

### INNOVATOR INSIGHT

# Facilitating gene therapy development with solutions to four capsid analytical challenges

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Access to robust analytical processes for viral vectors that support both the development, and the production phase is a challenge today, given the short turnaround time for obtaining analytical results. The functional cell-based assays and infectivity studies that have been used in the first years of gene therapy development can take days or weeks to generate results. However, in the downstream process, the decision to progress a batch must often be made within 24 hours or less. Key decisions about the affinity capture and anion exchange steps require analytics to answer the question about whether to progress a batch and how so. Today, manufacturers must use limited information for the decision to move ahead with a process and thus risk losing time and costly product.

With several gene therapies already on the market and hundreds more advancing rapidly through the clinic, next generation methods are essential to ensure successful commercialization. Access to simplified and rapid biophysical assays would provide more comprehensive information in a quicker manner to facilitate decision making on the right time scale.

New robust assays based on mass spectrometry (MS) and capillary electrophoresis combined with laser-induced fluorescence (CE-LIF) detection and other detection methods, such as UV, can rapidly provide accurate and reproducible results for both the protein and genetic components of viral vectors. Below are discussed techniques for capsid protein analysis and purity determination, viral vector genome integrity analysis, and the determination of empty and partial versus full capsids.

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## NEED FOR ROBUST ANALYTICAL SOLUTIONS

Most gene therapies use viral vectors such as adeno-associated virus (AAV), lentivirus and adenovirus to deliver genetic material into cells. Whether delivered to the cells *ex vivo* or *in vivo*, the new genetic material replaces or restores the normal phenotype of missing, non-functional or incorrectly functioning genes to treat a range of diseases.

AAV is widely used as a gene delivery vehicle because it is non-pathogenic, exhibits low immunogenicity, and can readily enter a variety of cell types. This small, icosahedral virus (~20–25 nm, ~5.9 megadaltons) comprises a protein shell (i.e., capsid) encompassing a single-stranded DNA that is approximately 4.8 kilobases in size. The viral capsid is a 60-mer typically made up of three viral protein monomers (VP1, VP2, VP3) with respective molecular weights of 87, 73, and 61 kilodaltons assembled in a ratio of approximately 1:1:10.

A common process for making therapeutic recombinant AAVs (rAAVs) involves transfecting host cells with three plasmids, one of which contains the entire rAAV genome and two helper plasmids that contain *Rep* and *Cap* genes that enable the host cells to make virions. After manufacture, the rAAVs are purified by immune purification or ion exchange chromatographies and lysis of the host cells, followed by dialysis and buffer exchange, then aseptic filling.

During AAV production, the capsid viral proteins participate in the packaging of both the capsid and genome. They also determine the efficacy of a gene therapy, playing roles in receptor binding during cell entry, intracellular trafficking and genome release.

Correct expression of the viral vector capsid with the right size, peptide sequence and post-translational modifications (PTMs) is essential. The purity of the capsids with respect to host-cell protein and other genetic contaminants is critical to avoid the potential for immunogenicity and off-target effects. It is also important to minimize the number

of empty and partial capsids, which can lower infectivity and thus lead to low protein production.

Many experimental conditions can influence the overall outcome of the production process. Rapid and robust analytical methods are therefore needed for effective in-process monitoring and final product release to ultimately produce a homogenous product that meets safety, strength, identity, and purity requirements.

## CAPSID PROTEIN ANALYSIS

The AAV capsid is the primary interface between host and virus. Since post-translational modifications have the potential to impact the binding and subsequent infectivity of capsid proteins to a host cell, any imperfection affects the performance of the viral vectors. The three viral proteins produced in the viral vector manufacturing process differ only slightly in length and the N-terminus. They can also be generated in multiple variants due to different PTMs, which can impact efficacy. A rapid, robust method is therefore needed to fully characterize the capsid proteins, including their ratios and the presence of desirable and undesirable PTMs, regardless of concentration and often using small sample quantities.

Liquid chromatography (LC) combined with mass spectrometry (MS) can be used to characterize capsid viral proteins. Specifically, quadrupole time-of-flight MS (Q-TOF MS) enables rapid characterization of AAV capsid proteins. SCIEX has developed a simple digestion strategy that eliminates the need for dialysis or spin filters for sample preparation.

Digested samples analyzed using a SCIEX X500B QTOF System coupled to an ExionLC™ System provided MS and MS/MS data for low-abundance peptides and PTMs (glycopeptides, deamidation sites, disulfide bonds, etc.) at the required sensitivity to achieve nearly complete sequence coverage, thus allowing confirmation of both C and N-termini and identification of modifications, along

with their localization and relative quantitation [1,2]. Such robust analytics deliver rapid, accurate results to strengthen gene therapy development and commercialization [3].

Proprietary SCIEX SWATH®-based LC-MS/MS can also identify and quantify thousands of host-cell proteins and other contaminants in a single run.

## CAPSID PURITY DETERMINATION

A rapid, robust, reproducible, and highly sensitive biophysical method is required for in-process evaluation of capsid protein purity at the low AAV concentrations found in most gene therapies (~50 ng/mL). The traditional method for determining AAV capsid viral protein purity involves SDS-polyacrylamide gel electrophoresis (SDS-PAGE) technology. There are severe shortcomings to this method, including a limited quantitation capability due to inherent sample preparation artifacts, a slow migration time and staining variability. Migration times for reversed-phase high-performance liquid chromatography (HPLC), meanwhile, vary significantly with serotype.

CE-SDS using a capillary gel electrophoresis mode, which has been used extensively for the purity analysis and quantitation of therapeutic proteins, offers advantages over conventional slab gel technology including high resolving power, better quantitation, excellent reproducibility, and automated operation, even for the lower concentrations of viral proteins found in AAV samples. It can also provide higher resolution than HPLC for protein separation.

For purity of AAV products with titers greater than  $1 \times 10^{13}$  genome copies per mL (GC/mL) or lower titer but sufficient sample volume, a PDA or UV detector can be used. Ultra-high sensitivity can be achieved using a fluorescent dye for sample labeling and laser-induced fluorescence (LIF) detection, enabling rapid analysis (~15 minutes) of in-process samples with AAV titers as low as  $1 \times 10^{10}$  GC/mL and limited sample amounts. In both cases, the sample prep is straightforward, and the method offers excellent resolving power, good repeatability, and high linearity of absorbance response to sample concentration.

## GENOME INTEGRITY ANALYSIS

The ability to determine the integrity of the genomes used in viral vectors for gene therapies is crucial, as their efficacy and safety depend on the presence of the intact genome in the carrier capsid. For AAVs, the transgene in the AAV genome cassette could be missing (empty or partial capsid) or truncated, or the capsid could contain contaminant products instead of the transgene.

There are several technologies currently in use for this determination, such as denaturing agarose gel electrophoresis, Southern blot, quantitative polymerase chain reaction (qPCR), HPLC, and Next Generation Sequencing. While these techniques all have specific strengths and some are low cost, they are time-consuming, have low precision, and all of them generate large amounts of toxic waste. Some cannot detect fragments that do not contain the target sequence, do not provide size determination, or are very expensive to implement.

Here again, CE in the capillary gel electrophoresis mode with LIF detection is a rapid, automated biophysical method for genome size analysis of double-stranded DNA (dsDNA), including restriction fragment analysis of its vectors, as well as single-stranded DNA (ssDNA) and RNA, and offers higher resolution than HPLC. Fragments differing by as few as 10 base pairs can be separated and detected using UV or LIF identification. Reconstitution of the gel to a larger volume allows for determination of plasmid stability via plasmid isoform analysis (relative abundance of supercoiled and open circular isoforms over time).

SCIEX PA 800 Plus allows for a simple sample preparation method, CE-LIF, designed to digest contaminant fragments outside of the AAV capsid without degrading the

viral proteins or causing interference. This is an ideal rapid biophysical analytical method for AAV genome integrity and purity analysis that can be done in four steps.

As part of its CE portfolio, SCIEX offers robust and accurate tools for rapid genomic analyses using the GenomeLab GeXP™ system, which is capable of Sanger DNA sequencing and quantitative polymerase chain reaction in one system. It can do genotyping and single nucleotide polymorphism analysis, as well as short tandem repeat analysis and DNA profiling. The ability to conduct these analyses in-house rather than sending them out to a lab provides better data control and affords more rapid decision-making.

## EMPTY VS FULL CAPSID DETERMINATION

In addition to the AAV full capsid containing the transgene, product-related capsid impurities can include an empty capsid, or virus protein shells without the vector genome, a partial capsid containing transgene fragments, and an 'other' capsid, which contains any sort of nontarget, extraneous host-cell nucleic acid. The contamination of packaged genome-related impurities affects the efficacy and the safety of the vector product, increasing the potential immunogenicity and can inhibit the transduction of the full capsid by competing for vector binding sites on the cells.

The analysis of empty and partial versus full capsids is thus one of the critical quality attributes for AAV products. There are multiple technologies used for determining the ratio of AAV full and empty viral capsids. The quick and easy methods (qPCR/ELISA and spectrophotometry) suffer from poor accuracy, while electron microscopy is too time-consuming, ion exchange chromatography (IEX) does not provide good resolution and charge-detection, and MS is not yet commercially available. Analytical ultracentrifugation (AUC) is the gold standard, but requires large sample sizes, is high cost and requires highly trained operators.

Capillary isoelectric focusing (cIEF), on the other hand, is a fast, easy-to-perform and robust biophysical method that is effective for the reliable separation and quantitation of full, partial and empty AAV capsids. Separation is achieved based on the charge variance of the isoelectric points, with full capsids having lower pI values than empties. SCIEX has developed a robust cIEF-based method for AAV full and empty capsids analysis that can be completed in less than an hour. This method shows excellent resolution between full, empty, and partial capsid peaks and is also capable of analyzing different AAV serotypes [4].

CE-LIF can also be used for full/empty capsid analysis in combination with genome integrity analysis. SCIEX has developed a fast, size-based screening workflow for AAV that involves purification of the AAV sample with the QIAquick PCR kit straight to nucleic acid, followed by separation and analysis. This method provides very good separation of intact and partial genome peaks and small size impurities in just 30 minutes (10 minutes for prep, 15 minutes for separation), allowing rapid analysis of in-process samples.

## CONCLUSION

Economical, rapid and robust biophysical methods for the analysis of viral vector capsids – both in-process samples and final products – is essential to ensuring safe and effective gene therapies. SCIEX has developed MS and CE-LIF solutions that provide the critical information required for characterizing AAV viral vector proteins, determining AAV capsid purity and genome integrity and separation and detection of full, partial and empty AAV capsids.

These methods offer excellent resolution and sensitivity with minimal preparation and can be automated for rapid analyses. While they have been developed specifically for AAV samples, including different serotypes, these methods could be modified to work with other viruses.

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