British Pharmacopoeia

Consultation

British Pharmacopoeia public consultation for draft guidance for Characterisation of the Capsid Particle Population in rAAV Products: Capsid Protein Characterisation.

Consultation period 18 December 2025 to 27 March 2026

Medicines & Healthcare products Regulatory Agency

British Pharmacopoeia

1. Patients, standards, and innovation

The quality of a medicine is critical to ensuring its safety and efficacy, and therefore the medicine's suitability for patients. Pharmacopoeial standards are part of an interlinked system, together with good practice guidelines and regulatory assessment, that form a foundation to ensuring medicines are of an acceptable quality. Additionally, standards have a place in supporting and enabling innovation through the availability of consistent and widely applicable quality requirements. Innovation in the field of medicines and healthcare has the potential to support patients throughout the world to live longer, healthier, and happier lives.

In recognition of the increasingly important role of biological medicines to healthcare worldwide, the Medicines and Healthcare products Regulatory Agency (MHRA) has developed and implemented a Strategy for pharmacopoeial public quality standards for biological medicines.[1] This strategy, adopted following consultation with stakeholders, laid out a vision of working collaboratively to explore and develop new standard setting approaches for biological medicines. It included a commitment to investigate and take forward standard setting opportunities for innovative Advanced Therapy Medicinal Products (ATMPs).

ATMPs have the potential to be transformative to patients and healthcare globally. However, development, characterisation, and production of these innovative medicines is challenging due to their high complexity, their product specificity, and the still-emerging technologies that support them. Publications such as the Advanced Therapies Manufacturing Taskforce Action Plan,[2] the Medicines Manufacturing Industry Partnership's Manufacturing Vision for UK Pharma[3] and stakeholder feedback have emphasised the important role that standards can have in the development of these medicines. This includes a focus on the value of widely applicable standards that could support knowledge building and facilitate analytics and characterisation.

This draft guidance was written by experts in the ATMP community to support those involved in the development of analytical methods throughout the product lifecycle, and therefore contribute to the quality assurance of innovative medicines for patients.

The MHRA and British Pharmacopoeia would like to recognise and thank the numerous experts in the BP's Working Party for ATMPs that have contributed to the development of this text. The work has been supported by a joint-staff secondment scheme between the BP and the UK's Cell and Gene Therapy Catapult.[4]

2. The draft document

As part of the MHRA strategy for the creation of pharmacopoeial public quality standards for biological medicines, the British Pharmacopoeia Expert Advisory Group for ATMPs, established in March 2020, has engaged with groups across the cell and gene therapy

[1] https://www.gov.uk/government/consultations/strategy-for-pharmacopoeial-public-quality-standards-for-biological-medicines

[2] http://www.abpi.org.uk/publications/advanced-therapies-manufacturing-action-plan/

[3] https://www.abpi.org.uk/publications/manufacturing-vision-for-uk-pharma-future-proofing-the-uk-through-an-aligned-technology-and-innovation-road-map/

[4] https://ct.catapult.org.uk/

community to develop non-mandatory guidance for key analytical technologies to ensure quality throughout the product lifecycle. The working party has developed guidance to support ATMP development across a wide range of organisations, laboratory settings, and therapy types. As such, guidance is product-agnostic and does not provide a step-by-step protocol, nor constitute a prerequisite for product acceptance, but instead offers measures to ensure the production of robust, comparable, and reproducible data within and across organisations.

The ATMP industry continues to grow rapidly worldwide, with increasingly sophisticated scientific discoveries being translated into therapies. There are a variety of challenges in characterising these experimental living medicines. Any CGT product must be characterised in terms of identity, purity and potency and the choice of, and route to, validation of these assays largely lies with the developer and manufacturer. Establishing robust potency assays grows in importance throughout the development of a CGT product and becomes critical in the later clinical stages. As products move towards pivotal clinical trials and licensure, establishing the mechanism of action of the product becomes critical. This requires that potency assays yield rich data which informs the interpretation of outcomes in vivo, whether in models or in early human trials.

This document builds upon the previous British Pharmacopoeia document, ‘Characterisation of the Capsid Particle Population in rAAV Products’, which provided comprehensive recommendations for assessing and quantifying the various rAAV particle species present in therapeutic products, including full, empty, partially filled/intermediate, and overpackaged capsids. The present document provides recommendations for a characterisation framework for analysis of capsid proteins, encompassing their identity, purity, structure, PTMs, and interactions. The guidance is organised according to the principal analytical methodologies used to assess these attributes.

3. How to contribute

The draft guidance for Characterisation of the Capsid Particle Population in rAAV Products: Capsid Protein Characterisation will be posted online for public consultation for a period of two months. During this time, we are asking stakeholders to complete and return the response document, available on our website, to BiolStandards@mhra.gov.uk.

When reviewing the guidance, you may want to consider the following points:

- Do you agree with the technical recommendations made in the document?
- Are the key methods for particle characterisation covered?
- Are there any aspects which you think are missing from the document?
- Is there any terminology within the document that you think needs to be more clearly defined?
- Is the document understandable and are recommendations clear and unambiguous?
- Could the format/style of the guidance be improved?

In addition to the request for technical comments, the response form includes more general questions around the value of the guidelines and other work within the area of ATMPs where standards and standardisation could add value. This information will be used to help the BP to understand and prioritise future work related to ATMPs.

4. Confidentiality and Freedom of Information

Information we receive, including personal information, may be published, or disclosed in accordance with the access to information regimes (primarily the Freedom of Information Act 2000 (FOIA), the Data Protection Act 1998 (DPA) and the Environmental Information Regulations 2004).

Please let us know if you would like any information you provide to be treated in confidence, and please indicate any commercial sensitivities. We will maintain that confidence and resist disclosure under the access to information regimes where possible and in compliance with our legal obligations. We will also consult you and seek your views before any information you provided is disclosed.

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Annex 1 - Draft guidance: Characterisation of the Capsid Particle Population in rAAV Products Capsid Protein Characterisation

Medicines & Healthcare products Regulatory Agency

Characterisation of the Capsid Particle Population in rAAV Products: Capsid Protein Characterisation guidance (Part 3)

Contents

1. Abbreviations

Acronym	Meaning
AAA	Amino Acid Analysis
AAV	Adeno-Associated Virus
AFM	Atomic Force Microscopy
AGE	Agarose Gel Electrophoresis
ALCOA	Attributable, Legible, Contemporaneous, Original, Accurate (data integrity principle)
ATP	Analytical Target Profile
BGE	Background Electrolyte
CAPEX	Capital Expenditure (context-dependent)
CDMS	Clinical Data Management System
CE	Capillary Electrophoresis
CEMS	Capillary Electrophoresis Mass Spectrometry
CGE	Capillary Gel Electrophoresis
CGT	Cell and Gene Therapy
CQA	Critical Quality Attribute
CZE	Capillary Zone Electrophoresis
DDA	Data-Dependent Acquisition (MS technique)
DDM	n-Dodecyl β-D-maltoside (detergent for membrane proteins)
DIA	Data-Independent Acquisition (MS technique)
DNA	Deoxyribonucleic Acid
DSP	Downstream Processing
DTT	Dithiothreitol (reducing agent)
ELISA	Enzyme-Linked Immunosorbent Assay
ESI	Electrospray Ionization (MS ionization method)
FLR	Fluorescence (detection method)
GMP	Good Manufacturing Practice
HCP	Host Cell Proteins (impurity in biologics)
HPLC	High-Performance Liquid Chromatography
HRAM	High-Resolution Accurate Mass (MS technique)
ICH	International Council for Harmonisation
IEX	Ion Exchange Chromatography
LC	Liquid Chromatography

MALDI	Matrix-Assisted Laser Desorption/Ionization
MS	Mass Spectrometry
PAGE	Polyacrylamide Gel Electrophoresis
PTM	Post Translational Modification
QbD	Quality by Design
QC	Quality Control
RNA	Ribonucleic Acid
RP	Reverse Phase (chromatography technique)
SDS	Sodium Dodecyl Sulfate (for protein denaturation)
SEC	Size-Exclusion Chromatography
SPR	Surface Plasmon Resonance (binding assay)
TEM	Transmission Electron Microscopy
UHPLC	Ultra-High-Performance Liquid Chromatography
UV	Ultraviolet (spectroscopy)
USP	Upstream Processing
VP	Viral Protein
vp/mL	Viral Particle Titre/mL
VPS	Virus Particle Size (context-dependent)

2. Terminology

The terminology here is intended to be a glossary of terms for readers to use as a reference tool and for a clearer definition of repeated concepts.

Empty capsids: DNA or Genome-less particles

Serotype: Wild type clades

Automation: The use or introduction of automatic equipment in a manufacturing or other process or facility.

Reference standard: A reference standard is a highly purified compound with a known and verified potency, used as a benchmark for analytical testing in various fields, particularly in pharmaceuticals. It is essential for assessing the quality, safety, and potency of pharmaceutical products throughout their development and market life.

PTMs: Post translational modifications (see reference table)

Table 1 PTMs Commonly Observed in AAV Capsid Proteins

PTM Type	Impact	Relevance
Deamidation	Alters charge, affects capsid stability.	Occurs during storage or at sub-optimal pH.
Oxidation	Reduces stability, increases immunogenicity.	Introduced during purification or storage.
Phosphorylation	Affects capsid assembly and receptor interactions.	May occur naturally in host cell systems.
Acetylation	Impacts protein interactions and immune response.	Minor but can occur in mammalian expression.
Glycosylation	Alters receptor binding and immunogenicity.	May occur if glycosylation-prone sequences exist.
Proteolysis	Degrades capsid proteins, reducing integrity.	Indicates protease activity in the process.

Capsid: The protein shell of a virus, including AAV, that encloses the viral genome. Capsid integrity, purity, and identity are critical quality attributes (CQAs).

Critical Quality Attribute: A physical, chemical, biological, or microbiological property or characteristic that must be controlled to ensure product quality.

Capsid Protein ratios (VP1-2-3 variable ratios seen differently across serotypes)

Snapback genome: A snapback genome in the context of AAV (adeno-associated virus) refers to an aberrant or unintended DNA structure that can form during AAV vector genome replication or packaging. It is often considered a type of impurity or defective particle and is particularly relevant when assessing vector genome integrity during analytical characterisation - The genome "snaps back" into a hairpin-like or duplexed form, mimicking a double-stranded configuration even though it is incomplete.

Empty - Full Capsids: As rAAV is made/packaged during production, non-homogenous capsid forms can manifest. Often seen as impurities that can impact or lead to challenging QC or assay testing. The exact impact on the wide range of empty and full or partial full capsids needs to be explored for every developer.

Migration of gel systems where proteins migrate under and electric charge differential based on mass.

Analytical Target profile: Prospective summary of performance characteristics that defines the intended purpose of an analytical procedure (ICH Q14). It outlines measurement requirements for one or more critical quality attributes (CQAs), specifies performance criteria such as specificity, accuracy, precision, and robustness, and

guides technology selection based on the operating environment, whether at-line, in-line, or off-line. Additionally, the ATP serves as a foundational element throughout the analytical lifecycle, supporting method validation in accordance with ICH Q2(R2) and enabling continual improvement.

3. Introduction

Advanced Therapy Medicinal Products (ATMPs) represent a rapidly evolving class of biotherapeutics that includes gene therapy medicinal products (GTMPs), somatic cell therapies, and tissue-engineered products. Among these, recombinant adeno-associated virus (rAAV) vectors have emerged as one of the most widely adopted platforms for *in vivo* gene delivery due to their favourable safety profile, durable gene expression, and broad range tissue tropism. The increasing number of AAV-based therapies progressing through clinical and commercial stages has created an urgent need for robust, standardised, and scientifically justified approaches to vector characterisation, quality control, and regulatory compliance.

The inherent complexity of AAV-based gene therapies, owing to their biological variability, intricate manufacturing processes, and heterogeneity in final drug product, presents unique challenges that differ significantly from those encountered in the production of conventional biologics. Consequently, new analytical paradigms are required to comprehensively assess identity, purity, potency, genome integrity, capsid integrity, and content (empty/full ratios), as well as to control product- and process-related impurities such as host cell proteins (HCPs), residual host cell DNA, helper virus sequences, and packaging-related byproducts.

Modern AAV characterisation requires a platform of orthogonal, phase-appropriate analytical technologies, including but not limited to:

- Capillary gel electrophoresis (CGE) for capsid protein profiling
- Liquid chromatography with UV or fluorescence detection (LC-UV/FLR) for impurity and degradation analysis
- Quantitative PCR or droplet digital PCR (qPCR/ddPCR) for genome titre
- Analytical ultracentrifugation (AUC), charge detection mass spectrometry (CDMS), and cryo-electron microscopy (cryo-EM) for particle composition
- Mass photometry and interferometric techniques for single-particle analysis
- LC-MS-based peptide mapping and PTM profiling for detailed capsid characterisation

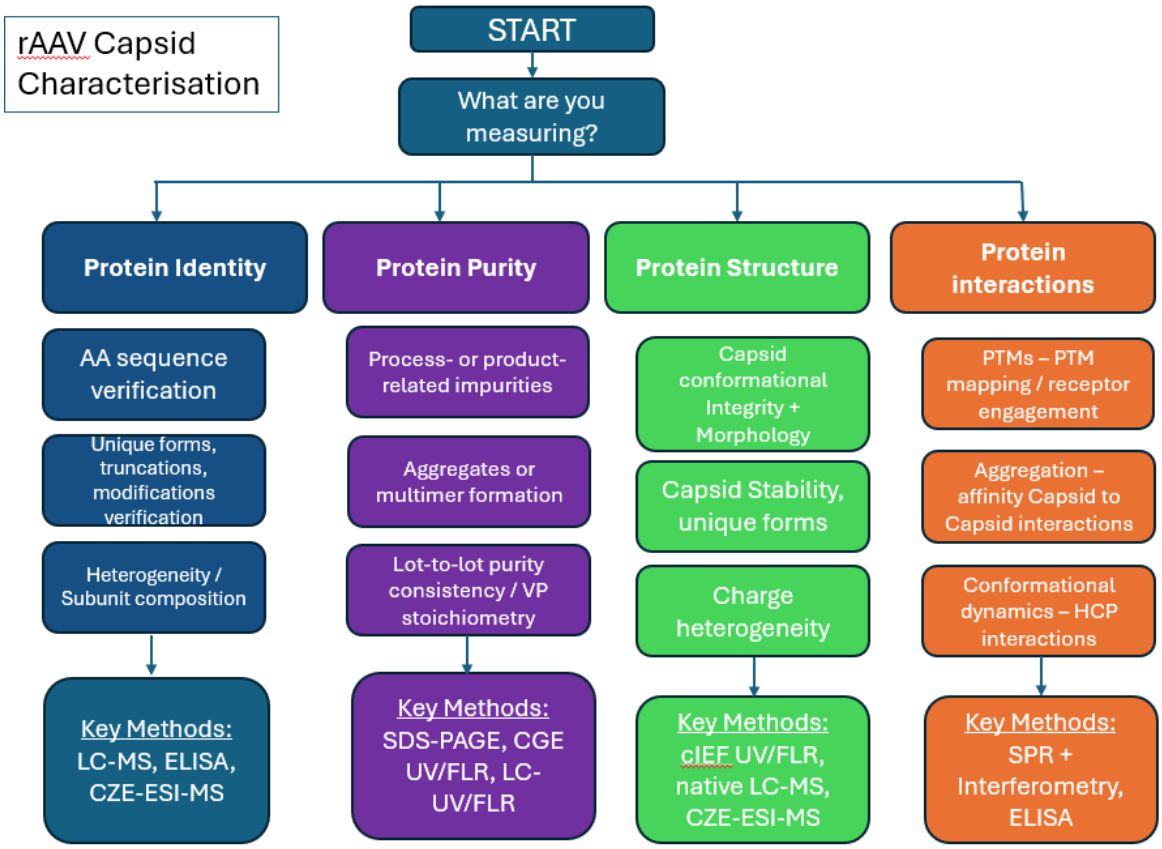
As the industry shifts towards lifecycle-based method development and embraces principles such as the Analytical Target Profile (ATP) and Quality by Design (QbD), regulatory authorities including the FDA, EMA, and NMPA expect data packages that demonstrate analytical robustness, specificity, and suitability for purpose, aligned with ICH Q6B, Q2(R2), Q14, and USP <1220> & <1225> guidelines.

This chapter aims to provide a harmonised, scientifically justified framework for the pharmacopoeia characterisation of AAV-based gene therapy products, intended to support developers, manufacturers, and regulators in ensuring the safety, identity, strength, purity, and quality (SISPQ) of AAV vectors at all stages of development. The guidance encompasses method selection, validation principles, system suitability

requirements, and emerging technologies that address current challenges in the characterisation of these complex modalities.

More specifically, AAV vectors capsid proteins play a crucial role in gene therapy. These proteins form the capsid, that encases the viral genome, ensuring its stability and facilitating its delivery to target cells. The capsid is composed of three viral proteins: VP1, VP2, and VP3, which assemble into an icosahedral structure (reference). The capsid's surface interacts with cellular receptors, determining the vector's affinity for specific cell types, also called tropism (reference), this tropism is typically linked to the capsid serotype numbering. Notably, there is a considerable cross-reactivity between tissue types and serotypes.

Modifications to capsid proteins can significantly impact the efficiency and specificity of the gene therapy product delivery. Post-Translational Modifications (PTMs) such as phosphorylation, deamidation, and glycosylation can affect the capsid's stability, immunogenicity, and interaction with cellular receptors (reference, reference).



4. Scope

This guidance builds upon the previous British Pharmacopoeia document, ‘Characterisation of the Capsid Particle Population in rAAV Products’, which provided comprehensive recommendations for assessing and quantifying the various rAAV particle species present in therapeutic products, including full, empty, partially filled/intermediate, and overpackaged capsids. The present document provides recommendations for a characterisation framework for analysis of capsid proteins, encompassing their identity, purity, structure, PTMs, and interactions.

The guidance is organised according to the principal analytical methodologies used to assess these attributes, including but not limited to LC–Mass Spectrometry, Liquid Chromatography–UV/FLR, antibody-based techniques (ELISA), affinity-based techniques (SPR and interferometry), SDS–PAGE, capillary gel electrophoresis, and emerging orthogonal methods. While many of these techniques can provide information on multiple attributes, some are more suitable for particular aspects of capsid protein characterisation. A summary table is provided in section 5.9 to compare the capabilities and limitations of each method, with further detail in the main text for readers less familiar with these technologies. Method-specific considerations for assessing different capsid protein attributes are also discussed.

Efforts have been made to include the most widely adopted and scientifically justified methodologies for capsid protein characterisation, though the technologies described are not exhaustive. Users should select the most appropriate method(s) for their intended purpose, considering the unique properties of their rAAV product and manufacturing process.

Although this guidance is focused on the characterisation of capsid proteins, it is recognised that some impurities or product variants may arise from other components (e.g., host cell proteins, truncated or misassembled capsids) and may require additional analytical approaches. The suitability of each method for in-process, intermediate, or final product testing is considered, supporting process development, characterisation, and comparability assessments. Where an attribute is deemed a CQA, it should be included in release specifications with appropriate acceptance criteria, and validated in accordance with ICH Q2 (R2) and Q14. For non-release tests, fitness for purpose must be demonstrated.

Assessment of nucleic acid content (e.g., vector genome titre, encapsidated DNA impurities), as well as other non-proteinaceous contaminants, is addressed in separate guidance documents and is outside the scope of this document. This guidance does not cover aspects such as viral infectivity, potency assays, or clinical efficacy.

5. Methods for analysing capsid protein characterisation

Comprehensive characterisation of AAV capsid proteins, including the major subunits VP1, VP2, and VP3, as well as their post-translational modifications (PTMs), degradation products, and stoichiometry across the many serotypes of AAV, *which can all display unique characteristic*s, is a cornerstone of quality assessment for recombinant AAV-based gene therapy products. The ability to reliably identify and quantify these components supports the establishment of CQAs tied to product safety, efficacy, and consistency, in alignment with global expectations outlined in ICH Q6B, Q2, Q14, and guidance for Advanced Therapy Medicinal Products (ATMPs).

Looking ahead, the landscape of capsid protein characterisation is evolving in response to regulatory and environmental drivers, such as the European Union's REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) framework, which is expected to phase out or restrict certain dyes, detergents, and heavy-metal-based stains historically used in electrophoresis and protein visualization. As these reagents are withdrawn, analytical laboratories are adopting greener, REACH-compliant alternatives, including non-toxic fluorescent stains, biodegradable surfactants, and label-free optical detection techniques, to future-proof their workflows.

Simultaneously, the field is experiencing a shift toward next-generation analytical technologies capable of higher sensitivity and reduced sample consumption. These include label-free mass photometry, single-molecule interferometric techniques, and advanced LC-MS workflows with intact mass and PTM profiling, which are poised to complement or replace some legacy approaches, particularly those with high reagent burdens or limited resolution.

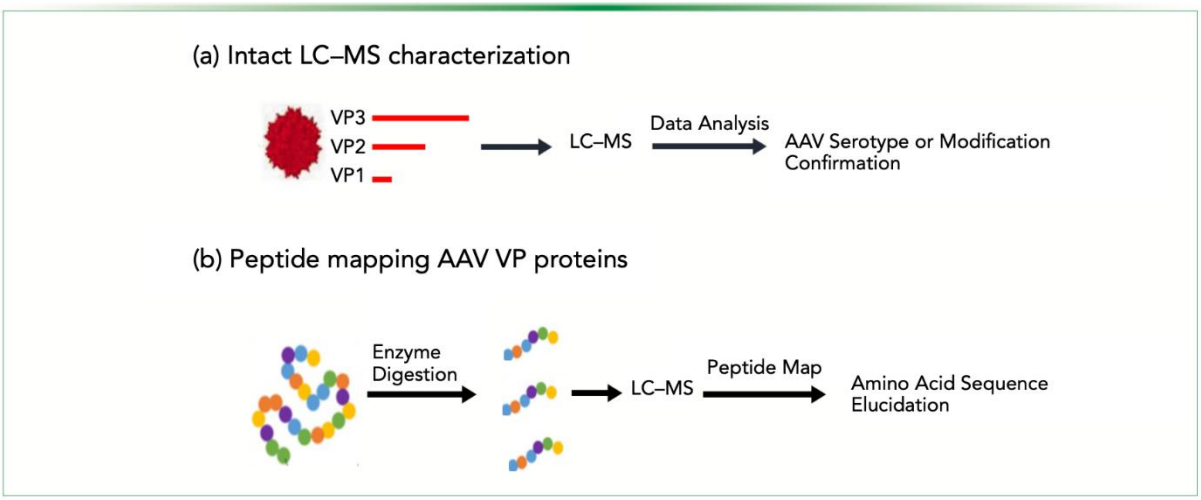
The following sections outline the principles, strengths, limitations, and regulatory considerations for each major method used in capsid protein characterisation today, while also highlighting the emerging trends and innovations shaping the future of AAV analytics.

5.1. LC-Mass Spectrometry

5.1.1. Introduction

Liquid Chromatography–Mass Spectrometry (LC-MS) has emerged as a powerful analytical tool for in-depth capsid characterisation. This method enables precise identification, assessment of capsid integrity, and quantification of capsid proteins (VP1, VP2, VP3) by intact mass analysis, as well as detection and relative quantification of PTMs by peptide mapping. Overall, LC-MS delivers comprehensive characterisation of the capsid heterogeneity. The technique offers high-resolution molecular data, enabling quality control, comparability studies, and insights into how manufacturing and stability conditions impact the capsid, making it a useful tool for AAV gene therapy development and production.

5.1.2. Method description



(Reference, to be redrawn: Characterization of Adeno-Associated Virus (AAV) Gene Therapy Products | LCGC International)

Liquid Chromatography-Mass Spectrometry (LC-MS) is an analytical technique used to characterise Adeno-Associated Virus vector (AAV) capsid proteins. The technique involves separating the VPs using generally reverse-phased or hydrophilic liquid chromatography and then identifying and quantifying them using mass spectrometry.

This method allows for the measurement of intact proteins and resulting peptides, providing detailed information about the capsid proteins primary sequences and stoichiometry (reference, reference, reference). For the analysis of intact proteins, Top-down mass spectrometry (MS) is used.

To achieve deeper VP characterisation, peptide mapping, which is a well-established technique in the biopharma industry, is used in the identification and quantification of PTMs and amino acid sequence analysis. It involves the proteolytic digestion of VPs followed by LC-MS analysis. The technique can achieve a high level of sequence coverage (>90% of VP sequence characterised) (reference, reference, reference).

5.1.3. Points to consider

Accurate identification of PTMs requires thorough data interpretation and often necessitates orthogonal validation. High-resolution mass spectrometry instruments, such as Orbitrap or Q-TOF, are generally required to distinguish closely related peptides and detect low-abundance species. Robust bioinformatics tools are essential for handling complex datasets, with clearly defined database search parameters, false discovery rate management, and manual validation steps.

For peptide mapping specifically, efficient capsid disruption and protein digestion are essential for obtaining reproducible results in sample preparation. The selection of

buffer, enzyme, and denaturation conditions can significantly influence data quality. When determining quantification strategies, it is important to align the approach with the specific goal, such as VP ratio determination or post-translational modification (PTM) profiling. Appropriate methods may include label-free quantification or using internal standards.

5.1.4. Advantages

An advantage of LC-MS is its ability to analyse different AAV serotypes, engineered variants and truncated species (impurities). It offers rapid analysis, making it suitable for high throughput needs. Top-down MS allows for detection of degradation products and sequence variants and mapping PTMs with greater precision that can be a challenge using bottom-up approaches. With sample preparation requiring fewer steps than bottom-up approaches (such as not requiring reduction and alkylation), the number of experimental artefacts is reduced as no chemical modification is required. This can help avoid modifications introduced during sample preparation in comparison to enzymatic digestion.

5.1.5. Limitations

The sample preparation for peptide mapping can be complex and time-consuming and may result in sample loss, causing high variability for quantification. Gradients may take up to 90 minutes, leading to extended analysis times. Another challenge to perform peptide mapping as a routine analysis is material availability because about 100µL of material at 1E+13 VP/mL is required per analysis.

For intact protein analysis, the signal from low abundant proteins may be masked from the signal of high abundant proteins within a sample. Therefore, larger sample quantities, enrichment techniques such as protein capture or immunoprecipitation, and high-resolution mass spectrometers are required. Efficient protein separation is required due to co-elution of proteoforms and complex mass spectra with overlapping peaks, which can make analysis difficult.

Additionally, two other factors may limit access to this technology: the high capital expenditure (CAPEX) required for utilities, equipment, and software licenses, and the expertise needed to develop and run LC-MS-based assays. Furthermore, the range of LC mobile phase compositions is limited, as the ionisation process necessary for MS analysis is sensitive to non-volatile salts (e.g., sodium, Tris) and adduct formation, which can affect sensitivity due to ion suppression.

From a method performance assessment perspective, the evaluation of the accuracy of the method is challenging because of the lack of standards and orthogonal techniques. This makes the relative quantification challenging.

5.1.6. Sample preparation

Two types of sample preparation are considered depending on whether the LC-MS is meant to analyse intact VPs or generated peptides of VPs.

Intact mass analysis

Intact mass analysis is simplified because proteolytic digestion is not required, reducing mixture complexity. Protein extraction and purification are still necessary.

Proteins are first extracted using a lysis buffer. To prevent protein degradation and protein modifications, appropriate lysis buffers containing protease/phosphatase inhibitors should be used. Keeping samples at low temperatures prevents post translational modifications during extraction. After extraction, proteins are solubilised in a suitable buffer, often containing salts or detergents such as SDS or DDM.

Many buffers are not MS compatible, so a buffer exchange step is required to remove interfering components and remove volatile salts using ultrafiltration and centrifugation for an MS compatible sample. Immunoprecipitation can be used to isolate target proteins, particularly useful for a targeted approach.

Finally, protein separation and purification separate the target protein either through liquid chromatography (LC) or by SDS-PAGE.

Peptide mapping

The first step involves denaturing the capsid to expose VPs' peptide bonds, making them accessible to proteases. This is typically achieved using denaturing agents like urea or guanidine hydrochloride, followed by incubation at an appropriate temperature, such as 37°C, for 30 minutes to 2 hours.

After denaturation, reducing agents such as DTT or 2-mercaptoethanol are added to break disulphide bonds, preventing aggregation and ensuring complete digestion. The sample is then incubated again at the denaturation temperature. To prevent the reformation of disulfide bonds, the free thiol groups of cysteine residues are alkylated using an alkylating agent like iodoacetamide.

Next, the sample undergoes buffer exchange, generally by using de-salting cartridge, into a buffer compatible with the selected protease and subsequent chromatographic steps. A common buffer is ammonium bicarbonate. Finally, enzymatic digestion is performed using a protease that cleaves the protein at specific amino acid residues. Trypsin is a popular choice due to its specificity for lysine and arginine residues. Nevertheless, the use of other proteases such as chymotrypsin or pepsin has been reported in the literature (reference, reference).The protease-to-substrate ratio is optimized to ensure complete digestion while minimizing enzyme autolysis.

5.1.7. Method set-up and execution

The method consists of the trapping and the separation of compounds of interests (VPs or peptides) using a gradient of eluting phase. Eluting compounds are then ionised and their mass-to-charge ratio measured.

Intact mass analysis

Intact proteins are fragmented into the MS, typically by ESI for efficient fragmentation. The intact mass of the protein is firstly analysed by MS1. Following identification of the intact protein, the protein is then fragmented in the mass spectrometer using MS/MS to create product ions. The product ions generated are compared to databases and used for protein identification and characterisation.

For specific parameters, a high resolution such as 15,000 at 200 m/z is required for accurate identification of fragment ions and a scan range that is appropriate for the intact mass of the proteins between a few hundred to a few thousand m/z, which can be further narrowed down for specific fragments. Sample amount usually required is 1-10 µl injection volume at a concentration range usually around 0.5-1 µg.

Peptide mapping

Ions, corresponding to the eluted peptides, are guided into the mass spectrometer and analysed ultimately to give a mass-to-charge ratio for each ion. One peptide may result in several ions. To detect as many ions of interest (ionised peptides) as possible, the mass-to-charge ratio range is typically from 200 to 2000 m/z, which may be modified based on the expected masses of the peptides after digestion using in silico digestion. Ion isolation and fragmentation, and analysis of the resulting fragments (MS/MS analysis) are recommended to improve confidence of the level and identification of peptide. High resolution mass spectrometers can reach more than 60,000 (at 200 m/z) resolution in full scan mode (“MS only”). However, in MS/MS analysis, it is recommended to limit the resolution to 15,000 m/z, for example (at 200m/z) to limit the duty cycle of the instrument and so to ensure MS/MS analysis speed scan.

5.1.8. Data acquisition and analysis

Two acquisition strategies exist: Data-Dependent Acquisition (DDA) and Data-Independent Acquisition (DIA) for selecting parent ions for fragmentation.

Data acquisition and processing are usually performed using separate software packages. Modern tools can process raw data from multiple instrument types, offering broad flexibility (reference, reference).

The first step in the data processing is the deconvolution of the mass spectra (raw data) into a list of masses. Those masses are then confronted with theoretical masses

of compounds archived in a database. The matching of experimental masses and theoretical masses results in the identification of the VPs or the peptides, in the case of peptide mapping experiments. A mass tolerance and, in some cases a false discovery rate, is set to prevent identification errors. The identification relies on the matching of theoretical and experimental masses. Additionally, to confirm protein identity, fragmentation and matching of the fragment masses may be considered.

5.1.9. System selection and suitability

High-resolution analysers from multiple manufacturers meet scientific requirements; no single platform is exclusive. This guidance applies to instruments equipped with electrospray ionisation sources.

5.1.10. Method development, qualification, and validation

Method development is required for sample preparation, separation, MS Parameters and data analysis.

Sample preparation method development requires purification of proteins to improve sample complexity which will help to improve signal to noise ratio.

For separation optimisation of LC is required for efficient separation including column type selection, mobile phase and flow rate. The sample needs to be compatible for ionization and fragmentation techniques. This requires the selection of the correct solvent. For example, for ESI, common solvent examples include methanol, acetonitrile and water, alone or in combination.

Validation is required to distinguish between different proteins and modifications. This is important for specificity, which can impact misidentification. The method also needs to be repeatable across different experiments and reproducible in different laboratories, where applicable.

5.2. Liquid chromatography-UV/FLR

5.2.1. Introduction

Liquid Chromatography with UV and Fluorescence (LC-UV/FLR) is widely used in GMP-compliant gene therapy QC analytics, including rAAV capsid protein characterisation, amino acid analysis, and impurity profiling.

5.2.2. Method description

Liquid Chromatography (LC) is used for the separation, identification, and quantification of components in rAAV samples. Two types of detection are commonly used: UV and fluorescence.

UV detection is commonly used to monitor the presence of nucleic acids and proteins in rAAV preparations. It provides an accessible and reliable method for quantifying these components based on their absorbance at specific wavelengths (280nm for protein and 260nm for nucleic acids). In certain applications, fluorescence detection offers higher sensitivity compared to UV detection. For example, it is particularly useful for detecting certain proteins by exciting aromatic amino acid residues (e.g. tryptophan). Therefore, it is recommended to know the excitation properties of the proteins of interest prior to selecting the method.

For characterising viral vector proteins, two main column stationary phases may be considered: reversed phase, which separates the capsid proteins based on their hydrophobicity. Alternatively, hydrophilic interaction can resolve capsid protein structures based on their hydrophilic properties. These two column chemistries are orthogonal, and the selection should be assessed on the serotypes of interest and amino acid sequences, which give information on capsid proteins hydrophobicity and pI. The LC-UV/FLR technique may be used to elaborate an identity testing as each vector protein's physicochemical properties, given by the composition of amino acid residues, result in a specific retention time of each vector protein. Usually, VP3 protein is well separated from the two other VPs. However, separating VP1 and VP2 proteins may be challenging due to the intrinsic property of AAV where alternative splicing of the *cap (VP1 protein)* gene meaning VP2 and VP3 are created from the same transcript, as such resulting in a higher sequence homology and so, and a higher similarity of their physicochemical properties. It is important to control for this during LC testing.

Method development activities are expected for any new engineered capsid. In reversed phased separation, various mobile phase solutions such as solutions of trifluoroacetic acid in water and acetonitrile (*e.g.,* 0.1% volume/volume). Although hydrophilic-based methods require greater development on mobile phases composition: buffer salts concentration in the aqueous phase (*e.g.,* from 5 to 100mM) and pH range (*e.g.,* from 2 to 7.5).

5.2.3. Points to consider

Sample integrity and formulation should be carefully considered for capsid characterisation, as properties such as high salt, excipients, aggregates may quench fluorescence and alter sample readouts (UV absorbance, emission). UV detection may not be sensitive enough for low-abundance impurities or trace protein fragments, especially in diluted viral vector samples. Additionally, quantification relies on the Beer-Lambert law, which assumes a consistent chromophore response - not always true for heterogeneous capsid populations.

5.2.4. Advantages

LC technology is widely used in biopharma industries and well-established in quality control laboratories. Development work is still required but the method implementation from development to routine testing laboratories is accessible and rapid.

5.2.5. Limitations

Due to the overlapping homologous regions over the three capsid proteins (VP1, VP2 and VP3), a coelution of VP1 and VP2 may occur. This should be assessed on a case-by-case basis, as it depends on the amino acid sequence of the VP proteins, which varies by serotype.

Because LC-UV and LC-FLR rely on VP's elution times, it is required to characterise all the peaks observed during the development by an additional technique like LC-MS. This will ensure that any new peak observed in testing will have to be investigated using the same relative methodology.

5.2.6. Sample preparation

The sample preparation is limited to sample dilution if needed and the denaturation of the rAAV capsid by using a chemical chaotropic reagent, temperature denaturation (note: artefactual modification may be generated) or any unfolding mean to make VPs available prior to their separation.

5.2.7. Method set up and execution

No specific recommendations are needed compared to classic proteins separation assay. Pure and UHPLC-grade solutions are recommended.

5.2.8. Data acquisition and analysis

Most of the chromatography data systems enable the acquisition, processing, and reporting of LC data. The analysis of data relies usually on peak integration and quantification based on peak area, system selection and suitability.

To achieve optimal separation, it is recommended to use UHPLC systems. Additionally, the optical cell length should meet UHPLC specifications to avoid any peak broadening that could compromise resolution. Those requirements are common for proteins separation methods in general; no further recommendations for rAAV VPs are expected.

5.2.9. Method development, qualification, and validation

The main activity in development is to identify the best chromatographic conditions to separate the three VPs and other potential subspecies by screening column chemistries, determining mobile phases compositions and defining the chromatographic gradient.

5.3. Antibody-based techniques: ELISA

5.3.1. Introduction

Enzyme-Linked Immunosorbent Assay (ELISA) is a plate-based immunological method used for the detection and quantification of proteins, antigens, or antibodies. In AAV analysis, ELISA is commonly employed to quantify AAV capsid proteins i.e., total viral particles.

5.3.2. Method description

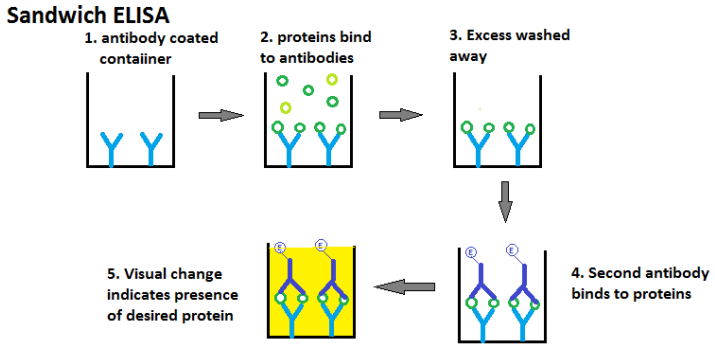


Figure 1. Sandwich ELISA

https://commons.wikimedia.org/wiki/File:Sandwich_ELISA.png

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ELISA relies on the specific binding between an antigen and an antibody, with detection achieved through enzyme-mediated signal generation, typically using colorimetric, fluorescent, or chemiluminescent readouts.

The sandwich ELISA is the most common type of ELISA utilised in AAV analysis, owing to the high degree of specificity imparted by its methodology. Typically, capture antibodies targeted against the antigen of interest are precoated onto a plate. Samples, containing the antigen of interest, are then added to the plate and incubated to allow binding before a wash step to remove unbound antigen. A detection enzyme-conjugated or fluorophore-conjugated antibody targeted against the antigen is added, in effect forming a sandwich. After a further wash, the subsequent steps are dependent on the readout method.

For colorimetric readout, the substrate is converted by the enzyme leading to a quantifiable colour change. For fluorometric based assay it can be read directly, whilst for luminescence-based assay a substrate is catalysed by an enzyme to generate light which can be quantified using a plate reader.

5.3.3. Points to consider

When deciding on performing ELISA, special attention should be paid certain aspects such as number of samples, is automation required, methodology to be chosen and if it is appropriate for the work from a time and cost perspective. ELISA methods quantify all capsids and will not discriminate between full or empty i.e. those particles which contain DNA or not respectively. Other analytics will need to be performed in conjunction with ELISA methodologies to determine these readouts. The specific details covering these elements are discussed in the sections below.

5.3.4. Advantages

With high sensitivity, ELISA methods can detect even low concentrations of target analytes, making it ideal for impurity and potency assays in CGT. It is very versatile, it can be adapted for a wide range of analytes, including total capsid proteins, VP subunits, or neutralizing antibodies. There are many commercially available ELISA kits streamline implementation for common AAV serotypes, making lab to lab standardisation easier and more robust. ELISAs have been utilised historically to measure protein concentrations and are a well-established methodology, hence their wide adoption as a means of measuring AAVs.

5.3.5. Limitations

High dependency on well characterised or high-quality antibodies. Requires highly specific antibodies, which may not always be available for novel AAV serotypes, these can be developed but may extend the drug development lifecycle. A common challenge is that certain sample components/impurities/artefacts can interfere with antibody binding and/or readout, leading to variability, which may require a minimal sample dilution factor for accurate quantification.

From the therapy producer's perspective, consideration must be paid to the kit itself, specifically the comparability of the AAV serotype standard provided and how similar/not this native particle will be to their own particles as there could be differences. A related point is the capacity of the kit itself to recognise the correct conformation of the target antigen when it is intact and present on the AAV capsid itself rather than as free antigen.

Special attention must be paid to the type of detection method, with colorimetric and luminescence protocols available. Colorimetric detection tends to have a narrow quantification range, whilst luminescence (inclusive of fluorescence) although having a broader range does not display a strong dose response.

5.3.6. Sample preparation

Prepare samples by diluting in the assay buffer to fall within the assay's dynamic range. Prepare a standard curve using a known concentration of the target analyte. It

is important to ensure consistent sample preparation to minimise variability. Consider additional clarification where appropriate, consider using a depth filter or centrifuge samples to remove particulates that might interfere or cause matrix inhibition with the assay.

5.3.7. Method set up and execution

Typically, manufacturers will provide their own protocols which come with their kits, but the processes tend not to deviate from kit to kit and the level of automation. The key element will be the generation of a standard curve using the appropriate standard material. The protocol between different ELISA kits rarely differs in the steps but rather with the incubation times and reagent dilutions, with capture antibody pre-coated plates a common feature.

Briefly, a capture and conjugated detection antibody are used which can recognise the specific AAV serotype capsid proteins, preferably in the particle conformation i.e. specific to protein on capsids rather than free capsid proteins. Subsequent steps will follow the standard sandwich ELISA procedure dependent on the specific readout method (section above, *Method description*).

5.3.8. Data acquisition and analysis

A standard curve is usually performed in every run, from which the data from the samples are interpolated from. Plot a standard curve based on known concentrations of the analyte and corresponding signals, from this you can calculate sample concentrations by interpolating from the standard curve. Automated rather than manual calculations can be less error prone.

5.3.9. System selection and suitability

When choosing a system, if using engineered or non-standard capsids, special attention will need to be paid to ensure the feasibility of implementing a method compatible with custom capsid antibodies.

Microplate reader capable of measuring the required signal (e.g., absorbance, fluorescence, or luminescence). Plate washer for consistent washing steps. For ensuring an acceptable dynamic range, ensure the assay covers the expected analyte concentration range by performing representative pre-studies and better understanding the sample/analyte. It is important to consider the level of automation associated with the protocol/kit, for example an automated plate washer would enable more consistent washing steps, while end-to-end automation solutions exist from some vendors.

5.3.10. Method development, qualification, and validation

Identify the correct ELISA kit or generate and quantify a standard which is relevant to your analyte. Optimise antibody concentrations, incubation times, and washing steps for maximum sensitivity and specificity, and robustness against temperature and incubation time. Demonstrate linearity, precision, and accuracy using representative samples. Assess the compatibility of the assay to an orthogonal method with different sample matrices.

Note: Some off the shelf antibody kits may not be suitable for engineered AAV variants due to lack of validated epitope development. To develop an ELISA method for non-standard capsid variants, this may require the generation and incorporation of custom antibodies specific for the capsid variant.

5.4. Affinity-based techniques: SPR and interferometry

5.4.1. Introduction

Surface Plasmon Resonance (SPR) and Bio-Layer Interferometry (BLI) are analytical techniques that preserve native biological interactions by eliminating the need for fluorescent or radioactive labels.

5.4.2. Method description

Affinity-based technologies such as SPR and BLI are powerful, label-free analytical tools for studying molecular interactions. They are increasingly applied in rAAV characterisation, including:

- Capsid–receptor binding, antibody binding, and neutralisation assays
- Potency correlation and stability studies
- Capsid ligand screening (e.g., heparin, HSPG, TfR interactions)

Both techniques measure binding kinetics in real time and are widely used in nonclinical development, comparability, and QC studies, especially for characterising AAV–host receptor or antibody interactions.

Affinity-based methods such as SPR and BLI are invaluable tools in the analytical toolkit for AAV characterisation. They provide a functional measure of capsid–ligand interactions, which correlates with biological activity, transduction potential, and batch-to-batch consistency.

5.4.3. Points to consider

Both SPR and BLI are highly sensitive to temperature and vibration, which may not be adequately controlled in all laboratory environments. Cycle to cycle drift can be observed if these environmental aspects are not managed, making assay validation challenging. Newer disruptive technologies (e.g., nano-plasmonic sensors, high-throughput BLI arrays) promise miniaturisation and multiplexing; however, regulatory emphasis on biosensor comparability and system cross-calibration for biologics remains critical.

5.4.4. Advantages

SPR and BLI offer real-time binding analysis without the need for fluorescent or radioactive labels, preserving native biological interactions. They are capable of measuring the strength and kinetics of AAV capsid interactions with receptors, antibodies, or other ligands, providing critical insight into biological function and product consistency. SPR, in particular, delivers high-resolution kinetic measurements and is considered the gold standard for characterising complex binding events. BLI,

while somewhat less sensitive, offers greater throughput and operational simplicity, making it highly suitable for comparability or batch testing in QC environments.

These methods are especially helpful for understanding AAV-receptor interactions and monitoring capsid integrity and potency through ligand binding, which is directly linked to in vivo transduction.

5.4.5. Limitations

Despite their utility, both techniques have inherent limitations. One of the key challenges is sample matrix interference - buffer composition, serum components, or excipients in AAV formulations can affect signal stability or lead to non-specific binding. Moreover, immobilisation of the ligand (e.g., antibody or receptor protein) on the sensor surface must be optimized to avoid orientation artifacts, which could distort binding profiles.

SPR systems typically require more complex instrument handling, buffer stringency, and regeneration conditions compared to BLI, making them less ideal for routine, high-throughput QC. On the other hand, BLI's lower sensitivity can limit its ability to detect weak or transient interactions, and sensor variability can affect inter-laboratory reproducibility if not carefully controlled.

Neither method provides information about structural integrity or heterogeneity of the capsid itself, unlike orthogonal methods such as mass spectrometry or electron microscopy. Instead, they provide functional binding data, which complements but does not replace structural characterisation.

5.4.6. Sample preparation

For optimal performance, AAV vector samples should be well-purified and free from interfering substances such as detergents, high concentrations of salts, or serum proteins. Ideally, samples are formulated in a neutral buffer such as PBS or HEPES. Prior to analysis, they should be filtered (0.22 µm) and buffer-exchanged if needed to match the assay conditions.

Ligands, such as purified antibodies or recombinant receptor fragments, are used either as immobilised capture reagents (e.g., anti-capsid antibodies bound to a chip or sensor) or as analytes in solution that interact with immobilised rAAV particles.

5.4.7. Method set up and execution

In SPR, a ligand is typically immobilised onto a gold-coated sensor chip via amine coupling or biotin-streptavidin interactions. The AAV sample is then injected over the chip surface under controlled flow, allowing measurement of association and dissociation phases. The same surface may be regenerated multiple times using acidic or salt-based buffers, enabling repeated sample injections.

BLI operates by dipping a ligand-loaded biosensor (such as Protein A or Ni-NTA-coated tips) into wells containing AAV samples. Binding interactions are monitored in real time by tracking shifts in light interference patterns. Assay setup is compatible with 96- or 384-well plates for increased throughput.

5.4.8. Data acquisition and analysis

Both SPR and BLI generate sensorgrams that depict binding in real time format. From these, kinetic parameters such as the association rate (ka), dissociation rate (kd), and the equilibrium binding affinity (KD) can be calculated. These values provide insight into the strength and duration of interactions, which is particularly useful when characterising receptor tropism, assessing changes due to capsid mutations, or evaluating the impact of stress or formulation on AAV potency.

In comparability settings, overlaying sensorgrams from different batches or serotypes allows developers to assess consistency in binding behaviour - a critical attribute to measure.

5.4.9. System selection and suitability

System suitability is essential, particularly in a GMP setting. It typically involves running controls to verify baseline stability, regeneration efficiency, reproducibility of binding curves, and sensor integrity. For SPR, this may include confirming chip activity after multiple regeneration cycles. For BLI, sensor re-use and tip-to-tip variability must be managed carefully, with calibration standards used to maintain consistency.

These methods must demonstrate adequate specificity (e.g., distinguishing between related capsid variants), and reproducibility/robustness (e.g., consistent performance across temperature, buffer, and operator changes).

5.4.10. Method development, qualification, and validation

For regulatory use, method development should define critical assay parameters such as ligand concentration, immobilisation density, regeneration conditions, and analyte injection profiles.

Validated SPR or BLI assays can be used in potency assessments, stability testing, serotype comparability, and functional lot release testing for gene therapy vectors.

5.5. SDS-PAGE

5.5.1. Introduction

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is a versatile, cost-effective, and accessible method for analysing AAV capsid proteins. While considered a mature and relatively low-complexity method, SDS-PAGE continues to play a crucial role in confirming capsid stoichiometry (1:1:10 ratio), detecting protein impurities, and supporting lot-release characterisation alongside more advanced orthogonal methods such as LC–MS or CGE.

5.5.2. Method description

SDS-PAGE is a widely used electrophoretic technique for the separation of proteins based on their molecular weight. Proteins are denatured and coated with SDS, a detergent that imparts a uniform negative charge, allowing separation solely by size as they migrate through a polyacrylamide gel under an electric field. SDS-PAGE is commonly used for qualitative and semi-quantitative analyses of protein composition, including capsid proteins (VP1, VP2, and VP3) in AAVs.

5.5.3. Points to consider

SDS-PAGE is low-resolution compared to LC–MS or CGE, and not suitable for detecting post-translational modifications (e.g., phosphorylation or truncations). However, emerging microfluidic and automated SDS systems (e.g., LabChip, Simple Western) improve reproducibility and enable use as semi-automated in-process controls.

5.5.4. Advantages

Versatile staining options, such as being compatible with Coomassie Blue, silver staining, or fluorescence-based dyes for visualisation. It is inexpensive compared to advanced techniques like CGE and has a very broad application across protein analysis workflows.

5.5.5. Limitations

This method provides lower resolution and is semi-quantitative; protein quantitation is less precise due to variability in staining and loading practices. SDS-PAGE is highly manual and time-consuming compared to automated techniques. Consequently, the method often requires extensive qualification and validation to meet regulatory QC standards.

5.5.6. Sample preparation

Sample preparation can be divided into three stages:

1. Protein Denaturation: Mix the sample with SDS-containing loading buffer and a reducing agent (e.g., beta-mercaptoethanol or DTT), then heat at 95°C for 5–10 minutes to fully denature proteins.

2. Loading Preparation: Centrifuge samples briefly to remove debris or bubbles. Load equal amounts of protein per lane to ensure comparability.

3. Loading Control: Select and include a protein ladder (molecular weight marker) to estimate protein sizes, ensuring the ladder matches the sample composition for accurate analysis.

5.5.7. Method set up and execution

Method set up involves four stages:

1. Gel Preparation: Cast polyacrylamide gels with a stacking gel (low %) and a resolving gel (higher %) or use pre-cast gels. Gel percentage depends on protein size (e.g., 12% gel for AAV capsid proteins).

2. Sample Loading: Load prepared samples and molecular weight markers into wells. Repeat any samples that may have leaked to ensure equivalence.

3. Electrophoresis: Submerge the gel in running buffer and apply an electric field (e.g., 100–200 V). Run until the dye front reaches the gel end for adequate separation.

4. Staining/Destaining: Stain with Coomassie Blue or alternative dyes, then destain to remove background for clear band resolution.

5.5.8. Data acquisition and analysis

After staining, capture gel images using an appropriate gel camera and documentation system. Identify protein bands based on their migration relative to the molecular weight marker. Estimate VP1, VP2, and VP3 stoichiometry by comparing band intensities (typically 1:1:10). Use densitometry software for quantitative analysis.

5.5.9. System selection and suitability

Standard SDS-PAGE setup including a gel casting system, electrophoresis chamber, and power supply, plus a gel imaging system (e.g., UV transilluminator or fluorescence scanner). Ensure proper gel polymerisation (absence of bubbles or inconsistencies) for reliable results.

5.5.10. Method development, qualification, and validation

VP protein ratios vary across serotypes, so confirm stoichiometry aligns with serotype-specific properties, capsids may deviate from the 1:1:10 ratio under different manufacturing conditions.

Optimise denaturation conditions, gel composition, and voltage for maximum resolution. Validate suitability for detecting VP proteins and impurities across process variations. Confirm fluorescent labelling if required for sensitivity enhancement.

5.6. Capillary Gel electrophoresis UV/FLR

5.6.1. Introduction

Capillary Gel Electrophoresis with UV or Fluorescence detection (CGE-UV/FLR) provides high-resolution data for determining VP1–VP2–VP3 capsid protein stoichiometry and detecting impurities or viral degradation products. It is more sensitive than conventional Agarose Gel Electrophoresis (AGE), capable of resolving small differences in molecular weight or charge heterogeneity. In contrast, AGE is better suited for assessing plasmid topology, genomic DNA size, or RNA integrity in gene therapy workflows.

5.6.2. Method description

CGE-FLR CGE-UV/FLR uses a polymeric sieving matrix within a fused-silica capillary to separate denatured proteins based on size-to-charge ratio. It is a robust, automated technique for separating, analysing, and quantifying AAV capsid proteins (VP1, VP2, and VP3).

Proteins are denatured under reducing conditions, then separated by molecular weight using a gel-with a thin capillary//lane, allowing migration through the matrix/lattice. Detection occurs via UV absorbance or fluorescence emission following appropriate labelling.

5.6.3. Advantages

This method provides high resolution and, unlike AGE-based methods, can offer simultaneous quantitation and detection of impurities or degradation products. It also permits higher reproducibility and reduced manual handling/steps. Depending on the type of detection/fluorescence it can enhance sensitivity for low-abundance proteins.

5.6.4. Limitations

Performance depends on the quality of the gel matrix used. Similarly, if the sample has an unusual number of impurities, these impurities may interfere with accurate quantitation. Voltage and temperature adjustments may be necessary to achieve optimal resolution for certain samples.

5.6.5. Sample preparation

Sample preparation typically involves a denaturation buffer containing SDS, a reducing agent (e.g., dithiothreitol), and, if fluorescence detection is used, a suitable fluorescent dye for enhanced sensitivity. The samples are typically clarified/centrifuged to remove insoluble components and minimise bubble formation prior to injection.

5.6.6. Method set up and execution

Key steps include:

1. Capillary Conditioning
2. Sample Loading
3. Electrophoretic Separation
4. Detection

Proper capillary conditioning is essential to ensure reproducible and high-quality separations in CGE, especially when analysing proteins, which are sensitive to surface interactions and buffer inconsistencies. Conditioning prepares the inner surface of the fused-silica capillary, removes residual contaminants, and stabilises the electroosmotic flow (EOF), which is critical for consistent migration times and peak shapes. Separation should be maintained at a constant temperature (e.g., 25°C) for reproducibility, and a high electric field (typically 15–20 kV) is applied.

Between runs, a short rinse with background electrolytes (typically 1–2 minutes) is performed. If protein adsorption or peak distortion is observed, an intermediate rinse with NaOH and ddH20 may be added.

At the end of the run, the capillary is flushed with NaOH, water, and stored in background electrolytes or water depending on the system recommendation to prevent drying and preserve capillary integrity.

5.6.7. Data acquisition and analysis

CGE systems integrate with software for data acquisition and processing. In regulated environments, a chromatographic data system ensures compliance with ALCOA++ and GMP principles. Data analysis involves peak integration and relative quantification of major capsid proteins (VPs) and subspecies such as truncated or oligomeric VP species.

5.6.8. System selection and suitability

The selection of capillary systems is limited. Different options exist and will depend mainly on the required throughput, as well as sample volume.

5.6.9. Method development, qualification, and validation

The first consideration is the detection type (UV or fluorescence), as fluorescence detection may require sample labelling during preparation. Fused-silica capillaries are the most used, and their dimensions should be evaluated even when ready-to-use options are available. To ensure consistency and reproducibility, it is recommended to use pre-assembled capillaries rather than custom-made ones where possible. Coated

capillaries, such as those with polyacrylamide, help minimise analyte interactions with the capillary wall, improving reproducibility and peak shape.

Because capillary electrophoresis is sensitive to buffer composition, buffer exchange may be necessary to achieve efficient separation and consistency across runs. Buffer compatibility should also be assessed to maintain stable electroosmotic flow and reliable migration behaviour. Finally, optimisation of electrophoresis conditions is essential and will depend on rAAV samples, as VP charge profiles can vary across serotypes.

5.7. cIEF UV/FLR

5.7.1. Introduction

Capillary Isoelectric Focusing (cIEF) is an electrophoretic separation technique that resolves proteins and protein complexes based on their isoelectric point (pI).

5.7.2. Method description

In cIEF, a sample is loaded into a capillary filled with a pH gradient medium containing ampholytes. When an electric field is applied, proteins migrate and focus at the position where their net charge is zero (pI). Detection is typically performed by UV absorbance at 280 nm or by fluorescence (FLR) if proteins or ampholytes are labelled. For AAV characterisation, cIEF is used to evaluate capsid charge heterogeneity, detect PTMs such as deamidation or sialylation, assess product-related variants, and verify capsid stability and batch comparability.

5.7.3. Points to consider

Unlike molecular-weight-based separations such as SDS-PAGE or CGE, cIEF resolves proteins by charge heterogeneity, enabling detection of PTMs, oxidation, deamidation, or capsid assembly variants that may alter surface charge distribution. It is often used alongside LC–MS or peptide mapping workflows to confirm findings and provide orthogonal data.

5.7.4. Advantages

cIEF offers high-resolution separation of charge variants, including PTM-induced isoforms, and provides pI values that can be tracked for comparability and stability studies. It requires very low sample consumption (typically less than 10 µL per injection) and is orthogonal to mass and size-based techniques such as CGE and LC-MS, enhancing overall characterisation depth. It is automation-friendly for QC laboratories using commercial cIEF platforms and is highly sensitive to minor charge shifts that may indicate degradation or manufacturing variability.

5.7.5. Limitations

Requires specialised instrumentation and trained analysts. Sample matrices must be free of particulates, salts, and non-volatile buffers, as these can impair focusing. PTMs that alter charge, such as sialylation or deamidation, may complicate interpretation if not confirmed by LC-MS. Ampholytes can introduce batch-to-batch variability and may be subject to regulatory scrutiny (e.g., REACH compliance). Due to complexity and interpretation requirements, cIEF is not widely used as a lot-release test and is primarily applied for extended characterisation.

5.7.6. Sample preparation

Samples should be exchanged into a low-salt, volatile buffer such as 10–20 mM ammonium acetate using ultrafiltration or buffer exchange. Typical input is 1E10–1E11 VP, corresponding to 0.5–5 µg total protein per injection. The sample is mixed with carrier ampholytes covering the expected pH range (e.g., pH 3–10 or 5–8). Fluorescent dye may be included for FLR detection, provided it is validated and REACH-compliant. Finally, samples should be degassed or centrifuged briefly to remove bubbles and particulates.

5.7.7. Method set up and execution

Key steps include:

1. Capillary Conditioning: Flush capillary with conditioning solution (e.g., NaOH, water, then ampholyte solution) to prepare surface and prevent carryover.

2. Sample Loading: Introduce sample-ampholyte mix via pressure or electrokinetic injection.

3. Focusing: Apply a stepwise or constant high-voltage field (typically 15–30 kV) to allow proteins to migrate and focus on their pI.

4. Mobilization and Detection: After focusing, proteins are mobilised (e.g., by chemical mobilisation or pressure) and detected online via UV (280 nm) or FLR.

5. Calibration: Use pI markers for internal calibration and alignment across runs.

5.7.8. Data acquisition and analysis

Data is recorded as an electropherogram showing focused peaks at specific migration times or pH positions. pI values are assigned using calibration standards, and peaks are integrated to assess the relative abundance of charge variants, including acidic and basic species. Profiles are compared against reference standards for batch release or comparability. Shifts in pI or the appearance of minor species may indicate instability, PTM changes, or manufacturing variability.

5.7.9. System selection and suitability

Platforms capable of cIEF analysis must undergo qualification (IQ/OQ/PQ). System suitability should be verified by achieving resolution between adjacent pI markers (Rs ≥ 1.5), repeatability of migration time or pI (≤2% RSD for the main peak across replicates), and detection sensitivity meeting validated thresholds (S/N ≥ 10:1 for main

VP peaks). Each run should include system suitability samples such as a reference standard, a pI ladder, and a blank.

5.7.10. Method development, qualification, and validation

Development focuses on optimising ampholyte range (broad vs. narrow pH), determining voltage profiles and mobilisation strategies for best resolution, and confirming compatibility of fluorescent dyes and buffers (REACH-compliant where possible). Validation follows ICH Q2(R2) or USP <1225> guidelines, assessing specificity (ability to resolve major and minor species), accuracy and precision (migration time/pI reproducibility and peak area quantification), linearity across relevant VP concentration ranges, and limits of detection and quantitation for minor variants. Robustness testing should include evaluation of pH gradient stability, ampholyte batch variation, and minor voltage or temperature fluctuations.

5.8. CZE-ESI-MS

5.8.1. Introduction

Capillary electrophoresis encompasses methodologies such as CGE, capillary zone electrophoresis (CZE), and capillary isoelectric focusing (cIEF). This section focuses on CZE, the most widely used CE technique coupled with mass spectrometric detection. CZE-ESI-MS allows for the separation and detection of proteins and peptides based on analyte electrophoretic mobility in a free-solution buffer under an electric field.

5.8.2. Method description

CZE-ESI-MS is well established for biotherapeutic analysis and is orthogonal to other separation techniques such as reversed-phase, HILIC, HIC, and ion-exchange chromatography. Direct coupling of CE to MS enables accurate mass identification of proteins or peptides and determination of PTMs.

5.8.3. Points to consider

Although not yet widely adopted, CZE-ESI-MS is increasingly recognised as a next-generation orthogonal method. It is complex but robust and reproducible, making it ideal for deep characterisation, process development, and capsid engineering studies. Data interpretation requires expertise in both electrophoretic and mass spectrometric domains, necessitating collaboration between analytical and bioinformatics specialists. As a hybrid technique, CZE-ESI-MS is best suited as a confirmatory tool in comparability or process characterisation studies rather than routine quality control.

5.8.4. Advantages

CE-ESI-MS is both sensitive, fast and can provide good resolution between species bearing charge altering PTMs. Characterisation of intact protein and protease treated peptide mapping approaches are both possible. Relatively short run times allow for higher sample throughput and sensitivity applies well to the small volumes of AAV material available for testing at multiple steps in the manufacturing process. The maturity of both CE and MS in application to biopharmaceutical characterisation means that a wealth of expertise is available. Multiple vendors have developed MS compatible CE systems compatible with a range of vendor platforms, including systems that comprise both spray device and automated sample handling functionality. Software is available for the analysis of the complex datasets generated, either from the vendors of the MS platform or third-party vendors aiming to cover multiple platforms.

The mechanism of separation lends itself to superior resolution of charge variant species, allowing for improved confidence of quantification and identification.

Compared to the widely deployed RP-LC-MS approach, CE-MS does not suffer from the same issue regarding potential loss of small hydrophilic peptides.

5.8.5. Limitations

Though both capillary electrophoresis and mass spectrometry are mature techniques for biopharmaceutical characterisation, their hyphenation requires a sound technical understand of both. The hardware capital expense and range of platforms on offer may complicate selection of a system. Buffer composition must be compatible with the MS based detection which requires volatile components, typically meaning a change from background electrolytes usually deployed for CE separations. Quantification based on MS detection can be influenced by ion efficiencies difference between analytes and matrix effects.

Coupling separation technologies and detection technologies from different vendors may present challenges in terms of hardware/software interfaces, maintenance, and system suitability assessments/qualifications.

5.8.6. Sample preparation

Typically, a buffer exchange step is required to remove salts, which can interfere with both the separation and ionisation of analytes. The presence of process related impurities, such as host cell protein, may obfuscate results. Affinity purification of process samples may be necessary for meaningful analysis.

Proteolytic digestion may be performed to enable a bottom up, peptide mapping, analysis.

5.8.7. Method set up and execution

Capillaries are selected to enable MS compatibility and to minimise protein adsorptive losses. Conditioning is performed prior to sample injection and between samples, to regenerate the capillary surfaces and minimise the effect of adsorbed protein on method reproducibility. BGE (background electrolyte) composition and pH should allow sample solubilisation and minimise degradation of capillary coating, organic solvents may be added to aid sample solubility and ionisation but may cause denaturation. Sample is injected and an electrical field applied to drive sample migration through the capillary and into the MS detector.

5.8.8. Data acquisition and analysis

Data acquisition takes place within the mass spectrometry data acquisition software; processing can be performed using either vendor produced analysis software or through third part options. Both top-down and bottom-up experiments can be analysed and provide data informing on protein/peptide masses and PTMs.

5.8.9. System selection and suitability

A key consideration when working with AAV is the requirement for high sensitivity due to low protein concentration and smaller batch sizes. Both sheath flow and sheathless systems are available with the latter operating at nanoflow rates and providing higher sensitivity and lower sample consumption. Due to the capital expense of CZE-MS systems it may be preferrable to choose a system based on currently available equipment i.e., select a CZE device or mass spectrometer based on existing setup.

System suitability should be assessed using appropriate model molecules, peptides or proteins depending on the objective of the method. These should assess performance of the CZE-MS hyphenated system in terms of the expected parameters used for analysis of AAV samples.

5.8.10. Method development, qualification, and validation

Method development should include assessment of sample pre-treatment, such as denaturation of the AAV capsid into its constituent proteins for intact protein analysis or digestion of the AAV capsid into peptides for a peptide mapping analysis. Optimisation of background electrolyte composition and additives may be necessary to obtain satisfactory resolution of analytes. Non-volatile components, surfactants, and high buffer concentrations may lead to ion suppression, noisy background, and contamination of the mass spectrometer source.

Optimisation of MS settings should be performed to maximise ionisation, desolvation, and adduct removal and for peptide mapping to optimise fragmentation of peptides. Depending on the system being used it may also be necessary to optimise the position of the ESI sprayer and spray stability.

5.9. Summary table comparing methods

Comparison of Analytical Methods for AAV Capsid Protein Characterisation							
Criteria	**LC–Mass Spectrometry (LC-MS)**	**Liquid Chromatography – UV/FLR**	**Antibody-Based (ELISA)**	**Affinity-Based (SPR & Interferometry)**	**SDS-PAGE**	**Capillary Gel Electrophoresis (CGE, UV/FLR)**	**Capillary Zone Electrophoresis – ESI-MS (CZE-ESI-MS)**
Sample Volume	50–200 µL	50–200 µL	50–100 µL	10–100 µL (depends on chip or sensor)	10–30 µL	5–20 µL	5–20 µL
Sample Concentration (Range)	~1E10–1E12 VP (≥5 µg protein for peptide mapping)	~1E10–1E12 VP (~1–20 µg protein)	≥1E9–1E11 VP/mL	≥1E9 VP/mL (purified)	~1E10–1E12 VP (1–10 µg protein)	~1E10–1E11 VP (~0.5–5 µg protein)	~1E10–1E11 VP (~0.5–5 µg protein)
Time to Result	4–12 hours (prep + run)	1–3 hours	4–8 hours (batch)	1–4 hours (setup + run)	4–6 hours	1–2 hours (including prep)	2–6 hours (sample conditioning + MS run)
Expertise Required	High (MS specialist)	High (chromatography and GMP-trained analyst)	Low–moderate (standard QC skillset)	High (specialist for assay and data interpretation)	Low–moderate (common lab skill)	Moderate (trained analyst)	Very High (specialist in electrophoresis-MS interfacing)
Suitability as In-Process Control (IPC)	No (extended characterisation only)	Yes (can monitor process impurities)	Yes (for capsid titre)	Potential (affinity kinetics during development)	Limited (not ideal for rapid IPC)	Yes (for VP ratios, identity, impurities)	No (mostly research-level, not routine QC yet)

Matrix Effects / Purity Requirements	Requires highly purified capsid protein (low salt/detergent)	Needs purified sample; crude feeds risk column fouling	Tolerates moderate impurities but binding inhibited by detergents/proteins	Requires clean sample (buffered, low particulates)	Tolerates moderate impurities; high detergents can interfere	Requires clean, denatured protein; tolerates some impurities	High purity essential; non-volatile buffers incompatible
Complexity of Equipment Qualification (GMP)	Very High (MS calibration, contamination controls, IQ/OQ/PQ)	High (detector calibration, system suitability, column performance checks)	Medium (kit qualification, plate reader validation)	High (sensor chip qualification, instrument PQ)	Low (basic validation; gels reproducible)	Medium (capillary performance checks, polymer validation)	Very High (MS and CE interfaces both require extensive PQ)
Resolution	Very High (PTMs, sequence variants, truncations)	High (intact VP proteins, degradation products)	Low (bulk VP capsid detection, no subunit resolution)	Moderate (binding kinetics, epitope-specific)	Moderate (VP bands resolvable, low precision vs. CGE)	High (VP1/VP2/VP3 ratios, ~1 kDa resolution)	Very High (charge-based resolution + MS mass accuracy)
Detects Aggregation?	No (focus on peptides or intact proteins, not particles)	Indirect (peak broadening possible)	No (bulk readout only)	Indirect (binding response anomalies)	No (denaturing method)	No (denaturing method)	Indirect (can detect complexes if optimized)

5.10. Other methods worth mentioning

5.10.1. MALDI-MS

Matrix-Assisted Laser Desorption Ionisation Mass Spectrometry (MALDI-MS), also known as Electrospray Ionisation Mass Spectrometry (ESI-MS), is an alternative to LC-MS. Both techniques have been widely used for protein characterisation and are considered complementary. MALDI-MS can be applied for intact mass analysis of capsid proteins as well as for peptide analysis following protein digestion.

In both cases, no preliminary separation is necessary, resulting in a single mass spectrum that simplifies interpretation. Additionally, one significant advantage of MALDI-MS is its ability to generate single ion species in most cases, making data interpretation easier compared to LC-MS. The mass spectrum of the peptide mapping sample is called peptide mass fingerprint as it is unique to the proteins thanks to endopeptidase cleavage specificity.

Despite these advantages, MALDI-MS has several limitations. The absence of separation means multiple proteins or peptides are analysed simultaneously, leading to ionisation competition and potential ion suppression caused by salts or highly abundant species. This can reduce robustness and specificity. Furthermore, coupling MALDI-MS with LC systems is challenging, and the technique requires manual steps such as sample/matrix co-crystallisation and plate installation into the mass spectrometer. These factors have limited its adoption in the biopharmaceutical industry, where LC-MS remains the gold standard for protein characterisation.

5.10.2. CDMS

Charge Detection Mass Spectrometry (CDMS) is an emerging technology that enables direct determination of both charge and mass by analysing samples at the single-ion level. Direct measurement of charge eliminates the need for complex deconvolution algorithms and their associated processing artefacts, greatly simplifying the analysis of complex samples where charge envelopes would otherwise overlap.

CDMS has been implemented using different approaches by various research groups; however, it remains an immature technique, with limited adoption, a small body of supporting literature, and few software solutions for data acquisition optimisation and analysis. Although this technology may be primarily suited towards empty: full type analysis, there is the potential to obtain capsid higher order structural information based on capsid charge state distributions.

6. Concluding remarks

This guidance addresses the development and manufacture of recombinant adeno-associated virus (rAAV) medicinal products, with a restricted focus on ensuring the

quality, safety, and efficacy of these therapies at the protein level through robust capsid characterisation.

Achieving these goals requires:

- Comprehensive analysis of rAAV capsid proteins, including the identification and quantification of VP subunits, assessment of post-translational modifications (PTMs), and evaluation of capsid integrity and purity.
- Minimising the presence of product- and process-related impurities, such as host cell proteins, truncated or misassembled capsids, and other contaminants that may impact product safety or efficacy.
- Applying orthogonal and validated analytical methods to confirm the identity, composition, and consistency of capsid proteins across batches and throughout the product lifecycle.

The analytical approaches and best practices described in this guidance reflect the current state of technical development. As the field evolves, new methods and insights will continue to shape best practice in capsid protein characterisation.

This document is intended to support developers and manufacturers of rAAV-based medicinal products in meeting regulatory requirements and ensuring product quality. However, ultimate responsibility for product quality and compliance remains with the developer, manufacturer, or sponsor. All decisions regarding analytical strategies and quality control should be based on sound scientific principles and risk-based approaches.

Feedback and collaboration from the scientific and regulatory community are encouraged to ensure this guidance remains relevant and effective as technologies and standards advance.