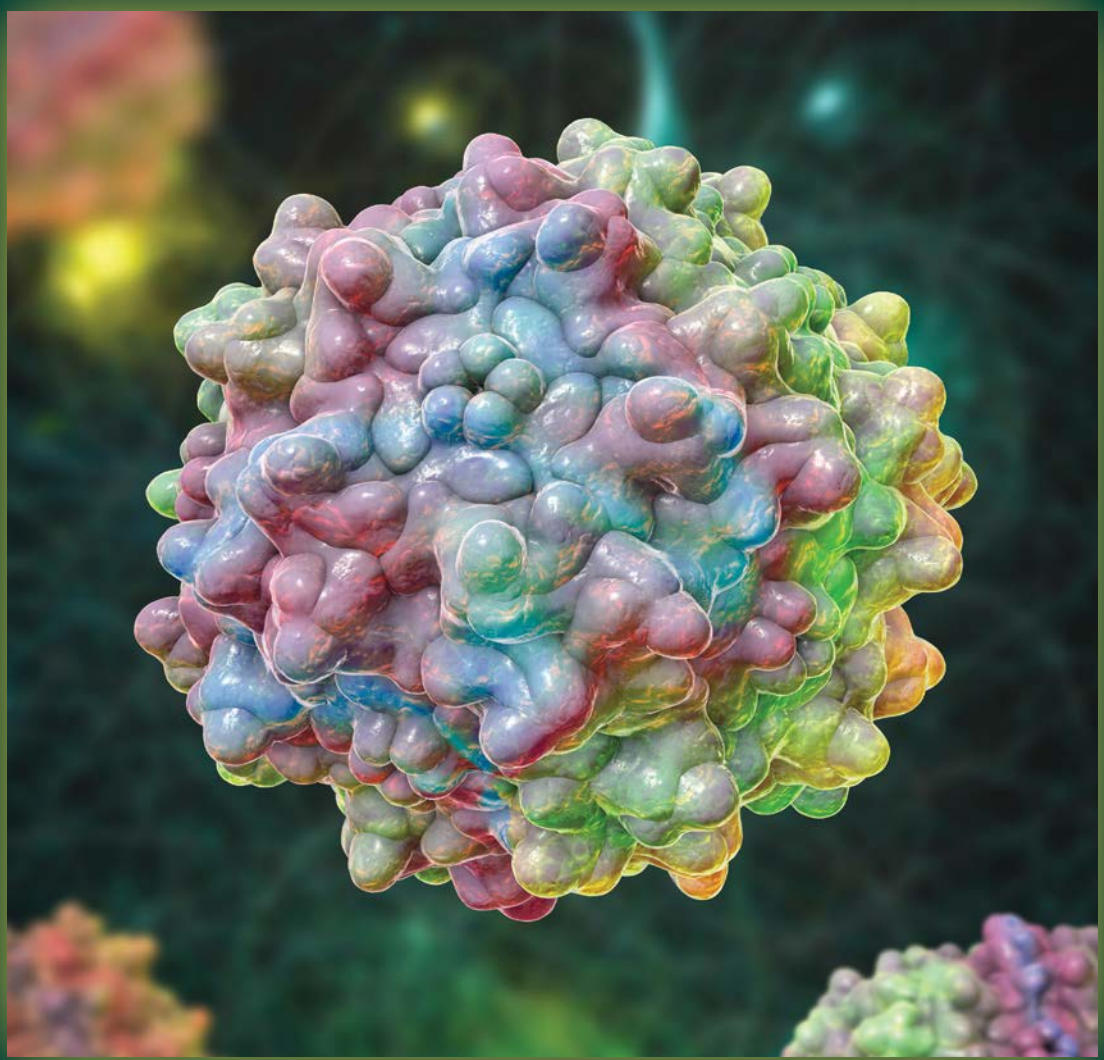
AAV Downstream Process and Product Characterization



Integrating Advanced Purification and Analytical Tools into the Workflow

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SPECIAL REPORT

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AAV Downstream Process and Product Characterization

Integrating Advanced Purification and Analytical Tools into the Workflow

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Adenoassociated virus (AAV) vectors are a leading platform for gene delivery in the treatment of many human diseases. Efficient production of high-yield, high-quality AAV vectors is essential for continued advancement of the gene-therapy field, which can deliver profound and curative outcomes for patients. AAV vector mediated gene delivery has been approved for treating inherited blindness and spinal muscular atrophy, and long-term therapeutic effects have been achieved in patients with other rare diseases, including hemophilia.

As the gene-therapy field rapidly expands, regulatory guidance is evolving to help ensure the safety of such complex therapies and driving the need for efficient and effective methods of process and product characterization. To ensure proper characterization and meet regulatory expectations for product quality and safety, vector production workflows must integrate advanced purification and analytical tools. As Figure 1 shows, vector quality and purity should be monitored throughout the entire workflow using a number of methods.

Regulatory guidance recommends monitoring mycoplasma and other contaminants in upstream processes, which span a set of unit operations from plasmid development through viral vector production. For downstream processes, guidance increasingly focuses on removal of empty or incomplete capsids and clearance of adventitious viruses in addition to residual host-cell proteins and host-cell/plasmid DNA. Lot-release test expectations are comprehensive, including demonstrated clearance of process-related impurities to ensure patient safety and product quality (see box above). The final release step is governed by specifications for residual host-cell DNA and plasmid removal.

Lot-Release Test Expectations

Identity
- Capsid/serotype and transgene

Strength
- Viral genome titer
- Total viral particles

Potency
- Infectious titer
- Functional analysis

Purity
- Host-cell protein (HCP) and DNA clearance
- Residual bovine serum albumin (BSA), endonucleases, ligands, plasmids, transfection reagents, and detergents
- Genome integrity
- Protein purity
- Aggregation status
- Ratio of full to empty capsids

Compendial Assays
- Appearance
- pH
- Osmolarity

Safety
- Absence of adventitious/replication-competent viruses
- Sterility (mycoplasma, endotoxin, bioburden)

Here, we describe advanced purification strategies for AAV capture and polishing steps along with analytical tools that can be integrated seamlessly into vector production processes for simplified upstream and downstream workflows.

Mycoplasma Testing

A known contaminant of mammalian cell cultures, mycoplasma can affect the safety, quality, and efficacy of biotherapeutic products. Given the risks associated with this contaminant and the need to comply with regulatory requirements, it is critical to

Figure 1: A typical adenoassociated virus (AAV) vector production workflow showing key points in the process at which analytical methods are integrated

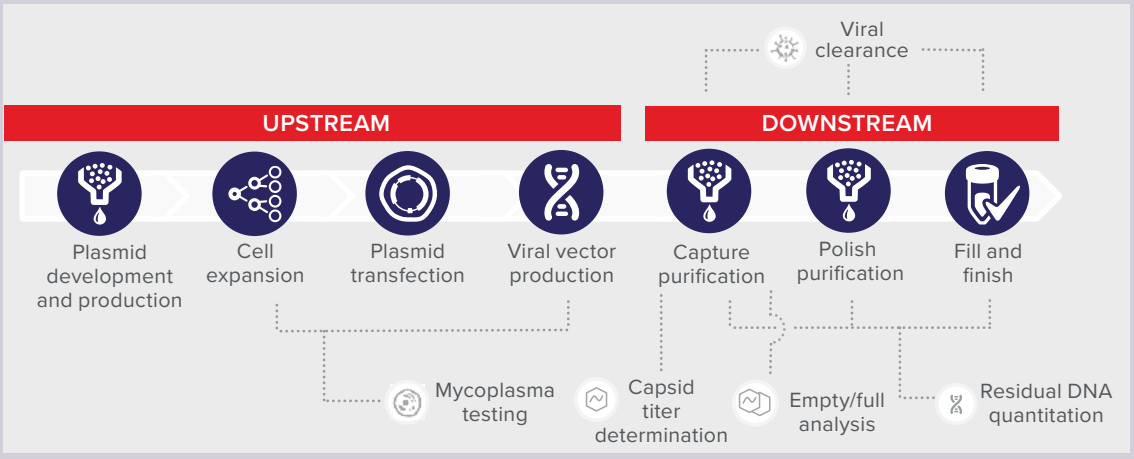
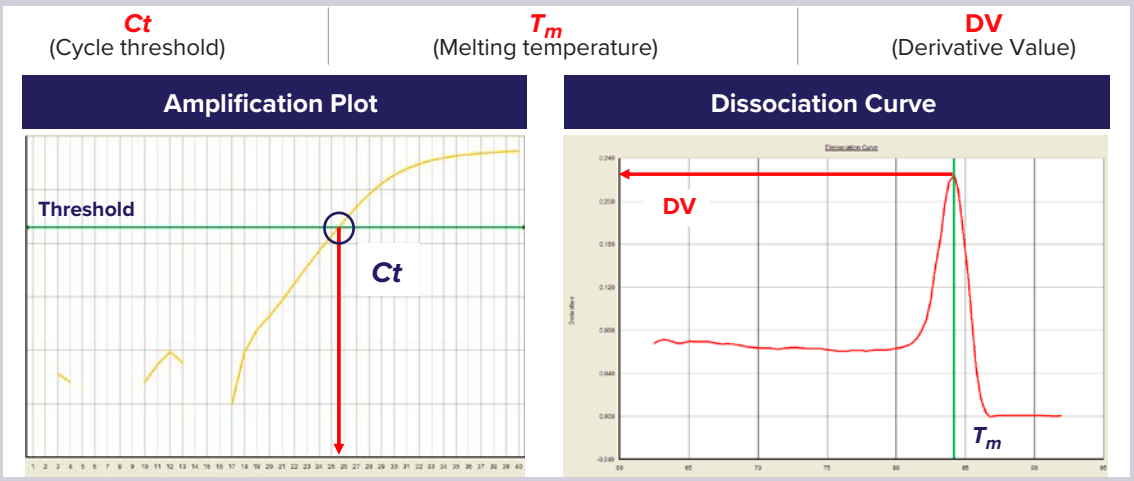


Figure 2: Analytical parameters



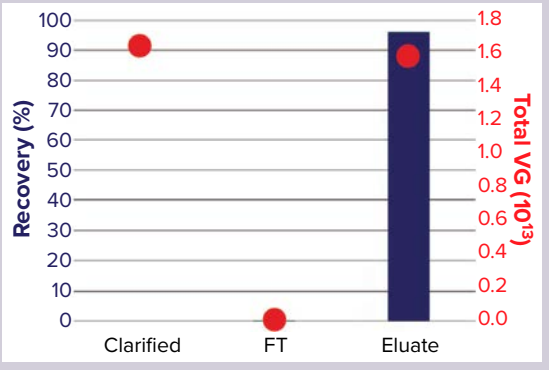
ensure that upstream cell cultures are free of mycoplasma before feeding bulk harvest material into a downstream process.

Historically, the only test method for mycoplasma accepted by regulatory agencies was based on a 28-day cell culture. Such a long testing cycle could delay lot disposition, so the industry has moved toward using nucleic-acid amplification techniques (NATs) as a faster alternative. One example with an extensive regulatory acceptance track record is the Applied Biosystems® MycoSEQ™ mycoplasma detection system, which incorporates a highly sensitive and specific real-time polymerase chain reaction (PCR) assay that delivers mycoplasma contamination results in under five hours.

Because the MycoSEQ system was designed to fulfill regulatory requirements, more than 40 marketed biologic manufacturing processes have received acceptance from regulatory agencies worldwide to use this assay for testing applications across multiple therapeutic modalities, including gene-therapy applications (following validation, regulatory filing, and review). More than 30 customers are now in the process of validation and regulatory submission using the MycoSEQ assay as a lot-release test.

The MycoSEQ system can detect more than 90 different species of mycoplasma and related species. Sensitivity has been demonstrated in both internal and external validations showing that it can detect fewer than 10 mycoplasma genome copies or colony-forming unit (CFU) equivalents. That is the required sensitivity for mycoplasma NAT detection methods according to regulatory guidelines. For a sample to be considered positive for mycoplasma, it must meet three objective analysis parameters established during validation (Figure 2):

Figure 3: Purification of a synthetic AAV serotype using the AAVX resin; recovery (blue bar) and yield are >90%.



• cycle threshold (*Ct*), a measure of the target DNA level at the start of the PCR reaction

• derivative value, a measure of specific amplicon quantity generated during the PCR reaction

• melting temperature (T_m), a measure of amplicon size and base composition that is known for mycoplasma using this assay.

A unique discriminatory positive control (DPC) significantly reduces the possibility of false-positive and false-negative results. It is used to confirm extraction and PCR performance without risk of false positives from accidental cross-contamination. The DPC facilitates amplification of a modified PCR product with a T_m well outside the range of real mycoplasma amplicons. With post-PCR melting analysis, users can determine whether samples contain mycoplasma or a positive control. Because a DPC maintains the same extraction behavior as genuine mycoplasma DNA, it can be used as a sample-extraction positive control for spiking test samples without risk of unrecognized cross-contamination. In addition, the DPC can be used as a surrogate for mycoplasma DNA during method optimization and early qualification, mitigating the requirement to use live mycoplasma and thus serving as a precaution against introduced mycoplasma contamination at early stages of method adoption. For validation, mycoplasma genomic DNA provides a suitable alternative to live mycoplasma.

The MycoSEQ system's AccuSEQ™ real-time detection software generates automated presence or absence results during data analysis. Automated calls are made based on the *Ct*, T_m, and derivative values of a test sample and inhibition control, as previously described. The software's security, audit, and electronic-signature capabilities are designed to enable 21 CFR part 11 compliance required in a good manufacturing practice (GMP) environment.

Capture Purification

The most common first purification step in an AAV downstream process is capture of capsids from cell lysate using affinity chromatography. POROS® CaptureSelect® affinity resins for AAV purification offer broad selectivity, high capacity and elution recovery (>90% recovery and 90% purity from a single step), and excellent scalability (Figure 3). The affinity ligands immobilized onto POROS beads are VHH camelid antibody fragments recombinantly expressed in yeast. Because their production process is free from animal-derived components, the resins are compatible with commercial processes. Two such ligands are serotype specific (AAV8 and AAV9), and a third ligand acts as a universal capsid affinity resin (AAVX). The AAVX resin serves as a platform solution for manufacturers developing therapies that include a range of serotypes.

The POROS backbone is a rigid, polystyrene-divinylbenzene–based solid support that allows for robust chemical stability and a linear pressure flow curve, independent of column diameter. The large pore structure reduces mass transfer resistance and results in an increased surface area, which in turn raises the binding capacity. The 50-µm bead size

Figure 4: Viral clearance capabilities of AAVX affinity resin; LRV = log reduction value

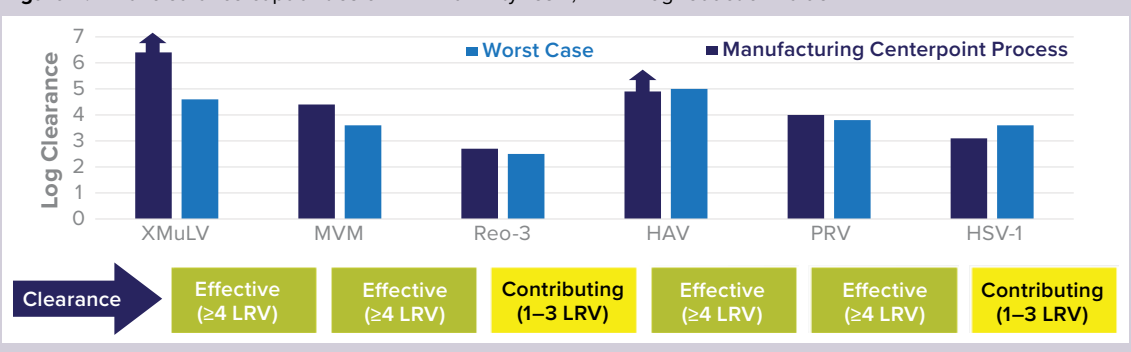
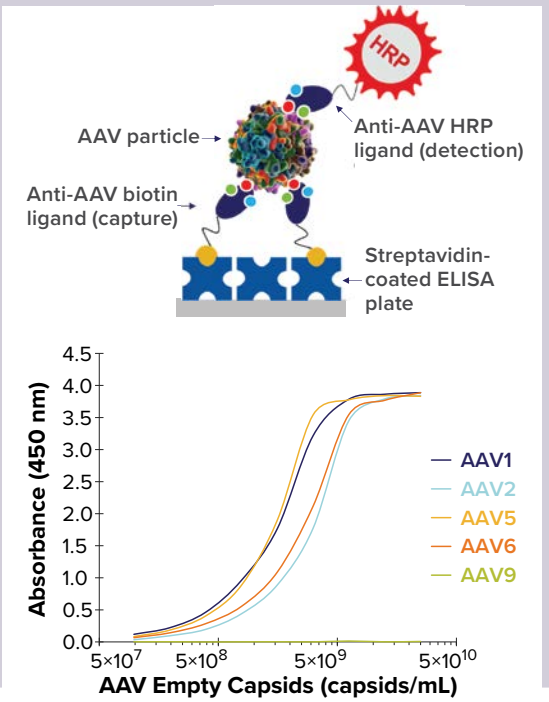


Figure 5: (TOP) Total AAV capsid enzyme-linked immunosorbent assay (ELISA) and (BOTTOM) results; HRP = horseradish peroxidase



gives increased resolution without compromising process efficiency.

Viral Clearance

In addition to host-cell and process-related impurity clearance, the AAVX resin has been demonstrated to provide effective viral clearance. Figure 4 shows robust clearance of model viruses achieved using AAVX affinity chromatography. The process was tested with a panel of six RNA- and DNA-based enveloped and nonenveloped viruses of different sizes. The AAVX resin achieved >4 log reduction of four of those and 1–3 log reduction of the remaining two viruses on the panel.

Capsid Titer Determination

CaptureSelect ligands also come in free conjugated forms. Biotinylated and horseradish-peroxidase (HRP)–labeled AAVX ligands can be used to develop enzyme-linked immunosorbent assays (ELISAs) for determination of total capsid titer. The method can be applied to both in-process and purified samples and used for monitoring the mass balance in harvest and capture unit operations.

Figure 5 illustrates use of AAVX-conjugated ligands in a highly sensitive total–capsid-titer ELISA for multiple AAV serotypes. Streptavidin-coated plates bind the biotinylated capture ligand, and the HRP-labeled ligand is used for detection. Standard curves of each serotype were prepared separately for AAV1, AAV2, AAV5, AAV6, and AAV9, then aliquoted onto the coated wells. Following a one-hour incubation, the wells were treated with a diluted preparation of the anti-AAVX HRP detection ligand and incubated for 10 minutes with a 3,3′5,5′-tetramethylbenzidine (TMB) substrate. The reaction was stopped by addition of acid. This method is intended as a starting point, and method optimization is always recommended. Under most conditions, it yields a valid assay covering the ranges of 1×10^8 and 1×10^{11} capsids/mL for most serotypes.

Table 1: Chromatography settings and mobile phases for analysis of empty and full capsids

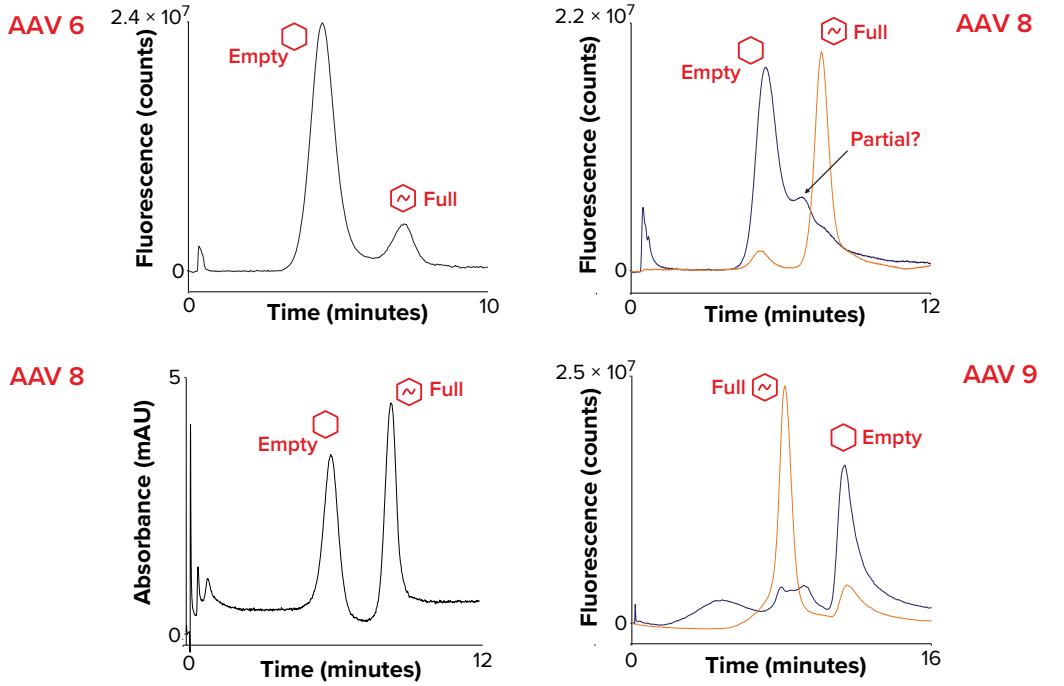
Column Format	4 × 50 mm or 2 × 50 mm
Detection	**UV:** Full (260 nm) and empty (280 nm) capsids **Fluorescence:** higher sensitivity, more accurate quantitative data, full capsids only
Temperature	30 °C (temperature and pressure can affect capsid structure stability)
Mobile Phase: Salt Gradient	**A:** 20 mM Bis-Tris propane at pH 9.5 or 8.5 (depending on serotype) **B:** 20 mM Bis-Tris propane at pH 9.5 or 8.5, 1 M tetramethylammonium chloride or tetraethylammonium chloride (for better resolution with a higher background signal)
Mobile Phase: pH Gradient	**A:** 20 mM ammonium bicarbonate and 15 mM ammonium hydroxide at pH 9.2 **B:** 30 mM acetic acid and 15 mM formic acid at pH 2.8

Empty/Full Capsid Analysis

Another critical step in vector-production workflows is analyzing the ratio of empty to full capsids, which can be determined using a ProPac™ SAX-10 high-performance liquid chromatography (HPLC) column. Robust separation of full and empty particles enables users to determine the ratio following both affinity purification and polishing steps. The results can be used to confirm successful removal of empty capsids and provide a baseline for further downstream purification.

ProPac SAX-10 columns are packed with polymer resin coated with a hydrophilic layer that prevents unwanted hydrophobic interactions, and the grafted polymer chains carry strong anion-exchange functional groups. Either salt or pH gradient elution can be used. With a salt gradient, protein samples bind to the stationary phase through charge interaction and elute with an increase in the salt concentration. With a pH gradient, negatively charged AAV particles become neutral as pH

Figure 6: Salt (TOP ROW) and pH (BOTTOM ROW) gradient analysis of full and empty particles



decreases, so they elute from the column. Small differences between the isoelectric points (pI) of full and empty particles allow for such separations.

Either UV or fluorescence can be used for detection. With UV detection, information on full and empty capsids is provided by the intensity of signals at 260-nm and 280-nm wavelengths, respectively. The sensitivity of UV detection is lower than that of fluorescence detection, which also provides better quantitative data because it monitors the AAV capsid signal, derived from the tryptophan residue of the capsid protein.

Table 1 summarizes conditions and mobile phases recommended for salt and pH gradients. Baseline separation of empty and full capsids from different serotypes was achieved using those conditions with tetraethylammonium chloride as the salt (Figure 6, TOP ROW). The pH gradient also provided good baseline separation for both AAV8 and AAV9 serotypes (Figure 6, BOTTOM ROW).

Polishing Purification

A range of weak and strong POROS anion-exchange resins can be used for removal of empty capsids in polishing chromatographic operations. The 50-µm POROS base bead is made of polystyrene-divinyl benzene, which provides rigidity for a stable column bed and enhanced chemical stability. As described above, these resins provide high binding capacity and linear pressure-flow curves without compromising on resolution during scale-up.

Qu et al. described empty and full capsid separation using POROS HQ resin (**3**). Capsids purified by cesium chloride gradient were applied to a POROS HQ column and then eluted using a linear sodium-acetate gradient. Empty capsids, characterized by a higher absorbing A_{280} peak, eluted sooner than the full capsids, characterized by a higher absorbing A_{260} peak. When the empty and full capsids were combined at a ratio of 16:1 and applied to the same column with the same gradient conditions, the empty capsid peak presented first followed by the full capsid peak with baseline separation, demonstrating the resin's resolving power and providing a feasible and scalable process for AAV empty capsid removal.

Residual DNA Quantitation

Purification workflows for AAV processing must remove residual host-cell DNA and plasmid DNA impurities effectively. The World Health Organization (WHO) requires documented residual DNA per therapeutic dose to be <10 ng; the US Food and Drug Administration (FDA) requests that host-cell DNA should be as low as possible and that a highly sensitive method be used to determine DNA levels. The agency also encourages companies to conduct vigorous clearance studies throughout their

Figure 7: Standard curves of (LEFT) plasmid DNA with a kanamycin-resistance (KanR) gene and (RIGHT) human embryonic kidney cell (HEK293) assays

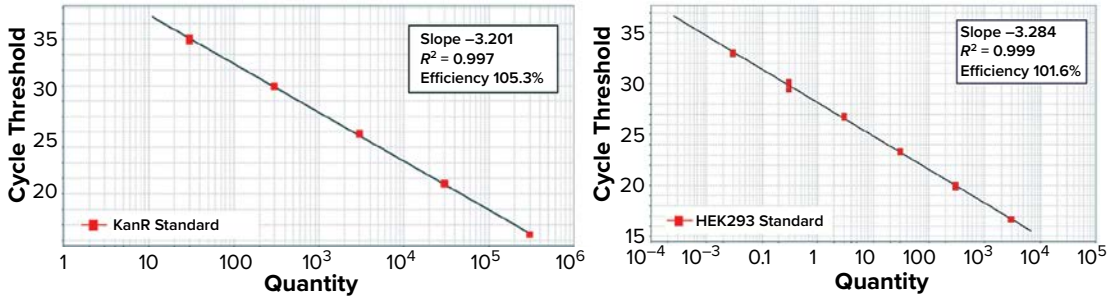


Table 2: Residual DNA kit specifications used in common AAV production platforms

Specification	Kanamycin-Resistance–Gene Plasmid DNA Kit	HEK293 DNA Kit	*Sf*9 Baculovirus DNA Kit
Linearity	$R^2 > 0.99$	$R^2 > 0.99$	$R^2 > 0.99$
PCR Efficiency	100% ± 10%	100% ± 10%	100% ± 10%
Precision	≤10% CV	≤10% CV	≤10% CV
LoD	15 copies	30 fg	30 fg
LoQ	30 copies	300 fg	300 fg
Range	300,000 copies to 30 copies	300 fg to 3 ng	300 fg to 3 ng

PCR = polymerase chain reaction **Sf9** = *Spodoptera frugiperda* cell line 9 **LoD** = limit of detection **LoQ** = limit of quantitation

downstream processes both to demonstrate removal of the vast majority of DNA from product streams and to monitor for process deviations.

To confirm successful removal of host-cell DNA according to regulatory requirements for lot-release testing, AAV product developers should incorporate a simple and reliable analytical kit that measures residual DNA into their workflows. To monitor DNA clearance, quantitation can be performed at different stages throughout downstream processing, from cell-culture harvest through to the final drug substance.

The Applied Biosystems resDNASEQ™ quantitative DNA system provides an effective, fully integrated, and all-inclusive approach to determining levels of residual DNA. Sample preparation, a sensitive and accurate DNA quantitation method, highly characterized DNA standards, all necessary reagents, and data analysis are included. Residual DNA testing assays are available for a number of cell lines, including two commonly used systems for AAV manufacturing: human embryonic kidney (HEK) 293 host-cell DNA quantitation and simultaneous quantitation of both *Spodoptera frugiperda* (*Sf*9) host-cell DNA and baculovirus vector DNA.

To address the need to quantitate residual vector DNA in AAV production using HEK293 cells, the resDNASEQ kit was developed for quantitative plasmid DNA with a kanamycin-resistance (KanR) gene.

The resDNASEQ assays offer ultrahigh sensitivity with a limit of quantitation (LoQ) down to 0.3 pg/reaction for HEK293 DNA, *Sf*9–baculovirus, and 30 copies for plasmid DNA with the KanR gene. A rapid, streamlined workflow with optional automated sample preparation provides results in under five hours. Table 2 summarizes the specifications of resDNASEQ quantitative HEK293, *Sf*9, baculovirus DNA, and quantitative plasmid DNA KanR gene kits. Standard curves in Figure 7 demonstrate the high sensitivity and broad dynamic range of two of those assays as examples.

The resDNASEQ quantitative plasmid DNA kanamycin-resistance–gene kit was designed to detect and quantitate the vast majority of currently known kanamycin-resistant–gene families. Careful analysis of conserved regions led to creation of a multiprimer assay to target all alleles with the same sensitivity. A number of common commercially relevant plasmids were spiked into the matrix in quantities of either 100 or 100,000 copies, then manually extracted and quantified using a resDNASEQ quantitative plasmid DNA kanamycin-resistance–gene kit. Each plasmid was recovered at >85%, and similar results have been observed for resDNASEQ quantitative HEK293 DNA kits.

To demonstrate specificity of the resDNASEQ quantitative plasmid DNA kanamycin-resistance–gene kit, a series of experiments used unrelated DNA directly spiked into the PCR reaction (Table 3) and

Table 3: Standards and reagents tested in the kanamycin-resistant plasmid kit exclusion panel (Figure 8)

Crossreactant H	3-ng spike *Escherichia coli* DNA
Crossreactant I	3-ng spike human embryonic kidney (HEK293) cell DNA
Crossreactant J	3-ng spike adenovirus 2
Crossreactant K	3-ng spike murine leukemia virus (MuLV)
Crossreactant L	3-ng spike rabbit antibody
Crossreactant M	3-ng spike bovine antibody
Crossreactant N	3-ng spike chicken antibody
Crossreactant O	3-ng spike pAV1 (AAV genome)
Crossreactant P	3-ng spike Madin–Darby Canine Kidney (MDCK) cells
Crossreactant Q	3-ng spike Chinese hamster ovary (CHO) cells
Crossreactant R	3-ng spike murine myeloma (NS0) cells
Crossreactant S	3-ng spike *Pichia pastoris*
Crossreactant T	Ampicillin (300,000 copies)
Crossreactant U	Blasticidin (300,000 copies)
Crossreactant V	Hygromycin (300,000 copies)
Crossreactant W	Puromycin (300,000 copies)
Standard	428std6 15 copies
NTC	No template control

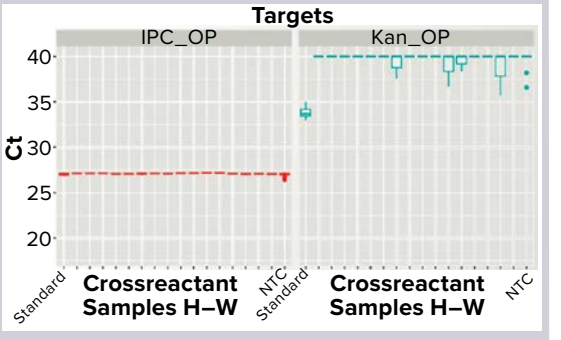
included an internal PCR control to monitor PCR inhibition (Figure 8). All reactions provided the same *Ct* value, which indicated that the PCRs performed as intended. None of the DNA in the exclusion panel was amplified by the resDNASEq kanamycin assay. The first lane in the graph shows amplification of the DNA standard included in the kit at 15 copies (*Ct* ≈ 34). Most of the other reactions provided a nonpurification curve; others provided a range of *Ct* values well above the limit of detection (LoD).

Enabling Technologies

In addition to the AAV gene therapies approved thus far, a robust pipeline of clinical candidates reinforces the potential of this modality to treat a wide range of diseases caused by single-gene defects and more complex conditions such as cancer, neurological, cardiovascular, and infectious diseases. AAV vectors are likely to remain a gene-delivery mechanism of choice for many such treatments.

Sustained growth of the AAV industry sector and the safety of gene therapies both depend on a combination of high-quality purification tools and analytical methods that are orthogonal to titer and recovery testing and are capable of meeting evolving regulatory requirements. Here, we have outlined analytical methods that work in conjunction with both capture and polish chromatography steps to

Figure 8: DNA specificity exclusion-panel test results from a kit for plasmid DNA with a kanamycin resistance gene, including standard at limit of detection (LoD) of 15 copies



create a simplified and streamlined AAV downstream process. Workflows that incorporate these advanced technologies will help ensure the quality and safety of gene therapies for their intended recipients and build confidence in this powerful therapeutic modality throughout the healthcare infrastructure.

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