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Current Opinion in Biotechnology

Analytical methods to characterize recombinant adeno-associated virus vectors and the benefit of standardization and reference materials

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Recombinant adeno-associated virus (rAAV) is an increasingly important gene therapy vector, but its properties present unique challenges to critical quality attribute (CQA) identification and analytics development. Advances in, and ongoing hurdles to, characterizing rAAV proteins, nucleic acids, and vector potency are discussed in this review. For nucleic acids and vector potency, current analytical techniques for defined CQAs would benefit from further optimization, while for proteins, more complete characterization and mapping of properties to safety and efficacy is needed to finalize CQAs. The benefits of leveraging reference vectors to validate analytics and CQA ranges are also proposed. Once defined, CQA specifications can be used to establish target parameters for and inform the development of next generation rAAV processes.

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Current Opinion in Biotechnology 2021, **71**:65–76

This review comes from a themed issue on **Analytical biotechnology**

Edited by **Michael J Betenbaugh** and **William E Bentley**

https://doi.org/10.1016/j.copbio.2021.06.025

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Introduction

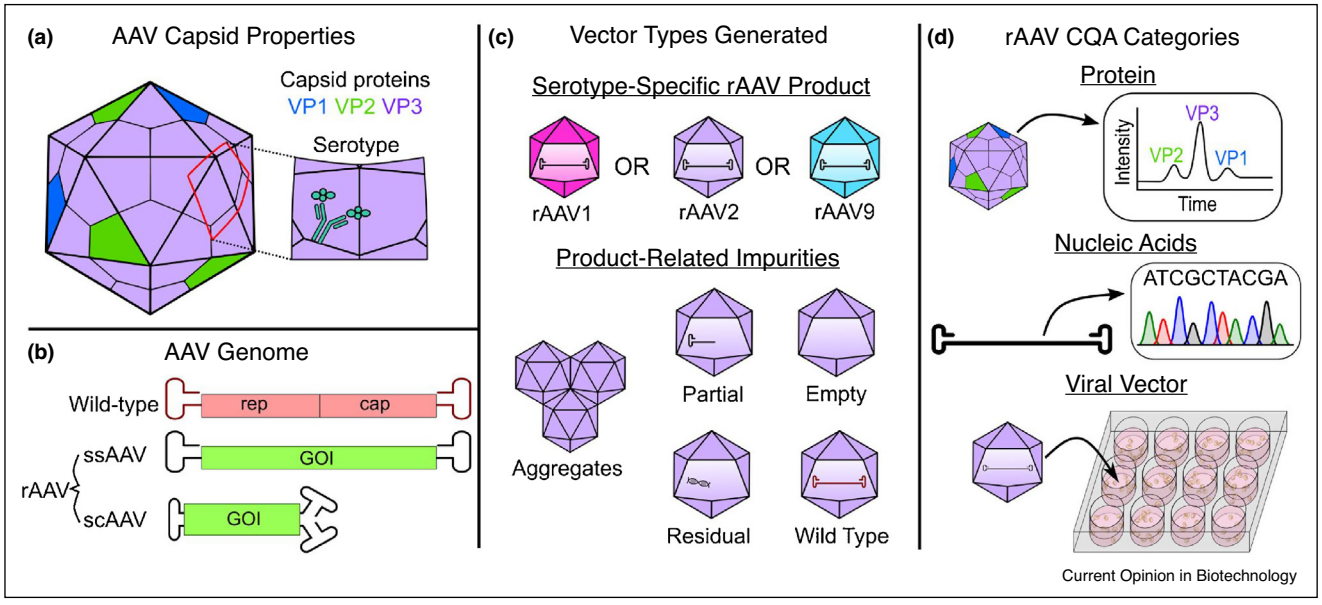
Adeno-associated virus (AAV) has become an increasingly important platform vector for *in vivo* gene therapy due to its safety profile [1,2]. In contrast to other viral vectors, it does not cause any known diseases in humans, has a lower immunogenicity, primarily expresses episomally, and has a lower integration frequency [3]. Additionally, it is naturally replication-deficient without a helper virus, and its capsid can be of different serotypes that have distinct tropisms to enable tissue-specific therapeutic delivery [1,2]. The wild-type AAV capsid consists of three types of viral proteins (VP1, VP2, and VP3), and nine variable regions determine serotype (Figure 1a) [2,4•].

The capsid contains a 4.7 kb single-stranded DNA genome encoding serotype-specific open reading frames essential for genome replication and packaging (*rep*), and capsid protein synthesis (*cap*) that are flanked by two inverted terminal repeat (ITR) regions that self-prime genome replication [2,4•]. Recombinant AAV (rAAV) vectors have a native or engineered serotype-determining capsid and retain the ITRs, but *rep* and *cap* are replaced with a therapeutic gene. While wild-type AAVs always have single-stranded genomes, rAAVs can have single-stranded (ssAAV) or double-stranded self-complimentary (scAAV) DNA genomes (Figure 1b) [2]. These variations in genome structure and serotype between rAAV vectors necessitate many quality control (QC) tests to confirm product safety and efficacy, defined as the absence of serious adverse effects and conference of the desired therapeutic outcome, respectively, before lot release [5•,6,7].

The complex nature of manufacturing rAAV necessitates further QC monitoring. Helper genes from a second virus (most commonly Adenovirus or Herpes Simplex Virus) and AAV *rep*/*cap* must be expressed in *trans* to facilitate rAAV genome replication, capsid protein synthesis, and genome packaging. These genes can be expressed on plasmids co-transfected into the production host, delivered by helper viruses, or integrated into the host cell and activated with an inducer [8–10]. Depending on the production platform, specifications for vector critical quality attributes (CQAs) and process residuals that must be monitored may vary [11••,12].

The approvals of three therapies to date, Glybera® (alipogene tiparvovec), Luxturna® (voretigene neparvovec), and Zolgensma® (onasemnogene abeparvovec), suggest that current analytical methods sufficiently characterize established CQAs to demonstrate the safety and efficacy of rAAVs to regulatory agencies [5•]. Safety testing evaluates the formulation integrity, confirms the absence of adventitious agents, and monitors process-related impurities [5•,13,14]. rAAV product efficacy testing evaluates levels of defective particles (product-related impurities) and CQAs crucial for therapeutic effectiveness. These efficacy-conferring CQAs include genome/capsid titers, genome/capsid sequences and structures (identity), and the ability of the vector to deliver a genome translated into a functional therapeutic protein (potency) [5•,14]. However, recent research has further detailed variability in capsid protein properties, types of

Figure 1



Overview of AAV properties, vector types generated during rAAV production, and rAAV CQA categories. **(a)** An illustration of a 60-sided AAV icosahedral capsid comprised of VP1, VP2, and VP3 proteins. One of the nine variable regions that binds serotype-specific antibodies is also shown. **(b)** Wild-type AAV and rAAV genomes. The wild-type AAV genome has two open reading frames, *rep* and *cap*, flanked by ITRs. In a rAAV genome, *rep* and *cap* are replaced with a therapeutic gene of interest (GOI) cassette. **(c)** Vector types that can be produced during rAAV manufacturing. The desired rAAV product is of a single wild-type or engineered serotype, contains a full-length genome, and is a non-aggregated capsid monomer. Product-related impurities arise when two or more capsids aggregate, or when the capsid contains a partial recombinant genome, no genome, residual process DNA, or a wild-type genome. **(d)** High-level categories for CQAs of the desired rAAV product and product-related impurities. A chromatogram of separated intact VPs represents protein analysis, a sequencing chromatogram depicts nucleic acid analysis, and an *in vitro* colorimetric protein activity assay shows viral vector analysis.

product-related impurities, and misencapsidated DNA profiles between vector serotypes and manufacturing processes [5•,15,16]. Further studies relating safety and efficacy to product physical, chemical, and biological property variations will therefore be essential to standardizing CQAs to monitor during product development and production [5•,6,7]. Additionally, since product potency is transgene and serotype dependent, more versatile potency assays are needed.

Here, we review recent advancements in methods to characterize rAAVs and product-related impurities (Figure 1c) [5•,6,16,17]. While important, methods to test product formulation, sterility, and process-related contaminants are generally compendial or easily adapted to rAAV applications [5•,6,13] and are not discussed in depth. Emphasizing assays for rAAV proteins, nucleic acids, and vector potency enables a more focused consideration of how capsid and genome properties, and associated CQAs, may affect therapeutic outcomes (Figure 1d). We also discuss specific properties that vary between serotypes, their process dependence, and their effect on safety and efficacy. The utility of and need for reference materials in analytics development and CQA definition is also considered. Overall, advances in nucleic acid and vector potency analysis are primarily limited by advancements in analytics, while protein analysis method development is challenged by both the constraints of techniques and understanding of how some properties affect therapeutic efficacy (Box 1).

Protein analysis

The characterization of rAAV protein-related properties focuses on serotype identity and product-related impurities. This analysis is multifaceted because it ranges from amino acid sequence modifications to whole-particle properties (Figure 2) [5•,6,7]. While the link to safety and efficacy is well-defined for many protein-related properties, the connections for a few are not. For example, post-translational modifications (PTMs) vary by serotype and host cell [11••,18•]. There is evidence linking-specific PTMs and clinical efficacy, but further investigation is needed [11••,18•,19]. As a result, converging on core methods to characterize PTMs and other protein properties presents a unique challenge. Characterization of rAAV therapies in development against reference materials could assist in identifying which variations in vector properties require monitoring as CQAs. Once links to safety and efficacy for key protein properties are further established, and a set of CQAs are finalized, standardized methods best for routine QC versus exploratory analyses can be determined.

Box 1 Abbreviations used in this review

Abbreviation	Definition
AAV	Adeno-associated virus
ATCC	American Type Culture Collection
AUC	Analytical ultracentrifugation
BLI	Bio-layer interferometry
CDMS	Charge detection mass spectrometry
CE	Capillary electrophoresis
CE-MS	Capillary electrophoresis-mass spectrometry
c(s)	Sedimentation coefficient distribution
CQA	Critical quality attribute
cIEF	Capillary isoelectric focusing
ddPCR	Digital droplet polymerase chain reaction
DSF	Differential scanning fluorimetry
DLS	Dynamic light scattering
dPCR	Digital polymerase chain reaction
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
FLR	Fluorescence
GFP	Green fluorescent protein
GOI	Gene of interest
HCP	Host cell protein
HEK293	Human embryonic kidney 293
HAE	Human airway epithelial
HeLa	Henrietta Lacks
HILIC	Hydrophilic interaction-liquid chromatography
HTS	High-throughput sequencing
IEX	Ion exchange chromatography
ITR	Inverted terminal repeat
LC	Liquid chromatography
LC–MS	Liquid chromatography–mass spectrometry
LC–MS/MS	Liquid chromatography–tandem mass spectrometry
LRS	Long-read sequencing
MADLS	Multi-angle dynamic light scattering
MALS	Multi-angle static light scattering
MRM-MS	Multiple reaction monitoring-mass spectrometry
MS	Mass spectrometry
m/z	Mass-to-charge ratio
NIST	National Institute for Standards and Technology
nm	Nanometer
PCR	Polymerase chain reaction
PTM	Post-translational modification
QC	Quality control
qPCR	Quantitative polymerase chain reaction
rAAV	Recombinant adeno-associated virus
RFU	Relative fluorescence units
scAAV	Self-complementary adeno-associated virus
SEC	Size exclusion chromatography
SRS	Short-read sequencing
ssAAV	Single-stranded adeno-associated virus
$TCID_{50}$	Median tissue culture infectious dose
T_m	Melt temperature
USP	United States Pharmacopoeia
UV	Ultraviolet
VG	Vector genome
VP	Viral protein

rAAV capsid identity

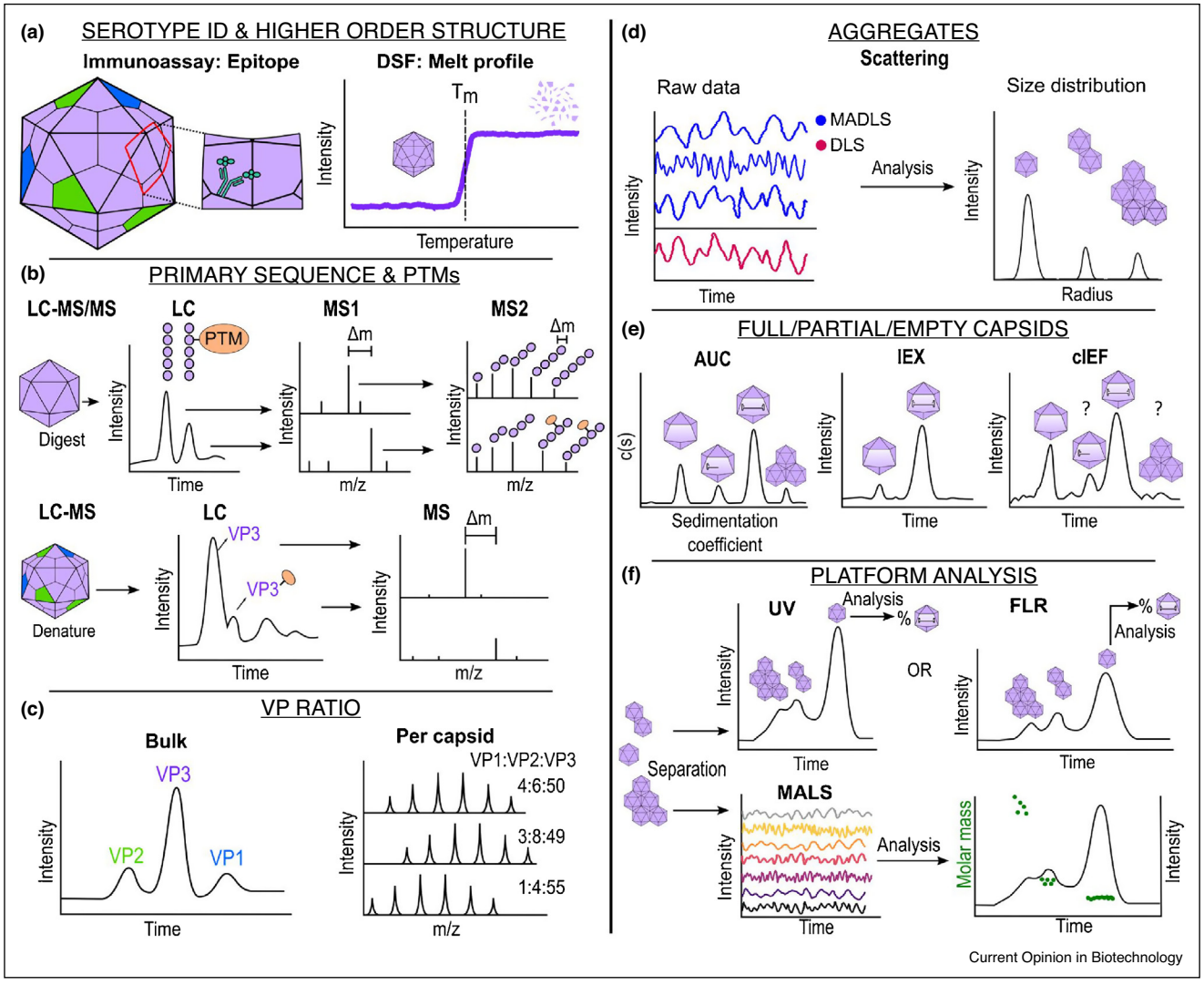
It is important to confirm serotype identity and verify proper capsid assembly (Figure 2a–c). However, consensus is lacking around which properties and corresponding methods for amino acid sequence and higher-level structure most effectively define identity [15]. AAV serotype identity is defined by capsid antigens that bind to specific antibodies; therefore, rAAV identity is often confirmed by immunoassays like enzyme-linked immunosorbent assay (ELISA) [20]. Bio-layer interferometry (BLI) [21] can also be employed for serotype identification. In both approaches, serotype-specific antibodies that only bind to assembled capsids are generally used, confirming epitope sequences and capsid assembly (Figure 2a), and enabling their dual-purpose use as total capsid titer assays [20]. ELISA and BLI can also be employed for host cell line-specific total host cell protein (HCP) analyses [5•,8,17].

Differential scanning fluorimetry (DSF) has recently been used to analyze capsid structure and confirm serotype identity. Unfolding is monitored by changes in fluorescence with heating, and melt temperature (T_m) is used to determine serotype (Figure 2a) [22–24]. Preliminary work shows DSF is sensitive enough to detect T_m differences between rAAV1 and rAAV6 capsids with a single amino acid mutation in variable region VI [22]. However, further validation studies are needed to determine if changes in PTMs that affect efficacy cause detectable shifts in melt profiles to enable widespread use for routine testing.

Immunoassays and DSF do not thoroughly probe primary structure, but liquid chromatography-tandem mass spectrometry (LC–MS/MS) peptide mapping (Figure 2b) can confirm amino acid sequence identity. Here, a sequence-specific proteolytic enzyme generates capsid-derived peptides [25]. Different MS-based approaches can then assess the peptide map and corresponding PTMs. In one study, a variety of fragmentation methods were employed to characterize the PTM profiles of rAAV1 and rAAV8 [11••]. Orthogonal separation and ionization methods can also confirm an identified PTM. Tandem MS and MS^E identified acetylated residues of six serotypes [25], and high resolution tandem mass spectrometry methods confirmed the location of glycosylation moieties on rAAV8 [26]. LC–MS/MS methods can also be used to monitor specific residual HCPs for product purity analysis [5•,8,17].

Further work is needed to fully understand how the plethora of observed PTMs may affect efficacy [11••,18•,19,25,26]. Recent work shows that mutation of glycosylation and SUMOylation sites on rAAV2 decreases gene transfer [18•], deamidation of asparagine residues decreases transduction efficiency [19], and that insect-derived rAAVs have more residues with PTMs and show decreased *in vivo* potency compared to human-derived vectors, despite sharing the same types of PTMs (acetylation, phosphorylation, and so on) per serotype [11••]. As PTMs that impact efficacy are identified, intact protein LC–MS or capillary electrophoresis-MS (CE-MS) methods can be used for routine monitoring (Figure 2b). Techniques applied thus far include reverse-phase LC

Figure 2



Protein-related properties and associated characterization methods. **(a)** Serotype identification (ID) and capsid assembly validation. Immunoassays detect capsids at antibody binding epitopes and total capsid titer is determined against a standard curve. DSF monitors capsid unfolding and T_m can be calculated. **(b)** Primary amino acid sequence determination and PTM identification by analysis of peptides or proteins with varying mass-to-charge (m/z) ratios. LC–MS/MS is employed for peptide mapping to identify the amino acid location of a PTM. LC–MS is used for intact protein analysis to determine whether a VP1, VP2, or VP3 protein is modified or clipped. Both methods determine mass shifts (Δm) against the expected unmodified protein sequence to confirm whether a PTM is present. **(c)** Capsid VP ratio determination. Bulk sample VP ratio analysis is performed using LC or CE. Per-capsid VP ratio can be determined by native MS. **(d)** AAV aggregate profiling. DLS and MADLS collect data over time from one or several angles, respectively. The data is autocorrelated to determine particle size distribution but cannot analyze capsid contents. **(e)** Full, partially full, and empty capsid population analysis. AUC is the gold standard method to resolve empty, partial, full, and aggregated capsids on a plot of sedimentation coefficient distribution (c(s)) versus the sedimentation coefficient. IEX can only differentiate full and empty capsids, but is more scalable than AUC. cIEF, an emerging alternative to IEX, may be able to resolve partial and aggregated capsids. **(f)** SEC for aggregate and capsid content analysis. Full capsid populations are determined by analysis of UV or FLR traces. Aggregate size distribution is determined using MALS, in which data collected at different angles (depicted by the colored intensity versus time traces) is analyzed to generate a plot of molar mass (green dots) and intensity (black trace) versus time.

[25], microfluidic CE [27], and hydrophilic interaction LC (HILIC) [28•].

The ratio of the VP1, VP2, and VP3 capsid proteins also affects efficacy (Figure 2c). Growing evidence shows that per capsid VP ratio is heterogeneous without a singular bulk VP composition [29,30,31••,32•]. VP ratio is determined in bulk by digesting capsids, separating VP1, VP2, and VP3 using a polyacrylamide gel [33], LC [25,28•] or CE [27,34,35], and measuring relative abundances from

band intensities or peak areas. However, bulk ratios are compositional averages and do not capture the variability in VP ratios for individual capsids. The per capsid VP ratio is measured on intact particles, and this has been achieved using full particle native MS analyses [29,30,31••,32•]. Interestingly, one dominant per capsid VP ratio was not observed in these studies, suggesting that incorporation of VP1, VP2, and VP3 into each capsid depends on temporal VP expression levels [29,31••,32•]. The effect of VP ratio heterogeneity on efficacy is not yet determined. If the variability in VP ratios affects product efficacy, high mass-to-charge ratio native MS methods should be more widely adopted, or other methods for full particle analysis with adequate sensitivity will need to be developed.

Product-related impurities

Capsid that are aggregated or contain incomplete genomes can compromise safety and efficacy because they cannot transduce target cells efficiently or deliver a therapeutic genome [5•,16]. These inefficiencies can necessitate higher total capsid doses to deliver sufficient therapeutic cargo, which can trigger undesired immune responses from a patient [4•,5•]. The consensus threshold for non-aggregated capsids (i.e. monomer populations) is ~95%, but such a limit is not established for full capsids [5•]. Aggregates can be detected by scattering methods (Figure 2d), and dynamic light scattering (DLS) is commonly used for determining relative size populations [36]. Multi-angle DLS (MADLS), an emerging alternative, can obtain absolute concentrations of different capsid populations because data collected at multiple angles over time are fit to yield more accurate results [37]. While DLS and MADLS characterize aggregate sizes and distributions, they do not address whether capsids are empty, partially full, or full.

Full, partial, and empty capsids can be resolved by analytical ultracentrifugation (AUC) [38], charge detection MS (CDMS) [30], and electron microscopy (EM) [39], but these techniques are low-throughput and require specialized equipment. Ion exchange chromatography (IEX) is more efficient and can analyze full capsid populations in-line or off-line. While the use of IEX is becoming more widespread in both research and QC settings, partially full capsids elute with full capsids, which, if unaccounted for, leads to overestimation of filled capsids [40–42]. Capillary isoelectric focusing (cIEF) is an emerging QC-friendly method that shows promise for separating partial and full capsids [43], but further validation will be needed to confirm sensitivity. These separation methods may resolve peaks representing aggregates, but aggregate size cannot be determined (Figure 2e).

Aggregates and full/partial/empty capsid distributions can be monitored in the same QC workflow by coupling size exclusion chromatography (SEC) to multiple detectors (Figure 2f) [44,45••]. Ultraviolet (UV) or fluorescence (FLR) detectors can determine relative aggregate populations [44], and multi-angle static light scattering (MALS) can measure capsid diameters and particle counts [46]. Additionally, 260/280 nm UV absorption ratios correlate well with AUC for assessing full capsid percentage [16,47]. This platform approach efficiently measures aggregate sizes and percentage of full capsids for release testing, and can also be used to quantify capsid and genome titers [45••]. However, if partial/empty capsid release thresholds are established, higher resolution methods will be required for routine testing, or appropriate correction factors for partial capsids must be validated for current methods [45••]. Use of reference vectors that contain known amounts of full, partial, and empty capsids could be useful for the validation of such assays.

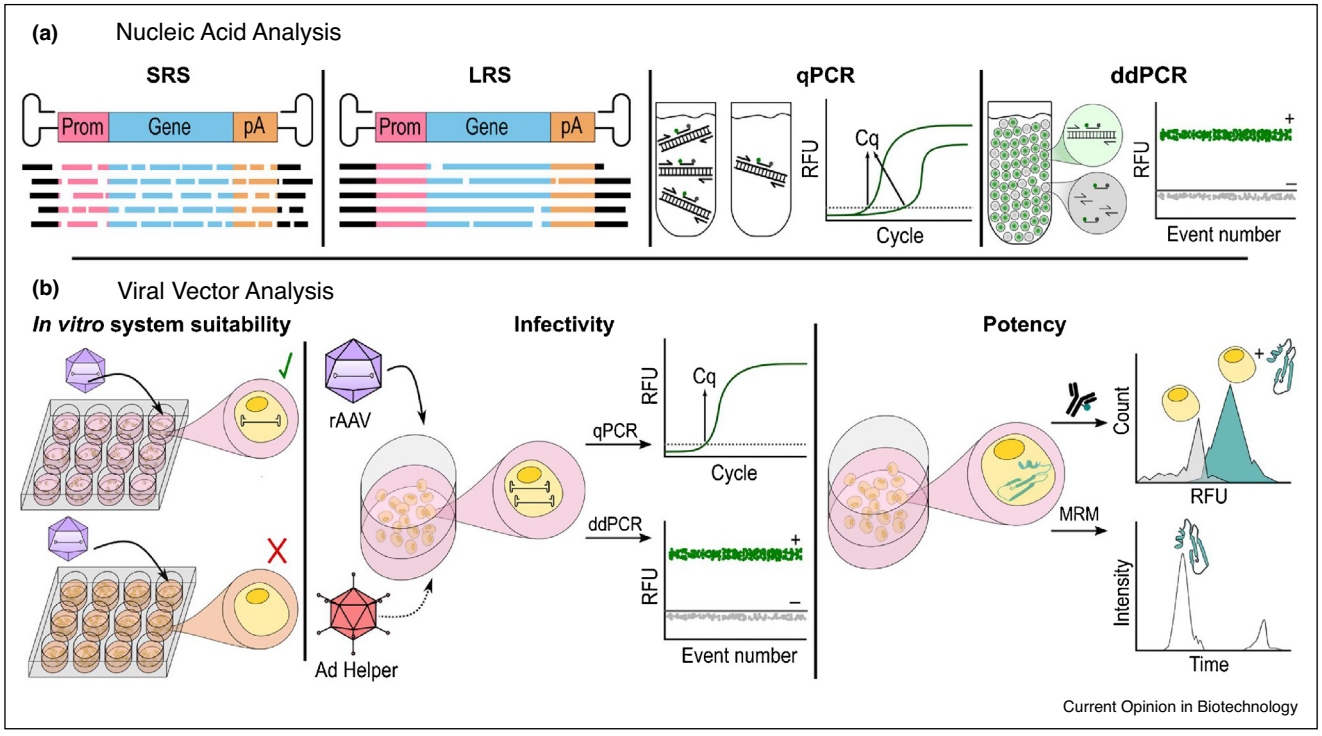
Nucleic acid analysis

The nucleic acid-related rAAV CQAs are genome identity and dose-determining vector genome (VG) titer (Figure 3a). Polymerase chain reaction (PCR) methods measure VG titer [48] and approximate incorrectly packaged (misencapsidated) DNA amounts [49], but quantitation is amplicon-specific and may not be representative of a full sequence. High-throughput sequencing (HTS) identifies full VGs versus misencapsidated sequences, but only assesses relative read counts [50,51••]. PCR and HTS provide complementary information, and can also be used to monitor non-encapsidated residual DNA impurities [5•,8,13,16], but sample preparation can be tedious and introduce bias or error. Optimization of HTS and PCR workflows and validation against reference materials with known VG titers and residual DNA profiles could standardize VG and purity analyses across laboratories and rAAV products.

Vector genome identity

HTS generates 50–300 base pair (bp) short reads, or thousands of bp long reads. Short-read sequencing (SRS) is faster, less error-prone, and requires 200−1000× less DNA than long read sequencing (LRS) [52]. Comparatively fast analysis of relative sequence frequencies by SRS enables efficient confirmation of VG sequence fidelity [50,53,54]. LRS evaluates whole DNA molecules, enabling identification of VG defects and misencapsidated sequences [51••,55]. Such analysis has revealed misencapsidations can occur when chimeras form between non-VG and ITR-containing sequences [51••]. However, SRS and LRS would benefit from streamlined sample preparation to improve input DNA libraries and generated reads. Sample preparation has been improved by: PCR-free library preparation for better relative quantification of misencapsidated sequences [50]; ssAAV genome annealing to eliminate the second-strand synthesis step [51••,54]; and streamlined sequencing adapter ligation [53,55].

Figure 3



Techniques used to analyze rAAV nucleic acids and vector potency. **(a)** Methods for characterizing VG identity and titer. Identity of the genome across the ITR, promoter (prom), therapeutic gene (gene), and polyA tail (pA) regions is confirmed by SRS or LRS. VG titer and misencapsidated DNA amounts are determined using qPCR based on the quantitation cycle (Cq) where the fluorescence (RFU) of a sample crosses the baseline during amplification, or ddPCR based on absolute counts of fluorescent droplets after amplification. **(b)** Workflows to develop and perform *in vitro* rAAV potency assays. A cell line and suitable culture conditions (culture medium, additives, and so on) must first be selected. Infectivity can be determined using a helper-free VG transfer assay or infectious titer assay with adenovirus (Ad) co-infection. qPCR or ddPCR can be used for VG quantitation. Assays for the therapeutic protein measure expression and/or activity. Immunoassays such as flow cytometry and separations/MS methods such as MRM can detect the therapeutic protein or an associated reaction product.

Vector genome and misencapsidated DNA quantification

Amplicon detection by real-time quantitative (qPCR) or endpoint digital PCR (dPCR) can measure VG titer and misencapsidated DNA. qPCR is more cost efficient but requires standards for relative quantitation, while dPCR is more costly but performs absolute quantitation on partitioned samples without a standard curve. Method selection balances cost and throughput, but both techniques could benefit from sample preparation workflow optimization. Sample preparation improvements have increased the efficiency of genome release from capsids via detergent-mediated lysis [49,56•], reduced genome association with DNA or protein impurities by adding surfactants [48], and optimized digestion steps to remove PCR inhibitors [57]. Selection of representative target amplicons is also crucial to method development. Target amplicons for misencapsidated DNA can be selected from abundant sequences in HTS analyses, but VG amplicon choice is more challenging, as truncated genome packaging may lead to inaccurate results [58•,59,60•]. qPCR error also depends on standard curve preparation, as results can vary depending on whether circular plasmid, linearized plasmid, or a rAAV reference vector is used [58•,61]. dPCR addresses these challenges, as there is evidence that dPCR, particularly digital droplet PCR (ddPCR), is more accurate than qPCR [58•,62]. Specifically, ddPCR is reportedly less affected by reagent interference and amplicon choice [58•,63], and may retain better accuracy at low concentrations [64]. Comparison of methods through collaborative studies on reference vectors to compare qPCR and ddPCR results could support standardization of both methods [65].

Viral vector potency evaluation

rAAV potency depends on whether vector transduction and VG expression leads to therapeutic protein activity. *In vivo* assays are easier to correlate with clinical outcomes [66], but *in vitro* cell-based assays are preferred because they are less labor intensive, faster, cheaper, and do not use animals [67,68•]. The main challenges to *in vitro* assay development are choosing a representative cell line and selecting quantifiable outputs that represent the endpoint therapeutic effect when validated against *in vivo* data

[69,70]. Infectious titer has historically quantified rAAV efficacy by measuring VGs delivered *in vitro* [36]. Recently, assays for the therapeutic protein have gained traction because measuring protein expression and activity better represents clinical efficacy (Figure 3b) [67,68•]. Optimization of such assays could be done more efficiently for new products using serotype and/or transgene-specific reference vectors as a starting point.

In vitro system selection

Selecting representative cell lines for infectivity or therapeutic protein assays is challenging because immortalized cell lines that are easily cultured are not always permissive to rAAV serotypes used for therapeutics [71,72]. Products with tissue-specific promoters create an additional constraint because the promoter must be sufficiently active in the selected cell line [67,68•,72]. Green fluorescent protein (GFP) expressing vectors can be used for cell line screens, as exemplified by testing transduction of a rAAV2.5 capsid targeting lung cells. GFP expression in HeLa, human embryonic kidney 293 (HEK293), and human airway endothelial (HAE) cells was compared. The HEK293 and HAE cells had similar GFP expression, suggesting easy-to-culture HEK293 can be used for *in vitro* assays for rAAV2.5 lung-targeting products [73]. In contrast, rAAV8-GFP inefficiently transduces HeLa and HEK293 [71], so potency assays using HepG2 [68•] and HuH-7 [67] liver carcinoma cell lines were developed for liver-targeting products. *In vitro* transduction efficiency and/or transgene expression can also be enhanced by adding compounds such as hydroxyurea during rAAV infection [68•], or by overexpressing relevant surface receptors [72,74].

Correlation of *in vitro* expression with therapeutic efficacy

The other challenge when designing an infectivity or therapeutic protein assay is selecting a method to quantify DNA or protein expression. Because rAAV infection does not result in cytopathic effect, VG replication, or genome integration, the development of a representative infectivity assay with a sufficient detection limit can be difficult [36]. The traditionally used median tissue culture infectious dose ($TCID_{50}$) assay overcomes this issue by measuring rAAV genome signal following rAAV and adenovirus co-infection of HeLa cells stably expressing AAV *rep/cap* [36]. Inducing genome replication increases VG signal, but these conditions are not representative of *in vivo* rAAV administration, nor is the method suited for all serotypes. Measuring helper-free genome copy number [47] or RNA expression [75] in a cell line selected for serotype-specific transducibility would be more representative.

Assays for the therapeutic protein measure expression and/or activity. Protein expression can be quantified by immunoassays, most commonly ELISA [66] or flow cytometry [67]. Both methods require protein-specific antibodies, but flow cytometry assays sometimes require additional optimization because wild-type and target protein-expressing cells must have sufficient separation for accurate quantitation [67]. Therapeutic protein activity can be determined by colorimetric [68•], LC [67], or multiple reaction monitoring (MRM)-MS [72,76] assays for an associated reaction product. While activity assays better represent efficacy, their specificity to the therapeutic protein's mechanism of action can make them challenging to develop. Additionally, depending on the properties of the therapeutic protein and associated reaction product, assays to measure expression and activity in one assay could be developed. Working groups such as the United States National Institute for Standards and Technology (NIST) Flow Cytometry Standards Consortium can help accelerate the development of robust protein characterization workflows.

Conclusion and future directions

Recent advances in rAAV analytics have improved how thoroughly product properties and associated CQAs can be characterized (Table 1), but the strengths and limitations of different methods must be considered for their use in process development versus QC [77••]. Nonetheless, there are gaps in the understanding of how certain protein properties (e.g. PTM profile, partial/empty capsids, VP ratio) affect safety and efficacy. Mapping PTM profiles via LC–MS/MS and linking them with *in vivo* potency studies on capsid variants will reveal which PTMs affect transduction efficiency and elicit immune responses [11••]. It is generally believed that partial and empty capsids pose safety risks [5•,16,40]; thus, a minimum full capsid threshold should be defined. Studying per-capsid VP ratio distributions will determine whether bulk VP ratios are well-correlated with efficacy, or if new methods for per-capsid VP ratio are needed for QC testing [29,30,31••]. Additional investigations will inform the definition of CQAs and associated specifications for the aforementioned properties.

CQA ranges established to guarantee efficacy and safety will likely vary between products due to rAAV serotype and/or host cell-imposed differences [11••]. Exploratory studies to define release criteria for CQAs and relate rAAV properties to safety and efficacy often require high-resolution analytical methods that may not be optimal for routine QC. Once a CQA is well-characterized, more streamlined analytical approaches can be used. Some newer techniques can be validated with few modifications (e.g. BLI), while others need to be expanded upon to detect relevant variations in rAAV CQAs. Two methods that require further validation for rAAV applications are DSF [22–24] and cIEF [43].

Regardless of method, inter-laboratory variability is an issue when characterizing rAAVs. Multiple groups

Table 1

Summary of properties that characterize the rAAV product and product-related impurities and comparison of methods used for analysis

Category	Property	Method	Sensitivity[a]	Throughput[a]	Cost[b]
Protein (Identity)	Serotype	ELISA	++	+++	$$
		BLI	++	+++	$$
		DSF	+	++	$
	PTM profile	LC-MS/MS	+++	+	$$$
		LC-MS	++	++	$$
		LC	+	++	$
	VP ratio	CE	++	++	$
		Native MS	+++	+	$$
		CDMS	+++	+	$$$
Protein (Product-related impurities)	Aggregates	DLS	++	++	$
		MADLS	+++	++	$$
		SEC-MALS	++	++	$$
		AUC	++	+	$$
	Full, partial, empty capsid ratio	CDMS	+++	+	$$$
		IEX	+	+++	$$
		cIEF	+	++	$$
		SEC-UV or SEC-FLR	+	++	$$
Genome	Identity	SRS	++	++	$$
		LRS	+++	+	$$$
	Titer	qPCR	++	+++	$
		ddPCR	+++	+++	$$
Viral vector potency	Infectivity	$TCID_{50}$ (infectious titer)	+	+	$$
		Helper-free VG transfer	+	++	$$
	Therapeutic protein expression/activity	ELISA	++	+++	$$
		Flow cytometry	++	++	$$
		LC	++	++	$
		MRM	+++	+	$$$
		Colorimetric assay	++	+++	$

[a] Sensitivity/Throughput ratings: + = low, ++ = medium, +++ = high.
[b] Cost ratings: $ = low, $$ = medium, $$$ = high.

characterized rAAV2 and rAAV8 reference materials developed via collaborative efforts between American and European laboratories and distributed by the American Type Culture Collection (ATCC) [78], and qPCR VG titer, ELISA capsid titer, and $TCID_{50}$ infectivity results varied between laboratories [79,80]. The range of measured VG titers was particularly wide for rAAV8, spanning two orders of magnitude [80]. This diversity highlights the need for well-characterized external reference vectors beyond what is currently available from ATCC for validation of both new assays and product-specific internal reference material [77••]. Companies including Vigene Biosciences, Virovek, and AMSBIO sell AAV reference vectors, and the United States Pharmacopoeia (USP) is considering development of new AAV9 and empty vector reference standards [65].

Reference materials will be particularly valuable for validating new platform assays that measure multiple CQAs in one workflow. For example, relative quantitation of nucleic acid-related CQAs by LRS could become feasible using reference genome spike-ins. Quantification of misencapsidated DNA by nanopore sequencing has been piloted [51••], and similar methods could be applied to rAAV titering. However, before implementation, sample preparation would require optimization to avoid DNA shearing or sequence rearrangements [51••,55], and lower error rates would be needed [81]. Additionally, reference vectors should be routinely used in platform potency assays that measure both gene transfer and protein expression/activity [67,76]. The inherent variability of biological assays necessitates comparison against standards that meet potency specifications [68•].

Because rAAV gene therapy is a maturing field, increased availability of reference materials can accelerate advancement in three critical areas: analytical method development, CQA definition/characterization, and process innovation. After CQAs for release testing are defined and off-line release methods are well-established, complimentary relationships between CQA specifications and critical process parameters may be used to integrate on-line analytics into commercial manufacturing [82•,83•]. This work could decrease off-line testing load and reduce the time from rAAV production to dose administration. Reference standard availability can also lower barriers to participation for academic laboratories and smaller companies, increasing the pace these innovative methods can be developed. Overall, acceleration of analytics development and a better understanding of rAAV properties and

associated CQAs will enable more efficient product commercialization, which can help close the gap between the availability of and demand for rAAV therapeutics.

Conflict of interest statement

Nothing declared.

Acknowledgements

The authors thank Melissa Anderson (Pfizer) for insightful discussions on rAAV analytics.

This work was supported in part by the National Science Foundation (1624698) and in part by the financial assistance award 70NANB17H002 from U.S. Department of Commerce, National Institute of Standards and Technology. EAG was supported in part by NIH NIGMS T32GM133395 from the National Institute of General Medical Sciences. The content of this publication is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of General Medical Sciences or the National Institutes of Health.

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