c&en BRANDLAB

Waters™ | WYATT TECHNOLOGY

Light-scattering techniques to characterize therapeutic gene vectors

Table of Contents

Introduction

The idea that we could rewrite our genetic code to cure diseases once seemed purely in the realm of science fiction. Yet scientific advances over the past 2 decades are rapidly making gene therapy a reality.

Over 1,000 clinical trials involving gene therapy are underway, according to clinicaltrials.gov. The US Food and Drug Administration has predicted that, starting in 2025, between 10 and 20 cell and gene therapy treatments could reach the market each year.[1]

The advent of safe and effective delivery systems, or vectors, that contain and deliver nucleic acids is being hailed as an "inflection point" for gene therapy.[1] This advent has created a surge of new development activity for vectors such as adeno-associated viruses (AAVs), lentiviruses, adenoviruses, and lipid nanoparticles (LNPs).

Because gene therapy technologies are inherently heterogeneous products, they present unique characterization challenges. For example, gene therapy production yields particles of varying sizes, and not all particles end up filled with nucleic acid cargo.

The success and safety of gene therapy programs depend on analytical methods for quality control across all stages of development and production. Ideally, these methods are rapid, cost effective, easy to use, reproducible, and highly reliable.

Advanced techniques for nanoparticle analysis are coming of age. Light-scattering analysis—either as a stand-alone method or combined with separation techniques—can quantify critical quality attributes (CQAs) such as particle size, concentration, and loading. The fast characterization of small lot-to-lot variations that these methods provide supports quick and thorough product development, process optimization, and quality control to help ensure safe and effective gene therapies.

This e-book will illustrate how light-scattering and size separation methods are poised to help accelerate and safeguard gene therapy programs throughout development and into the clinic.

References

1. US Food and Drug Administration, "Statement from FDA Commissioner Scott Gottlieb, MD, and Peter Marks, MD, PhD, Director of the Center for Biologics Evaluation and Research, on New Policies to Advance Development of Safe and Effective Cell and Gene Therapies," press announcement, Jan. 15, 2019, https://www.fda.gov/news-events/press-announcements/statement-fda-commissioner-scott-gottlieb-md-and-peter-marks-md-phd-director-center-biologics.

Chapter 1

Overview of gene vectors and analytical techniques for their characterization

Gene therapies fall into two primary categories. In the first category, a patient directly receives therapeutic nucleic acids, delivered in vectors such as adeno-associated viruses (AAVs), adenoviruses, and lipid nanoparticles (LNPs).

In the second category, ex vivo therapy, a patient's cells—most often from blood or bone marrow—are isolated, genetically manipulated in the laboratory, and then returned to the patient. Lentiviruses are commonly used to introduce genes to cells in ex vivo therapies.

Despite the many advances in gene therapeutics, the efficient, reliable, and effective delivery of genetic material using viruses or LNPs remains technically challenging. To ensure safety and reproducibility, several key parameters need to be monitored and fully understood throughout development and production. These characteristics may become critical quality attributes (CQAs) for the purposes of regulatory filings with the US Food and Drug Administration.

Robust, easily implemented analytical techniques are thus a core need in gene therapy development and production. The separation and light-scattering techniques described in this chapter—especially powerful in combination—characterize and analyze gene therapeutics at all stages of production, from research and product development to process development, manufacturing, and quality control.

Conventional analytical techniques

Whether genetic material is to be delivered via a viral vector or by LNPs, assessing total titer and payload is critical for accurate and repeatable dosing. The following are techniques that are conventionally employed for gene vectors, along with some benefits and limitations. These methods can be used alone (in batch) or in combination with a separation such as size-exclusion chromatography or field-flow fractionation.

UV–vis absorbance

Ultraviolet–visible (UV–vis) methods typically measure absorbance at 280 nm

or 260 nm to quantify protein and nucleic acid content. These techniques are useful for detecting nucleic acid cargo in capsids or LNPs. However, they have been shown not to correlate perfectly with payload analysis, such as AAV capsid content or LNP loading, and can suffer from artifacts for particles larger than ~50 nm in diameter, such as AAV aggregates and LNP or other viral vector products.

Fluorescence

Fluorescence is helpful in detecting specific compounds, especially those in low abundance. As with UV–vis, it can be difficult to quantify quality attributes by fluorescence spectroscopy because the response can vary in a size- and composition-dependent manner.

PCR-based methods

The concentration of nucleic acid in viral and nonviral gene vectors can be determined by quantitative polymerase chain reaction (qPCR) or droplet digital PCR (ddPCR). For payload analysis, including AAV capsid content and LNP encapsulation efficiency, PCR-based methods must be combined with other techniques that measure total titer or total particle concentration.

Microscopy-based methods

Electron microscopy can directly visualize gene therapy vectors and may be able to differentiate between loaded and unloaded particles. Sample preparation can be time consuming and require specialized equipment and personnel.

Separation techniques

Because of the inherent heterogeneity of preparations, separation is often the first step to characterizing a gene therapeutic. While many separation approaches exist, this e-book will focus on the particular utility of size-exclusion chromatography and field-flow fractionation, both of which are amenable to coupling with light-scattering instrumentation.

Size-exclusion chromatography

Size-exclusion chromatography (SEC) is widely used for the analysis of biologics and is also applicable to smaller gene vectors. Separation by SEC works best for particles smaller than 40–60 nm in diameter.

Field-flow fractionation

Field-flow fractionation (FFF)[1] is a separation technique that uses a narrow channel without a stationary phase and is effective for separating species that are 1–1,000 nm in diameter. Particles suspended in liquid are gently focused against a semipermeable membrane by a cross flow, perpendicular to the membrane. A second channel flow moves solvent along the channel with a parabolic flow profile (figure 1). The combination of forces separates particles by size. Because smaller particles diffuse higher in the channel than larger particles do, they elute first; larger particles elute later.

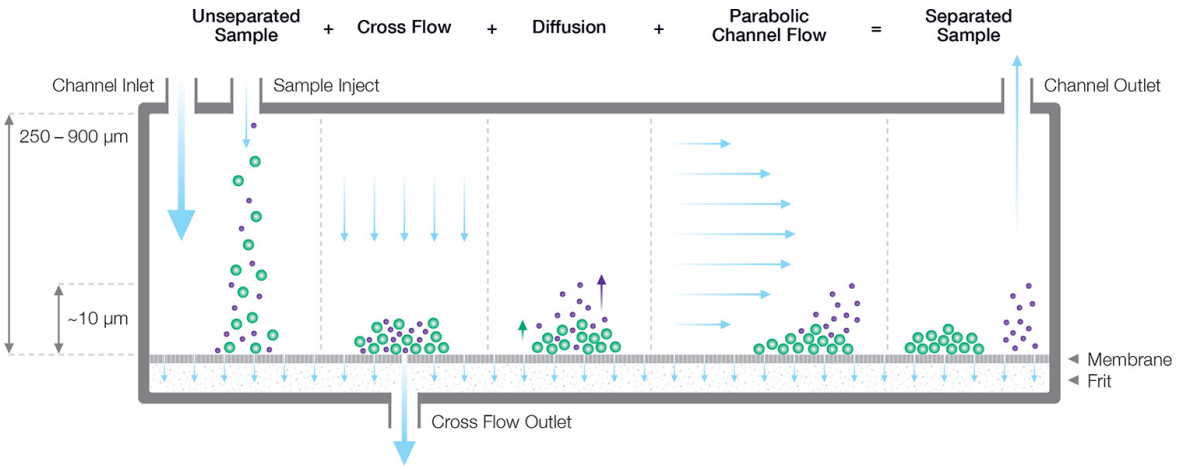


Figure 1: Field-flow fractionation separates particles by size using cross flow, diffusion, and a parabolic solvent front. Smaller particles (purple) diffuse higher in the channel against the cross flow compared with larger particles (green), thus experiencing a faster channel flow and eluting earlier. *Credit: Wyatt Technology*

Light-scattering analytical techniques

Light-scattering offers simple, automated, and robust methods for characterizing and quantifying gene therapy vectors. Light-scattering measurements can be employed independently, but their use on line and downstream of a separation technique like FFF or SEC, combined with UV and differential refractive index (dRI) detection, offers significant advantages over conventional analytical techniques. These methods can be implemented to quantify particle size, concentration, payload, and stability.

Dynamic light scattering

Dynamic light scattering (DLS)[2] measures fluctuations in light-scattering intensity resulting from the diffusion of molecules and particles in solution. It is frequently used in batch mode (i.e., without a separation technique) to measure hydrodynamic radius (R_h) from less than 1 nm and up to several microns. Even without separation, DLS can provide information about the presence of aggregates and other contaminants in as little as 30 s or less. DLS combined with simultaneous static light scattering (SLS) can provide particle concentration.

Multi-angle light scattering

Multi-angle light scattering (MALS)[3,4] measures the intensity of scattered light at multiple angles to determine the absolute molar mass and size of molecules and particles in solution, as well as particle concentration. MALS is most frequently combined with a separation technique, like SEC or FFF, and one or more concentration detectors, including UV–vis and dRI, as shown in figure 2. Both SEC-MALS and FFF-MALS can provide detailed quantitation of gene therapy vectors, payload analysis, and other quality attributes in a single 30–60 min assay.

Figure 2: Examples of systems combining size-based separation with in-line light-scattering analysis. Left: an SEC-UV-MALS-dRI system consisting of industry-standard high performance liquid chromatography modules, a DAWN MALS instrument, and an Optilab dRI detector. The size-exclusion column is located between the HPLC system and the detectors. Right: an FFF-UV-MALS-DLS-dRI system, consisting of industry-standard HPLC modules, an Eclipse™ FFF controller and separation channel, a DAWN MALS instrument with embedded DLS module, and an Optilab dRI detector. *Credit: Wyatt Technology*

Wyatt DLS and ELS instruments for quantifying and screening gene vectors

Wyatt Technology offers three models of DLS and electrophoretic light-scattering (ELS) instruments:

- DynaPro® NanoStar® measures particle size and concentration in sample volumes as small as 2 µL in microcuvettes. Its unique onboard touchscreen app provides simple stand-alone measurements.
- DynaPro Plate Reader measures particle size and concentration in standard 96, 384, or 1,536 microwell plates. The convenient plate format integrates with plate-based workflows for screening formulations, process conditions, or process fractions.
- Mobius™ simultaneously measures particle size and zeta potential for sample volumes as small as 60 µL. The low applied voltage prevents damage to fragile bionanoparticles.

DYNAMICS® software provides smart data quality assessments, powerful data analysis features, and 21 CFR Part 11 compliance for all three models. Learn more at www.wyatt.com/products/DLS.

Light-scattering analyses for quality attributes

Already well established for characterizing biopharmaceuticals and vaccines, SEC-MALS is regularly cited in regulatory filings for products like glycoconjugates and other biologics. Table 1 summarizes some key gene therapy attributes and how light-scattering tools can be applied to study them. Measurements of average size, particle concentration, and payload can also be performed in real time. The following chapters, which are categorized by specific gene therapy vectors, will explore how light-scattering measurements, with or without size-based separation, can be used to enhance gene therapy research, development, and production.[5–10]

Table 1. Summary of gene vector attributes quantified by light-scattering techniques

Attribute	Quantities	Light-Scattering Techniques
Total titer, total particle concentration	• AAV: Capsid particle titer (Cp) • LNP: Number of LNP per mL, total lipid and nucleic acid concentration • Other gene vectors: Total number per mL	• Batch DLS/SLS provide rapid, low-volume, high-throughput quantitation. • SEC-MALS or FFF-MALS provide concentration as a function of particle size.
Payload and payload distribution	• AAV: Vg (viral genomes per mL), Vg/Cp (full:total ratio) • LNP: Size-based payload distribution, encapsulation efficiency	SEC-MALS and FFF-MALS can be used to simultaneously quantify payload and total concentration in a single, automated assay.
Purity and aggregation	• Aggregate size and concentration • Quantitation of other impurities or released payload	• High-throughput DLS is ideal for formulation, stability, quality control, and other product and process development. • FFF-MALS provides detailed quantitation of aggregates that may be missed by SEC and conventional techniques
Routine and extended characterization	• Size • Molar mass • Stability • Formulation	Light-scattering techniques provide additional characterization simultaneously in the same assay as other quality attributes.

Source: Wyatt Technology

References

1. "Understanding Flow Field-Flow Fractionation," Wyatt Technology, accessed Mar. 17, 2023, https://www.wyatt.com/library/theory/flow-field-flow-fractionation-theory.html.

2. "Dynamic Light Scattering (DLS)," Wyatt Technology, accessed Mar. 17, 2023, http://www.wyatt.com/DLS.

3. "SEC-MALS," Wyatt Technology, accessed Mar. 17, 2023, https://www.wyatt.com/solutions/techniques/sec-mals-molar-mass-size-multi-angle-light-scattering.html.

4. "FFF-MALS: Advanced separation and characterization of macromolecules and nanoparticles," Wyatt Technology, accessed Mar. 17, 2023, https://www.wyatt.com/solutions/techniques/fff-mals-characterization-of-nanoparticles-colloids-macromolecules.html.

5. Judit Bartalis, Michelle Chen, and Daniel Some, *AN2004*: *Why and How to Quantify AAV Aggregates by FFF-MALS*, Wyatt Technology, https://wyattfiles.s3.us-west-2.amazonaws.com/literature/app-notes/fff-mals/AN2004-How_to_quantify_AAV+aggregates_by_FFF-MALS.pdf.

6. US Food and Drug Administration, *Guidance for Industry PAT—A Framework for Innovative Pharmaceutical Development, Manufacturing, and Quality Assurance*, September 2004, https://www.fda.gov/media/71012/download.

7. Amanda K. Werle et al., "Comparison of Analytical Techniques to Quantitate the Capsid Content of Adeno-Associated Viral Vectors," *Mol. Ther.—Methods Clin. Dev*. 23, 254–262 (Aug. 30, 2021), https://doi.org/10.1016/j.omtm.2021.08.009.

8. Nagarathinam Selvaraj et al. "Detailed Protocol for the Novel and Scalable Viral Vector Upstream Process for AAV Gene Therapy Manufacturing," *Hum. Gene Ther.* 32, no. 15–16 (Aug. 17, 2021): 850–861, https://doi.org/10.1089/hum.2020.054.

9. Nicole L. McIntosh et al., "Comprehensive Characterization and Quantification of Adeno-Associated Vectors by Size Exclusion Chromatography and Multi-Angle Light Scattering," *Sci. Rep.* 11 (Feb. 4, 2021): 3012, https://doi.org/10.1038/s41598-021-82599-1.

10. Xiujuan Jia et al, "Enabling Online Determination of the Size-Dependent RNA Content of Lipid Nanoparticle-Based RNA Formulations," *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.*, 1186 (Dec. 1, 2021): 123015, https://doi.org/10.1016/j.jchromb.2021.123015.

11. US Food and Drug Administration, *PAT — A Framework for Innovative Pharmaceutical Development, Manufacturing, and Quality Assurance*, September 2004, https://www.fda.gov/media/71012/download.

Wyatt solutions for separating, quantifying, and characterizing gene vectors

Both SEC–MALS and FFF–MALS employ the DAWN® MALS instrument combined with HPLC modules and an Optilab dRI detector. ASTRA® software provides powerful data analysis features with application-specific modules for viral vectors and LNPs. Both ASTRA and VISION,™ Wyatt's software for designing and controlling FFF-MALS methods, offer 21 CFR (Code of Federal Regulations) Part 11 compliance.

- SEC-MALS for AAV: Quantify multiple quality attributes simultaneously in a single, 30-min, quality-control-ready assay.[7–9] Wyatt's SOP Guidance Manual, and Method Implementation and Training program, provide tools for adopting this platform method. Visit www.wyatt.com/solutions/aav-characterization.html for details.
- FFF-MALS for aggregate quantitation: Eclipse™ FFF provides automated FFF separation with excellent resolution and repeatability for gene vectors and their aggregates.[5]
- SEC-MALS and FFF-MALS for LNP: Both SEC-MALS and FFF-MALS can provide size-dependent RNA content for LNP formulations using ASTRA's unique Lipid Nanoparticle-Nucleic Acid Analysis, which determines payload content information equivalent to fractionation and offline quantitation.[10] Visit www.wyatt.com/LNP for more information.

The FDA has also issued guidance for process analytical technology (PAT), which emphasizes timely analysis of critical process parameters and CQAs throughout production.[11] After implementing MALS for product development, real-time MALS can be employed in-line using an ultraDAWN™ for process development and to monitor CQAs during production. Visit www.wyatt.com/RT-MALS to learn more.

Chapter 2

Adeno-associated viruses

Many gene therapy treatments have become possible thanks to developments in adeno-associated viruses (AAVs). With the rapid growth in AAV-based therapies, there is a heightened need for efficient and precise methods for characterizing and quantifying these vectors throughout product and process development.

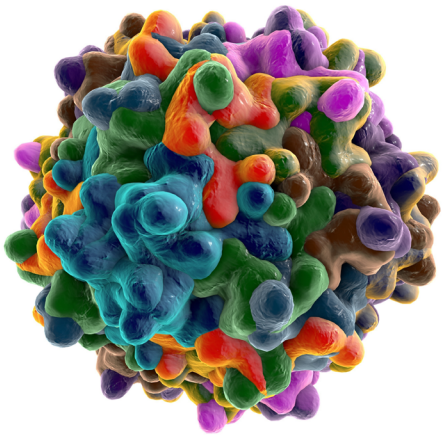


Figure 1: The outer protein coat of an adeno-associated virus, illustrated here, is icosahedral and about 25 nm in diameter. *Source: Kateryna Kon / Shutterstock*

"Determining vector genome titer using a detection method specific for the genetic cargo is an essential metric for dosing," says Darren Begley, principal scientist on the analytical development team in the Gene Therapy Franchise at Resilience. "But we also need to inform clinicians of the total capsid titer, and check for the presence of aggregates, for safety." Researchers measure these qualities during product research, development, manufacturing, and as part of quality control (QC); depending on the chosen method, the process can be time and labor intensive. High-throughput methods may be particularly helpful during the early stages of AAV therapeutic development, when researchers need to screen large numbers of candidates and formulations. Streamlining the measurement of critical quality attributes (CQAs) can accelerate therapeutic development.

Conventional approaches

The fraction of full and empty capsids is commonly quantified by combining two techniques: polymerase chain reaction (PCR)-based analysis for the genome concentration and a second assay, such as the enzyme-linked immunosorbent assay (ELISA), for protein concentration.[1] This approach often results in low accuracy and presents challenges in terms of throughput, reagent quality, and manual handling of samples.[2]

UV–vis spectroscopy, either in batch or in combination with separation, has been applied to estimate the overall capsid

titer and empty-full capsid ratio. As a stand-alone method, it cannot reliably quantify full and empty capsids when other species—such as free protein, free nucleic acid, and aggregated particles—are present. "Measuring the ratio of UV absorbance at 260 nm and 280 nm is insufficient for determining capsid content and can cause significant underestimation or overestimation of the amount of encapsidated nucleic acid," Wyatt's director of analytical sciences Sophia Kenrick says. Along with absorbing light, AAV aggregates and other large species scatter light, which can introduce significant error and make batch UV–vis absorption methods unsuitable for in-process AAV or stressed samples, she adds.

Other more sophisticated techniques are also available. Analytical ultracentrifugation (AUC) reliably differentiates empty, full, and partially filled capsids but requires specialized equipment, purified samples, and extended time for analysis.[3] Though charge detection mass spectrometry, a relatively new methodology, produces reliable data and is amenable to high-throughput processes, it requires specialized equipment and may have limited dynamic range and solvent compatibility.[3]

Light-scattering methods

Light-scattering methods are uniquely suited to quantify multiple attributes simultaneously in a single assay. The analytical platform utilized by Begley's team includes size-exclusion chromatography (SEC) on a high-performance liquid chromatography (HPLC) system with UV, multi-angle light scattering (MALS), and differential refractive index (dRI) detectors. "SEC-MALS gives us accurate, quantitative readout of total capsid titer on most downstream samples and a sense of encapsidation efficiency by both UV260:UV280 ratio and the total mass by MALS," he says. "It is a serotype-independent method, which means we deploy a single method, regardless of AAV serotype, with much higher precision than ELISA. It has some limitations but is very well suited to our purposes, both for supporting process development and as an analytical method for release of final materials."

CASE STUDY
High-throughput formulation and stability screening

A key early step in the development of AAV therapies is optimizing formulation conditions to maintain capsid stability. Batch dynamic and static light scattering are ideal here for measuring changes in apparent hydrodynamic radius (R_h) and particle concentration in each formulation condition or in response to stress. For example, large aggregates may appear as separate populations with R_h much larger than that of the monomer. Alternatively, the generation of small oligomers (e.g., dimer and trimer) may lead to an increase in average R_h and polydispersity.

In this study, researchers screened multiple AAV preparations with the DynaPro Plate Reader, which simultaneously measures dynamic and static light scattering (DLS and SLS).[4] Analysis of size and particle concentration as a function of different buffers revealed conditions that

maintained the desired AAV structure and conditions that promoted aggregation. In addition, the researchers investigated whether varying capsid content impacted thermal stability (figure 1).

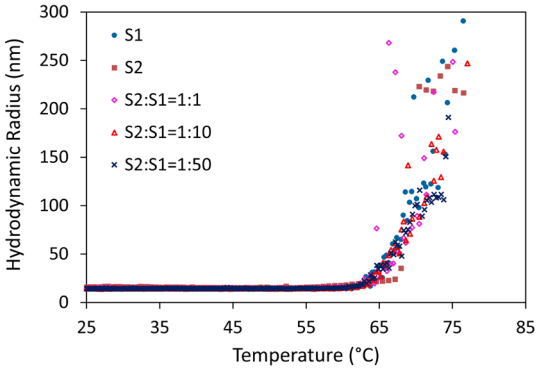


Figure 1: Samples containing different ratios of empty AAV capsids (S1) and full AAV capsids (S2) were exposed to increasing temperatures in a DynaPro Plate Reader. Empty, full, and different ratios of empty and full capsids exhibited similar thermal stability. *Credit: Wyatt Technology*

CASE STUDY
Comprehensive characterization

"Low-throughput, labor-intensive analytical methods such as AUC or electron microscopy are not a great fit for rapid AAV process development and optimization," Begley says. To demonstrate an alternative approach, researchers assessed AAV samples with a method combining size-exclusion chromatography (SEC) with triple detection—MALS, UV, and dRI.[5]

"The main advantages of SEC-MALS for the analysis of AAV samples—and other biologics—are ease and precision," Kenrick says. "SEC-UV-MALS-dRI does not require standard curve preparation, reagents, or prior knowledge of structure or content." In the study, researchers generated two AAV samples, one with full capsids and the other with empty capsids. They mixed the samples at ten different ratios and assessed these mixtures with SEC-MALS to determine the total number of viral capsid particles, the ratio of empty and full capsids, and the percentage of monomers or aggregates. The data from 30-min SEC-UV-MALS-dRI runs were sufficient to quantify the full and empty capsids (figure 2) as well as the contributions of individual components (i.e., DNA

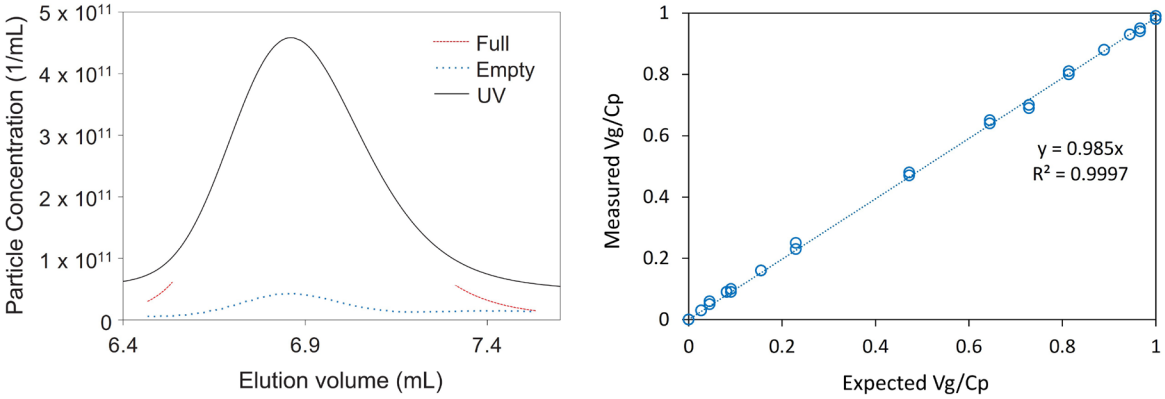


Figure 2: Left: empty and full virion concentrations of an AAV sample eluting from SEC, calculated once per second and overlaid with the UV chromatogram. Right: correlation between expected and measured viral genome concentration to capsid titer (Vg/Cp), obtained with SEC-MALS measurements on mixtures of known empty and full AAVs. *Credit: Wyatt Technology*

and capsid) to the molar mass of viral particles. The researchers used that information to calculate the empty: full ratio and found excellent agreement between calculated and measured values (R^2 = 0.9997).

The unique combination of ease of use, automation, speed, serotype independence, accuracy, and repeatability of the SEC-MALS approach has been well received in the AAV gene therapy field. Several reports by authors in the biopharmaceutical industry discuss the advantages of this method—as well as those of ion-exchange chromatography (IEX)-MALS—to comprehensively characterize AAV preparations.[3,6,7]

Large aggregates

Identifying aggregates is an important step in AAV quality control because large aggregates have increased potential to trigger an immune response. However, the SEC stationary phase can disrupt or filter larger aggregates, which might then go undetected.[5] "FFF-MALS is an ideal tool for characterizing AAV aggregates," Kenrick says. "These larger species may be removed by the SEC column but are not removed by the FFF channel. The same UV-MALS-RI detection platform employed for SEC-MALS can be used with FFF to ensure complete quantitation of the AAV aggregates in addition to the monomer, fragments, and process impurities." FFF-MALS can quantify the large aggregates more reliably than SEC-MALS can. In a study conducted by scientists at Novartis Gene Therapies and Wyatt, FFF was able to retain aggregates that were filtered out by the SEC column. Furthermore, only MALS was able to quantify aggregates accurately.[8] Simple quantitation by UV or fluorescence peak area significantly overestimated the amounts of these impurities.

References

1. Christina Wagner et al., "Biophysical Characterization of Adeno-Associated Virus Vectors Using Ion-Exchange Chromatography Coupled to Light Scattering Detectors," *Int. J. Mol. Sci.* 23, no. 21 (October 22, 2022):12715, https://doi.org/10.3390/ijms232112715.

2. Jürg M. Sommer et al., "Quantification of Adeno-Associated Virus Particles and Empty Capsids by Optical Density Measurement," *Mol. Ther.* 7, no. 1 (January 2003): 122-8, https://doi.org/10.1016/S1525-0016(02)00019-9.

3. Amanda K. Werle et al., "Comparison of Analytical Techniques to Quantitate the Capsid Content of Adeno-Associated Viral Vectors," *Mol. Ther.—Methods Clin. Dev.* 23 (Dec. 10, 2021): 254–62, https://doi.org/10.1016/j.omtm.2021.08.009.

4. Xujun Zhang, Wade Wang, and Sophia Kenrick, *AN5007: Characterization of AAV-Based Viral Vectors by DynaPro DLS/SLS Instruments*, Wyatt Technology, accessed Mar. 17, 2023, https://wyattfiles.s3-us-west-2.amazonaws.com/literature/app-notes/dls-plate/AN5007-AAV-quantitation-and-stability-analysis-by-batch-DLS.pdf.

5. Michelle Chen and Anatolii Purchel, *AN1617: Quantifying Quality Attributes of AAV Gene Therapy Vectors by SEC-UV-MALS-dRI*, Wyatt Technology, accessed Mar. 17 2023, https://wyattfiles.s3-us-west-2.amazonaws.com/literature/app-notes/sec-mals-proteins/AN1617-AAV-CQA-Analysis-by-SEC-MALS.pdf.

6. Nicole L. McIntosh et al., "Comprehensive Characterization and Quantification of Adeno Associated Vectors by Size Exclusion Chromatography and Multi Angle Light Scattering," *Sci. Rep.* 11, no. 1 (2021), https://doi.org/10.1038/s41598-021-82599-1.

7. Nagarathinam Selvaraj et al., "Detailed Protocol for the Novel and Scalable Viral Vector Upstream Process for AAV Gene Therapy Manufacturing" *Hum. Gene Ther.* 32, no. 15-16 (August 2021): 850–861, https://doi.org/10.1089/hum.2020.054.

8. Judit Bartalis, Michelle Chen, and Daniel Some, *AN2004: Why and How to Quantify AAV Aggregates by FFF-Mals*, Wyatt Technology, accessed Mar. 17, 2023, https://wyattfiles.s3.us-west-2.amazonaws.com/literature/app-notes/fff-mals/AN2004-How_to_quantify_AAV+aggregates_by_FFF-MALS.pdf.

Chapter 3

Lipid nanoparticles

Lipid nanoparticles (LNPs) consist of ionizable lipids, phospholipids, and functionalized lipids that encapsulate nucleic acids, forming spherical packages ranging from 30 nm to 250 nm in radius (figure 1). LNPs were first approved as a drug delivery vehicle in 2018 for the small interfering RNA (siRNA) drug Onpattro. More recently, they have received global recognition as the delivery system for mRNA in COVID-19 vaccines developed by Moderna and BioNTech. This technology holds much promise for a variety of advanced therapeutic applications, including transient gene suppression, transient gene expression, and gene editing.[1]

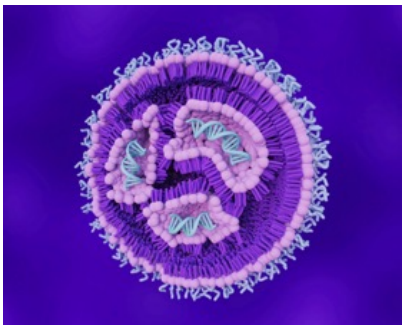


Figure 1: An illustration of a lipid nanoparticle, where lipids form a spherical coating around smaller collections of lipids that hold therapeutic genetic material. *Credit: Wyatt Technology*

Unlike AAVs, which are uniform, well-defined structures, LNPs are polydisperse in size and nucleic acid loading. This variability can impact dosing and biodistribution.[2] "Quantifying not only the total amount of lipid and nucleic acid but the full size-based payload distribution as well is key to formulating the optimal effective dosage," Kenrick says.

Traditional approaches

In addition to the techniques described in chapter 1, an array of techniques is available to quantify LNP quality attributes, each with its distinct advantages and disadvantages.[3–5] Zeta potential measurements determine surface charge for stability. Analytical ultracentrifugation can assess encapsulation efficiency, but, as with viral vectors, this technique requires specialized equipment and personnel. Batch DLS and nanoparticle tracking analysis (NTA) can reveal LNP size and concentration but may miss key details of the size distribution, and the dilute conditions required for NTA may destabilize the particles. Fluorescence measurements to assess encapsulation can be complicated by the presence of common salts or functionalized lipids.

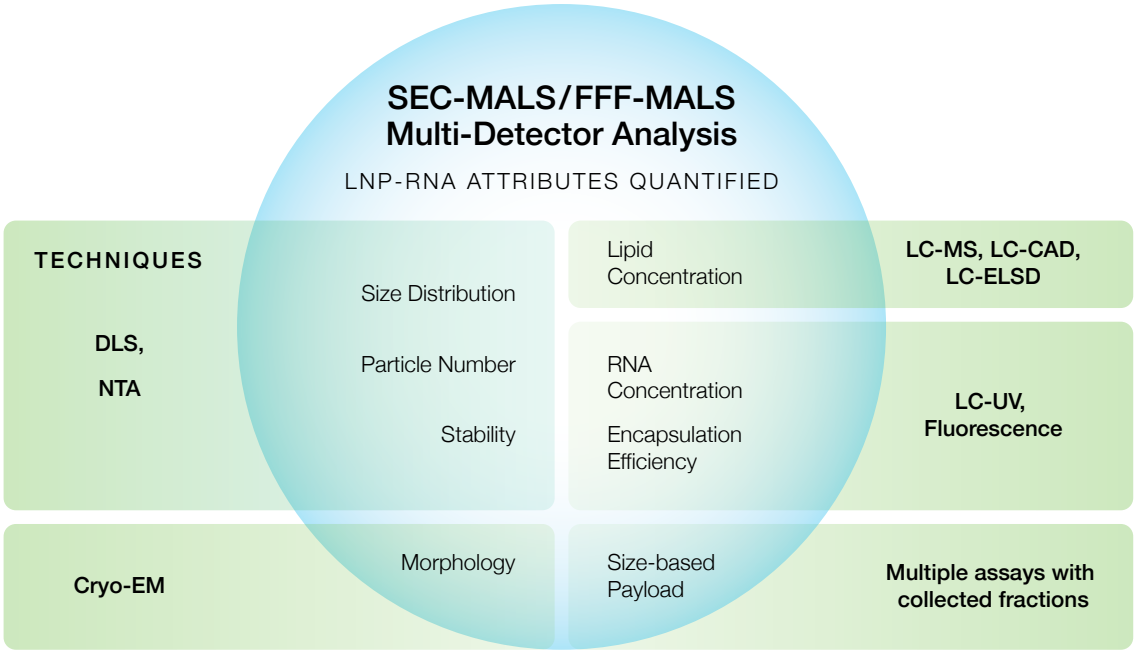


Figure 2: A collection of techniques to measure critical quality attributes and other key properties of lipid nanoparticles carrying nucleic acid for gene therapy. Most techniques only measure some of these properties. SEC or FFF followed by MALS measurements can quantify all these attributes in one analysis. *Credit: Wyatt Technology*

Light-scattering methods

While DLS is a first-line tool for many labs to estimate LNP size distribution and stability,[6] size-based separation combined with MALS provides multi-attribute quantitation of LNP-based gene vectors in a single run (figure 2). SEC-MALS is often the best place to begin, but FFF-MALS may be required for certain formulations—for example, to eliminate shear and address larger size ranges.[7]

Scientists at Wyatt and the Norwegian research organization SINTEF collaborated to establish a robust protocol outlining the use of a multidetector FFF for LNP analysis.[7,8] Using an Eclipse FFF instrument and a DAWN MALS detector, they demonstrated how FFF–MALS can be implemented to comply with regulatory requirements for LNP characterization (figure 3). Results from their method correlated with those from nanoparticle tracking analysis (NTA) and extended the quantitation beyond the range that is measurable with NTA. “This analytical technique is set to take on the standards of reproducibility, accuracy, and reliability needed in the pharmaceutical regulatory space,” Kenrick says.

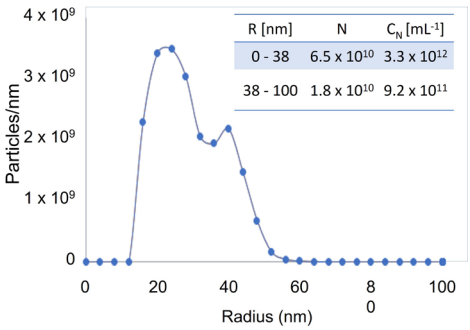
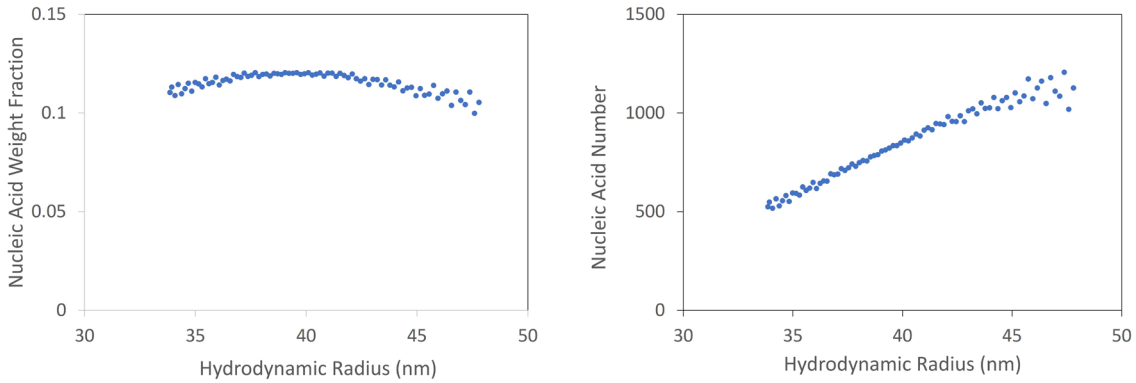


Figure 3: With simultaneous determination of particle size and concentration during size-based separation, FFF-MALS can provide fully quantitative size distributions. *Credit: Wyatt Technology*

CASE STUDY
Payload and payload distribution

SEC- or FFF-UV-MALS-DLS-dRI enables online quantification of the nucleic acid cargo as a function of particle size in LNP-based gene therapy. “Traditional methods using only UV cannot accurately quantify the payload of intact LNPs,” Kenrick says. “Particles larger than 50 nm in diameter scatter UV light in a manner that depends on particle size, shape, and encapsulated payload. Thus, for LNPs, the total measured UV absorbance at 260 nm is the combined result of absorption by the cargo and scattering.” Wyatt’s proprietary method accounts for this phenomenon, quantifies the correct nucleic acid concentration, and determines the encapsulated payload as a function of LNP size along with the encapsulation efficiency.

Researchers at Merck and Wyatt developed a protocol to validate the SEC- or FFF-MALS approach against tedious offline methods that require rupturing the particles with surfactants to extract the nucleic acids prior to UV measurement.[9] The online method eliminates the need for offline sample handling and analysis, successfully revealing the size-dependent RNA payload distribution (figure 4). “Using SEC or FFF coupled with MALS, DLS, UV, and RI detection, scientists can quickly and easily quantify size-dependent payload distribution and get a more in-depth understanding of their intact drug product,” Kenrick says. “Having this kind of a robust technique that provides multiple quality attributes can give them new insights into their dosing and toxicity profiles.”



	Nucleic acid Mw (kDa)	Lipid conc. (mg/mL)	Nucleic acid conc. (mg/mL)	Encapsulation efficiency (%)	Nucleic acid number
F1a	12780 ± 0.5%	10.67	1.42	97.8	819 ± 0.5%
F2a	8800 ± 1.2%	7.36	0.97	97.8	564 ± 1.3%
F3a	13200 ± 0.9%	7.95	1.07	97.5	845 ± 0.9%

Figure 4: Combining size-based separation with on line MALS, DLS, UV, and dRI detectors can quantify encapsulation efficiency and the amount of lipid and nucleic acid encapsulated in particles of a given size. Shown here are the results of two LNP-RNA preparations, which exhibit quite different size dependencies. *Credit: Wyatt Technology*

CASE STUDY
Process development for in-line monitoring of LNPs

Often during manufacturing processes, specific quality information is determined only at the end of the process. In contrast, real-time MALS can provide a constant readout of particle size, concentration, and other key attributes online or in-line with the manufacturing product stream. Product meeting specifications can be collected for further processing, and triggers can be set up to flag the operator and divert the stream when the product falls out of specification.

To demonstrate the utility of real-time MALS, Wyatt researchers simulated an undesirable process change in the homogenization of a liposome solution.[10] At the 25-min point in the production process, a sudden pressure change was introduced, which resulted in a decrease in the liposome radius. Figure 5 shows the radius measured in real time; the change in liposome size at the point of increased pressure is visible. The red vertical line at ~28 min indicates where the liposome crossed the user-defined threshold (2 nm from the target size) and a signal was sent from the ultraDAWN to trigger diversion of the product. Had the deviation been observed in bulk with final QC testing alone, an entire batch would likely have been discarded. This immediate feedback can also enable more rapid process optimization.

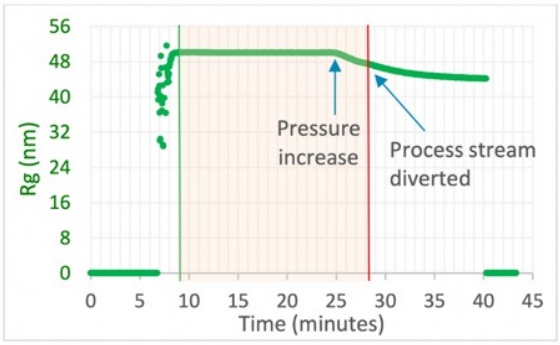


Figure 5: Real-time monitoring of liposome size (radius of gyration, R_g) during homogenization with RT-MALS, showing a constant, in-spec size until a pressure increase causes a change. When the average measured size dropped out of the permissible range, a trigger was issued to divert the stream to waste. *Credit: Wyatt Technology*

References

1. Ryuichi Mashima and Shuji Takada, "Lipid Nanoparticles: A Novel Gene Delivery Technique for Clinical Application." *Curr. Issues Mol. Biol.* 44, no. 10 (October 2022): 5013–27, https://doi.org/10.3390/cimb44100341.

2. Sam Chen et al., "Influence of Particle Size on the In Vivo Potency of Lipid Nanoparticle Formulations of siRNA," *J. Controlled Release* 235 (Aug. 10, 2016): 236–44, https://doi.org/10.1016/j.jconrel.2016.05.059.

3. Amy Henrickson et al., "Density Matching Multi-Wavelength Analytical Ultracentrifugation to Measure Drug Loading of Lipid Nanoparticle Formulations," *ACS Nano* 15, no. 3 (March 23, 2021): 5068–76, https://doi.org/10.1021/acsnano.0c10069.

4. Christina M. Bailey-Hytholt et al., "Formulating and Characterizing Lipid Nanoparticles for Gene Delivery Using a Microfluidic Mixing Platform," *J. Visualized Exp.* no. 168 (Feb. 25, 2021): e62226, https://doi.org/10.3791/62226.

5. Supandeep Singh Hallan et al., "Challenges in the Physical Characterization of Lipid Nanoparticles," *Pharmaceutics* 13, no. 4 (April 14, 2021): 549, https://doi.org/10.3390/pharmaceutics13040549.

6. "Dynamic Light Scattering (DLS)," Wyatt Technology, accessed Mar. 17, 2023, http://www.wyatt.com/DLS.

7. Fanny Caputo and Christian Sieg, *AN1617: WP2612: Meeting Regulatory Needs in the Characterization of Lipid Nanoparticles (LNPs) for RNA Delivery via FFF-MALS*, Wyatt Technology, accessed Mar. 17, 2023, https://wyattfiles.s3.us-west-2.amazonaws.com/literature/app-notes/fff-mals/WP2612-Meeting-regulatory-needs-LNP-RNA-via-FFF-MALS.pdf.

8. Robert Mildner et al., "Improved Multidetector Asymmetrical-Flow Field-Flow Fractionation Method for Particle Sizing and Concentration Measurements of Lipid-Based Nanocarriers for RNA Delivery," *Eur. J. Pharm. Biopharm.* 163 (June 2021): 252–65, https://doi.org/10.1016/j.ejpb.2021.03.004.

9. Xiujuan Jia et al., "Enabling Online Determination of the Size-Dependent RNA Content of Lipid Nanoparticle-Based RNA Formulations," *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* 1186 (December 2021), https://doi.org/10.1016/j.jchromb.2021.123015.

10. Wyatt Technology, *AAN8006: In-Line Monitoring of Liposome Size by RT-MALS*, accessed Mar. 17, 2023, https://wyattfiles.s3-us-west-2.amazonaws.com/literature/app-notes/rt-mals/AN8006-In-line-monitoring-of-liposome-size-by-RT-MALS.pdf.

Chapter 4

Lentiviruses and adenoviruses

While AAVs and LNPs are the most popular gene therapy vectors, other viral vectors are under investigation for in vivo or ex vivo gene therapy. Lentiviruses are a subcategory of retroviruses. They are about 100 nm in diameter, spherical, enveloped with a lipid bilayer, and they contain single-stranded RNA (figure 1). They have particular utility in cell-based gene therapies, including cancer immunotherapies such as Kymriah.[1] Adenoviruses, which are nonenveloped viral particles 90 nm in diameter, generally deliver DNA and because of their immunogenicity are often employed as vaccines (figure 2).[2]

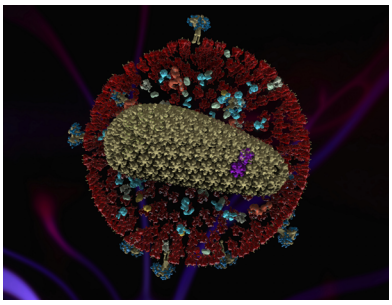


Figure 1: Illustration of the structure of an enveloped retrovirus. *Credit: bekirevren/Shutterstock*

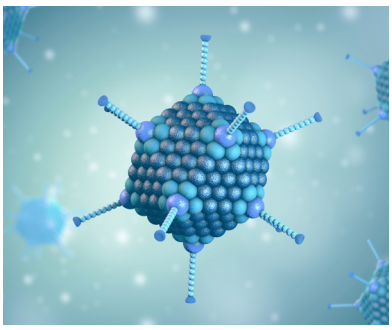


Figure 2: Illustration of the structure of adenovirus. *Credit: Christoph Burgstedt/Shutterstock*

Lentiviruses and adenoviruses pose additional challenges for analysis because of their large size, complex morphology, and polydispersity. “Their size typically makes SEC a poor option for separation, and the polydispersity of lentivirus means that batch techniques, like DLS, can miss key information,” Kenrick says.

CASE STUDY
Optimizing retroviral vector purification

Scientists from Baylor College of Medicine and Wyatt Technology conducted a study to identify how retroviral vector (RVV) purification methods can influence the critical quality attributes of purity, size distribution, and concentration.[3] In this study, one production run of lentivirus particles was

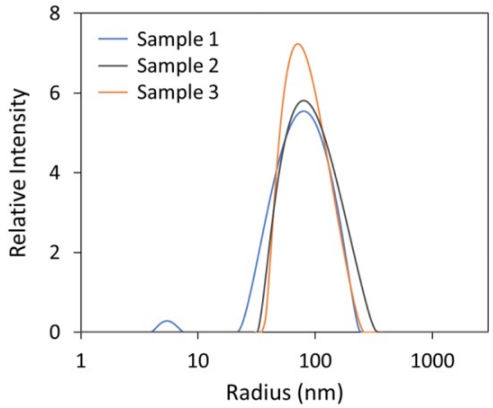


Figure 3: Size distributions of a retroviral vector purified under three different conditions, as analyzed with batch DLS. *Credit: Wyatt Technology*

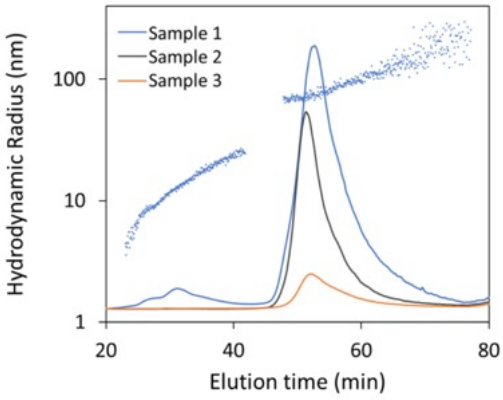


Figure 4: FFF-MALS analysis of retroviral vector purified under three different conditions. Total light scattering is shown as smooth lines, and dots show measured R_h. *Credit: Wyatt Technology*

purified using three different methods. First, researchers used batch DLS analysis as a quick screen of particle size and concentration. A wide size range was common to all three preparations (figure 3).

A separation method was required for detailed size distribution and concentration. In a head-to-head comparison of SEC versus FFF for retroviral analysis, recovery was significantly reduced under SEC, with particles larger than 87 nm either degraded or removed.[3] FFF-MALS revealed the entire size distribution with high resolution.

Figure 4 shows the FFF-MALS fractogram of the three retrovirus preparations. The desired particles elute between 48 and 68 min with radii ranging from 50 to 150 nm; the measured hydrodynamic size of the particles from sample 1 is overlaid in blue dots.

Sample 1 displays additional peaks at ~26 min with R_h of ~3.5 nm and at 27–40 min with sizes ranging from 10 nm to 45 nm. These impurities correspond to small proteins in the formulation buffer, likely residual cell culture components. Sample 3 shows the greatest purity but at the expense of the lowest concentration. Table 1 shows the RVV titer measured by batch DLS compared with more detailed quantitation by FFF-MALS for each of the three samples.

Table 1: Hydrodynamic radii ($R_{h,z}$ ¬and R_h) and particle concentration (PC) compared between measurement by FFF-MALS and stand-alone DLS for retroviral preparations purified by three different methods.

Sample	$R_{h,z}$ from FFF-DLS (nm)	PC from FFF-MALS (mL^{-1})	R_h from DLS (nm)	PC from DLS (mL^{-1})
1	99.2 ± 0.6	$(1.62 \pm 0.01) \cdot 10^{10}$	79.2 ± 0.8	$(1.3 \pm 0.2) \cdot 10^{10}$
2	94.9 ± 0.4	$(9.68 \pm 0.02) \cdot 10^{9}$	85.3 ± 1.1	$(5.1 \pm 0.1) \cdot 10^{9}$
3	114 ± 2	$(1.98 \pm 0.01) \cdot 10^{9}$	92.0 ± 0.9	$(1.5 \pm 0.1) \cdot 10^{9}$

Source: Wyatt Technology

CASE STUDY
Real-time monitoring of downstream adenovirus processing

While offline techniques such as DLS, SEC-MALS, and FFF-MALS provide detailed assessment of product attributes, the time lag between production and measurement precludes their use for immediate feedback on process changes and real-time process control. Implementation of MALS as a PAT tool helps accelerate downstream process development and ensure product quality by discriminating viral vectors from free proteins and nucleic acids, identifying potential degradation and determining titer in real time.

Process development scientists at Janssen Vaccines and Prevention demonstrated how MALS can provide essential data on relevant adenoviral vector attributes during ion-exchange purification and ultrafiltration/diafiltration unit operations.[4] Figure 5 compares the UV chromatogram obtained during ion-exchange

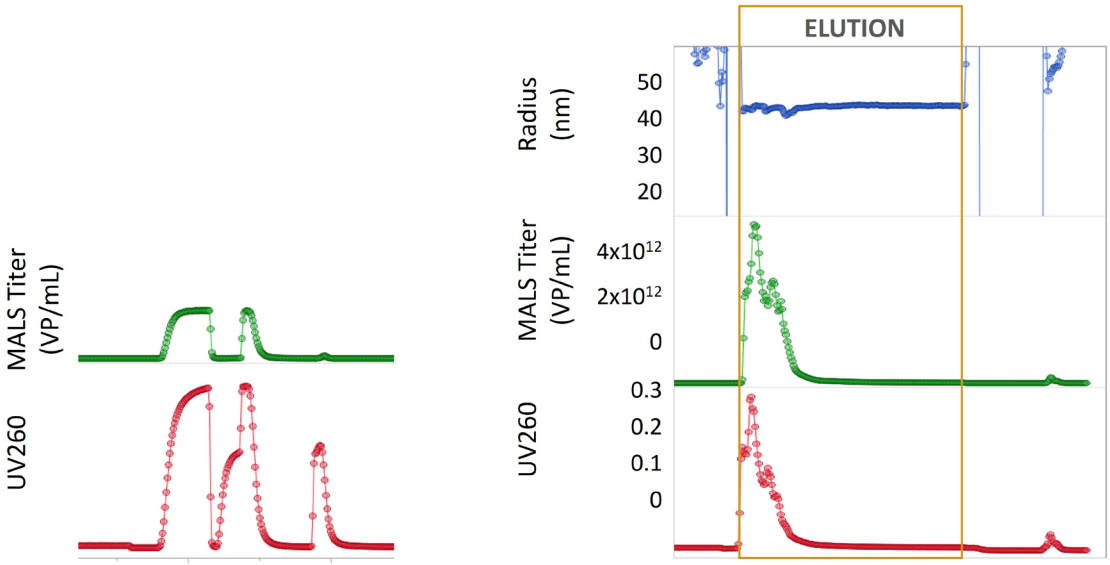


Figure 5: Left: UV and MALS titer (viral particles/mL) traces during the loading and wash phases of IEC purification. Right: RT-MALS (size and titer) and UV measurements during the elution phase. The real-time titer values can be integrated to obtain overall titer in the pool. *Credit: Wyatt & Janssen*

purification with MALS-derived size and titer traces. On the left side of figure 5, portions of the loading and wash phases that have strong UV absorbance with no corresponding MALS titer indicate free biomacromolecules, whereas the during the load phase, matching UV and MALS peaks just before 14 min point to viral breakthrough.

On the right side of figure 5, the entire eluting peak is seen to consist of particles of a constant size corresponding to the monomeric virus, with no appreciable aggregates. Instantaneous titer values track closely but are not identical to UV extinction; the titer is integrated over the collection period to determine the number of virions in the final pool. Size and

titer determined by in-line RT-MALS are compared with off-line measurements in table 2, indicating close correspondence. Similar corresponding size and titer values were obtained between on line RT-MALS measurements during tangential-flow filtration and samples taken in the course of the operation.

Table 2: Comparison of in-line, real-time and offline measurements of adenovirus size and titer in the pool collected from ion-exchange purification

	Size (nm)	Titer (1/mL)
RT-MALS	R_{geom}: 45.7	7.31 x 10^{11}
Reference offline method	R_h: 48.6	6.07 x 10^{11}

Source: Wyatt and Janssen

References

1. Altar M. Munis, "Gene Therapy Applications of Non-Human Lentiviral Vectors," *Viruses* 12, no. 10 (Sept. 29, 2020): 1106, https://doi.org/10.3390/v12101106.

2. Nia Tatsis and Hildegund C.J. Ertl, "Adenoviruses as Vaccine Vectors," *Mol. Ther.* 10, no. 4 (2004): 616–29, https://doi.org/10.1016/j.ymthe.2004.07.013.

3. Austin Seal et al., *AN9010: Characterizing Lentiviral and Gammaretroviral Vectors by Batch DLS and FFF-MALS*, Wyatt Technology, accessed Mar. 17, 2023, https://wyattfiles.s3.us-west-2.amazonaws.com/literature/app-notes/fff-mals/AN9010-Characterize-retroviral-vectors-DLS-FFF-MALS.pdf.

4. Clara Pérez Peinado and Dan Some, "Real-time analysis of product attributes in downstream processing of viral vectors," Wyatt Technology, https://www.wyatt.com/library/gated-webinars/real-time-process-analytics-using-multi-angle-light-scattering-rt-mals-request.html.

Conclusions

Analytical techniques based on light scattering, stand alone and in conjunction with size-based separations, offer versatile and powerful capabilities for quantifying and characterizing gene vectors. Plate-based screening of formulation conditions and process fractions by DLS/SLS rapidly evaluates size and titer. In-depth separation and analysis of gene vectors to determine accurate size distributions, concentration, and payload can usually be accomplished in a single run using detectors with standard HPLC components for SEC-MALS or an Eclipse FFF system for FFF-MALS. An ultraDAWN RT-MALS detector serves to perform in-line and on line monitoring of product attributes during process operations such as viral vector purification or LNP production. Increasing utilization of these techniques may improve productivity and quality while accelerating the time to market of these novel and promising therapeutic modalities.