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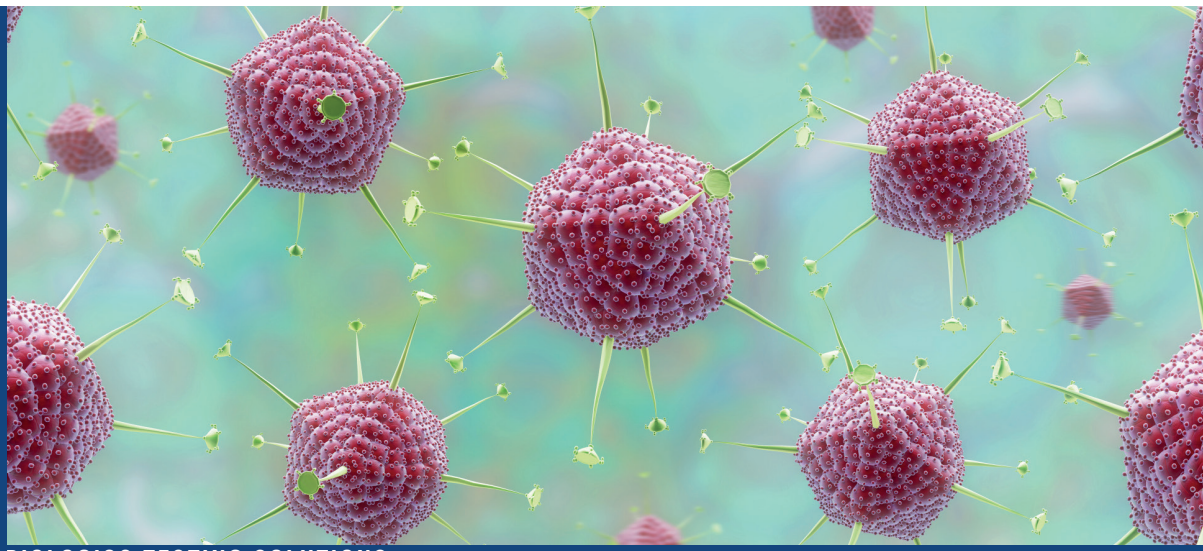
Summary
Recombinant AAV products are increasingly important in the realm of gene therapies. Charles River provides specialized testing packages for the characterization and quality control testing of these products.



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Recombinant adeno-associated viruses (rAAVs) are becoming the vector of choice for delivering therapeutic transgenes. The resulting products are currently being investigated for the treatment of various conditions, including cancer, ocular, pancreatic, and central nervous system diseases. rAAV therapies work by introducing new genes into a patient's cells to replace a malfunctioning gene. rAAV-mediated gene therapies are ideal since, after infection of the host, the delivered genes do not incorporate into the host genome, but the DNA instead remains in the extrachromosomal state. This DNA is then transcribed as the other genes in the cell but does not replicate when the cell undergoes division. Especially of interest to the CNS field is the rAAV's ability to infect not only replicating but also quiescent cells, particularly neurons. Recombinant AAV gene therapies have shown strong evidence of efficacy and safety in a large number of animal models and clinical trials.

This unique therapeutic approach requires a specialized testing package for the characterization and quality control (QC) testing of AAV particles and constituent capsid protein assemblies. Some of these methods are already available for use, while others may require optimization and ICH validation prior to being implemented in a QC testing program. Testing that is part of a standard characterization package for rAAV gene therapies is described below.

Mass Spectrometry

Many different mass spectrometry methods are employed to characterize rAAV therapies, ranging from strain confirmation by LC-MS analysis of intact viral proteins, profiling/quantitation of post-translation modifications (PTMs) by peptide mapping, to the identification of residual host cell proteins by LC-MS based proteomics methods. Mass spectrometry is also being employed in selected cases to assess efficacy of rAAV therapies by detecting the upregulation of desired molecular targets, including proteins expressed in various tissues.

Peptide mapping by LC-MS/MS of the proteolytically digested AAV is commonly used both for amino acid sequence confirmation to characterize an rAAV therapy, and as a stability-indicating QC method. Quantitation of each virus particle may also be performed through a digestion/mapping approach in which a unique virus particle peptide is quantified after normalization by means of a spiked,

synthetic heavy peptide as an internal standard (IS), performed using a triple quadrupole MS. Additionally, once the viral capsid proteins are dissociated from one another, intact molecular weight (MW) via LC-MS provides a highly useful overview about the type and amounts of PTMs to complement peptide mapping.

Glycan profiling of viral envelope proteins (e.g., VSV-G) on the surface of enveloped virus particles can also be included in a characterization package. Glycan profiling can include O-linked glycosylation as well as N-linked. Quantitative sialic acid analysis and quantitative monosaccharide analysis are also commonly employed to characterize the glycan population of glycosylated gene therapy products.

Biophysical Characterization (Higher Order Structure Analysis)

Biophysical methodologies may be used for characterization as well as QC testing of rAAV based products. Specially developed analytical ultracentrifugation (AUC) methods can be used to determine empty versus full capsid ratios, as this is a key quality measurement for gene therapy products.

Stability-indicating methods will focus on particle quality and size, using methods which can resolve particle aggregation and particle degradation. Differential scanning calorimetry (DSC) is used as a stability-indicating method, demonstrating stability as a function of formulation and for comparability of batch-to-batch. Dynamic light scattering (DLS) is also useful for particle size distribution and characterization.

Analytical Testing

Along with mass spectrometry and biophysical methods, there are a variety of other analytical techniques that can be used for analysis of rAAV-based gene products. These include chromatographic, electrophoretic, and ELISA-based assays. The unique nature of rAAV products requires a customized testing package to meet clients' specific needs. Some testing that may be included in this package is described below.

A variety of HPLC methods can be incorporated into an rAAV testing plan. For example, reverse-phase high-performance liquid chromatography (RP-HPLC) may be used to QC the previously characterized capsid proteins. Following dissociation of the capsid proteins from one another, RP-HPLC is used to calculate percent ratios of the virus particle proteins. Anion exchange HPLC is employed for separation and quantification of supercoiled versus non-supercoiled plasmids. Percent supercoiling is often requested by the FDA, as it is believed to correlate with plasmid infectivity.

ELISA assays may be applied as an orthogonal method to LC-MS to quantify viral coat or capsid proteins. This can be coupled with a prior step to fractionate the analyte based on size to remove monomers or fragments of the capsid protein which may give a positive ELISA response. Quantitation of proteins by amino acid compositional analysis is also common.

N-terminal sequencing by Edman degradation is an industry standard method commonly used to verify the N-terminal integrity of capsid proteins. Initial separation of capsid proteins via SDS-PAGE or RP-HPLC may be used, and additional information such as capsid N-terminal truncations may be evaluated.

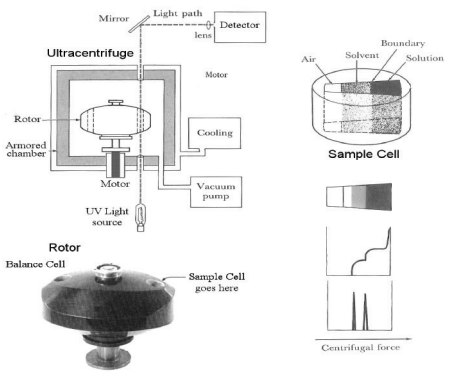

Figure 1: Sedimentation velocity analytical ultracentrifugation (SV-AUC) is a promising method as applied to measurement of empty-full AAV ratios, because both empty and full particles are in principle resolved in the same experiment, and requires minimal sample manipulation. *Image courtesy of Beckman Coulter.*

Other techniques that can be incorporated into testing plans include SDS-PAGE or CE-SDS for analysis of non-reduced versus reduced virus-like particles (VLPs). Residuals testing should also be performed for these gene therapy products. Polyethylenimine (PEI) is often used as a transfection agent in gene therapy to promote plasmid entry into cells, and it is important to analyze for residual linear as well as branched PEI, along with other residuals and impurities that may be introduced during your process.

rAAV Vector Genome Titer by Real-time PCR (qPCR) and Droplet Digital PCR (ddPCR)

Since AAV typically will not replicate without the presence of a helper virus, vector genome titer, rather than infectious titer, has been commonly used for clinical dosing. Historically, rAAV vector genome titer has been measured by qPCR technology in real time using fluorescent dye-labeled probe based on the standard curve derived from amplification signals of target DNA from a dilution series of a known quantity of DNA standard.

Recently, Droplet Digital PCR (ddPCR) technology has gained acceptance by both the gene therapy industry and regulatory agencies to measure rAAV vector genome titer. ddPCR technology is based on water-oil emulsion droplet technology to fractionate a sample into 20,000 droplets, with target-specific PCR amplification occurring in each individual droplet. Following PCR, each droplet is analyzed to determine the presence (as positive droplet) versus the absence (as negative droplet) of the intended target. The overall droplet data (number of positives and negatives) are then used to calculate the absolute target quantity by Poisson statistics in the original sample.

Comparing to qPCR, ddPCR can not only have a higher tolerance of matrix interference since low-efficiency PCR reactions in any droplets due to inhibition are still counted as positive droplets but also achieve higher degree of precision in the absolute quantification of intended target without the requirement of any known quantity of DNA standard.

rAAV Vector Infectious Titer by TCID50 (Median Tissue Culture Infectious Dose) Assay

In addition to AAV genome titer, it is important to determine its infectious titer, which is typically determined by TCID50 assay with endpoint read-out based on qPCR measurement of replicating rAAV genome DNA. HeLa RC32 cells expressing AAV2 Rep/Cap gene have been widely used to support rAAV vector replication in the presence of human adenovirus type 5 (Ad5). Typically, a serial dilution of rAAV vector is used to infect HeLa RC32 cells in the presence of human Ad5 helper virus in 96-well plate with multiple replicates for each dilution. After 72 hours incubation, infected cells in all replicates of all dilutions are lysed and subjected to target-specific qPCR assay for quantification of rAAV genome DNA. The background signal in the target-specific qPCR assay is determined based on the appropriate control samples (including uninfected cells and cells only infected by human Ad5). Any replicate resulting in target-specific qPCR signal above the determined background is counted as “positive”. rAAV vector TCID50 titer is then calculated based on the overall positive versus negative wells at each dilution by Karber’s method.

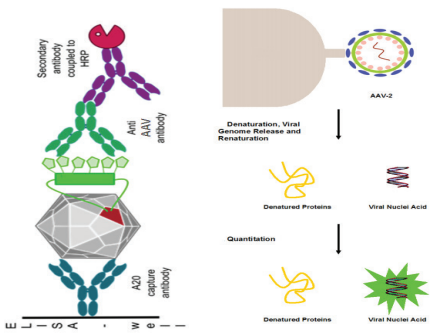

Figure 2: Present methods for quantifying the amount of AAV protein and nucleic acid, and therefore the empty-full ratio. Protein is often quantified by ELISA, while nucleic acid is quantified by qPCR. A more recent method utilizes the A260/A280 ratio of processed AAV samples. *Image courtesy of www.progen.com.*

rAAV Vector Residual Host Cell DNA Testing by Real-time PCR (qPCR)

Plasmids transfection into HEK293 cells is an established process to manufacture rAAV vectors. Due to the rather delicate nature of rAAV vectors, their purification options can be limited comparing to recombinant proteins. As a result, a significant amount of residual host cell DNA and residual plasmid DNA could be present in the final rAAV vectors, even after benzonase treatment followed by affinity and chromatograph purification steps.

To ensure the biosafety of a rAAV vector, the quantities of residual host cell DNA (including total amount along with fragment sizing distribution and any specific viral sequences present in the production cells) as well as residual plasmid DNA used in the production process, needs to be determined. Typically, qPCR assays targeted to 18s rRNA genes are used to determine both the total amount and the size of residual HEK293 cells DNA, while a qPCR assay targeted to a kanamycin resistance gene, commonly present in plasmids as a selection marker, is used to determine the total amount of residual plasmids. Since Ad5 E1a sequences, a known oncogene, are present in HEK293 cells, an additional qPCR assay needs to be performed to quantify the residual E1a sequences in the final rAAV vector.

For any other rAAV vector manufacturing process, qPCR assay(s) targeted to the representative gene for specific production cells and/or helper virus would be used to determine residual host cell and/or helper virus DNA presented in final rAAV vectors.

Replication-Competent AAV (rcAAV) Testing

Even though rAAV vector manufacturing processes have been specifically designed to reduce the risk of rcAAV generation, presumably through a recombination event, there is still a possibility of rcAAV present in the final product. Therefore, rcAAV testing is required by guidance documents. For a typical rcAAV assay, permissive cells for an intended serotype AAV are infected with either wild type AAV virus (if available), or a chimeric AAV positive control with the intended capsid, along with rAAV vector of the same serotype, all in the presence of human Ad5 helper virus to support rcAAV replication in cell culture. After three rounds of cell-based amplification, each sample is processed and subjected to Rep2-specific (or any other appropriate target-specific) qPCR assay. The background signal in the target-specific qPCR is determined based on the appropriate control samples (including uninfected cells, cells only infected by human Ad5, and cells only infected by AAV positive control in the absence of human Ad5). Any target-specific qPCR signal above the determined background indicates the presence of rcAAV. The intended clinical dosing of rAAV vector can be used to determine the input vector amount in this assay.