British Pharmacopoeia

Consultation

British Pharmacopoeia public consultation for guidance on the Characterisation of the particle population in AAV products

Consultation period 23 November 2022 to 31 January 2023

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This draft guidance was written by experts in the ATMP community to support those involved in the development of analytical methods throughout the product lifecycle, and therefore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 Working Party for ATMPs, established in March 2020, has engaged with groups across the cell and gene therapy community to develop

[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/

non-mandatory guidance for key analytical technologies to ensure quality throughout the product lifecycle. The working party has developed two sets of guidance to support ATMP development across a wide range of organisations, laboratory settings, and therapy types. As such, the 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.

This guidance addresses the use of methods for the characterisation of viral particles in AAV-based therapies and will provide current best practice to determine the distribution of viral particles containing DNA and those which do not. The bulk of the guidance offers a description of methods for determination of the recombinant AAV particle population. Focus has been put on established methods and for each method a brief description of method principle and its use in AAV particle characterisation are discussed, including strengths, weaknesses, and considerations for sample preparation and system selection. The methods are not necessarily exhaustive but include:

- Vector genome to capsid titre ratio
- Analytical Ultracentrifugation
- Transmission Electron Microscopy
- Size Exclusion Chromatography
- Mass Photometry

The guidance also includes an approach to the development of a control strategy across a product lifecycle with considerations for the selection of product release methods and definition of acceptance criteria. The draft Characterisation of the particle population in AAV products best practice guidance is included as Annex 1 to this document.

3. How to contribute

The draft Characterisation of the particle population in AAV products best practice guidance 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 Guidance on the Characterisation of the particle population in AAV products

Medicines & Healthcare products Regulatory Agency

Characterisation of the Particle Population in AAV Products

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1 Abbreviations

rAAV	Recombinant Adeno-Associated Virus
AEX	Anion Exchange
ATMP	Advanced Therapy Medicinal Product
AUC	Analytical Ultracentrifugation
BSA	Bovine Serum Albumin
CDMS	Charge Detection Mass Spectrometry
cGMP	Current Good Manufacturing Practise
CMA	Critical Material Attributes
CPP	Critical Process Parameters
CQA	Critical Quality Attribute
DLS	Dynamic Light Scattering
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EM	Electron Microscopy
FMEA	Failure Mode and Effects Analysis
GMP	Good Manufacturing Practice
HMW	High Molecular Weight
HPLC	High Pressure Liquid Chromatography

ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IPC	In-Process Control
IS	Internal Standard
ITR	Inverted Terminal Repeat
LMW	Low Molecular Weight
LOD	Limit of Detection
LOQ	Limit of Quantification
MALS	Multi-Angle Light Scattering
MS	Mass Spectrometry
PBS	Phosphate Buffer Solution
PCR	Polymerase Chain Reaction
QC	Quality Control
Q-ToF	Quadrupole Time-of-Flight
QTTP	Quality Target Product Profile
RI	Refractive Index
RMSD	Root Mean Square Deviation
SEC	Size Exclusion Chromatography
SV	Sedimentation Velocity
TEM	Transmission Electron Microscopy
TPP	Target Product Profile
UV	Ultraviolet

2 Terminology

For the purpose of this guidance, the totality of capsid species potentially present in Recombinant AAV-based products are referred to as particle population. Although the "*full : empty ratio*" is a common term referring to rAAV species, it should be noted that this reduction of the particle distribution is a simplification which may not be appropriate to describe all rAAV-based products. A convenient terminology due to its brevity the term "*full : empty ratio*" lacks clarity of additional capsid species potentially present in a product. Hence, use of the "*full : empty ratio*" should be clearly defined to avoid ambiguity.

Throughout this guidance document the term rAAV particle population will be used to refer to *all* rAAV species which may be generated and be present in a product:

Full particles: rAAV particles containing DNA matching the expected size of vector DNA (i.e., size of transgene sequence plus regulatory elements and inverted terminal repeats). The total mass of full particles is close to the theoretical sum of the mass of capsid proteins and vector DNA mass. It should be noted that full particles may *not* equate to particles carrying therapeutic effect. As discussed in section 3, further characterisation on the capsid protein and nucleic acid level of encapsidated DNA is required to determine structure – function relationship.

Empty particles: rAAV particles containing little to no encapsidated DNA. The particle mass closely matches the expected mass of the rAAV capsid proteins.

Partially filled / intermediate particles: rAAV particles in which encapsidated DNA is smaller than in full particles. The particle mass lies between empty and full particles.

Overpackaged particles: rAAV particles containing DNA greater than the DNA packaged in full particles (up to, and potentially slightly above, the theoretical packaging limit of approx. 4.7 kb of ssDNA). The particle mass is greater than that of full particles.

3 Introduction / scope

This guidance addresses the use of methods for the characterisation of viral particles in AAV-based therapies and will provide current best practice. There is a growing body of evidence pointing towards a diverse population of viral particles at the capsid and nucleotide level as illustrated in Figure 1. To thoroughly assess the makeup of various sub-species, the characterisation of AAV-based therapies requires an understanding of multiple attributes:

- **General particle population**
  Distribution of viral particles containing DNA and those which do not.

- **Encapsidated DNA**
  Nature, size, distribution, and potential methylation of vector DNA and product/process related DNA impurities encapsidated within viral particles.

- **Capsid modifications**
  Confirmation of primary amino acid sequence, presence of variants, and post-translational modifications of the viral capsid proteins.

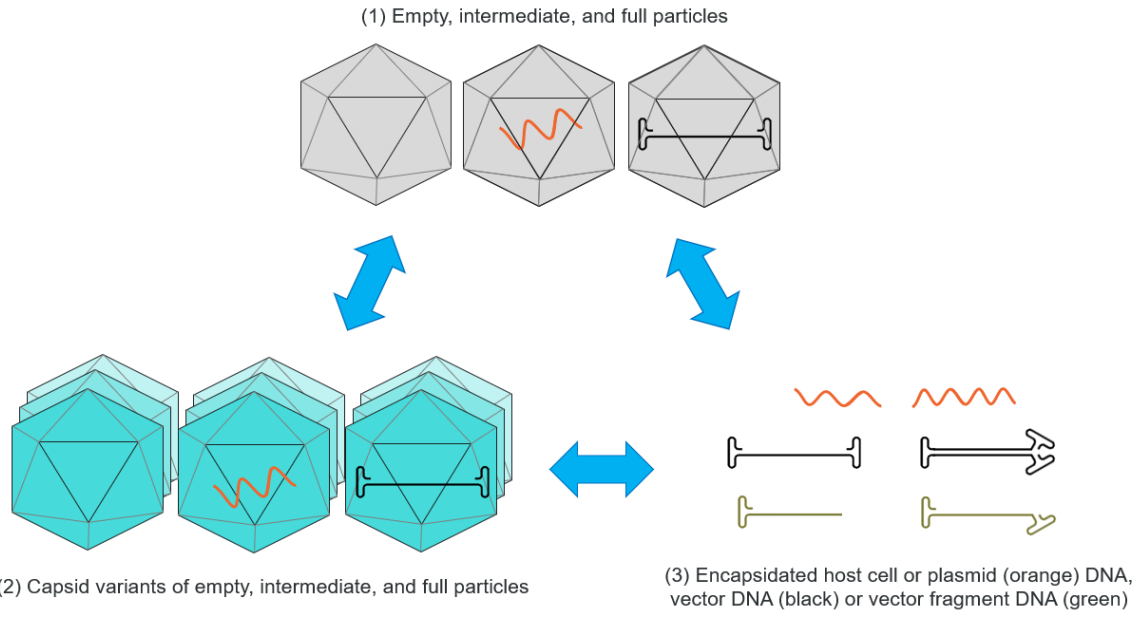

Figure 1 rAAV products potentially contain (1) a heterogenous population of particles carrying vector DNA, product- and process related DNA impurities, and empty particles. (2) For each particle species, additional capsid variants due to difference in the post translational pattern of viral proteins may be present. (3) Across all partial and full particles there is likely a diverse population of process- and product related DNA impurities in addition to the encapsidated single-stranded or self-complementary vector DNA sequence.

Because of the wide scope, guidance for characterising the above noted attributes has been split into three documents. Encapsidated DNA and Capsid modifications guidance will be released separately to this document. In this document, methods for characterisation of rAAV particle population and their use during the development and quality control of rAAV-based

therapies are outlined. When developing a control strategy for these therapies, companion parts on the characterisation of encapsidated DNA in rAAV particles and the characterisation of the viral capsid structure should be considered also.

The present document is structured into two parts. Firstly, a description of methods for determination of the rAAV particle population. Focus has been put on established methods and for each method a brief description of method principle and its use in AAV particle characterisation are discussed, including strengths, weaknesses, and considerations for sample preparation and system selection. Secondly, an approach to the development of a control strategy across a product lifecycle with considerations for the selection of product release methods and definition of acceptance criteria are provided.

The adoption of this guidance is envisioned to help promote standardisation of the discussed techniques within the cell and gene therapy community. Several aspects of the framework are applicable to all users and should be given due consideration irrespective of the context in which assays are being developed; their purpose is primarily to facilitate reproducible, high-quality data generation. In the context where an assay is intended for use as part of a human medicine's development program, then it is important to recognise that it will need to conform to pertinent regulatory guidelines appropriate to the stage of development, for example for product licensing all assays are required to conform to the requirements of ICH Q2(R1).

4 Points to consider

The present guidance is seen as one step towards standardisation of the AAV-based gene therapy field. Information herein should be interpreted with consideration of the latest scientific information, applicable regulatory guidelines and standards, and product specific requirements.

4.1 Evolving understanding of AAV safety profile

AAV-based gene therapy has demonstrated safety and efficacy in many clinical trials and commercial products. As more sponsors are evaluating treatments for different diseases there is a recognition that understanding of how viral vector dose and observed adverse events, particularly how toxicities observed in animal and human studies using rAAV-based therapies are linked, is still limited. Although severe reactions to treatments are likely a combination of multiple factors (as shown in Figure 2), the presence of full, empty, and partial particles is considered a key element. This is because the capsid, its genome, the transgene product, and potential encapsidated host cell and plasmid DNA impurities are the main potential immunogenic components of AAV vectors. The ability to ensure highest product quality across all stages of product development is therefore key to reducing potential adverse events.

Figure 2: Adverse events in AAV-based gene therapies may be influenced by one or more attributes. Assurance of consistently high product quality is crucial for risk mitigation of adverse events.

4.2 Developing AAV characterisation assays

It should be noted that this document has been created to be read in conjunction with applicable guidance for method development and validation. Published guidance documents on method development and validation apply for the methods discussed herein. This document provides a summary of established methods for the determination of rAAV full, empty, and intermediate particle species and a discussion of their individual strengths and weaknesses in the context of viral particle characterisation.

It is acknowledged that the information contained herein gives a snapshot in time only. New approaches for upstream and downstream production are being proposed regularly – including but not limited to stable producer cell lines, improved plasmid designs and transfection reagents, novel capsid designs, and improved purification techniques. To support these development efforts appropriate analytical technologies are required. Currently, significant method development efforts are being made by the AAV community to ensure the analytical toolbox for rAAV particle characterisation reaches maturity and can be standardised across the field. Hence, this document should not be taken as a complete list of feasible methods for characterising AAV particle population.

Scientific understanding of the various elements potentially causing product toxicity is rapidly evolving. To further the development of AAV-based therapies thorough characterisation of viral capsids and their genetic payload are required. This creates a need for more standardisation to compare the quality of AAV-based therapies across products and manufacturing platforms. But it also drives innovation to improve on the current standard of analytical characterisation. Hence, it is understood that improvements in the understanding of critical quality attributes relating to product toxicity will result in an improved approach to product characterisation.

4.3 Reference material

There is currently no physical International Standard (IS) to support the harmonisation and standardisation of full : empty testing to assure the consistency of measurement of key attributes of AAVs. An international effort was made to provide reference materials for AAV2 and AAV8, these materials were made using methods that have been superseded by today's technologies and stocks are being depleted. The materials were characterised to provide a particle number and not a full : empty particle ratio.

Multiple programs are under way to establish well characterised particle standards. It is expected that availability of one or more physical standards will significantly aid the development of AAV characterisation assays. Developers of AAV-based therapies are encouraged to evaluate the performance of their methods against such external standards when available.

In the absence of an established reference material product, developers should consider the use of commercially available full : empty standards and the use of product specific in-house standards.

In-house reference materials should be produced as a single batch and stored at an appropriate volume and temperature to ensure stability over time. If batches need to be mixed then it should be ensured that the resulting material is homogeneous. Subsequent replacement batches should be calibrated to the preceding batch to retain consistency and provide traceability. The material should be characterised to provide a full : empty ratio and provide sufficient stock of materials that can be used across different testing sites and for the

duration of work. Consideration should be given to the matrix in which the reference material is produced which should preferably be comparable to the variety of samples being assayed to prevent matrix effects on analyses.

The use of an in-house reference material allows product attributes to be consistently assessed over time for that specific product. A generic reference material from an external provider enables the same product attribute to be compared across products as it is available to all manufacturers.

For first principal methods or methods agnostic of serotype and encapsidated vector DNA it is expected that such evaluation of the method with an external reference will lead to a greater standardisation of testing. Furthermore, it will provide users of the physical standard additional data on analytical capability even if the serotype of the reference material is different to the developed therapy. In conjunction with product specific development work this can be used to support the scientifically sound set up of the method.

Ultimately, the standardisation and an understanding of method capability with the external reference material will allow better comparison of rAAV characterisation results across different products. Which in turn supports the scientific community to improve the understanding of safety risks potentially associated with empty and intermediate particles.

5 Empty / full characterisation

5.1 Overview

Selection of methods utilised for rAAV particle profile determination should be tailored to each process and product and how the method(s) sit within the overall control strategy (refer to section 6). This selection should be informed by product specific requirements based on the knowledge obtained from process and product characterisation studies. The type of testing performed (in-process vs product release), test sample characteristics, evidence of method suitability for the sample matrix should be considered as well as latest scientific knowledge.

A summary of methods discussed in this guidance is provided in the table 1.

Table 1 Comparative summary of methods used to determine full : empty capsid ratio covered in this guidance.

	MassP	AUC	nsTEM	CryoTEM	SEC-MALS	ELISA to q/dPCR ratio	CDMS
Sample Requirements	low	high	low	low	medium	low	medium
- **Material**	low	high	low	low	low	low	low
- **Concentration**	medium	medium	high	high	medium	low	low
Time to result	fast	slow	slow	slow	fast	medium	medium
Expertise	low	high	medium	high	medium	medium	high
IPC	yes, depending on sample matrix	yes, depending on sample matrix	yes, depending on sample matrix	yes, depending on sample matrix	yes, depending on sample matrix	yes, depending on sample matrix	yes, depending on sample matrix
QC	yes	yes	yes	yes	yes	yes	yes
Transferability	high	low	medium	medium	medium	high	medium
E:F ratio	yes	yes	yes	yes	yes	yes	yes
Resolve intermediate particle species	Intermediates distinguishable	fully resolved	no	fully resolved	no	no	fully resolved
Aggregation	partially distinguishable	fully resolved	fully resolved	partially distinguishable	partially distinguishable	no	no

5.2 Methods

5.2.1 Ratio of vg/cp titre

5.2.1.1 Method description

A conventional method of assessing full : empty ratio of an AAV sample is by obtaining a ratio between the viral genome (vg) titre and the capsid titre (cp). Quantitative polymerase chain reaction (qPCR) or digital PCR (dPCR) are the current industry standards for quantifying AAV viral genome (vg) titres. Details on the application of qPCR and dPCR for vg copy number quantification have been published by British Pharmacopeia - ATMP Guidance – Vector Copy Number.

Enzyme-linked immunosorbent assay (ELISA) is a typical method in industry to quantify titres of assembled and intact AAV capsid particles, regardless of the genomic content within the particles. ELISA is based on antibodies binding specific to conformational AAV capsid epitopes, allowing the generation of photometric activity via the catalysis of substrates.

5.2.1.2 Strengths

qPCR, dPCR and ELISA equipment are benchtop, do not require extensive training to operate, and accommodate multiple samples per run. As conventional methods to determine full : empty ratio, these techniques have been extensively assessed and qualified/validated across the AAV industry. Sample requirements for qPCR, dPCR and ELISA are low; samples typically require serial dilution prior to assay setup, depending on the sensitivity and the linear range of the developed assays. With qPCR and dPCR, the reactions can be multiplexed for different targets, increasing assay throughput as well as robustness, e.g., by multiplexing with sample preparation controls in the same reaction.

qPCR, dPCR and ELISA workflows can be automated to various degrees from sample preparation to data acquisition, depending on the equipment of choice for each assay. Data analyses are relatively simple, and calculation of titres can be automated from raw data to ensure data integrity. These advantages make the assays suitable for process development and in-process characterisation.

5.2.1.3 Weaknesses

qPCR, dPCR and ELISA are considered off-line measurements for full : empty ratio estimation and are not suitable for on-line/at-line assessments of processes. Each assay requires half to a full day to generate titres, due to multiple steps in the workflows with incubations, or time required to complete the thermocycling PCR steps. Dependent on the developed methods, a vg titre from q/dPCR and an estimated full : empty ratio may be required prior to ELISA to ensure the samples are diluted within the ELISA linear range.

Estimation of full : empty ratios by q/dPCR and ELISA compounds the inherent errors of the individual assays. Samples of vg and cp titres close to the lower limits of quantification of PCR and ELISA will incur larger compounding errors, as well as requiring larger sample volumes to perform the assays. dPCR is known to be more sensitive and precise than qPCR, however the assay variability can be increased by buffer matrices and impurities (e.g., residual non-encapsidated plasmid, host cell DNA and protein), especially for more crude upstream samples.

5.2.1.4 General considerations

5.2.1.4.1 Sample preparation

AAV from different stages of production will be in different buffers with varying degrees of impurities, which are parameters that can increase the variability of vg and cp assessment. Buffer exchange and/or removal of impurities should be considered prior to analytical characterisation. ELISA is more robust to matrix interference as the technique involves

capturing AAV particles and diluting out unbound entities prior to quantification. Any manipulation of the samples prior to analytical characterisation must be assessed for impact on the data generated.

5.2.1.4.2 Method set up & execution

When assessing the full : empty ratio of samples at the different stages of an end-to-end process, all process step samples should be analysed on the same plate for q/dPCR or ELISA to minimise variability attributable to plate variation rather than the process. Alternatively, an inter-plate control can be used to normalise for plate-to-plate differences. It will be advantageous to analyse the same aliquot of rAAV on both q/dPCR and ELISA; if different aliquots are used, the number of freeze-thaw cycles of the same sample should be controlled. During plate setup, a reference standard should be analysed in each run of d/qPCR and ELISA – to control for the experimental setup as well as providing as a trending/performance control to monitor the variation of full : empty ratio across runs.

If an automated workflow for setting up the q/dPCR and ELISA is used, prior assessment of manual versus automated setup should be performed to determine the impact on the data generated. Comparison of full : empty ratio should be standardised on either the automated or manual workflow and should not be compared across the platforms.

5.2.1.4.3 Data acquisition & analysis

Controls should be in place during q/dPCR and ELISA data analysis with appropriate acceptance criteria to increase assay precision and minimise the compounding error of the full : empty ratio. Only if the appropriate controls pass their acceptance criteria should the samples be analysed for their vg and cp titres; otherwise, the q/dPCR or ELISA plate should be repeated.

It is important to note that during data interpretation, q/dPCR methods do not consider the integrity of the viral genome, as it specifically amplifies for the target sequence. Capsids considered as full by PCR and ELISA may not have the complete viral genome sequence (i.e., partially filled capsids), which can affect the infectivity and functionality of the virus. This can have an impact on the assessment of product potency and safety, as these attributes may differ significantly between partially filled capsids and ones with the complete viral genomes.

5.2.1.5 System selection/suitability

Cp titration by ELISA is limited by the serotype specific nature of the assay and an appropriate assay will need to be considered for the therapy product. Commercial kits and antibodies are available which are either specific to a certain serotype or have binding to a conserved epitope and are suitable for a wider range of AAV serotypes. When using alternative natural or engineered serotypes care will be required to ensure that available reagents are suitable.

Vg titration is carried out by a quantitative PCR approach, there are many available vendors in this area for both real-time PCR and digital PCR. Care should be taken to ensure that the equipment is suitable to the assay requirements, for example the number of samples that can be analysed in a day and the number/colour of probes that can be used for multiplexing if required.

5.2.1.6 Method development/qualification/validation

Guidance on the development and validation of vector copy number titration in AAV-based therapies by qPCR and dPCR has been published by the British Pharmacopeia (A framework for standardising vector copy number quantification within the cell and gene therapy community). It is recommended to use a primer/probe combination that targets the middle of the vector cassette and to avoid using the ITR regions for quantification.

For the ELISA, ICH guidelines should be followed. If using a bespoke serotype, specific antibodies may need to be generated if commercial antibodies are not suitable, as well as generation of AAV particles for reference material, standards, and internal controls. In addition, commercial ELISA kits depend on the use of standard curves made from reference materials, which can create bias batch to batch. Bias in cp titres can also be observed between ELISA kits and other orthogonal methods, the differences between which should be assessed during development of the assays.

To understand the full : empty ratio a suitably pure control material is required. Following repeated analysis by ELISA and PCR the variability ratio can be determined, this is likely to be higher than for an individual assay due to compounding of errors. Once the method is established a trend control should be incorporated into analysis and revalidation may be required when there is a change in the batch of the standard material in the assays.

5.2.2 Analytical ultracentrifugation

5.2.2.1 Method description

Analytical ultracentrifugation (AUC) is a biophysical method that measures the sedimentation coefficients of species present in a sample. The sedimentation coefficient depends on the molecule's molecular weight, hydrodynamic radius, and diffusion coefficient as well as on the viscosity, density, temperature, and physio-chemical properties of the matrix. Two types of AUC experiments are commonly used for the characterisation of biological samples. In the first, termed sedimentation velocity (SV)-AUC, cells containing double-sector centerpieces, in which the sample is loaded in one sector and the sample buffer is loaded in the other, are lodged inside a rotor. The rotor is spun at a constant high speed (for AAV products, 10,000 to 15,000 rpm typically) to induce a constant gravitational field that will cause the different species to sediment to the bottom of the cells. The sedimentation event is monitored by recording radial scans of the AUC cells with either UV absorbance or interference detection. The scans take the shape of sigmoidal curves and delineate the movement of the solutes' boundary with time. The raw data is fit with the Lamm equation to produce a sedimentation coefficient distribution that can quantitatively report on the different species present in a mixture. Small particles are associated with small sedimentation coefficients and take longer to sediment whereas large particles reach the bottom of the cell more rapidly and are therefore associated with higher sedimentation coefficients. The other AUC experiment is termed sedimentation equilibrium (SE), in which the rotor is spun at lower speed (typically ~ 3,000 rpm for rAAV particles). The goal here is to allow the gravitational force to reach an equilibrium with the opposing diffusion force and therefore distribute the solutes of the mixture along a boundary that has the shape of an exponential curve. In this case, collection of temporal radial scans is not required. Instead, recording of one scan (or a few to increase signal-to-noise ratio) once equilibrium is reached is sufficient. Fitting of the data to single or multiple exponentials allows calculation of molecular weights independently from diffusion coefficients.

5.2.2.2 Strengths

Compared to chromatography-based methods and other biophysical/biochemical techniques, SV-AUC has the highest resolving power and is capable of reporting on multiple critical quality attributes at once, in a single experiment. Given that full rAAV particles have almost twice the molecular weight of empty particles, SV-AUC readily separates them and identifies them with unique sedimentation coefficients which can be used as an identity test. In addition, SV-AUC can be used to determine and characterize impurities found in AAV samples, such as low molecular weight species (free nucleic acid, free proteins, etc…) and high molecular weight impurities such as aggregates and higher-order oligomers. Furthermore, because of its superior resolving power, SV-AUC is the only method capable of identifying, separating, and quantitating partially filled and/or overfilled particles in their intact form, i.e., without the need

to denature and disassemble the capsid to release their internal nucleic acid, with subsequent isolation and characterisation by gel electrophoresis, for example. Because these two impurities have the same hydrodynamic radius as the empty and the full species, their separation and characterisation is only possible by SV-AUC. One understated benefit of the SV-AUC method which constitutes a major advantage over other techniques is that the analysis occurs in the sample's own formulation buffer without dilution, buffer exchange, or any sample-preparation-induced changes which might alter the original sample matrix.

5.2.2.3 Weaknesses

The major drawback of the SV-AUC analysis is that it requires large amounts of material (0.5 mL at 5 x 10^{12} vg/mL for optimal signal) which are not always available for rAAV samples. However, if rAAV samples are formulated at higher concentrations (~ 10^{13} or 10^{14} vg/mL), the required volume is no longer taxing because the sample must be diluted to reach the optimal concentration and avoid saturating the absorbance detector. The technical details or expertise associated with setting up an SV-AUC experiment, collecting the data, and choosing among a plethora of modelling software programs to analyse it can be intimidating factors for non-subject-matter-experts. For instance, because the sedimentation coefficient distribution is a result of the fit of the raw data to the Lamm equation, using slightly different fitting parameters and/or different software programs might generate slightly different results.

5.2.2.4 General considerations

5.2.2.4.1 Sample preparation

Sample preparation in SV-AUC experiments is heavily dependent on the type of detection desired. UV absorbance detection is preferred over interference detection during an SV-AUC experiment because rAAV products are typically produced at low concentrations. In fact, proper interference measurements by SV-AUC require exact buffer matching between the sample matrix found in one sector and that of the reference found in the other. This objective can be achieved through dialysis which adds more sample manipulation during sample preparation. Moreover, matching the column heights between both sectors of the centrepiece, which is imperative for a meaningful result by SV-AUC and interference detection, requires highly specialized meniscus-matching centrepieces which are often not readily available. Consequently, UV absorbance is the preferred detection method for the characterisation of rAAV products. UV absorbance does not have the same stringent conditions as interference detection and sample preparation is limited to a dilution of the product (if needed) to reach the optimal concentration of 5 x 10^{12} vg/mL which results in the optimal UV signal of 1 OD. Once the sample is prepared at the optimum concentration, it is loaded in the sample sector of the centrepiece and an equal volume of the matched buffer is loaded in the reference sector. The cells are closed tight and aligned properly in the rotor before initiation of the experiment.

5.2.2.4.2 Method set up & execution

While sample preparation is relatively simple, the choice of UV absorbance wavelength requires additional consideration. While measuring UV scans at 3 different wavelengths is possible on older instruments, this practice should be avoided. This is because the monochromator imprecision of ± 3 – 5 nm is causing slight variations in the collected data between scans as the wavelengths is unlikely to return to the exact same value. Consequently, data suffers from low signal-to-noise ratio. Newer ultracentrifuge models are more precise (± 0.5 nm) thus enabling multi-wavelength recording of radial scans. The two most important wavelengths to measure UV absorbance scans of AAV sedimentation profiles are 260 and 280 nm to capture the nucleic acid and protein content, respectively. In addition, UV absorbance measurements at 230 nm exhibit a UV signal that is higher by a factor of ~0.8 (when compared to that at 280 nm) because the extinction coefficient for both particles is higher at 230 nm. This feature is especially advantageous for the analysis of process

intermediate samples in which high concentration is not always possible. Ideally, the SV-AUC experiments would be set up to collect UV absorbance data at all three wavelengths and interference data at set intervals of time (typically a scan every 3 min). In general, for rAAV samples, the method is set to collect 75 – 100 scans which are sufficient to capture the complete sedimentation of the empty and full particles at 15,000 rpm. The rotor and the cells are set for a temperature equilibration period of 2 hours prior to initiation of the experiment.

5.2.2.4.3 Data acquisition & analysis

Interference scans and UV absorbance data at each of the three wavelengths are collected and stored automatically. Once the experiment is complete, the data is loaded onto an analysis software in which the raw data is fit to the Lamm equation. Special care is required when loading the data to avoid uploading more scans than needed and biasing the fit towards later scans which do not contain any useful sedimentation information beyond outlining the baseline. Once the fit converges, it is essential to evaluate the quality of the fit by assessing the root mean square deviation (RMSD) and the absence of any anomalous patterns in the residual plots. Typically, an RMSD value of 0.005 – 0.007 on the older ultracentrifuge models and 0.002 – 0.005 on the newer ones are indicative of a good fit. Integration of the areas under the peaks in the sedimentation coefficient distribution, which gets generated because of the fit, enable's calculation of each of the species percentage amount in the mixture. However, empty and full particles have significantly different extinction coefficients at 260 and 280 nm. Therefore, when integrating the areas under the peaks in the sedimentation coefficient distribution plots of these two wavelengths to obtain the amounts of each species present in the mixture, it is imperative to correct these percentages for the differences in extinction coefficients. On the other hand, UV absorbance measurements at 230 nm have generated full : empty particle ratios similar to those obtained by interference measurements, thus implying that the extinction coefficients of the empty and full particles are comparable at 230 nm. In other words, UV measurement at 230 nm is not associated with the same extinction coefficient bias observed at 260 and 280 nm and therefore removes the need to correct the integration areas as described above and provides a direct measurement of the empty-to-full ratio. The 230 nm data is used to determine the empty-to-full ratio from the sedimentation coefficient distribution without correction for extinction coefficients (pending confirmation from the orthogonal interference data), whereas the absorbance data at 260 and 280 nm (or rather their ratio) would be used for advanced product characterisation and enhanced product understanding.

5.2.2.5 System selection/suitability

System suitability is assessed prior to temperature equilibration at the beginning of every run by spinning the rotor at very low speed (usually 3,000 rpm) and by collecting 2 or 3 scans only on each cell. The intent here is to ensure that there are no leaks in any of the cells and that the profile looks as expected. Radial calibration needs to be performed frequently and especially when a new rotor is used or when switching between rotors. Occasionally, the instrument needs to run a standard to ensure that its monomer sedimentation coefficient is in line with historical values.

5.2.2.6 Method development/qualification/validation

The following method parameters need to be evaluated during method development and qualification: linearity, repeatability, intermediate precision, and LOD/LOQ. Linearity is dictated by the dynamic range of the UV absorbance detector. The concentration range is typically a narrow one (ideally between 0.5 and 1.0 OD). Below the lower end (0.5 OD), species present at low levels in the mixture will be below the LOQ of the assay. Above the upper end (1.0), higher concentrations will saturate the detector. Repeatability can be achieved in the same run by loading the same sample in different AUC cells, however, intermediate precision

requires analysis by different analysts on different days. The LOD and LOQ can be determined by spiking small amounts of an impurity (such as HMW species) into the sample and assessing their recovery, or by determining them from the linearity experiments according to ICH Q2(R1) guidelines.

5.2.3 Transmission electron microscopy

5.2.3.1 Method description

Transmission electron microscopy (TEM) is a microscopy technique in which a beam of electrons is transmitted through a sample to form high-resolution images. The digitised images can subsequently be processed to extract quantitative or semi quantitative information about the specimen. There are two TEM techniques that have been used routinely to characterise rAAV capsid content; negative stain transmission EM (nsTEM/TEM) and cryoTEM (also referred to as cryo-EM). Both methods facilitate analysis through direct visualisation of the rAAV particles and allow an assessment of capsid size and morphology. Other contaminants and impurities may be identified using TEM such as broken AAV capsids, non AAV debris and residual DNA as well as aggregates.

In nsTEM a heavy metal salt is used to enhance the contrast of the specimen and to protect the specimen from the vacuum inside the electron microscope. Although nsTEM primarily enhances the surface structures of the AAVs, it can also be used to distinguish full, intact capsids from empty or broken capsids. Broken and destabilised empty capsids will take up the stain internally or collapse and accumulate stain on top of the particle. The empty particles can thereby be distinguished from full / intact capsids by contrast analysis, with empty capsids having a darker interior appearance.

For cryoTEM, particles are not stained but instead the sample is applied to a microscopy grid which is then flash frozen to cryogenic temperatures allowing assessment of particles. This method allows visualisation of the capsids in a hydrated frozen format, closer to the native state and therefore requiring less sample manipulation than with negative stain TEM. Since cryoTEM will resolve internal structures as well as the capsid surface (by differences in grey scale), the genomic material can be directly visualized.

5.2.3.2 Strengths

The material requirement for TEM analysis is relatively low, only a few microlitres are required per analysis run. TEM is a direct visualisation technique, making it possible to assess sample purity, capsid integrity, and aggregation apart from determining the level of genomic content. Advancing technology and image analysis software make quantification more robust and more user friendly.

In cryoTEM the capsids are visualised in their native state without any additives, thereby facilitating accurate determination of full, intermediate, and empty capsids. CryoTEM is less likely to be impacted by matrix interference than nsTEM as background proteins and other impurities can be eliminated during data processing.

nsTEM is a simple to learn, straight-forward preparation technique that is good for relative quantification and is a powerful method for analysing sample purity.

5.2.3.3 Weaknesses

The TEM technique requires advanced equipment and is a comparably slow technique. Although sample preparation is straight-forward, the assessment of the preparation and its suitability for analysis requires experienced operators.

Preparation of samples for cryoTEM requires extensive operator training and has a low throughput without automation. Although broken particles can be visualised using cryoTEM,

the technique is generally not suitable for detecting small assemblies of host cell and viral proteins. CryoTEM requires specialised software for quantification and QC/GMP implementation.

In nsTEM, formulation components, e.g., sugars, may stabilize the particles and thereby prevent the contrast agent from staining the empty capsids, which impacts the accuracy of the analysis. In addition, the sample preparation and drying of sample onto EM grids may impact capsid integrity and aggregation and thus compromise data analysis. nsTEM is generally less sensitive for quantification of intermediates compared to cryoTEM and can be challenging for QC/GMP implementation.

5.2.3.4 General considerations

Initially EM analysis of rAAV preparations was manual, requiring an experienced analyst to count a defined number of particles and determine the percentage of empty capsids in the sample based on visual appearance. This analysis is time-consuming and subjective, requiring specialised skills. More sophisticated preparation, detector technology and data analysis tools are evolving for TEM-based analysis; automation of TEM improves efficiency which allows for more accurate and robust analysis. In some cases, this advancing technology may facilitate GMP compliance.

5.2.3.4.1 Sample preparation

In TEM a small volume of sample, normally a few microliters, is absorbed onto a hydrophilized electron microscopy grid. The EM grid is typically a single-use fenestrated metal disc overlaid with a thin carbon film. The excess liquid is blotted off using a filter paper, either manually or by using an automated robot. In nsTEM the specimen is subsequently stained using a heavy metal contrast agent e.g., uranyl acetate, phosphotungstic acid or commercially available stains. In cryoTEM the specimen is instead embedded in vitreous ice by rapid immersion freezing in a secondary cryogen, typically liquid ethane.

5.2.3.4.2 Method set up & execution

The determination of full : empty AAV capsids using TEM is generally not sensitive to small differences in magnification, nevertheless it is advisable to periodically calibrate the magnifications using a standard. A field of view (pixel size multiplied by the number of pixels) in the range of 800-1200 nm would usually be used for data acquisition. The TEM should be aligned according to the manufacturer's instructions before use.

In cryoTEM, no additional contrast agents are added to the specimen. To enhance the contrast, comparably high defocus values can be used (4-12 µm) since the high frequency information is not necessary for the full : empty analysis. An electron dose of approximately 20-50 $e^-/Å^2$ is appropriate. The thickness of the vitreous ice can impact the analysis. A too thin ice film may expose the particles to the vacuum inside the column thereby negatively impacting the integrity of the particles and a too thick ice film will lead to a lower contrast in the images. Measures should be taken to select areas of appropriate ice thickness without biasing the sampling.

In nsTEM, the stain will provide sufficient contrast already at defocus values close to zero, and defocus values in the range of 1-6 µm and an electron dose of approximately 20-50 $e^-/Å^2$ are typically appropriate for the analysis. Since the stain needs to penetrate the capsid, or the capsid needs to collapse, to visualize the empty particles, an additional preparation procedure to destabilize the capsids may be required. This is especially true if the formulation contains ingredients that stabilized the particles, e.g., sugars. The thickness of the amorphous stain may have a major impact on the analysis. A too thin staining may expose the particles to the vacuum inside the column thereby negatively impacting the integrity of the particles and a too

thick amorphous stain film will lead a lower contrast in the images. Measures shall be taken to select areas of appropriate stain thickness without biasing the sampling.

5.2.3.4.3 Data acquisition & analysis

TEM analysis requires small volumes of highly concentrated sample. To reach a statistically significant result, typically 1 to $5x10^3$ particles need to be included in the analysis dataset. Images shall be acquired from different areas of the EM grid to mitigate impact from potential sample heterogeneity. Experimental testing is required to verify that the dataset is sufficiently large to reach the required precision. This can be done by calculating the full : empty ratio for each analysed area of the grid and determine when the mean value converges.

The gray scale values for each particle in the cryoTEM images are used as the metric to determine if the particles are full, partial, or empty. An image analysis method that can differentiate and determine the greyscale value of the background (Bg) surrounding the particles and the shell (Sh) and core (Co) of each individual particle is used, cf. Figure 1. For all particles, the shell appears dark in the cryoTEM images. If the core has a grey scale value equal to or darker than the shell, the particles are classified as full. If the background and core have an equal grey scale value, the particles are classified as empty. Particles with a core that is darker than the background but brighter than the shell are regarded as partially filled capsids.

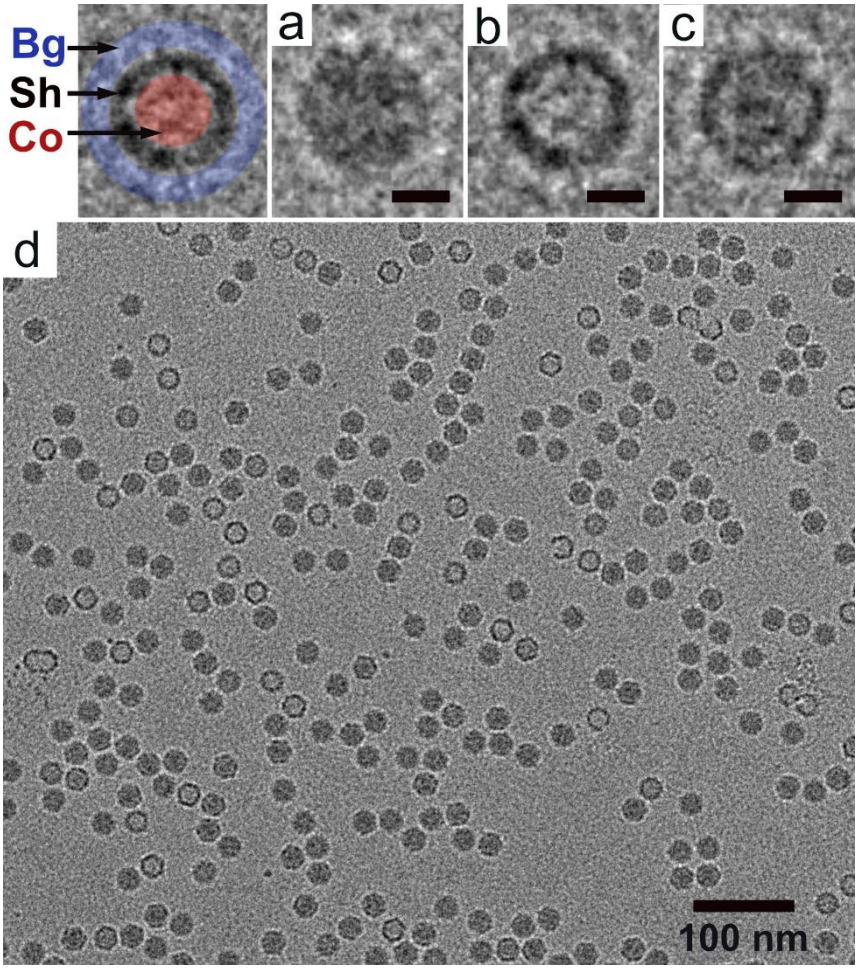

Figure 1. A cryoTEM image displaying a full (a), empty (b) and partially filled (c) AAV capsid. Panel d shows a representative cryoTEM image of a typical AAV sample. The scale bars in panel a, b and c represents 10 nm.

5.2.3.5 System selection/suitability

There are a small number of manufacturers of cryo-enabled transmission electron microscopes and automated robots for rapid immersion freezing (vitrification). Since the spatial resolution requirement for determining the percentage of full, partial, and empty rAAV particles in a sample is comparably low, in terms of hardware, practically any TEMs and detectors (cameras) designed for imaging of vitrified biological specimens can be used. In a GMP regulated quality control setting measures should be taken to ensure the integrity of the data e.g., by access control, audit trail, automated and controlled transfer of images and controlled retention of images and meta data.

Apart from the commercially available vitrification robots, manual systems are commonly used. The rapid immersion freezing does not have a direct impact on the determination of percent full capsids, but it is a prerequisite for generating vitreous ice of suitable thickness.

It is advisable to run a system suitability control sample in parallel with the test samples in each analysis to ensure that the system and method generates consistent and reliable results over time.

5.2.3.6 Method development/qualification/validation

Validation of cryoTEM for the intended purpose of determining the percentage of full, empty, and intermediate AAV capsids has successfully been performed in accordance with the principles of ICH guideline Q2 (R1). Since cryoTEM determines the ratio of full, empty, and intermediate particles, not all parameters described in ICH Q2 (R1) are applicable, e.g., linearity. Moreover, since there are no commercial standards for genomic content readily available, orthogonal techniques are needed to determine the accuracy of the cryoTEM method for any given product. Apart from small deliberate changes to method parameters, the minimum sample size (i.e., the number of included particles in the dataset) is determined as part of the robustness testing.

The TEM method applied to one product may not be directly transferable to other products as the length of the genome and the composition of the formulation may impact the specificity, precision, and accuracy of the method.

5.2.4 Size Exclusion chromatography - Multi-angle light scattering

5.2.4.1 Method description

Size exclusion chromatography (SEC) coupled with UV/vis absorbance, multi-angle light scattering (MALS), and differential refractive index (dRI) detectors is a commonly used platform to characterize macromolecules. The SEC-UV-MALS-RI setup can be used to determine AAV capsid content by measuring the contribution of protein and nucleic acid. Additionally, this analysis can provide comprehensive characterisation information of rAAV capsid, such as particle concentration, capsid molar mass, and encapsidated DNA molar mass. Hereafter, SEC-MALS will be used to represent the SEC-UV-MALS-RI platform.

5.2.4.2 Strengths

SEC-MALS provides a first principal and straightforward platform assay to determine the rAAV capsid content. The size-exclusion separation reduces the matrix interference from other impurities and the multiple detectors provide a wealth of data that can be used to fully characterise AAV capsids. The ease of sample preparation, the accuracy, and the precision of the results make the SEC-MALS method a compelling platform for rAAV analysis.

5.2.4.3 Weaknesses

Since the SEC-MALS cannot resolve the full, partial, and empty rAAV capsids, the information of intermediate species is blended into full and empty capsids. For the samples containing a high degree of partially filled rAAV capsids, orthogonal methods are required to have a better understanding of the AAV samples. Additionally, large rAAV aggregates might be present in the samples, and they might be filtered out from selected SEC columns. Lastly, rAAV product-specific extinction coefficients are required to be accurately determined. Standards of full rAAV and empty rAAV capsids are needed for the measurement.

5.2.4.4 General considerations

5.2.4.4.1 Sample preparation

Pre-purification might be required for AAV upstream samples. rAAV samples after the affinity column are suitable for this analysis. Due to the SEC separation method, few sample preparation steps are required. It is advisable to measure the samples using DLS technology to understand the aggregation level of the AAV product before submitting it for SEC-MALS analysis. Filtration using 0.45 nm filters, or a centrifugation method might be required to remove large particles from the AAV samples to protect the HPLC system.

5.2.4.4.2 Method set up & execution

Size exclusion columns

The size exclusion columns have significant impacts on peak resolution, signal-to-noise ratio, and system suitability in general. SEC-500 and SEC-1000 columns or equivalent columns are recommended to have an optimal resolution to separate high molecular weight (HMW) and low molecular weight (LMW) impurities from the AAV peak.

Size exclusion mobile phase

The mobile phase of size exclusion chromatography should be optimised for specific AAV products. The stability of the AAV product in the mobile buffer is critical for the analysis. PBS-based buffer with additional sodium chloride at different concentrations is recommended. The addition of surfactant is preferable for AAV samples.

System preparation and sequence

General HPLC system maintenance and preparation

The general practice for HPLC system maintenance and cleaning is required for good SEC-MALS analysis data. Follow the recommended procedures from HPLC system manufacturers.

Column preparation

The selected SEC column is connected to the HPLC system and the flow rate is gradually increased to the desired value. Overnight equilibration using an optimised mobile phase is recommended.

The SEC-MALS sequence

A sequence template containing blank, controls, AAV samples, and utility methods (e.g. column reconditioning) is recommended. The mobile phase or formulation buffer can be used as blanks to perform a noise check before the protein and AAV controls. BSA can be used as a general protein control to evaluate the system and AAV product-specific standards can be used as AAV controls to ensure the system's readiness for AAV analysis. Below is an example of the SEC-MALS sequence template.

5.2.4.4.3 Data acquisition & analysis

Since two concentration detection (UV260, UV280, and/or dRI) are required to distinguish the protein and nucleic acid contribution of AAV capsid content, the extinction coefficient of the protein capsid and the nucleic acid of the AAV products should be experimentally determined. Empty capsid and Full capsid standards are required to determine the product-specific extinction coefficient.

Table 2 Exemplar SEC-MALS sequence table

Vial No.	Sample name	Sample type	Inj. vol [µl]	Comments
1	Blank	Unknown	0	
2	Mobile phase	Unknown	30	
3	Equilibration	Unknown	30	Equilibration
4	BSA standard	Validation	30	SST
5	Standard	Standard	30	SST
6	AAV Sample 1 to 20	Unknown	30	Variable
7	Standard	Unknown	30	SST
8	Mobile phase	Unknown	30	Optional
9	BSA standard	Validation	30	Optional
10	Mobile phase	Unknown	30	Optional

Capsid protein extinction coefficient measurement

It is advisable to measure at least three ~100% empty AAV samples to determine the average value of the protein extinction coefficient. dRI detector and UV detector are used to measure and calculate the protein extinction coefficient. The capsid protein specific refractive index increment (dn/dc) is set to 0.185 mL/g, and the protein extinction coefficient for UV280 and UV260 is calculated respectively using the mass balance equation.

Nucleic acid extinction coefficient measurement

It is advisable to measure at least three ~100% full or AAV samples with a high percentage of full particles to determine the average value of nucleic acid extinction coefficient. dRI detector and UV detector are used to measure and calculate the nucleic acid extinction coefficients. The Nucleic acid intrinsic dn/dc is set to 0.170 mL/g and the measured capsid protein extinction coefficients at UV260 and UV280 are used to calculate nucleic acid extinction coefficients at UV260 and UV280.

Protein conjugation analysis

Protein conjugation analysis is used to calculate the percentage of capsid protein and encapsidated DNA for AAV samples, leading to the results of AAV capsid content. Two methods can be used to calculate the full/total ratio.

- Expected protein molar mass and expected nucleic acid molar mass.
- Measured protein molar mass and expected nucleic acid molar mass.

5.2.5 Anion exchange chromatography (AEX)

5.2.5.1 Method description

Anion exchange chromatography coupled with either UV/vis absorbance or fluorescence detection is commonly used to characterise rAAV to assess empty full ratios. It relies on the very small charge differences (~0.4) in isoelectric point (pI) arising from the presence or absence of negatively charged encapsidated DNA. Significant efforts have been made in recent years to apply new stationary phase column technologies and buffer systems to improve the resolution of this type of method for rAAV. There are now a variety of column technologies available to try but methods require optimising depending on the serotype and transgene. This is because subtle differences in pI arising from either the different DNA load or amino acid sequence can all impact the resolution of the chromatography.

5.2.5.2 Strengths

Analytical AEX for rAAV once setup for your product and sample type is a very robust and reliable method that has been employed with great success in the industry. It can be validated relatively easily using industry standard systems and software's that are 21CFR part 11 compliant.

5.2.5.3 Weaknesses

Analytical AEX for rAAV varies from serotype to serotype and transgene to transgene, so it is very difficult to setup as a platform method. It is very sensitive to process related impurities and buffer types, so is difficult to establish as an in-process method. Generally the method struggles to separate partial species, and is lower resolution than other methods such as AUC. But after optimisation, baseline resolution should be able to obtained for most rAAV products for empty and full species.

5.2.5.4 General considerations

5.2.5.4.1 Sample preparation

Were possible the sample should be buffer exchanged into the starting mobile phase conditions. AEX for rAAV is very sensitive to salt and pH of the sample, and this may impact the quality of the chromatography if the sample is not in the right buffer.

5.2.5.4.2 Method set up & execution

There are many different method setups and executions that can be used for analytical AEX for rAAV, its best to fit it with the stability of your product, concentration and detection method as some examples.

5.2.5.4.3 Data acquisition & analysis

This will depend on the system being used and detector.

5.2.6 Mass Spectrometry – Native and Charge detection

5.2.6.1 Method description

There are two main types of mass spectrometry method that can be used to measure the ratio of empty to full rAAV particles, native direct infusion mass spectrometry and charge detection mass spectrometry (CDMS). Native direct infusion mass spectrometry relies on either the use of known characterised "empty" and "full" standards or an assumption of the mass to charge ratio (*m/z*) range of the empty and full species.

CDMS is a variant of mass spectrometry in which both m/z and charge are detected, making it easy to determine the mass of ions in a sample. It has largely been used in research labs using home built mass spectrometers. In conventional CDMS, after molecular ions are created, one or a few highly charged ions enter a metal tube with electrodes at both ends. The

electrodes trap the ions, which oscillate back and forth through the tube. An amplifier connected to the tube detects the charges that these ions induce on the cylinder in what is known as image charge detection. More recently CDMS has been commercialised by one Mass Spectrometry vendor by adapting the Orbitrap detection. Multiple commercial instruments are now in development given the application to Gene therapy. Both Native and CDMS normally require a nano source and electrospray ionisation to maximise sensitivity.

5.2.6.2 Strengths

Advantages of CDMS include the ability to measure high mass structures, such as virus capsids, that are beyond the capability of conventional mass spectrometry. For these high mass structures, the particles are so heterogeneous that no charge state assignment can be made in a conventional native MS spectrum. In terms of empty versus full, CDMS can distinguish empty from partial capsids. Based on accurate masses of both the empty capsid and the packed virus, providing a means to quality control genome packaging. The advantage of using direct infusion native mass spectrometry is that the result can be captured in a short period of time compared to CDMS (<5min), and numerous commercial mass spectrometers can be used to acquire the data with only system setting optimisation required. Both CDMS and native MS have the advantage of requiring low volumes of material <10 µL although this is concentration specific. CDMS is more sensitive than native MS and can detect viral capsids down to 1 x 10^{10} vp/ml but the lower the concentration the longer the analysis time, and native MS is a log less sensitive in general, but it will vary from system to system and setup to setup.

5.2.6.3 Weaknesses

Weaknesses of CDMS include the long run time of several hours to obtain a spectrum as ions must be measured one at a time if a sample is a low concentration, significantly longer data acquisition times compared to direct infusion native MS. There is a requirement for a buffer exchange step, sample stability needs to be considered, particularly with unstable serotypes, as the buffer exchange is into an MS friendly buffer. Direct infusion native MS has the weakness of not giving an actual mass, but a theoretical mass can be calculated based on a reference standard. Another weakness is that both direct infusion native MS and CDMS require expert users that understand the complexities of the analysis of high molecular weight highly heterogeneous complexes.

5.2.6.4 General considerations

5.2.6.4.1 Sample preparation

The serotype and the concentration of the sample to be analysed should be carefully considered. It is easy to induce sample aggregation and subsequent sample loss if the sample concentration is too high or low for certain serotypes. Generally, samples are buffer exchanged into 100-200 mM ammonium acetate at pH 7.5, but this needs to be optimised to maximise the sensitivity and quality of the data.

5.2.6.4.2 Method set up & execution

This is very system specific and there is only one commercially available CDMS system available. Both CDMS and direct infusion native mass spectrometry require the optimisation and setup of the direct infusion and source ionisation conditions. One of the biggest challenges when analysing viral capsids and their large size is the ability to efficiently ionise the particles. With direct infusion native mass spectrometry this can be done on both orbitrap, Q-ToF and ToF systems, and depending on the vendor require different instrument setups.

5.2.6.4.3 Data acquisition & analysis

The only commercially available system using orbitrap technology comes with its own vendor specific data acquisition and analysis software. It provides relative ratios of species and direct mass measurements. Using a standard MS setup for direct infusion native mass spectrometry

you can use vendor specific integration software to calculation relative ratios of empty, partial, and full species.

5.2.6.5 System selection/suitability

As previously discussed, there is currently only one commercially available CDMS system using orbitrap technology but there are multiple others in development and its expected there will be more competition in this space in the next 2-3 years. In terms of direct infusion native mass spectrometry system selection, the system needs to be able to ionise and detect ions upwards of 35,000 m/z. The system also needs to be able to resolve full and empty species, and some systems may separate partial species as well.

5.2.6.6 Method development/qualification/validation

Significant method development is required for both a CDMS and a native MS method. Although once the direct infusion has been optimised it is applicable for both method types. Given the numerous parameters required to optimise these types of analysis and the lack of data integrity within the software used to acquire and process these analyses, it is impossible to validate this type of method. These types of methods are best suited to support product characterisation to better understand product related impurities. They are also excellent tools to support process development if there is an issue with incorrect genome packaging.

5.2.7 Mass Photometry

5.2.7.1 Method description

Mass photometry utilises the principle of interferometric light scattering to ”weigh” analytes at the molecular level. Interference between light reflected by the measurement surface and scattered by analyte molecules is measured. This interferometric contrast is proportional to the mass of any molecules present. Through calibration, the molecular weights of analytes can be determined at the single particle level.

AAV particles range in mass from approximately 3.7-5 MDa depending on payload status e.g., empty, partial, full, or overpackaged. The technology is optimised to detect particles across this mass range and is theoretically serotype agnostic. Through plotting of multiple particle measurements, it is possible to construct a quantitative representation of rAAV species (i.e., various payload status) present in a sample. The resolving power of the instrument allows for an estimation of relative abundance of empty, partially full and full capsids.

5.2.7.2 Strengths

Measurement is label-free, does not require analyte immobilisation, and full : empty ratios can be generated in minutes and with very low sample volume requirements. The technology is amenable to use by non-expert, minimally trained analysts, and without the need for complex sample preparation steps. The technology also has the potential for detecting protein impurities and potentially estimating AAV aggregation levels.

Mass photometry, as an optical technique, is truly orthogonal to other methods used to provide full : empty ratios. Compared to AUC and cryoTEM in preliminary testing, this technology has shown comparable full : empty estimation, without the high sample volume requirements of AUC or the expensive hardware and expertise required for cryoTEM. The low sample volume requirements, quick turnaround, ease of use, and low hardware capital expense make this technique a viable option for at-line process monitoring.

5.2.7.3 Weaknesses

As a disruptive technology within the rAAV analytical space, the performance of mass photometry (e.g., precision, accuracy) is yet to be tested extensively across the industry. The

assessment of levels of percentage full capsids at different particle concentrations in various matrices will be critical. When testing robustness, initial data has shown that impurities in crude AAV samples can interfere with the determination of empty and full capsids. In general, the system is more suitable for assessing samples after affinity capture. With upstream samples, the relatively high abundance of empty capsids can present further complications in identifying the full capsids. Therefore, the technology may be more suitably applied to at-line measurement of, at least, partially purified samples.

5.2.7.4 General considerations

5.2.7.4.1 Sample preparation

Certain buffer components may interfere with the full : empty ratio estimation, necessitating an additional sample preparation step, e.g., buffer exchange, prior to measurement. Alternatively, samples can be diluted in a compatible buffer prior to analysis to reduce the impact of interference. The extent to which a sample can be diluted is determined by the final particle count, based on the particle concentration, which must be sufficient to generate measurements with meaningful Gaussian distributions for each species present.

5.2.7.4.2 Method set up & execution

Calibration of the system is required to derive molecular masses from the contrast measurement. This is performed using a standard containing an appropriate number of species with known masses to generate a calibration curve. A linear response is expected across the mass range of interest for AAV analysis and standards should be protein based. Calibration curves should be prepared and applied to sample measurements taken within a predefined time window. It may be necessary to produce more than a single calibration curve per day of measurements; this should be determined experimentally. It is advisable to store the calibrants in single-use aliquots to avoid cross-contamination.

Considerable variability with respect to particle counts maybe be observed between technical replicates. Inconsistencies in particle number can be due to differences in sample loading volume and pipetting technique between measurements. Variability can also come from the number of particles binding and unbinding to the slide during sample loading. Adequate training of the operator during sample loading and performing replicates should be considered. Differences in particle counts do not necessarily impact on the relative proportion of full : empty measured.

5.2.7.4.3 Data acquisition & analysis

To distinguish between empty, partially filled, full, and overpackaged AAV particles the theoretical mass of the capsid and expected payload must be known. Analysis is automated to indicate empty and full capsids, however partial and overpackaged capsids, or aggregates, currently require manual identification.

5.2.7.5 System selection/suitability

Currently there is a single vendor for mass photometry with different models, one of which is optimised for AAV full : empty analysis.

5.2.7.6 Method development/qualification/validation

A reference AAV material should be developed and used across different measurements as a positive and trending control. The platform will need to be qualified and validated (e.g., precision, linearity) during the life cycle of a therapeutic product. Points to consider for qualification/validation will include availability of sample, as well as ways to reduce the variability e.g., adequate sample loading technique, or automation of sample loading.

6 Control strategy development for particle population

The control strategy is the sum of all mitigation activities which ensure the risk to process failure are kept at a minimum and that product quality remains consistent from batch to batch which in turn gives assurance of final product quality and safety throughout the development lifecycle. This section provides a brief introduction on key elements of a control strategy and gives stage appropriate considerations on the implementation of a control strategy for the particle population.

Development of the full Drug Substance and Drug Product control strategy and a detailed description of the items supporting a Quality by Design approach are outside of the scope of this document. Quality by Design principles, the selection of methods, and their implementation in the context of a product control strategy should follow latest scientific understanding, regulatory guidelines, ICH quality guidelines, and best practices.

6.1 Principles of using a Quality by Design approach

Manufacturers of rAAV-based therapies are encouraged to utilise Quality by Design principles and adopt a science- and risk-based approach early in the drug development process. Central to the Quality by Design approach is that quality is not reliant on end of product testing alone. Instead, quality is built into the process through a development program that addresses the needs of product design criteria.

Key elements of the Quality by Design approach are summarised in Figure 2 and briefly introduced in the following subchapters. Quality by Design is a systematic approach to identify and assess the criticality of material, process, and analytical parameters and their impact on critical quality attributes. Parameters which may adversely impact product quality are controlled and those which may be useful as monitoring tools are identified.

Risk assessments based on understanding of process and analytical capability inform the implementation of critical process controls and testing which in turn ensures that the final Drug Substance and Drug Product specification is met. In addition, establishing a robust manufacturing process and phase-appropriate control strategy from early development supports continuous improvement through all stages of development – including post approval changes.

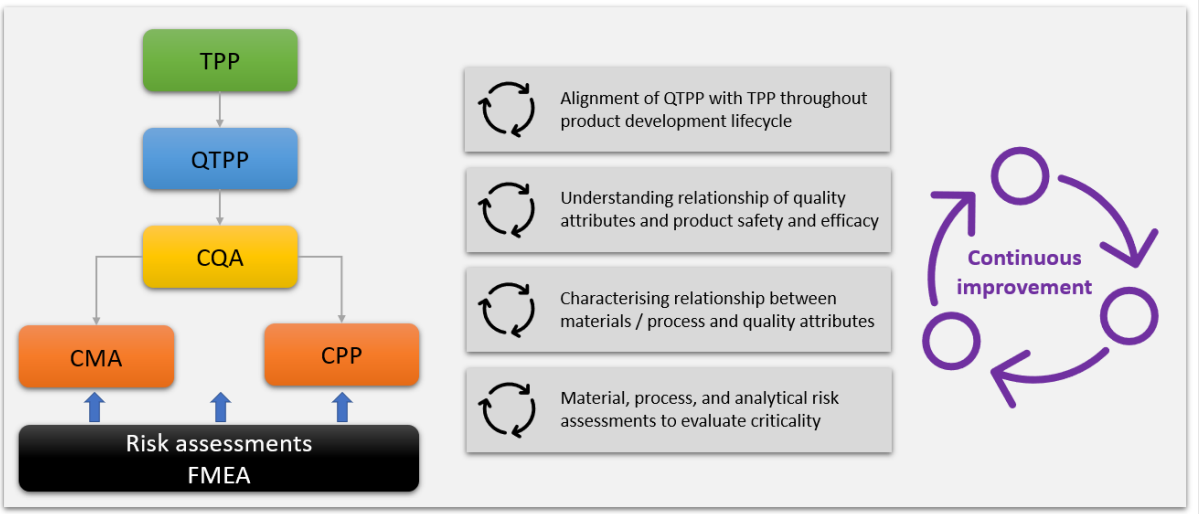

Figure 3 The control strategy development is a continuous process which builds on previous iterations. Product design criteria are specified in the target product profile (TPP). This provides the basis of the quality target product profile (QTPP) which in turn informs the definition of critical quality attributes (CQA) based on the product design criteria. Process development allows the definition of critical material attributes (CMA) and critical process

parameters (CPP). Through product characterisation studies the CMAs and CPPs are linked to the quality attributes.

Typical elements of a control strategy can be divided into i) product specific controls, such as material and reagent qualification, in-process and final product testing, control of storage and shipment conditions, and the in-use conditions of the final product up to the point of administration, and ii) support system controls, such as facility design, operator training, procedural controls, qualification and periodic maintenance of process and analytical equipment.

Benefits of utilising a Quality by Design approach are increased awareness of the critical links between process parameters and materials with clinically relevant product attributes. Which in turn ensures the control strategy is appropriate for the product throughout its lifecycle. The enhanced process understanding, and control reduces risk of process changes and/or transfers and minimises the risks of batch failures.

6.1.1 Quality target product profile

For all development work predefined objectives should be established which are aligned with the product's design criteria as defined in the quality target product profile which in turn is derived from the target product profile (TPP). This ensures the output of the development work addresses the identified risks and aids in process understanding and control. Hence, the quality target product profile underpins the entire control strategy. The design criteria should be established early in the development process and reviewed regularly to guide the development effort and ensure the overall product development, as defined in the target product profile, and quality development aspects remain aligned.

6.1.2 Critical quality attributes

Critical quality attributes are defined based on the agreed design criteria and informed by prior knowledge where available. As for the quality target product profile the critical quality attributes should be reviewed throughout the product development and be modified as necessary to reflect the latest knowledge and product understanding. A risk-based approach is used to identify those attributes which may impact quality, safety, and/or efficacy of the final product.

A criticality rating is then applied to each identified attribute to guide the development effort towards highest ranking attributes (based on current knowledge, process control, and testing capability). The criticality rating is informed by the severity/impact of the attribute on quality, safety, and efficacy and its detectability. Prior knowledge from product development studies or body of evidence in the literature should be used to justify the severity scoring where available. The detectability of a change in attribute which could impact the final product is defined by the capability of available analytical methods and the established link between process steps and final product quality, safety, and efficacy.

6.1.3 Critical process parameters and critical material attributes

Linkage of product parameters and the attributes of materials used in the manufacturing process with quality attributes typically requires a significant body of evidence from routine manufacturing and/or process characterisation studies. In some cases, specific linkage studies under worst case conditions may be performed to confirm the impact of a process parameter or material on product quality. In addition, process parameter and material risk assessments are used to stratify materials and process parameters into critical, key, and non-key groups.

To generate the necessary understanding of how product attributes and process steps are linked, a rational assessment on the understanding of product quality, safety, and efficacy as well as process- and analytical capability should be performed at regular stages in the product

development lifecycle. This allows a quantitative definition and ranking of quality risk which in turn informs process and analytical development work (and potentially pre-clinical studies) to mitigate the identified risks.

6.1.4 Failure mode and effects analysis

The failure mode and effects analysis is used to document the current knowledge of process controls, to identify potential failures, and to assess the risk to the process if a failure were to occur. This assessment is typically conducted in two stages. First, failures are scored based on their severity, frequency of occurrence, and their detectability and a final risk rating is calculated. In a second review of the identified failures the scoring is modified by the available controls. Scoring in a consistent manner allows the prioritisation of continuous improvement activities on the failures identified as those with the greatest impact on process performance. In addition, the available controls and their impact on reducing the risk of process failure are documented.

Considering the complexity of manufacturing rAAV-based therapeutics the failure mode and effects analysis should be conducted early in the process development phase and kept up to date as process understanding is gathered. This allows early identification of items which could have the greatest impact on process performance. And focuses the development work and implementation of process control activities accordingly.

6.2 Control of particle population

The rAAV particle population, i.e., the distribution of full, intermediate, and empty rAAV particles, is typically considered a critical quality attribute because of the potential impact on product quality and safety (as discussed in section 4). Hence, the control strategy needs to consider the product and process specific items which may impact the formation of different rAAV particle species and the parameters which ensure consistent generation of a highly similar distribution of rAAV particles.

Examples for control of the rAAV particle population include, but are not limited to,

- Starting and raw materials related to the upstream and downstream manufacturing of rAAV particles (e.g., plasmid DNA, helper virus, manufacturing cell line, transfection reagents, buffers, and solutions for purification and polishing steps),
- Process parameters used in upstream manufacturing and downstream purification & polishing steps which relate to enrichment of full rAAV particles, and
- Method capability for determination of full : empty ratio and determination of additional rAAV species.

Achieving batch to batch consistency is key in material destined for clinical and commercial rAAV-based therapies. It is recommended that critical parameters which can impact quality and safety of the rAAV particle population and thereby material are defined through a risk-based approach applying the principles of Quality by Designed as described in the previous section.

6.2.1 rAAV particle population as part of QTTP

The expected rAAV particle profile should be defined in the quality target product profile through an evidence-based process. Complexities concerning the capsid protein, source, and consistency of encapsidated DNA, the targeted cells, and planned route of administration need to be given due consideration when defining the target attributes. To define meaningful criteria for rAAV particle population use of the common term “*full : empty ratio*” requires a clear definition to avoid ambiguity (as discussed in section 2).

During early stages of product development there is usually limited information on the link between the rAAV particle population and their function. In the absence of product specific knowledge on the structure function relationship, developers should ensure that pre-clinical material used in safety assessment studies is representative of material destined for clinical use. For example, establishing a lower threshold for full rAAV particles together with an upper limit for total particles may be appropriate at this stage.

As product characterisation provides more insight into the range of particle species and their impact on product quality, efficacy, and safety, sponsors should assess defining upper thresholds for empty and intermediate particle species also. Design criteria set in the quality target product profile should consider product characteristics (e.g., self-complementary or single-stranded rAAV vector, intended dose, serotype/capsid modification), treatment population and target (e.g., age, tissue, organ), and the route through which the product will be administered (e.g., local/direct vs systemic delivery).

In addition, the treatment risk/benefit and relevancy of available prior knowledge from in-house development or the literature should be assessed also when defining product specific acceptance criteria for full, empty, and intermediate species.

6.2.2 Process development to link CPPs and CMAs with CQAs

In the early stages of process development, the primary purpose of characterising the rAAV particle population is to inform the next iteration of the manufacturing process. Often multiple conditions of the process are evaluated in parallel and at small scale. At this point sample throughput likely takes precedent over deeper characterisation of the particle profile present in each process condition.

Illustrative example 1 – Critical material attribute:

The percentage of supercoiled plasmid DNA was found to significantly improve the presence of full particles. Plasmid lots > 90% performed better compared to supercoiled plasmid DNA in the range of 80% to 90%. The specification for plasmid DNA lots was therefore defined as ≥ 90% supercoiled DNA.

Nevertheless, product characterisation through use of orthogonal methods is highly recommended. Especially at key stages where the output can have a significant impact on the future direction of the development work. This is because a first evaluation of process parameters and material attributes and their impact on the particle population are typically informed by this early-stage process development work. Literature and prior knowledge of the manufacturing process and materials can be used to further inform the assessment. The output is a first version of materials deemed critical for the manufacturing process and their specifications. And, as for material attributes, a first ranking of process parameters and their criticality is informed by process development work.

Illustrative example 2 – Critical process parameter:

In a process utilising Caesium chloride (CsCl) density gradient for rAAV material purification, the parameters around the ultracentrifugation step have been evaluated during process development to understand the operating window for time, temperature, and revolutions per minute. Upon scale up and preparation for routine manufacturing these conditions were confirmed as providing adequate coverage around the setpoint ± variability of the instrument.

As work on the process progresses the focus will gradually shift from establishing the criticality of parameters and materials to understanding the design space for each item. Using a risk-based approach focus should be on the items with the highest impact on the rAAV particle profile.

At the point where development converges towards a defined manufacturing process, determination of the rAAV particle profile throughout the process but particularly in the final product should be part of the product characterisation plan. In a stage appropriate manner and aligned with the quality target product profile the characterisation of the rAAV particle population should seek to understand the potential safety risk of each particle species.

Illustrative example 3 – Failure mode and effects analysis:

Recovery of density gradient fractions was initially captured as low risk because of good consistency during small scale development studies. Operating at scale and with different operator experience resulted in higher-than-expected variability in rAAV purity and bioactivity post ultracentrifugation. Additional training, procedural control, and automation of processing steps were introduced to mitigate the risk and control this manufacturing step.

Due consideration should also be given to complexity of particle diversity, the encapsidated DNA species, and which fraction of the total rAAV particle population carries the therapeutic effect. Risk assessments should be utilised to capture the knowledge and any gaps in the understanding of the structure-function relationship of the product.

Adherent cell culture systems coupled with small-scale gradient separation and ultracentrifugation are often used in the early stages of process/product development. These typically generate material with high percentage of full particles but are difficult to scale up and operate in a closed cGMP environment required for Drug Substance manufacturing. For products requiring large amounts of rAAV more scalable and enclosed manufacturing systems should be considered.

Changes to the manufacturing process between material generated for pre-clinical safety assessment and during clinical development require an assessment of the change and demonstration of product similarity pre- and post-change (refer to section 6.3.3).

6.2.3 Routine testing

For routine manufacturing of safety assessment batches, clinical supply, and commercial material the testing can be classed depending on its intended purpose. In-process testing performed by the Quality Control unit is typically executed to ensure process performance and final product quality. With the generation of batch history data action or acceptance limits should be defined for these tests, as appropriate. Release testing of Drug Substance and Drug Product lots is also performed by the Quality Control unit and ensures the final material meets the acceptance criteria defined in the product specification.

It is generally advisable to implement only methods of highest accuracy, precision, and robustness for the release testing of rAAV-based therapies. In addition to method performance, the selection of the release method should consider demonstrated suitability for the test sample, correlation with orthogonal methods, and compliance of the analytical platform with relevant guidelines.

Characterisation and extended characterisation testing may be performed by the Quality Control unit or in development labs. These assays are typically more complex and because of inherent characteristics challenging to implement in a Quality Control unit. Samples for characterisation may be taken from in-process steps or at the Drug Substance and Drug Product stage to gather information on the performance of unit operations or to further product understanding. Methods performed for characterisation are considered for information only, i.e., acceptance criteria generally are not defined. However, it is good practise to monitor results, investigate out of trend observations, and document the findings.

Characterisation vs extended characterisation:

Characterisation assays are typically performed on all routine manufacturing runs whereas extended characterisation methods are only applied for e.g., investigations or to demonstrate product comparability following a change in the manufacturing process or process transfer.

It is recommended that the rAAV particle population is monitored throughout the process. Not all sample types are conducive to testing crude materials at the end of upstream/beginning of the downstream process. Impurities and low titre reduce the range of methods available at these stages (as described in section 5). Use of orthogonal methods is recommended. This allows testing of in-process samples with quick turnaround methods whilst more resource intensive methods are performed on polished, high-titre material only. A method capable of resolving full, intermediate, and empty particles should be employed at least at one stage post purification and polishing.

The distribution of rAAV particle species is generally not considered stability indicating. However, the complex interactions of formulation buffer excipients, process and product related impurities, and the different charge variants or rAAV particles may lead to a different degradation rate of the various particle species. Hence, this assumption should be confirmed in development experiments before the rAAV particle testing is removed from formal stability programs.

6.3 Lifecycle management

6.3.1 Principles

The output of the process development and product characterisation work together with data from routine batch manufacturing runs will inform the continuous improvement of the manufacturing process, test methods, and sampling. Wherever possible method changes should be avoided or minimised during clinical development to prevent the challenges that can occur from changing an assay and generating a different result with a new assay format. However, it is recognised that changes to a method or introduction of a new testing facility during the lifecycle of a product may be required. While changes may be required to ensure continued product supply during clinical development. This may necessitate technology transfer activities of process and/or test methods to new/additional sites.

For minimal disruption during the development lifecycle, appropriate strategies to manage process and analytical changes should be defined early on – even in the case where no or limited changes are being anticipated. All change activities should be performed in a controlled manner to ensure minimal impact on final product quality, safety, and efficacy. Where changes are required the level of control and risk mitigation measures should be commensurate with the level of change and phase of development.

Life cycle management draws heavily on the understanding quality, process, and material attributes. Having established critical parameters and attributes through a risk-based process will provide the foundation on which change assessments can be made. A systematic

evaluation of the planned process or method changes will highlight the impacted attributes. This in turn allows the creation of a mitigation plan to reduce the risk associated with the planned change.

6.3.2 Monitoring method performance in early development

Method changes are common during the early process development phase. This may be due to limited access to established methods or because established methods are not suitable for the process/product. Hence, methods in support of process development work are often implemented with limited understanding on analytical capability.

The uncertainty of method robustness may partly be mitigated by implementing a trending control when testing process development material. In early development material representative of the final process is likely not available and there may be a preference to wait for a more suitable control sample in later stage of development. However, implementing a trending control which mimics quality of the current process can benefit both the process and analytical development work:

- Monitoring the results of the control sample in parallel to the test samples can give context to extreme values observed across runs.
- The impact of method changes which lead to a shift in the reported value of the trending control can be quantified and allows a correction factor to be applied on earlier test samples.
- The information from trending a control sample can inform the selection of which orthogonal methods are best suited for in-process and final product release testing.

6.3.3 Product comparability

Appropriate change management will ensure the final product pre- and post-change are comparable in product quality, safety, and efficacy. Depending on the size of change the required evidence of product similarity may be obtained from ongoing process development work or dedicated in-vitro and potentially in-vivo characterisation.

- For changes considered minor the available data from process development complemented with small scale evaluations of the planned change may be sufficient to mitigate the risk of the process change.
- Larger changes with potential impact on quality, safety, and efficacy should be evaluated through in-vitro characterisation.
- In-vivo characterisation, and potentially including clinical bridging studies, may be required where process changes are likely to result in a change of the product profile beyond what has been evaluated in early-stage clinical development.

Where changes to both process and analytical methods have been introduced the comparison of the product quality, safety, and efficacy needs to consider the difference in test method. Demonstration of method equivalency is typically performed by performing side-by-side testing of old and new method (e.g., pre- and post-site transfer). Side-by-side testing may not be feasible for all types of changes, however (e.g., use of different instrument platform). In such cases, retesting of retain samples from historic batches to either demonstrate similarity of methods or to establish a statistically valid correction factor between old and new method.

In addition to the type and size of change, several other factors require due consideration for the design of product comparability studies, e.g., stage of clinical development, available samples of relevant process batches, the age of any retain samples, the statistical approach to demonstrating method and product similarity. Sponsors are encouraged to engage with and seek feedback from health authorities early on to ensure a robust study design.

It is highly recommended to perform pivotal clinical studies with material representative of the commercial process. Significant changes, such as change from adherent to suspension or ultracentrifugation to chromatography based separation, should be implemented early in clinical development as these may impact multiple CQAs – including particle population. Large differences in the full : empty ratio between clinical samples and commercial product may necessitate additional clinical studies if equivalent safety/efficacy cannot be demonstrated using in nonclinical studies.

The complex relationship of the particle population, the potential capsid modifications, and the different DNA species which can theoretically be encapsidated should be given due consideration throughout the development lifecycle. Where changes are implemented which could result in differences to the particle population the characterisation of the particle population should be appropriate to determine the impact on

- *Particle distribution*
  An assessment of the total particles from the empty, intermediate, and full particle fraction should be made to ensure the total particles post-change do not exceed limits evaluated in safety studies.
- *Encapsidated DNA species*
  Nature and size distribution of the encapsidated DNA should be determined.
- *Capsid modifications*
  Primary sequence, presence of viral protein variants, and the presence of post-translational modifications should be assessed.
- *Structure-function relationship*
  The bioactivity of the final product pre- and post-change through use of a (surrogate) potency assay should be compared.