Analytical Procedures for Viral Vectored Vaccine Quality

Draft guidelines

Accelerating product development through a common understanding of quality

Viral vectors are an effective mechanism for delivering cargo DNA to targeted cells, and significant resources have been invested to develop and manufacture viral vectors for gene therapy and vaccine applications. In recent years, several viral vectored vaccines, including Zabdeno®, ERVEBO® and Mvabea®, have been developed for use against Ebola. Viral vectors are particularly useful for vaccines because they can induce a robust immune response to foreign or virus-infected cells without the need for adjuvants. Modified versions of adenovirus are the most commonly used viral vectors for vaccines, but modified measles and vaccinia viruses have also been used in vaccines. With the successful development of viral vectored vaccines to fight COVID-19, the global demand for these types of products is expected to grow.

Appropriate analytical techniques are critical. There are significant complex challenges in upstream and downstream processing of viral vectored vaccines. Viral vectored vaccines have two major components: the DNA transgene encoding the protein of interest responsible for eliciting an immune response, and the viral vector, which serves as a delivery vehicle to get the DNA into the cells. Each component has its own set of quality attributes and potential impurities that need to be analyzed. Furthermore, when the viral vector and DNA are combined, it is also important to assess the percentage of capsids that contain the transgene (full capsids) vs. empty capsids that lack the desired DNA cargo. Therefore, appropriate and discerning analytical methods need to be used throughout product development and manufacturing to ensure the quality and safety of the final product. Additionally, translating small-scale virus production into large-scale commercial production is a complex process, and developing a robust analytical strategy that supports the entire product development and manufacturing cycle is crucial to being able to deliver consistent, stable products to the patient.

A common set of methods is needed. Since the successful application of viral vector technology to vaccines is relatively new, regulatory guidelines and industry standards to guide non-proprietary aspects of viral vectored product quality during development are still evolving. Without a common set of methods for determining quality, developers and manufacturers must develop their own in-house methods and protocols, taking attention and resources away from advancing their innovations within the demands of accelerated development timelines.

Additional methods and input needed

To address this need, USP is developing a set of analytical methods for viral vectored vaccine quality to support developers, manufacturers, regulatory agencies, and national control laboratories worldwide. The goal is to create a shared understanding of viral vectored vaccine quality attributes overall, which aims to accelerate product development, guide successful scale-up of manufacturing and fuel regulatory confidence that manufacturers are employing best practices and appropriate quality controls when using this new modality.

These draft guidelines include methods that have been adapted from publicly available sources and were vetted by one of USP's scientific expert committees on biologics, which includes representatives from industry, government and academia. This document also incorporates best practices described in two of USP's existing standards on vaccines in the United States Pharmacopeia—National Formulary, General Chapters <1235> *Vaccines for Human Use—General Considerations and* <1239> *Vaccines for Human Use—Viral*.

To advance these draft guidelines, we call on industry, academic and government experts with experience or interest in viral vectored vaccines and viral vector technology to provide feedback on the methods detailed in these draft guidelines and recommend additional information to support the understanding of viral vectored vaccine quality. We encourage the submission of any alternative analytical methods as well as supporting documents (e.g., validation documentation). By collaborating with USP, participants play an active role in shaping standards and solutions that contribute to building the supply of safe, effective, quality medicines that people around the world can trust.

More information can be found on our website at usp.org/viral-vectors. To provide feedback on this proposed draft or inquire about other aspects of this work, contact **USPVaccines@usp.org.**

About USP

For more than 200 years USP has worked to build the public's trust in medicine. The standards and solutions we develop have been guiding the safety and quality of medical products through major transformations that have taken place in the biopharmaceutical industry. We do this by convening industry and scientific experts in emerging technology areas to codify best practices that will advance the understanding of specific quality issues as that technology continues to grow. This supports the development of new medical products using that technology and is an important part of the overall safety net for medicines that builds patient and provider trust.

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Introduction

Vaccines based on recombinant viral vector technology have been evaluated for the past few years and some of these received marketing authorization (e.g., Zabdeno, ERVEBO and Mvabea) for Ebola. Vaccines for SARS-CoV-2 have been developed using recombinant viral vectors with some receiving authorization for emergency use in a variety of countries. Examples of SARS-CoV-2 viral vectored vaccines include those manufactured by Oxford–AstraZeneca (Vaxzevria), Gamaleya Research Institute (Sputnik V), Janssen, and CanSino Biologics (Convidecia).

Viral vectored vaccines use a modified version of harmless virus as a vector to deliver genetic material coding for a desired antigen (for example the COVID-19 spike protein). These vaccines have two key components: 1) the viral vector, which is used as a vehicle to deliver 2) the DNA "cargo" that enables production of the antigen of interest. The viral vector used as the delivery vehicle is genetically altered so it can't cause illness and the genetic material does not integrate into a person's genome. The DNA cargo contains the coding sequences for the antigen(s) of interest, which is produced once inside the cells, triggering an immune response in the body.

Adenovirus, measles, and vesicular stomatitis virus (VSV) are the most common vectors used in viral-vectored vaccines given their strong immune response. Replication-defective adenovirus vectors are the most common viral vaccine vectors used in current investigational vaccines. Adenovirus vectors are nonenveloped icosahedral-shaped viruses of 70–90 nm, consisting of a protein capsid containing single-stranded DNA and have many known serotypes. In addition to carrying DNA into a host cell for production of antigenic proteins, the vector can enhance immunogenicity without an adjuvant and induce robust cytotoxic T lymphocyte responses to eliminate virus-infected cells. These vector particles activate innate immunity through TLR-dependent and TLR-independent pathways causing an upregulation of type I interferons and inflammatory cytokines.

These draft guidelines provide recommendations on analytical procedures for viral vectored vaccines, specifically replication-defective viral vectors. The quality of viral vectored drug substances and drug products is determined by their design and the specifications applied to them during the development and manufacturing process. This document provides recommended analytical methods for critical quality attributes for identity, purity, potency, quantity, physical state (integrity), and safety of the viral vectored vaccine drug substance and drug product.

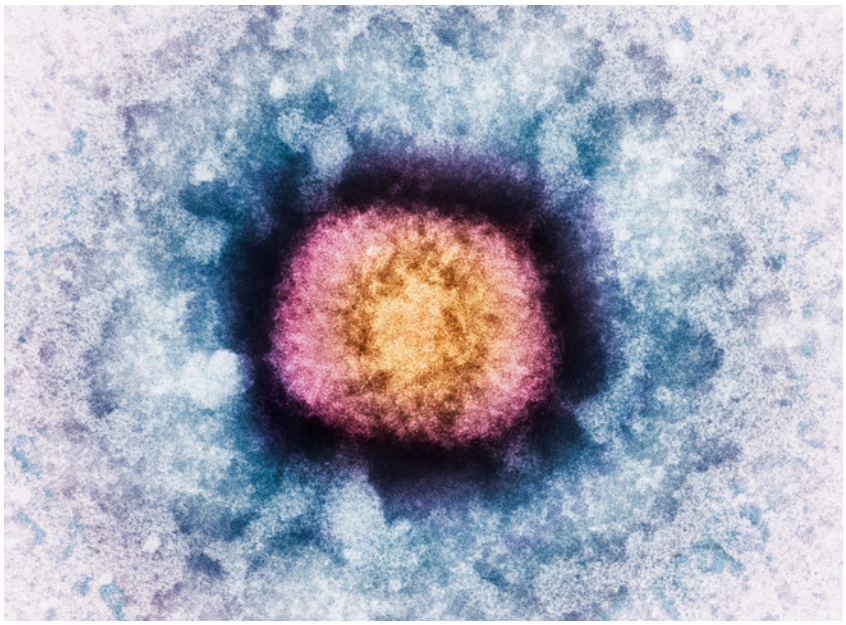

Table 1: Analytical Methods for Drug Substance and Drug Product

Quality	Attribute	Recommended Analytical Method
Identity	DNA sequence	Quantitative polymerase chain reaction (qPCR)
		Next generation sequencing (NGS)
	Viral Capsid	Western blot
		Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC)
		Liquid Chromatography - Mass Spectrometry (LC-MS)
Potency	Transgene expression	ELISA
	Physical viral titer	Quantitative polymerase chain reaction (qPCR)
		Infectivity assay (flow cytometry)
		Tissue culture infection dose ($TCID_{50}$) assay
		Vector genome titer assay (ddPCR)
Quantity	Total viral capsid particles	Size-exclusion chromatography with multi-angle light scattering (SEC-MALS)
		Anion-exchange High-performance Liquid Chromatography (AEX-HPLC)
Purity	Vector aggregates	Dynamic light scattering (DLS)
	Capsid content (empty vs. full)	Anion-exchange High-performance Liquid Chromatography (AEX-HPLC)
	Residual host cell DNA	Polymerase chain reaction (PCR)
Safety	Replication competent virus	Replication-competent adenovirus (RCA) assay
	Adventitious agents	In vitro adventitious virus assay
	Bioburden	USP <1115>
	Sterility	USP <71>
	Endotoxin	USP <85>
General tests	Appearance	USP <1>, <790>
	pH	USP <791>
	[a]Osmolality	USP <785>

[a]Only tested on drug product.

Identification

Because viral vectored vaccines consist of both the viral vector and the cargo DNA, it is important to test both components to confirm identity. Methods A or B may be used to establish the identity of the DNA. Methods, C, D, and E apply to the viral vector used for delivery.

Method A: Identification and quantification of DNA target sequence by qPCR

The quantitative polymerase chain reaction (qPCR) is a highly sensitive technique that can be used for the amplification and detection of deoxyribonucleic acid (DNA).

Resuspension solution: Dissolve tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) and ethylenediaminetetraacetic acid (EDTA) to obtain a solution of 10 mM and 1.0 mM, respectively. Add hydrochloric acid or sodium hydroxide to adjust to a pH of 8.0.

Primer and probe design: Target-specific primers and probes should be carefully designed. There are commercially available software packages (e.g., Primer3, PrimerQuest by IDT, NCBI Primer BLAST, and Primer Express) that can be utilized. Most of these packages include adjustable parameters, such as the primer, probe, or amplicon length, along with specific melting temperatures.

Viral DNA isolation: Treat viral vector samples with DNase I at 37° C for 30 min to degrade any non-encapsulated DNA that may be present followed by an inactivation at 95° C for 10 min.

DNA standard solutions: Dilute the DNA standard stock solution in Resuspension solution to obtain five or more suitable standards. Use a purified genomic DNA as standard serial diluted to the concentration range of 0.001–100 pg/µL.

Sample solution: Following DNase treatment, samples for testing may require dilution or reconstitution to 1) overcome matrix interference affecting the DNA recovery, 2) yield an appropriate starting volume, or 3) bring the analyte concentration within the quantitative range of the qPCR method. Sample solutions may be diluted in water or in Resuspension solution if necessary.

qPCR analysis: Perform qPCR analysis of a 25-µL volume mixture comprising the components in Table 2 under the thermal cyclic conditions provided.

Table 2. qPCR Assay Sample Preparation

Component	Amount
$MgCl_2$	2 mM
dNTP mixture	0.4 mM
Forward primer	0.2 µM
Reverse Primer	0.2 µM
DNA sample	50 ng
TaqMan DNA polymerase	up to 300 nM (1U)
Nuclease-free water	to a final volume of 25 µL

Table 3. qPCR Thermal Cycling

Step	Temperature (°C)	Time	Cycles
Enzyme activation	95	10 min	None
Denaturation	95	30 s	35 cycles
Annealing	55 to 65.5 (gradient)	30 s	None
Extension	72	1 min	None
Final extension	72	10 min	None

[Note: Some instruments and reagents require a preincubation step. Carefully follow specific instrument and reagent recommendations.]

Monitor the signal of the labeled probe using a suitable fluorescent detector. Determine the threshold value using the instrument-specific recommendations. Record the cycle thresholds (C_t) for each sample. Plot the log quantity of DNA of the DNA *standard solutions* versus the C_t. Calculate the slope and the intercept. Using these values and the following equation, calculate the quantity of DNA in each well.

Result = 10 (C_t-b/m)

C_t = cycle threshold for the *Sample solutions*

b = intercept of the line for the *Standard solutions*

m = slope of the line for the *Standard solutions*

Calculate the quantity of DNA in each of the *Sample solutions*. Correct for any dilution or concentration of the sample.

Method B: Genomic DNA Sequence by NGS

Multiple commercial instruments are available for next generation sequencing (NGS). Small sections of DNA are ligated onto the ends of fragments of vector DNA that are used to hybridize the DNA onto a flow cell. Once DNA is hybridized, millions of copies of the DNA are synthesized using polymerase in what is called "cluster generation". The cluster is then sequenced using nucleotide bases that are fluorescently labeled and have a terminator. The complimentary nucleotide binds in the proper position on the single DNA strand attached to the flow cell, but the terminator prevents additional nucleotides from being added. The fluorescent signal is read to determine which nucleotide was added and the terminator cleaved so the next base can be added to repeat the process of stepwise addition of labeled nucleotide base to sequence the entire DNA fragment. Computer analysis of the fluorescent signals from the DNA hybridized to the flow cell is used to determine the sequence of the starting DNA molecule.

Tris-EDTA buffer (TE buffer): Prepare a solution containing 1M of Tris-chloride (pH 8.0), 0.2 mL of EDTA (pH 8.0) to 98.8 mL of Milli-Q water.

Design primers: Choose an amplicon length that is suitable for your sequencing platform and the number of viral copies in the sample (for example 300–500 nt for MinION or Illumina platform). Resuspend lyophilized primers by pre-spinning tubes to make sure that the pellet is at the bottom of the tube and add TE buffer to make a primer stock concentration of 100 µM.

Preparation of primer pools (Primer Pool 1 and Primer Pool 2): To ensure complete viral genome coverage, prepare two pools of primers separating the overlapping amplicons. Add an equal volume of each 100-µM primer stock such that the forward and reverse primers for alternate regions are pooled together. Dilute these with nuclease-free water (1:10) for a working concentration of 10 µM.

Viral vector DNA extraction: The virus that carries its genetic material in the form of double-stranded DNA must be extracted from the viral capsid before it can be sequenced.

Extraction solution: Dissolve 7.5 Anson units of a broad-specificity serine protease in 1.4 mL of RNase-free water containing 0.04% sodium azide.

Lysis solution: Add 310 µL of 0.04% sodium azide in RNase-free water to a tube containing 310 µg of lyophilized carrier RNA to obtain a solution of 1 µg/µL. Dissolve the carrier RNA thoroughly. Mix 6.2 µL of the RNA solution with 220 µL of a 0.5% maleic acid buffer containing 40% (w/w) guanidium hydrochloride.

Wash solution 1: Prepare a 5 M guanidine hydrochloride solution containing 1.6 mM Tris-HCl (pH 7.5) and 50% ethanol.

Wash solution 2: Prepare a solution containing 1.6 mM Tris-HCl (pH 7.5) with 0.1% sodium azide and 70% ethanol.

Add 25 µL of *Extraction solution* to a 1.5-mL microcentrifuge tube. Add 200 µL test sample to the tube. Add 200 µL of Lysis solution, mix thoroughly and incubate at 56° C for 15 min. Centrifuge the tube briefly to collect the liquid in the bottom of the tube. Add 250 µL of ethanol to the sample and mix thoroughly. Let the sample stand at room temperature for 5 min. Centrifuge the tube briefly to collect the liquid in the bottom. Transfer all the lysate from the tube onto centrifugal silica membrane DNA purification column. Close the column and centrifuge at 6000 ×g for 1 min. Discard the filtrate. Add 500 µL of *Wash solution* 1 to the column and centrifuge at 6000 ×g for 1 min. Discard the filtrate. Add 500 µL of *Wash solution* 2 to the column and centrifuge at 6000 ×g for 1 min. Discard the filtrate. Add 500 µL of ethanol to the column and centrifuge at 6000 ×g for 1 min. Discard the filtrate. Centrifuge the column at 20,000 ×g for 3 min to dry the membrane. Discard any liquid recovered from the membrane. Open the column and incubate at 56°C OD for 3 min to completely dry the membrane. Add 150 µL of RNase-free water to the center of the membrane. Close the column lid and incubate at room temperature for 1 min. Centrifuge at 20,000 ×g for 1 min to collect the eluate containing the test DNA.

Measure the absorption spectra using a spectrophotometer. Pure DNA should have a OD 260/280 ratio of 1.8 and a OD 260/230 ratio in the 2.0–2.2 range.

PCR analysis

Reaction buffer (5X): Prepare a solution by adding 4.25 mL of 1 M potassium acetate, 1.25 mL of 1M tricine (pH 8.7), 4.0 mL of glycerol, 0.5 mL of DMSO and 120 µL of 500 mM magnesium acetate.

Prepare a master mix for each of the two primer pools in microcentrifuge tubes by combining 5 µL of *Reaction buffer* (5X), 0.5 µL of 10 mM dNTPs, 0.25 µL of DNA polymerase, 10 µM of *Primer Pool* 1 or *Primer Pool 2* (final concentration 0.015 µM/primer) to PCR-grade water. Mix thoroughly by vortexing and spin down in a microcentrifuge. Label 0.2-mL PCR tubes and add 22.5 µL of master mix to each tube. Add up to 10 µL (2-20 ng) of extracted DNA. Place the tubes in a thermocycler and run the programs as follows. For cycle 1: Denature at 98°C for 30 s. For cycles 2–40: denature at 98°C for 15 s. Anneal/extend at 65°C for 5 min.

PCR cleanup and quantification: Transfer the content of the tubes to 1.5-mL microcentrifuge tubes. Depending on the length, add the volume of AMPure XP beads[1]. If <500 bp, add 25 µL of beads; if 500–1000 bp, add 20 µL of beads; and if >1000 bp, add 15 µL of beads for a 25-µL PCR reaction. Perform washes following the instructions in the protocol provided in the kit and elute in 30 µL of EB buffer[1]. Quantify 1.0 µL of the cleaned product using PCR instrument following manufacturer's instructions. Concentration should be in the range of 5–50 ng/µL for each reaction. Negative control should be >1.0 ng/µL.

Library preparation and sequencing: Procedures are platform specific and should follow manufacturer's protocol. The following procedure is specific to MiSeq from Illumina, but other platforms may also be used.

Determine the number of samples per flow cell (two barcodes per sample are recommended, which means up to 47 samples plus a negative control on each sequencing run).

Normalization: Keep pools in individual 1.5-mL microcentrifuge tubes. Add 50 ng of sample and add nuclease-free water for a final volume of 50 µL.

End repair and dA-tailing: Perform end repair and dA-tailing according to the Hyper Prep Kit protocol (KAPA)[2].

Library preparation: Complete library construction with the KAPA kit according to manufacturer's instructions. Replace the KAPA adaptors[2] with SureSelectxt HS2[3] indexes adaptor ligation step. Perform a 0.8× instead of 1× SPRI cleanup during postamplification cleanup to remove potential adaptor dimers.

Validate library: Measure the size distribution of the library using the TapeStation 2200 or equivalent according to the manufacturer's instructions. Calculate the molarity of each library using the KAPA Library Quantification Kit[4] according to the manufacturer's instructions. Prepare library for loading onto MiSeq and start the sequence run. Complete instrument setup and start the sequencing run. Baseline and demultiplexing will be performed automatically by the instrument.

Alternatively, quantification of the libraries can be performed using qPCR, ddPCR, Bioanalyzer, MicroChIP or other method.

Analyzing the sequencing data: Check the coverage by reference to the alignment file. Compare the alignments with the positive and negative control alignments to help indicate problematic samples or regions. Also, use the variant frequency plot to help determine the allele frequency of mutations in the sample.

Method C: Viral Protein by Western Blot

Western blot assay is a method in which viral proteins are separated according to size, transferred onto a membrane, and then incubated with antibody. Negative and positive samples are run simultaneously to allow identification of viral proteins.

Lysis buffer: Prepare a solution with 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl, pH 7.4), 150 mM NaCl, 5mM ethylenediaminetetraacetic acid (EDTA), 1% NP-40, and 1 tablet of protease inhibitor.

Loading buffer (2× Laemmli buffer): Prepare a solution containing 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, and 0.125 M Tris-HCl. Adjust the pH to 6.8.

Running buffer: Prepare a solution with 25 mM Tris, 190 mM glycine, and 0.1% SDS.

Transfer buffer: Prepare a solution with 25 mM Tris, 190 mM glycine, and 20% methanol.

Ponceau S staining buffer: Prepare a solution with 0.2% (w/v) Ponceau S and 5% glacial acetic acid.

Tris-buffered saline with Tween 20 buffer (TBST buffer): Add 150 mM NaCl and 0.1% Tween 20 to 20 mM Tris (pH 7.5).

Tris-buffered saline buffer (TBS buffer): Add 150 mM NaCl to 20 mM Tris (pH 7.5).

Blocking buffer: Prepare a solution with 3% bovine serum albumin (BSA) in TBST buffer.

Sample preparation: Place the cell culture dish on ice and wash the cells with cold TBS buffer. *[Note: The viral vector used for the experiment must be propagated in the infected cells in culture prior to sample preparation step.*] Aspirate the TBS buffer, then add cold *Lysis buffer* (1 mL per 100 mm dish). Collect the cells into a precooled microcentrifuge tube. Maintain constant agitation for 30 min at 4°C. If necessary, sonicate 3 times for 15 s to complete cell lysis. Spin at 16,000 ×g for 20 min in a 4°C precooled centrifuge. Remove the tube and place it on ice. Transfer the supernatant to a fresh tube on ice and discard the pellet. Use a small amount of lysate (10–20 µL) to determine the protein concentration. Take 20 µg of sample and add equal volume of *Loading buffer*. Boil the cell lysate in *Loading buffer* at 95°C for 5 min. Centrifuge at 16,000 ×g in a microcentrifuge for 1 min.

Protein separation: Load 20 µg of protein on a 4%–12% Bis-Tris gel along with molecular weight markers. Run gel for 5 min at 50 V. Increase voltage to 100–150 V and run the gel for about 1 h until the dye reaches the bottom of the gel.

Protein transfer: Place the gel in *Transfer buffer* for 10–15 min. Transfer the protein to a Poly (vinylidene fluoride) (PVDF) membrane.

Antibody incubation: Briefly rinse the blot in water and stain it with *Ponceau S staining buffer* to check the transfer quality. Rinse the stain with three washes of TBST buffer. Block in *Blocking buffer* at room temperature for 1 h. Incubate overnight in the primary antibody solution against the target protein at 4°C. Remove the primary antibody solution and rinse the membrane with secondary antibody for 1 h.

[Note: Fluorescent secondary antibody may also be used]

Imaging and data analysis: Depending on the label on the secondary antibody, add an appropriate chromogenic or chromogenic or chemiluminescent substrate to the blot according to the manufacturer's recommendation. Capture the signals using an imager and use image analysis software to read the band intensity of the target proteins.

Method D: Protein Fingerprinting by Reversed-Phase HPLC (RP-HPLC)

The identity of the viral capsid proteins can be determined using reversed-phase HPLC (RP-HPLC) and UV absorbance. This method can also be adapted as a quantitative method for the determination of the protein composition of adenovirus vector-based vaccines. Standard and samples should be diluted in the formulation buffer for drug product to a concentration of 2.5 × 10^{11} virus particles/mL.

Solution A: 5% acetonitrile with 0.1% TFA.
Solution B: 0.1% TFA in acetonitrile.

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	80	20
37	66	34
85	54	46
110	40	60
115	80	20
130	80	20

Chromatographic system
(See *Chromatography* <621>, *System Suitability*.)
Mode: LC
Detector: UV 280 nm
Column: Vydac 214TP C4, 2.1-mm ×250-mm, 300 Å, 5-µm packing
Column temperature: 40°
Sample tray temperature: 8°
Flow rate: 0.2 mL/min
Injection volume: 100 µL
System suitability
Samples: *Standard solution* and *Sample solution*
[NOTE—Condition the Chromatographic system by running at least two blank gradient programs before injecting standard or samples. Separately inject each sample bracketing with standard before and after. Record the responses of each peak.]
Suitability requirements: Peaks used to identify the viral vectored vaccine proteins should be present in each chromatogram. The absolute difference in retention time for each of the protein peaks between the two standards chromatograms should be ≤0.5 min. The difference in retention time for each of the protein peaks between the sample chromatogram and the average from the standard chromatograms should be ≤0.5 min. The relative difference in peak height of each of the protein peaks between the two Standard solution chromatograms should be ≤15%.
Acceptance criteria: The chromatogram of the sample should be similar to that of the standard. The relative difference in peak height between the normalized sample peak height (normalized by total peak height versus the average total peak height of the standard chromatograms) and the average standard peak height of each of the protein peaks must be ≤15%.

Method E: Protein Fingerprinting by liquid chromatography and mass spectrometry (LC-MS)

Mass spectrometry can be used to identify and characterize the primary structures of viral capsid proteins by intact mass analysis and/or peptide mapping analysis. Intact mass analysis by liquid chromatography mass spectrometry (LC-MS) can be used to confirm the masses and relative expression levels of the capsid proteins. Peptide mapping by liquid chromatography and tandem mass spectrometry (LC-MS/MS) can be used to confirm the sequences and major post-translational modifications (PTMs).

Sample preparation for intact mass analysis: The samples should be concentrated or buffer-exchanged to MS friendly buffer such as 100 mM Tris or ammonium bicarbonate at pH 8.0. Treat the concentrated samples with acetic acid at 10% (v/v) concentration for 15 min, then centrifuge at 12,000 rpm for 5 min to ensure complete capsid dissociation. The recommended sample concentration is around $1x10^{13}$ GC/mL for LC-MS intact mass analysis.

Solution A: Water containing 5% (v/v) acetonitrile with 0.1% TFA.
Solution B: 0.1% TFA in acetonitrile.
Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	80	20
1	68	32
16	64	36
20	20	80
21.5	20	80
22	80	20
30	80	20

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: MS

Column: Acquity UPLC Protein BEH C4 300 Å, 1.7 µm, 2.1×100 mm or equivalent columns

Column temperature: 80°C

Sample Tray temperature: 8°C

Flow rate: 0.2 mL/min

Injection volume: 10 µL (or ~ 1 µg of proteins)

Mass Spectrometer Setting: Different types of Mass Spectrometers can be used for intact analysis. The MS parameter settings and software are instrument dependent.

Data analysis: Use appropriate software to deconvolute the raw mass spectra and report molecular weights for all major species.

Acceptance criteria: The molecular weights of the proteins detected by MS are consistent with the sequence provided for the viral capsid proteins. Molecular weights can also be matched to a reference standard.

Sample Preparation for Peptide Mapping: Denature a 10 µL amount of sample containing 1.0×10^{12} viral genomes (~5 µg of protein) and reduce in 30 µL of 150 mM Tris-HCl (pH 8.0) containing 8M guanidine HCl plus 3.8 mg/mL TCEP for 60 minutes at 60°C. After cooling to room temperature, perform alkylation by adding 10 µL of 133 mM iodoacetamide and incubating at room temperature in darkness for 30 minutes.

[NOTE—Reduction and alkylation steps are not required but typically will provide enhanced sequence coverage of the proteins. If samples contain surfactant, denaturing SEC may be used to clean up the samples.]

Dilute sample with 210 µL of Tris-HCl, after which sequencing grade modified trypsin is added to digest the protein samples into peptides. Add sufficient amount of enzyme for a final trypsin:protein ratio of 1:20 to 1:100 (w/v). Vortex briefly, seal the tube with Parafilm, and incubate at 37°C for 4 to 18 hours with rotation. Stop reaction by adding 5 µL of 1.0% TFA.

[NOTE—The amount of trypsin needed will vary depending on the amount of protein sample present and the speed of digestion. Typically, a ratio of 1:20 (w/w) is sufficient for complete digestion of 10 µg of protein with 4 h.]

LC-MS/MS analysis: Many different reversed phase UPLC columns can be used for peptide separation, and various Mass Spectrometers can be used for peptide mapping data acquisition. A representative UPLC-MS/MS configuration is given below. It is recommended to make changes based on the requirements and guides for specific instruments and columns used.

Solution A: 0.1% formic acid in water.

Solution B: 0.1% formic acid in acetonitrile.

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	97	3
0.5	97	3
50	45	55
50.1	10	90
55	10	90
55.1	97	3
75	97	3

Chromatographic system

Column: XSelect Peptide CSH C18 XP, 2.1 mm ID × 150 mm, 2.5 µm particle, 130 Å pore or equivalent column

Column temperature: 40° C

Sample Tray temperature: 8° C

Flow rate: 0.25 mL/min

Injection volume: 10 µL

Perform data-dependent acquisition (DDA)analysis in positive ion mode. Set MS and MSMS scan parameters per manufacturer's recommendation.

Data analysis: List all the peptides detected, along with their corresponding capsid protein and amino acid range, detected m/z, mass errors and PTMs.

Acceptance criteria: Capsid protein identification by MS is a qualitative assay therefore as "positive" or "negative" for the viral protein of interest. You may include criteria for N-terminal, C-terminal and overall sequence coverage, and relative abundance of PTMs.

Potency

Method A: Transgene Expression by ELISA

Enzyme-linked immunoassay (ELISA) is a highly sensitive technique based on immunological reactions that combine the specific reaction of antigens and antibodies with the efficient catalytic action of enzymes on substrates. This method utilizes the sandwich ELISA format which is designed for detection of soluble antigens.

Blocking solution: PBS containing 0.05% Tween 20 (PBS-T) + 5% nonfat milk powder (w/v)

Analytical procedure

Step 1: Coat a 96-well ELISA plate with purified capture antibody at a concentration of 100 ng/well in 100 µL of PBS per well for 2 h at room temperature (RT). Lightly tap the plate against a surface to ensure the protein is evenly coated in every well. Seal the plate and incubate overnight at 4°C. Plates can be stored at 4°C for up to a week.

Wash the coated ELISA plate three times with PBS-T in an automated plate washer. Add 200 µL of *Blocking solution* to all wells of the plate. Seal the plate and place the plate in a RT incubator for a minimum of 1 h. Do not exceed 4 h.

Step 2: Remove the Blocking solution and add 100 µL of sample containing the viral protein of interest. Seal the plate and incubate for 1 h at RT.

[Note: Always compare the signals of unknown samples against those of a standard curve. Run standards (duplicates or triplicates) and blanks with each plate.]

Wash plate three times with PBS-T. Add 100 µL of detection antibody diluted in 1:1000 in 0.5% nonfat milk to each well of the plate. Seal the plate and place it in a shaker for a minimum of 1 h at RT.

[Note: In order to decrease antibody interference, be sure that the capture and detection antibodies do not bind to the same epitope of the target protein. In addition, if a secondary antibody is used for detection, then the capture and detection primary antibodies must be from different species.]

Wash plates three times with PBS-T. Add 100 µL of conjugated secondary antibody (e.g., horseradish peroxidase-labeled anti-rabbit IgG if a rabbit detector antibody was used), diluted in *Blocking solution* immediately before use. Seal the plate and place in a shaker for 1 h. Wash plates three times with PBS-T. Add 100 µL of a suitable substrate solution to each well (substrate solution should be at RT prior to use). The estimated incubation time for the enzyme-substrate reaction ranges from 20–30 min. Add 50 µL of stop solution. After stopping the enzymatic reaction, the plate should be read at an optical density (OD) suitable for the chromagen used within 15 minutes.

Analysis of data

Prepare a standard curve from the data produced from the serial dilutions of the standard solution with concentration on the x axis (log scale) vs absorbance on the y axis (linear). Determine the concentration of the unknown sample from the standard curve by the OD of sample.

Method B: Viral Titer Determination by qPCR

The qPCR technique is highly sensitive and can be used to assess the titer of the viral vector.

Primer and probe design: Target-specific primers and probes should be carefully designed. There are commercially available software packages (e.g., Primer3, PrimerQuest by IDT, NCBI Primer BLAST, and Primer Express) that can be utilized. Most software includes parameters that can be adjusted, such as the primer, probe, or amplicon length along with specific melting temperatures.

Viral DNA isolation: Treat viral vectored samples with DNase I at 37°C for 30 min to degrade any non-encapsulated DNA that may be present followed by an inactivation of the DNase by incubation at 95°C for 10 min.

Standard preparation: Digest the linearized form of the expression plasmid (plasmid that contain the gene of interest) with digestion enzyme by incubating the plasmid in the enzyme for 1 h at 37°C. Test the digestion reaction in a 1% agarose gel pre-stained with 10% ethidium bromide. Excise the linearized plasmid from the gel, and then purify using QIAquick Gel Extraction Kit[5] following manufacturer's instructions. Test the DNA yield and quality using Invitrogen Qubit Flurometer[6] and Qubit dsDNA High Sensitivity Assay Kit[7].

Prepare a six-point 10-fold serial dilution from a 1 ng/µL working stock solution yielding dilutions ranging from 1 ng/µL to 0.01 pg/µL.

Positive control solution: Prepare a positive control sample that is known to express the target by diluting the sample to a concentration appropriate for the assay (specification, or otherwise justified).

Negative control solution: Water or sample buffer used in the extraction procedures should be used as negative control. The *Negative control solution* is tested using the qPCR-based method to determine the background and to demonstrate that there is no cross-contamination during the assay. This is also known as the "no template control".

qPCR analysis: Perform qPCR analysis on a 25-µL volume mixture comprising the following mixture under the thermal cycling conditions provided below. Transfer 25 µL of the sample, *Positive control solution, Negative control solution*, and standard to corresponding wells of a 96-well qPCR plate.

Table 4. qPCR Assay Sample Preparation

Component	Amount
DNA sample	0–10^8 copies (up to 1000 ng)
Forward primer	900 nM
Reverse primer	900 nM
TaqMan probe	300 nM
2× TaqMan Universal Master Mix II or equivalent	1×
Nuclease-free water	to a final volume of 20 µL

Table 5. qPCR Thermal Cycling

	Temperature (°C)	Time	Cycles
	50	2 min	1
Denaturation	95	10 min	1
Denaturation	95	15 s	40
Annealing	60	60 s	None

[Note: Some instruments and reagents require a preincubation step. Carefully follow specific instrument/reagent recommendations.]

Monitor the signal of the labeled probe using a suitable fluorescent detector. Determine the threshold value using the instrument-specific recommendations. Record the cycle thresholds (C_t) for each sample. Plot the log quantity of DNA of the DNA standard solutions versus the C_t. Calculate the slope and the intercept. Using these values and the following equation, calculate the quantity of DNA in each well.

Result = 10 (C_t-b/m)

C_t = cycle threshold for the *Sample solutions*

b = intercept of the line for the *Standard solutions*

m = slope of the line for the *Standard solutions*

Calculate the quantity of DNA in each of the *Sample solutions*. Correct for any dilution or concentration of the sample.

Method C: Viral Titer Determination using a Flow Cytometry-based Infectivity Assay

Viral infectivity assays are used to determine the amount of infectious virus present in a virus-containing fluid that can produce cytopathic effects in tissue culture over a period of time during in which the cells in culture remain viable. However, not all virus types cause a cytopathic effect in tissue culture; therefore, cell line and virus must be carefully matched in order to see this effect. Alternatively, a 50% tissue culture infective dose ($TCID_{50}$) assays could be used to determine the infectious titer. The assay below uses HEK293 cells but alternatively, other cell lines can be used.

Incubation buffer: 1% bovine serum albumin in PBS

Analytical procedure: To quantify infectious vector titer by flow cytometry, infect the adherent HEK293T cells with serially diluted vector samples to induce expression of the transgene. Viral titer can then be detected by detection of the transgene-fusion protein on the cells. First seed 6 X 10^4 cells/well in 0.5 mL Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum in a tissue culture treated 24-well plate. Incubate cells at 37°C and 5% CO_2 in a static incubator for 24 hours. Remove the cell culture medium and then infect the cells by transferring 0.5 mL of diluted virus solution containing 8.0 µg/mL of polybrene. Make sure to include a negative control. Incubate the plate for 18 hours at 37° and 5% CO_2. After incubation, remove the medium and replace it with fresh culture medium. Incubate the plate for additional 48 hours at 37°C and 5% CO_2. Next, prepare the samples for flow cytometry. Detach the cells by incubating the cells with 200 µL of 0.05% trypsin-EDTA for 5 minutes at 37°C. Stop the enzymatic reaction by adding 500 µL culture medium. Centrifuge the plate at 300 X g for 5 minutes and remove the supernatant. Resuspend cells in 150 µL of PBS and transfer to a non-tissue culture 96-well plate with conical bottom. Centrifuge the plate and discard the supernatant. Add 100 µL of a 1:1000 diluted Zombie NIR fluorescent dye8 in PBS to each well to determine viable versus dead cells. Incubate the plate for 10 minutes in the dark. Centrifuge the plate and discard the supernatant. Add 100 µL of fixing solution to each well and incubate for 15 minutes. Remove supernatant from each well after centrifugation, then add 150 µL of PBS. Again, remove supernatant from each well after centrifugation, then add anti- virus

conjugated antibody at a 1:200 dilution in 40 µL of *Incubation buffer*. Incubate the plate for 30 min. Wash the cells twice with 100 µL of staining buffer. After each wash step, centrifuge the plate and remove the supernatant. Resuspend the cells in 40 µL of staining buffer and perform flow cytometry.

Analysis: Run samples in triplicate and analyze by flow cytometry on single cells gating sequentially on, cell population and positive cells. The percentage of positive cells of viable single cells can be determined by using the formula below.

Infectious titer = (P^1 × N × D)/ (V × 100)

P^1 = percentage of positive live cells

N = Number of cells at the time of infection

D = Dilution factor of virus used for infection

V = Infection volume (mL)

Method D: Viral Titer Determination by $TCID_{50}$

The measurement of infectious virus titer can be performed by using a $TCID_{50}$ endpoint dilution assay. This assay quantifies the amount of virus required to kill 50% of infected hosts or to produce a cytopathic effect in 50% of inoculated tissue culture cells. For adenovirus, HEK293T cells can be used.

Growth medium: DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S)

Serum-free medium: DMEM medium supplemented with 1% penicillin/streptomycin (P/S)

Virus titer in a 96-well plate: The day before transduction, seed a 96-well tissue culture plate with HEK293T cells at 2.5–3.0 × 10^4 cells/well in 100 µL growth medium. Leave the plate in the incubator for 24 h to let the cells attach to the wells. After 24 h, make a 5-fold serial dilution of viral stock in a separate round bottom 96-well plate using a serum-free media by adding 40 µL of media/well and 10 µL of virus stock to the column's first wells. Perform a 5-fold serial dilution by removing 10 µL from the first column to the next. Mix the dilution by pipetting contents of the well up and down 10–15 times. Discard pipette tips between dilutions. Next, remove the culture medium from each well of the tissue culture plate with HEK293T cells. Add 30 µL of the diluted virus to each well. Do not add virus to control wells (for example columns 11 and 12). Centrifuge plate at 2000 rpm at room temperature for 2 h, then incubate the plate at 37°C for 4–6 h. Remove the plate from the incubator and add 170 µL of growth medium to each well (total 200 µL/well). Continue to incubate the cells for an additional 72 to 96 h to monitor cytopathic effect (CP_E) in culture while cells remain viable.

$TCID_{50}$ calculation: After the incubation period, inspect cells for CPE or cell death and classify each well as infected or not infected. The dilution at which 50% of the wells showing a CPE is used to calculate the $TCID_{50}$ of the virus sample. The results of $TCID_{50}$ assays can be analyzed by Reed-Muench formula which is expressed as 50% infectious dose (ID_{50}) per mL after a define period of time.

I = [(percentage of wells infected at dilution above 50% − 50)/ (percentage of wells infected at dilution above 50% − the percentage of wells infected at dilution below 50%)]

50% endpoint titer = 10log total dilution above 50% − (I × $\log^h$)

Where I is the interpolated value of the 50% endpoint and h is the dilution factor.

Method E: Viral Titer Determination by VG Titer Assay by ddPCR

The exact titer of vector genome (VG) copies is critical for ensuring accurate and reproducible dosing in clinical settings. The ddPCR technique can directly quantify DNA copies with precision and without a standard curve.

DNA suspension buffer: 10 mM Tris (pH 8.0) with 0.1 mM EDTA

10x Pluronic F-68 solution: Add 10 µL of stock 100x Pluronic F-68 to 90 µL of nuclease-free water in a low binding DNA microcentrifuge tube. Prepare freshly.

Poly(A)+ solution: *DNA suspension buffer* with 100 µL/mL poly(A) with 1% Pluronic F-68

Digestion of non-encapsulated DNA: Prepare the following master mix containing all the components except the virus. Calculate the total volume required by multiplying the volumes by the required number of reactions plus at least 1 extra reaction. Add water, followed by 10x DNAse reaction buffer, 10× Pluronic F-68 solution, and DNase I to a low binding DNA microcentrifuge tube. Pulse mix the solution followed by brief centrifugation. Transfer 45-µL aliquots into individual tubes of 0.2-mL 8-tube PCR strips. Add 5 µL of virus sample and cap the 8-tube PCR strips. Flick several times to mix, then microcentrifuge to bring the solution to the bottom of the tubes. Incubate the samples in a thermal cycler for 30 min at 37°C, then cool to 4°C at 3°C/s.

Table 6. Digestion Reaction

Component	Volume/Reaction, µL
10× DNase reaction buffer	5 × *n*
10× Pluronic F-68 solution	5 × *n*
DNase I (2 U/µL)	5 × *n*
Sample	5 × *n*
Nuclease-free water (not diethylpyrocarbonate (DEPC)-treated)	30 × *n*
Total volume per sample	50

n, number of reactions

Sample dilution: For an unknown viral concentration sample, prepare 6 two-fold serial dilutions of the virus after DNase digestion in low DNA binding microcentrifuge tubes for testing.

Capsid lysis: For the last 4 dilutions, transfer 20-µL aliquots of each dilution into 0.2-mL 8-tube PCR strips and cap. Pulse the tubes in a microcentrifuge to ensure that the solution is at the bottom of the tubes. Lyse the capsid by incubating the tubes for 10 min at 95°C, then cool to 4°C at 3°C/s using a thermal cycler. Thoroughly mix the samples by flicking the tubes after removing them from the PCR instrument, then microcentrifuge to bring the liquid to the bottom of the tubes.

ddPCR reaction preparation: Depending on the number of reactions, prepare a master mix containing all of the components except the DNA following the table below. Add water followed by Supermix, 20x assay mixes and restriction enzyme to a low DNA binding microcentrifuge tube. Mix by vortexing the tube, then aliquot for DNA addition. Make 3 reactions for each viral DNA dilution.

Table 7. ddPCR Reaction Setup

Component	1:4 DNA Dilution Volume/Reaction, µL
ddPCR Supermix for probes (no dUTP)	10
20× FAM assay mix (ITR2)[9]	1
20× HEX assay mix (gene of interest)[10]*	1
DNA capsid lysis sample	5
5 U Restriction enzyme (20,000 U/mL)	0.25
Nuclease-free water (not DEPC-treated)	2.75
Total volume per sample	20

Droplet generation: For manual droplet generator, pipet 20 µL into the sample wells on the cartridge followed by 70 µL of droplet generation oil for probes into the oil wells. Generate droplets, then transfer them to a PCR plate, and seal with the foil plate sealer.

For automated droplet generator, add 20 µL into each well on the plate. Generate droplets, then transfer them to a PCR plate, and seal with the foil plate sealer.

PCR amplification: Follow the thermal cycling protocol below for PCR amplification.

Table 8. Thermal Cycling Protocol*

Cycling Step	Temperature (°)	Time	Ramp Rate (°/s)	Number of Cycles
Enzyme activation	95	10 min	2	1
Denaturation	94	30 s		40
Annealing/extension	55	1 min		
Enzyme deactivation	98	10 min		1
Hold	4	infinite		1

* Use a heated lid set to 105° and a sample volume of 40 µL

Read droplets and analyze data: Read the droplet fluorescence using a droplet reader and analyze the data and determine the concentration (copies/µL) for the ddPCR reactions. Use the appropriate dilution factor for each sample to back calculate the titer of the initial solution.

Quantity

Method A: Total Viral Capsid Particle by SEC-MALS

Quantification of particles based on the presence of the viral genome can be achieved using size-exclusion chromatography (SEC) coupled with multi-angle light scattering (MALS), UV, and differential refractive index (DRI) detectors. SEC-MALS can also measure relative capsid content (ratio of full to total capsids) and percentage of aggregates. By combining detection by MALS, UV absorption at 280 nm and 260 nm, and DRI, SEC–MALS can be used to quantify multiple viral vector quality attributes (QAs) simultaneously.

Phosphate buffer solution (2x PBS): Combine 9.0 g of sodium chloride, 1.09 g of disodium phosphate, 0.32 g of monopotassium phosphate to a final volume of 100 mL of distilled water

Solution A: 2× PBS + 10% ethanol

Sample concentration: Sample concentration range of 5×10^{10} to 1×10^{15} capsids/mL

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV absorbance 260 and 280 nm

Column: SEPAX SRT SEC-1000, 4.6-mm×300-mm

Column temperature: 25°C

Sample temperature: 4°C

Flow rate: 1.0 mL/min

Analysis

For SEC-MALS of viral vectored samples, the MALS and the DRI signals must be normalized, and the average and distribution of molar masses can be determined using intrinsic dn/dc values of 0.185 and 0.170 for the capsid protein and encapsulated DNA, respectively, and using a "sphere" model.

Method B: Total Viral Capsid Particle by AEX-HPLC

Quantitation of viral vector particles in a sample is determined using anion exchange high performance liquid chromatography (AEX-HPLC). This method can also be used for separation and quantitation of viral products of multiple serotypes.

Solution A: 20 mM Tris-HCl (pH 7.5)

Solution B: 1 M Sodium chloride in *Solution A*

Mobile phase: See the gradient table below.

Time (min)	*Solution A* (%)	Solution B (%)
4.5	100	0
25.5	30	70
1.5	30	70
3.5	0	100

Standard solution: Standard curves are constructed by injecting increasing amounts of a chromatographically purified Reference Standard (5×10^8 to 5×10^{11} total particles injected).

Sample solution: The sample is diluted 1:5 in *Solution A*

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 260 nm, FLR 280 nm excitation and 320 nm emission

Column: Amersham Pharmacia Biotech HR 5/5, packed with 1 mL of Q Sepharose XL adsorbent

Column temperature: 20°C

Flow rate: 1.0 mL/min

Injection volume: 10 µL

Analysis

Construct a linear standard curve by plotting the peak area versus the number of particles. Determine the dilutional linearity of each of the standard curve dilutions by analyzing the chromatograms for each injection for peak areas of empty and full peaks. Calculate the peak area response of the major peak (full capsid) and the minor peak (empty capsid) based on UV absorbance at 260nm. Using the peak area, quantify empty and full capsids in samples.

System suitability

Suitability requirements: The chromatographic profile of the *Sample solution* is similar to that of the *Standard solution*. The relative standard deviation of the peak area for replicate samples is NMT 5%.

Purity

Method A: Vector Aggregates by Dynamic Light Scattering

Dynamic light scattering (DLS) assay can be used to analyze viral vector aggregation with reproducible results. DLS can also help determine the size of the particles from scattered light in a solution as well as conformational changes due to intercalation. The assay can be used to monitor size and consistency of the viral particles. DLS can also be used to measure other attributes such as total concentration, size, and size distribution of particles.

Dialysis buffer: 50 mM Tris (pH 7.5) and 100 mM sodium chloride

Sample preparation: Dialyze all samples for 2 h at 4° against dialysis buffer. Filter samples through a 0.1-µm filter, followed by equilibration of sample at 20° prior to data acquisition.

Data acquisition: Examine the hydrodynamic radius (Rh) and homogeneity of each sample using a DLS system equipped with a 3-mW laser (λ = 633 nm). This method can be used to analyze size ranges from 0.5–1000 nm; 1 kDa to 1 MDa. Run each sample at different concentrations to determine the hydrodynamic radius. Three repeat measurements of each sample should be done at each temperature using backscatter detection and low volume quartz batch cuvette. The thermal ramps should cover a temperature range of 45°–75°C with 1° increments and collect size measurements at every 1° increase and take particle concentration measurement at every 5°. Each measurement should consist of five 5-s acquisitions. Collect data and determine Rh, content, and aggregate content, molar mass, and viral particle concentration using the software provided by the instrument.

Method B: Capsid Content, Empty vs. Full Capsid by AEX-HPLC

During the production of viral vectors, a population of viral particles will not include the DNA encoding the antigen, leading to an empty capsid. In addition, partially full capsids can be generated that contain truncated genomes in the capsid or an incorrect DNA (e.g., host cell DNA). Analytical separation and quantitation of empty and full capsid can be performed using anion exchange high performance liquid chromatography (AEX-HPLC). A two-step gradient is typically used to achieve acceptable peak asymmetry/tailing, signal-to-noise (S/N) ratio and theoretical plate counts for column efficiency.

[Note–Mobile phase gradient for separation of empty and full capsids for different serotypes may differ. Gradient adjustment maybe necessary.]

[Note–Before starting the experiment, the column must be cleaned with high salt (NaCl) for at least 15 min to achieve a steady baseline.]

Solution A: 20 mM bis[tris(hydroxymethyl)methylamino] propane (BTP) buffer (pH 9.0)

Solution B: 20 mM BTP buffer with 1 M sodium chloride (pH 9.0)

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	95	5
1	95	5
3	85	15
4	81	19
7	81	19
10	50	50
12	5	95
16	5	95
16.01	95	5
18	95	5

Sample solution: Dilute the sample in formulation buffer of the drug product to a concentration range of 4.70E+11 to 1.73E+13 capsids/mL, depending on the serotype.

Chromatographic system: HPLC system with multisampler, multicolumn thermostat with diode array detector (DAD) and fluorescence detector

Mode: LC

Detector: UV 260 nm and 280 nm with fixed slit width of 4 nm

Fluorescence

- **Excitation:** 280 nm
- **Emission:** 348 nm

Response time for UV and fluorescence signals: >0.25 s

Column: Monolith CIMac AAV Full/Empty 0.1 mL with 1.3-µm packing

Column temperature: 20°C

Flow rate: 1.0 mL/min

Injection volume: 10 µL

System suitability

Suitability requirements: The chromatographic profile of the *Sample solution* is similar to that of the Standard solution. The relative standard deviation of the peak area for replicate samples is NMT 5%.

Method C: Residual Host Cell DNA by qPCR

Residual host cell DNA can be quantitated using primers and probes specific for the DNA of the host cell used in manufacturing of the viral vector.

Sample preparation: Extract DNA from viral vectors using a commercially available DNA extraction kit.

Resuspension solution: Dissolve Tris-HCl and EDTA to obtain a solution of 10 and 1.0 mM, respectively. Add hydrochloric acid or sodium hydroxide to adjust to a pH of 8.0.

DNA standard stock solution: Dilute the purified and accurately quantitated genomic DNA from the host cell of interest (e.g., HEK293 or HEK293 or product specific cell line to a concentration of 1 µg/mL in *Resuspension solution*.

Sample solutions: Samples for testing may require dilution or reconstitution to 1) overcome matrix interference affecting the DNA recovery, 2) yield an appropriate starting volume, or 3) bring the analyte concentration within the quantitative range of the qPCR method. *Sample solutions* may be diluted in water or in *Resuspension solution* if necessary. For drug substance samples, *Sample solutions* should contain sufficient starting material to allow determination of the residual DNA content, if present at the specification limit.

Positive control solution: Prepare by spiking *DNA standard stock solution* into *Resuspension solutions* at a concentration appropriate for the assay (specification, or otherwise justified).

Negative control solution: Water or *Resuspension solution* is used in place of *Sample solutions* in the extraction procedures and will be extracted with any samples (if extraction is necessary). The *Negative control solution* is tested using the qPCR-based method to determine the background and to demonstrate that there is no cross-contamination during the assay. This is also known as the "no template control".

2× Master mix: A suitable buffer containing magnesium chloride, dNTP mixture (deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxycytidine triphosphate, deoxyuridine triphosphate, deoxythymidine triphosphate), and highly purified DNA polymerase. Mix well immediately before use.

DNA stock primers and probes: Determine appropriate primers and probes for the host cell of interest. Prepare individual 10-µM solutions of the primer pairs and probe specific to host cell, using DNase-free water.

DNA probe solution: Dilute *DNA stock probe* to 2.5 µM with DNase-free water.

Standard solutions: Dilute the *DNA standard stock solution* to obtain 5 or more suitable standards within the concentration range of 0.001–100 pg/µL.

Analysis

Samples: *Sample solutions*, *Positive control solution*, *Negative control solution*, and *Standard solutions*

[Note: If samples are extracted, then extracted Sample solutions and extracted Control solutions will be used.]

Transfer 25 µL of the 2× Master mix to each well of a 96-well qPCR plate. Add 5 µL each of the DNA stock forward primer, the *DNA stock reverse primer*, and the *DNA probe solution* of the appropriate species to each well. Add 10 µL of either (extracted) *Sample solutions, Standard solutions*, (extracted) *Negative control solution*, or (extracted) *Positive control solution* to their respective wells.

[Note: The qPCR reaction volume may be scaled as appropriate to accommodate different instruments.]

Mix, seal the plate tightly, and centrifuge for 1 min at 1000 ×g. Place the plate in a suitable qPCR thermal cycler. Incubate for 2 min at 50°C, then for 10 min at 95°C, followed by 40 cycles, with each cycle consisting of 95°C for 15 s and 60°C for 1 min.

[Note: Some instruments and reagents require a preincubation step. Carefully follow specific instrument/reagent recommendations.]

Monitor the signal of the labeled probe using a suitable fluorescent detector. Determine the threshold value using the instrument-specific recommendations. Record the cycle thresholds (C_t) for each sample.

Calculations: Plot the log quantity of DNA of the *Standard solutions* versus the C_t.

Calculate the slope and the intercept.

Using these values and the following equation, calculate the quantity of DNA in each well:

Result = 10 (*Ct – b/m*)

C_t = cycle threshold of the *Sample solutions*

b = intercept of the line for the *Standard solutions*

m = slope of the line for the *Standard solutions*

Calculate the quantity of DNA in each of the *Sample solutions*. Correct for any dilution or concentration of the sample.

Safety

Method A: Replication Competent Adenovirus

The presence of replication-competent adenovirus (RCA) is a safety concern for biologics based on recombinant adenoviruses. The RCA assay detects replication competent adenovirus viral vectors in vaccine active substance.

Culture medium: Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS)

Infection medium: *Culture medium* containing 1% penicillin–streptomycin.

Sensitivity control: T175 flask containing *Culture medium* inoculated with test article spiked with wild type adenovirus.

Negative control: T175 flask inoculated with *Culture medium*.

Interference control: 96 well plates spiked with 0.2, 1, 2.5, and 5 plaque forming units of wild type adenovirus per well in combination with test article or *Culture medium*

RCA assay procedure: One day (20–24 h) prior to infection, seed 96-well plates and T175 flasks with cells (e.g., A549 or HEK293 cells) at 3×10^5 cells/mL, using 100 µL per well and 53 mL per T175 flask. Test article and interference/sensitivity controls are inoculated separately to avoid cross-contamination. After 3 h the *Culture medium* is replaced by *Infection medium*. Cultures are passed after 7 days using frozen/thawed cell lysates onto freshly seeded A549 cultures. All plates and flasks are scored for cytopathic effects (CPE) 3 days after reinfection.

Acceptance criteria: If all flasks are CPE negative, the sensitivity control CPE positive, the negative control CPE negative, and the interference control valid, the test article is defined RCA free.

Method B: In Vitro Adventitious Agent Test

Adventitious agent tests are routinely used to assess safety and purity. In vitro assays for virus detection can be applied using multiple cell lines to which the sample is applied and subsequently observing for cytopathic effect (CPE), hemagglutination (HA), or hemadsorption (HAD).

The use of vaccine-naïve control cells is highly recommended when performing cellular tests as vaccine virus can interfere with some tests. Control cells must be handled in the same manner as the production culture and should be maintained in parallel using the same reagents.

Cells and viruses: The virus strain and cell line used to prepare virus stocks must be matched. Most of the adenovirus subtypes can be matched with A549, HEK293, or Vero cell lines. Run one of these standard indicator cell lines to detect the presence of the test viruses at low levels of infectious units.

Cells: Grow cells at 37°C in Eagle's Minimum Essential Medium (EMEM) media supplemented with 10% irradiated fetal bovine serum (FBS).

Seed virus: Store seed virus samples at –70°C.

In vitro adventitious virus assay: Check the virus stock for presence of contaminating bacteria and mycoplasma by PCR and titrate by either plaque assay or $TCID_{50}$ assay. To detect potential viral contaminants, perform 10-fold serial dilutions of each virus stock inoculated to cells grown in 6-well plates. Make sure to include a cell line that is capable of propagating the virus as a positive control in the assay. Dilute each virus stock in growth medium to achieve a range of 100–0.0001 infectious units of the viral stock per well. After 60 min, feed the cells with EMEM containing FBS and incubate at 36.0°C in 5% carbon dioxide. Observe for monolayers at regular intervals for 14 days and replenish with growth medium as required. On day 14, remove the cells from the incubator and test for HA or HAD. For HA, remove an aliquot of growth medium and test for HA by incubating with a suspension of chicken, guinea pig, or human erythrocytes. Incubate the cells at 4°C, followed by room temperature, and then 36.5°C. For HAD, wash the monolayers of the cells with PBS before overlaying the duplicate wells with a suspension of chicken, guinea pig, or human erythrocytes. Incubate plates for 30 min of sequential incubations at 4°C, room temperature, and 36.5°C. Perform three replicate assays for each.

Perform a blind passage of the growth medium from cells for all virus stock samples that appeared to be morphologically normal; without CPE on day 14. Observe monolayers for CPE at regular intervals for an additional 14 days while replacing with growth medium when required. After day 28, remove cells from incubator and test for HA or HAD.

[Note – The limit of detection (LOD) for each virus and cell line pair is defined as the lowest amount of virus that was detectable in all 3/3 repeat assays by CPE, HA, and HAD or any combination of those endpoints.]

Analysis: If at least 1 of the 6 wells shows evidence of viral infection, the set of dilution is considered positive. Stocks that scored positive can be serially diluted further and tested to establish an LOD.

Determine the infectious units per milliliter by using the standard indicator cell line to detect the presence of the test viruses at low levels (0.1 infectious units) per volume (0.5 mL) of inoculum.

Endnotes

1. AMPure XP – PCR Purification Kit can be obtained from Beckman Coulter, Product Code A63881 or equivalent.
2. MiSeq Sequencing KAPA Hyper Prep Kit can be obtained from Roche, Product Code 07962363001 or equivalent.
3. SureSelectxt HS2 Indexes, MSQ can be obtained from Agilent, Product Code G9622A or equivalent.
4. KAPA Library Quantification Kit for Illumina platforms can be obtained from Roche, Product Code 07960140001 or equivalent.
5. QIAquick Gel Extraction Kit can be obtained from QIAGEN, Product Code 28706X4 or equivalent.
6. Qubit Fluorometer can be obtained from ThermoFisher, Product Code R0104S or equivalent.
7. Qubit High Sensitivity dsDNA Kit can be obtained from ThermoFisher, Product Code Q32851 or equivalent.
8. Zombie NIR dye can be obtained from BioLegend, Product Code 423105 or equivalent.
9. FAM assay ITR2 can be obtained from BioRad, Product Code 12014589.
10. HEX assay can be obtained from BioRad, Product Code 12014590.

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