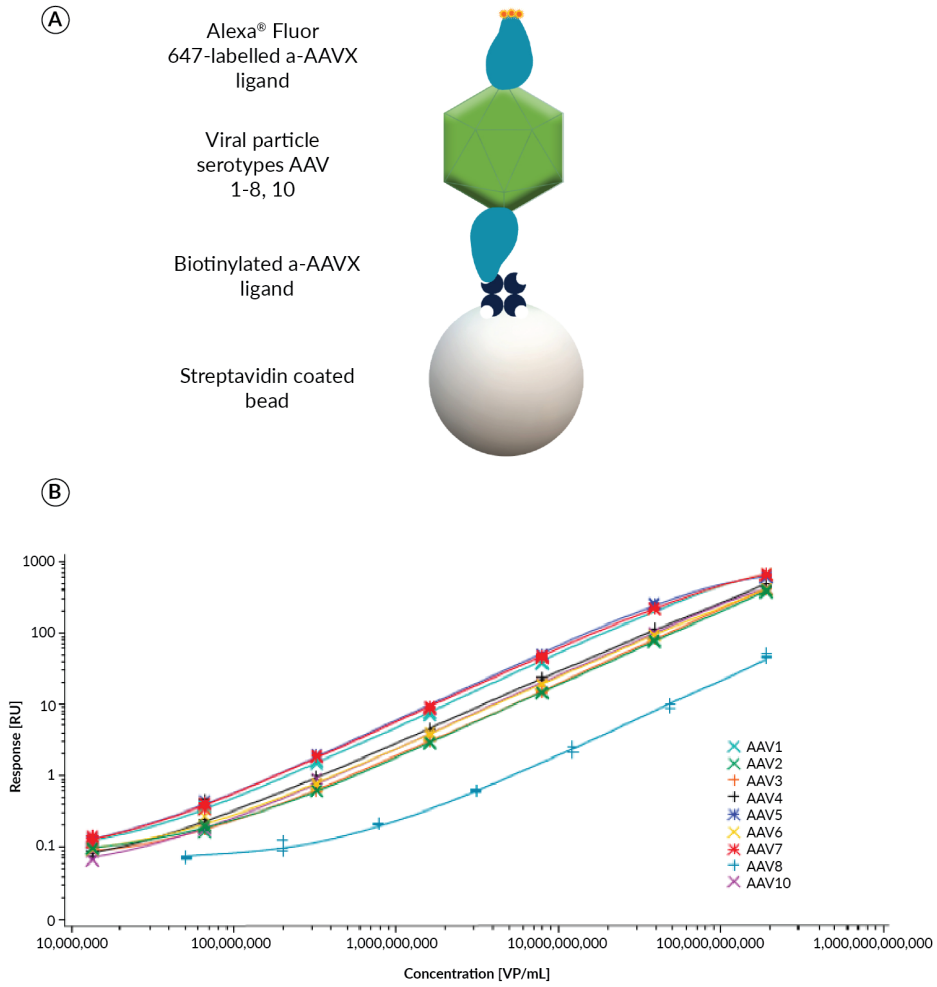
requirements for analysis of samples throughout manufacturing and bioprocessing.

Human embryonic kidney (HEK) 293 or HEK 293T adherent or suspension cell lines often are used to produce AAV and LV vectors for cell and gene therapies and for viral vector vaccines. ELISAs are the most commonly used method for HCP characterization of HEK 293 AAV or LV bioprocess samples but have drawbacks of high assay variability and low productivity. Thus, repeat analyses are common, delaying project decisions and lot approvals. Gyrolab immunoassays in a sandwich assay format using anti-HEK 293 HCP antibodies (Cygnus Technologies) as capture and detection reagents have been developed, delivering data for HCP analysis over a broad working range of 2–10,000 ng/mL. The broad assay range minimizes sample repeats, and the 1-hour assay time allows higher throughput

FIGURE 4

A) Gyrolab® AAVX Titer Kit with one set of capture and detection reagents were used B) for standard curves of AAV serotypes 1–8, and AAVrh10.



One of the challenges of measuring AAV capsid titer using ligand-binding assays has been the need for specialized detection reagents directed towards individual serotypes, and the difficulty in sourcing and evaluating these reagents for use. A) The Thermo Scientific™ CaptureSelect™ anti-AAVX (ThermoFisher Scientific) ligand incorporated into a sandwich immunoassay format as both capture and detection reagent provides binding and detection of 9 AAV serotypes (AAV1-8 and AAVrh10) in a single assay format with one set of reagents. B) Standard curves using commercially sourced AAV standards (Sirion Biotech GmbH) diluted in buffer for serotypes AAV1-8 and AAVrh10 demonstrated a 3.5 log dynamic range. Gyrolab AAV titer immunoassays were performed using the Gyrolab AAVX Titer Kit with duplicate data points according to the kit instructions.

analyses to keep up with the large number of samples generated during bioprocessing, with up to 960 datapoints/day.

SUMMARY

Improvements in bioanalysis approaches are urgently needed to meet the demands of compressed development timelines for gene therapies and backlogs in manufacturing pipelines. Immunoassays, traditionally using plate-based methods, are an attractive target for improvements in assay time, dynamic range, and sample volume requirements. Significant advances in these areas have been made by the microfluidic, nanoliter-scale Gyrolab immunoassay platform, producing assay data in one hour with a wide dynamic range, and consuming under 10 µL of sample. These dramatic immunoassay improvements are expected to alleviate the bioanalysis timeline delays in viral vector manufacturing.

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AUTHORSHIP & CONFLICT OF INTEREST

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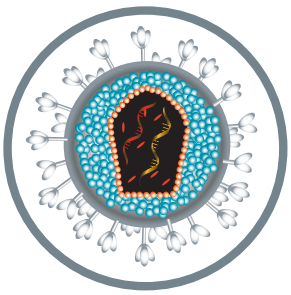
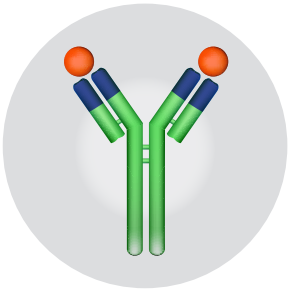


AAV and Lentiviral Titer Determination

Bioprocessing Impurity Analysis



Immunogenicity Analysis

Biomarker Panels

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