

EXPERT INSIGHT

Analytical approaches to characterize AAV vector production & purification: Advances and challenges

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Adeno-associated viral (AAV) vectors have been reported to be a great promise in a large number of clinical trials. Multiple AAV gene products will enter into early and late phase clinical trials. To sustain this, reliable, fast, robust, GMP compliant analytical methods and characterization protocols are needed. Specific analytical assays need to be performed to assess vector productivity, vector purity, biological activity and safety. Besides the potency of the vector, current advances appear to improve the methods for the quantification and characterization of AAV particles that are empty or contain DNA fragments other than the full vector genome of interest. This article describes the analytical methods available to measure the strength/dose of the AAV vector, to quantify full/empty particles and illegitimate encapsidated DNA, their limitations and recent improvements.

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In recent years, AAV vectors have been increasingly evaluated in various gene therapy clinical trials for treating a plethora of diseases. In parallel with the production of AAV vectors for clinical trials in human diseases, technological progresses have focused on the development of rigorous quality control assays in order to support regulatory

applications and favor new product licensing. In addition to the classical testing schemes as developed and implemented for biotech-derived products, additional AAV vector specific assays have to be put into place to prove the safety of the vector preparation, assure patient's health and avoid any undesired affect. These specific testing include

the establishment of the vector titer (vector genome titer, infectious particle titer, ratio of full to empty particles), its potency, the proof for the absence of replication competent virus ('replication competent' or wtAAV), the absence or presence of only an acceptable limited amount of residual contaminating DNA, residual proteins and chemical reagents from

the cell culture medium and the purification process. The testing of the final AAV products for pre-clinical and clinical lots, classified into four groups in function to their attribute is listed in **Table 1**.

For most of these “safety issues”, assays have been developed which are

routinely used for the characterisation and testing of each vector lot. However, some of these tests are not optimal, time consuming, of limited accuracy or cumbersome providing results of only limited precision or insufficient sensitivity, leading to a poor reproducibility

► **TABLE 1**

Example of release testing of AAV vector product.

Attribute	Assay	Method
Strength/dose	Vector Genome titer (VG)	Dot blot Spectroscopy/Fluorimetry qPCR/ddPCR
	Infectious Genome Titer (IG)	Infectious Center Assay (ICA) TCID50
	Total vector particles	ELISA or SDS-PAGE
	Activity (expression assay)	Cell based assay
	Potency (functional activity)	Cell based assay or in vivo assay
Identity	Genome DNA	Sequencing
	VP proteins	Western blot Mass spectroscopy
Purity	Host cell DNA	qPCR
	Helper-plasmids or helper viruses DNA	qPCR
	Host cell protein	ELISA
	Residual production reagents and raw materials (antibiotic resistance genes, detergent, benzonase, BSA, column leachables...)	qPCR ELISA HPLC Mass spectrometry
	Ratio Full/Empty particles	qPCR/ELISA HPLC Electron microscopy Analytical Ultracentrifugation
Safety	Sterility	EP 2.6.1, USP<71>
	Bacterial endotoxins	EP 2.6.14, USP<85>
	Mycoplasma	EP 2.6.7
	Adventitious viruses	EP 2.6.16
	Replication competent AAV (rcAAV)	Cell based assay
	Vector aggregates	DLS

ELISA, enzyme-linked immunosorbent assay; HPLC, high performance liquid chromatography; DLS, dynamic light scattering. The EP and USP references are chapter guidance of the European and United States Pharmacopeia providing procedures used to test products.

signifying that these tests have either to be optimized or replaced by novel analytical methods or assays. This review presents an update of the analytical methods used to measure the strength/dose of the AAV vector, to quantify full/empty particles and illegitimate encapsidated DNA, their limitations and recent improvements.

STRENGTH/DOSE OF AAV VECTOR

Measurement of strength/dose of purified AAV vectors typically includes assays to quantify the genome concentration, infectious concentration as well as functional activity of the transgene.

Vector genome titration methods

As vector genome (VG) is the key component involved in rendering the therapeutic effect in gene therapy, large attempts have been made to develop a fast, reliable and robust method for its titration.

The first titration method for determining VG quantity of AAV are based on dot blot assays on DNA genomes contained within the particles of recombinant AAV (rAAV) vectors [1,2]. This method was widely used in the past, in particular for proof of concept and dose response studies in small rodents. Later other methods were implemented such as Southern Blotting [3], UV spectrometry [4], fluorimetry with PicoGreen dye [5] and real-time quantitative PCR (qPCR) which has increased the range of quantification, sample throughput, precision and robustness of VG titration [6,7]. The qPCR has become

the most standard and accepted method to titer VG AAV.

Several papers have highlighted the cautious use of qPCR as variations in titer could reach up to 10 fold when compared to dot-blot and UV spectrometry [8,9]. Recommendations have emerged and the generation of reference standard materials (RSMs) which are available at the American Type Culture Collection (ATCC) allowed to normalize inter-laboratories titration assays. The AAVRSM Working group generated two reference standard materials, AAV2 RSM and AAV8 RSM. Despite using a common procedure, these studies showed inter-laboratory variations in qPCR VG titers of almost 2-log [10,11], highlighting difficulties to establish a standard procedure.

There is a high number of scientific recommendations for AAV titration by qPCR, and extreme care should be taken to validate the entire method. The use of RSMs is highly recommended to demonstrate that the overall process is controlled. Special attention must be paid on qPCR in addition to sample pre-treatments, such as DNase, proteinase K and restriction enzyme treatments, nucleic acid extractions and dilutions. Description of these critical steps is briefly discussed here.

As VG titer relies on quantifying the number of encapsidated vector genome, samples are firstly treated with DNase in order to remove non-packaged vector DNA that can result in an overestimation of VG titers. DNase and the vector capsid are generally then denatured with a proteinase K treatment followed by a heat denaturation before quantifying VG [6,12]. Proteinase K-treated samples could be directly quantified

without a DNA extraction step. However, performing a DNA extraction step is highly recommended especially when studies are performed to determine yields of downstream processes. Presence of high levels of impurities, inhibitory substances coming from the culture medium or excipients (detergent) in in process samples could lead to incorrect VG quantification.

In order to harmonize protocols among laboratories, Aurnhammer et al developed a universal qPCR method based on an AAV2 ITR specific sequence [13]. A direct comparison of AAV titration results between laboratories and the usefulness of a conserved sequence among rAAV vectors have simplified the workflow of laboratories. However, some discrepancies in qPCR VG titers are observed among different targeted regions with a higher variability in the region near the ITR. Genome concentrations generated when the amplified regions are close to the hairpin ITR are underestimated for ssAAV vectors and especially for scAAV vectors [14]. Amplifying distal region to the ITR could increase VG titers. VG titers are usually higher in the central part of the AAV genome and decrease on both sides until hairpin structures [9, 14, 15]. Some experts reported on the reliable use of circular DNA [16] while linearization of plasmids in a relaxed form has been advised to improve qPCR and titer accuracy [9,17]. Treating samples with restriction endonucleases (ED-PCR) such as SmaI, which cuts in the complex structure region of the ITR, eliminates the inhibitory action of the hairpin structure in proximal qPCR amplification. Treatment of standards and vectors with SmaI restriction enzyme before qPCR increases

the VG titers and reduces the titer variation among different targeted sequence [14].

Interestingly, a « free-ITR » qPCR was shown to deliver VG titers similar to those performed in the transgene cassette by linearizing plasmid standards just outside the ITR region [18]. Nevertheless, VG titers determined from ITR or ITR proximal amplification could be overestimated due to the amplification of truncated vector DNA containing terminal genome fragments (« Defective interfering genomes ») [14, 19,20] that are usually found in vector preparations containing both full and empty particles. Therefore, the vector quality and the purification process of vector could explain differences in titers from different AAV amplification region. For this reason and for accurate VG titration, qPCR performed on the transgene region, which is located in the central part of the genome, seems to be more relevant for at least 3 reasons : no or low impact of the ITR complex region on qPCR, higher confidence for quantifying full vs truncated vector DNA and higher vector specificity. Choice of an amplicon distal to the ITR region such as the transgene central region should therefore increase VG titer accuracy.

Despite these improvements, the quantification of AAV VG remains dependant on a standard curve which is generated from the dilution of standard plasmids containing the DNA sequence. Thus, its precision is influenced by the serial dilutions of this standard as well as of the serial dilutions of the sample to be quantified. Even if the qPCR method has significantly improved the precision of viral genome titration, accuracy is still one of the most difficult criteria

to achieve satisfactorily, in particular to compare AAV products and different production processes.

In conclusion for the qPCR steps, much attention must be paid when designing the region dedicated to perform the VG titer. qPCR standards preparation and stability are known to be key factors for increasing inter-assay precisions and the use and establishment of appropriate controls such as internal controls with a well-defined titer are highly recommended. Validating VG quantification method by using appropriate guidelines [21,22] and by comparing titers obtained with reference standard materials is therefore highly recommended.

The introduction of ddPCR offers multiple advantages for AAV vector titration. As there are no standards used in ddPCR, this reduces its bias in genome quantification. Titers obtained are similar to those obtained with optimal qPCR assay and as ddPCR is an end-point titration assay, it offers better resistance to inhibitors than qPCR. Mean VG titer of AAV2 RSM was determined at $3.28\text{E}+10$ VG/mL with qPCR and a similar titer ($3.6\text{E}+10$ VG/mL) was generated with the ddPCR technology [11,12]. As both methods generate similar titers and despite the high inter-laboratory variation observed in qPCR (2-log), the AAV2 RSM VG titer was accurately defined. Moreover, we demonstrate that the choice of the amplified region for VG titration have less impact than in qPCR, leading to generation of homogeneous titers using target independent region (unpublished data). The ddPCR technology has increased the intra-assay precision of VG titers with median CV lower than 2.21%, whereas median CV

for qPCR were lower than 5.35% [12]. Better inter-assay precisions are also achieved with CV lower than 8% for ddPCR vs 16.5% for qPCR [12].

In summary, as genome concentration is used to define the dose effect and therapeutic dose, VG titer precision is of major concern. ddPCR has proven to be the method of choice to solve this issue and tends to be used in near future in most laboratories.

Infectious genome titration – biological activity

Methods for quantifying rAAV infectious particles that can be applied to any vector, independently of the transgene product, rely on the detection of rAAV genome replication in the presence of AAV rep-cap genes and adenovirus. Two methods have been implemented, the replication center assay (RCA) and the TCID₅₀ (50% Tissue Culture Infective Dose) which involve inoculation of serial dilutions of the rAAV vector made on HeLa rep-cap-trans-complementing cells (i.e. HeLaRC32 or C12 cells) co-infected with adenovirus type 5 [23,24]. Vectors carrying reporter genes such as the green fluorescent protein (GFP) can be easily titrated by flow cytometry in transduction units (TU/mL). For therapeutic transgene, infectious genome titers (IG) can be given as IU TCID₅₀/mL using a qPCR on the *ITR2* gene. This method can quickly give accurate number of infectious particles of the vector stock and lead to a comparison of lot-to-lot consistency. Since, the ratio between VG and IG titers shows high variability among serotypes (around 10 for an AAV of serotype 2 and up to 10,000 for serotype 8), this assay is not adapted to compare infectivity titer of productions of

different serotypes. Moreover, there are some drawbacks to these methods. One problem is that they are not suitable for some AAV serotypes that are poorly infectious on HeLa cells, even though co-infection with Adenovirus 5 increases the virus titers up to 100 fold [25]. And secondly, this assay may not truly reflect the infectivity pattern of what will happen in different tissues and especially in the target cells (neurons, skeletal muscle, kidney among others) of the product.

An indirect way to determine the concentration of functional particles is to measure or detect mRNA expression or transgene protein expression (for example by western blotting or ELISA) in a dose-dependent manner following cell transduction with the rAAV vector. The indicator cells must be permissive to both rAAV entry and transgene expression, which can be problematic especially when the transgene is under control of a tissue-specific promoter that is likely to be poorly or not active in cultured cells. If, however, the method is quantitative and not only qualitative, it is easy to defend and suggest as an alternative method of the TCID₅₀/ICA assays. Furthermore, in the case of the expression of the genetic insert product is demonstrated using appropriated cells to the target tissue, the dose-response is correlated to its activity, this type of test could be assessed as an approach to establish the potency assay to support Phase III clinical trials.

PRODUCT-RELATED IMPURITIES

Product related impurities remaining after purification include but are not limited to nuclease resistant

nucleic acids (encapsidated DNA ie host cell DNA, helper-plasmids or helper-viruses DNA) and vector aggregates. Particles that are empty or have encapsidated nucleic acids fragments other than the therapeutic genome cassette are part of the product-related impurities. These impurities are inactive and may represent safety concerns. Therefore, reliable methods for their measurement and characterization have been suggested.

Particles quantification – ratio full and empty capsids

Empty particles are a non-desired AAV product, produced at a significant level during the biosynthesis of AAV vectors and can represent up to 90% of vector preparations [8]. As the impact of empty particles on product efficiency and safety is not clearly defined yet, empty particle is not a critical quality attribute of the AAV product.

Different indirect methods can be used to determine the relative amount of empty particles in AAV preparations, such the quantification of full capsids (vg) using qPCR method, the determination of total entire AAV particles by an ELISA method [26] using commercial antibodies specific to the capsid (as possible for some AAV serotypes) and establishment of the ratio between both values. However, the main drawback is that two different detection methods are used and that nobody is completely sure if the ELISA is only detecting complete vector particles or if it also interacts even weakly with free capsid proteins. The amount of particles in vector preparations could also be quantified by loading onto SDS-PAGE an identical vector genome quantity as an internal control

in which the concentration is well known - for example a vector preparation containing only full particles. Consequently, as the intensity of the VP bands is enhanced proportionally to the empty particle concentration, the particle concentration could be estimated. Regardless of the two methods used, the variability of each method must be evaluated (for example around 0.5 log10 for qPCR and at least 30% for ELISA), which shall be taken into account in the ratio value. Thus, the ratio of full/empty particles could only be an estimation by this approach.

To overcome this issue and achieve the ratio of full/empty particles in a single experiment, other techniques such as spectrometry, HPLC techniques or electron microscopy have been proposed. Sommer et al has developed a spectrometric approach that uses the absorbance ratio at 260nm/280nm to quantify the number of empty and full particles of AAV2 [4]. Nevertheless, it requires purified material and is very sensitive to impurities and the nature of buffer formulation. Some laboratories have already used HPLC as an analytical method to measure the quantity of AAV particles with SEC, Ion exchange and affinity columns and the quantitative amino acid analysis [25,27,28]. Ion-exchange chromatography has been presented as a method that may be extrapolated to multiple serotypes/variants. The quantification by cation-exchange chromatography also appeared promising but was only efficient on pure preparations [29,30] and only for few AAV serotypes (AAV1 and AAV8) [27,31,32]. Another proposed approach is electron microscopy (EM) [27,30]. However, it is a labor-intensive method, the sample could be damaged during the

experiment and it is complicated by the presence of particles containing truncated DNA or by aberrant particles that can be either be partially stained as full particles or unstained. Although today, it is easier to setup a robust and reliable EM method in the laboratory with the miniTEM equipment, a discrepancy could be observed between on-grid measurement and in solution. Therefore, this technique should be suitable only for comparative studies. Currently, the cryoTEM seems more appropriate to characterize the composition of AAV vector preparations since this method is able to discriminate and quantify particles containing partial genomes from full/empty particles [33]. In addition, this method is accurate, robust and GMP compliant. Very recently, the charge detection mass spectrometry (CDMS) method has been described for the characterization of AAV8 vector, ssAAV and scAAV [34]. The results are comparable to those obtained in EM with a higher sensitivity. Sedimentation velocity analytical ultracentrifugation (SV-AUC) is also a powerful tool to distinguish and quantify the different AAV species such as empty viral particles, full genome-containing viral vectors, and vectors with fragmented genomes or non-transgene-related DNA contaminants. The main advantage of AUC technique is that it can quantify AAV independent of the AAV serotype, the size and form of transgene (single/double stranded DNA, small or large transgene) [35]. AUC-SV is considered an accurate method because it does not require standards. Despite its advantages, AUC is not yet readily amenable for use in routine release testing of clinical lots because of the difficulty in validating its data analysis software. Nevertheless, this

method is becoming more prominent in process and product AAV characterization. The AUC technology is currently implemented in different laboratories as a routine assay to characterize the production process and define the strategies to remove or reduce empty particles, to monitor structural heterogeneity of AAV vectors or to demonstrate manufacturing consistency [35–37].

In conclusion, although empty particles are considered as impurities, to date their quantification is not mandatory in the guidelines but the regulatory agencies are increasingly asking to provide their percentage in the final product. In addition, with the risk of having a non-compliant lot, it is not recommended to perform the capsid content (ratio of full to empty) as part of lot release without conducting specific *in vivo* studies to demonstrate the impact of empty particles on the product efficiency.

Illegitimate encapsidated DNA

During recombinant AAV production, viral capsids are known to package not only their genomes flanked by two ITR but also various DNA fragments. Several types of illegitimate DNA encapsidation, helper virus sequences including Rep/Cap sequences, DNA fragments from plasmids and cellular genome, have been identified in purified AAV vector preparations [8,38–40]. With respect to the analysis of nucleic acid contaminations, in addition to total DNA amount, the relative level of only few specific DNA sequences that encode for oncogenes or transforming factor (e.g., E1A, SV40 large-T antigen), can be estimated from qPCR method using primers specific to the target sequence. The qPCR

method cannot however be used for detecting other AAV genomes, due to limitations including: 1) lack of specific-target qPCR assay for each contaminating sequence; 2) limited coverage of DNA contaminants; 3) inability to evaluate the presence of truncated genome. The use of single-molecule sequencing (SMS) technology can provide valuable information to analyze various forms of AAV genomes (truncated – incomplete DNA genome) as well as residual plasmid contamination level [19]. Given the fact that SMS is cost effective, this technic could be a good tool to directly analyze DNA packaging. Similarly, Lecomte et al. developed a method based on next-generation sequencing (NGS) to characterize DNA contaminant levels [41]. Using this non-targeted screening, the authors showed that AAV vectors produced by transient transfection contained around 0.30% of host cell DNA. Otherwise, identification and quantification of DNA species within AAV particles produced with baculovirus expression system has been recently estimated less than 2.1% of the DNA contents [42].

Another issue in the characterization of residual cellular DNA is the evaluation of the size distribution. To date, it has been defined only for vaccine products that the maximum size of residual DNA should be below the size of a functional gene and thus less than 200 bp. The French Medicines agency has been recently developed and validated a qPCR assay using primers targeted a highly repetitive and conserved gene among mammalian species (18S RNA gene) to quantify residual cellular DNA in vaccines [43]. Besides DNA content, amplified PCR fragments of 123bp and 254bp were used to achieve the relative abundance of both smaller

and longer fragment. Although it has been shown by Southern blot analysis that the host cell DNA encapsidation can occur up to 4700 nucleotides, the mechanism and frequency of this aberrant DNA packaging is still not known fully. Thus, this qPCR assay can be suitable and considered for analyzing DNA content of AAV vector derived from most mammalian cell lines.

Under the existing regulatory guidelines for vaccines regarding amount per dose (maximum tolerable amount of 10ng rcDNA/dose) and average size (<200bp), these specifications are not adapted for gene therapy products and may require re-consideration for gene therapy products based on the risk/benefit of the product.

TRANSLATIONAL INSIGHT

The implementation of optimized methods with validated analytical controls to support the Phase I through Phase III clinical studies as well as licensed product are critical to ensure patient safety and understand the risks associated with the impurities of the AAV products.

The ideal analytical method would be sensitive, accurate, linear over a broad range, resistant to sample-matrix interference, and allow high throughput. The methods remain applicable for use in research laboratories as well as GMP facilities for quality control of clinical products.

Because VG concentration is used for dosing, an accurate measure of VG titer is necessary for efficacy and safety considerations. The qPCR has been employed to address the need for a rapid and reliable assay for product lot release. Even if this assay

is the gold standard method which is widely used in research and GMP laboratories, qPCR method should be replaced by the ddPCR method for higher VG titer precision and accuracy.

In terms of impurities, the guidelines from the medicine agencies worldwide (EMA and FDA) do not provide established specifications for gene therapy products. It is worth mentioning that to date, no specifications are required regarding residual DNA content, these are usually established based on the results of development lots and from the amount of product injected to the patient. Assays can be assessed using more than one method and laboratories are encouraged to achieve deeper studies. In the development setting, the application of AUC, cryoTEM, NGS have been shown to provide useful and rapid information to identify AAV particle species related to the purification process. Because these technologies are emerging, and requires specific equipment and training, they are not yet applied as part of routine testing in laboratories but become a valuable contribution for facilitating studies on vector biology during the development phase of vectors.

FINANCIAL & COMPETING INTERESTS DISCLOSURE

The author has no relevant financial involvement with an organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock options or ownership, expert testimony, grants or patents received or pending, or royalties. No writing assistance was utilized in the production of this manuscript.



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