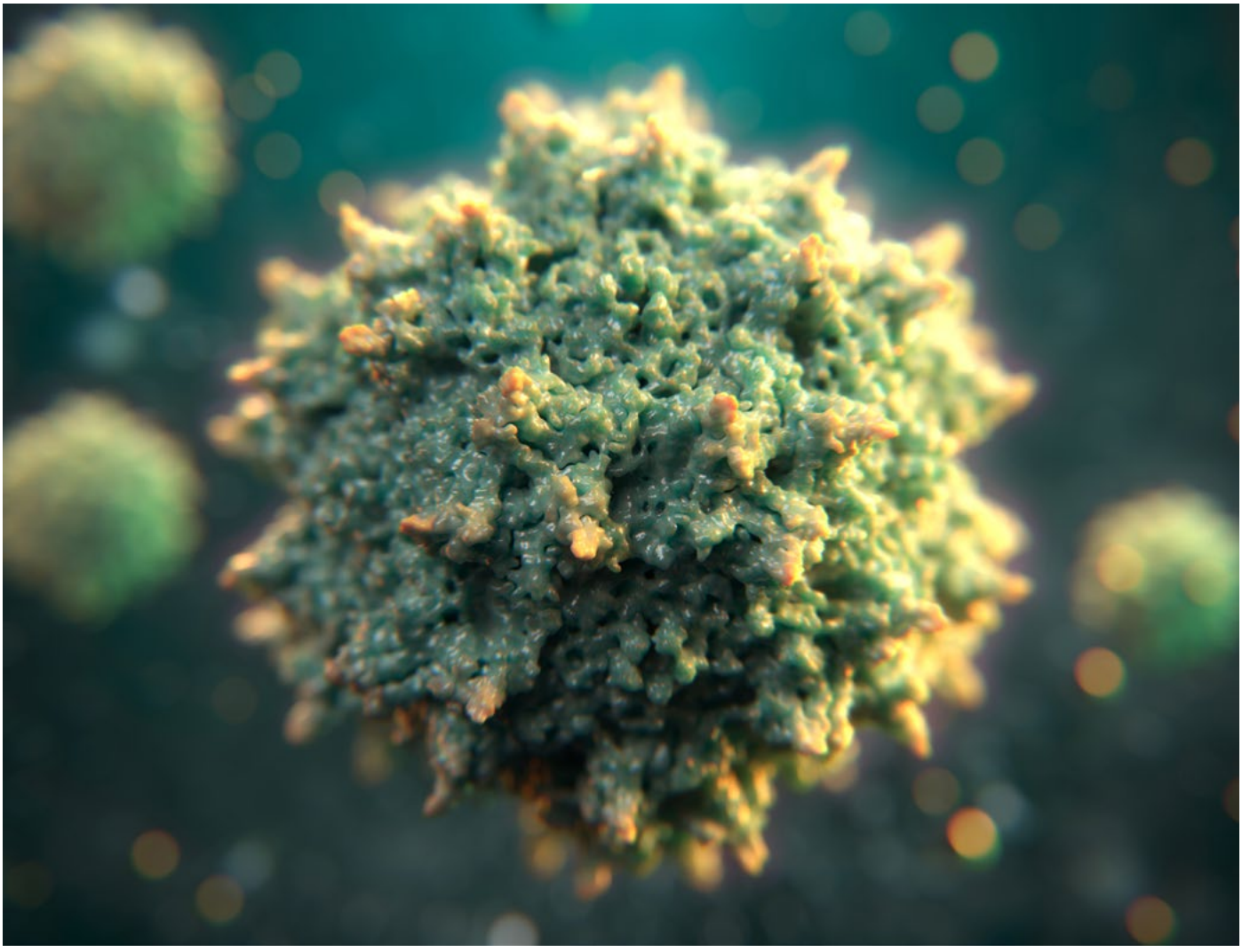
SCIEX

Empowered by nature driven by analytical innovation

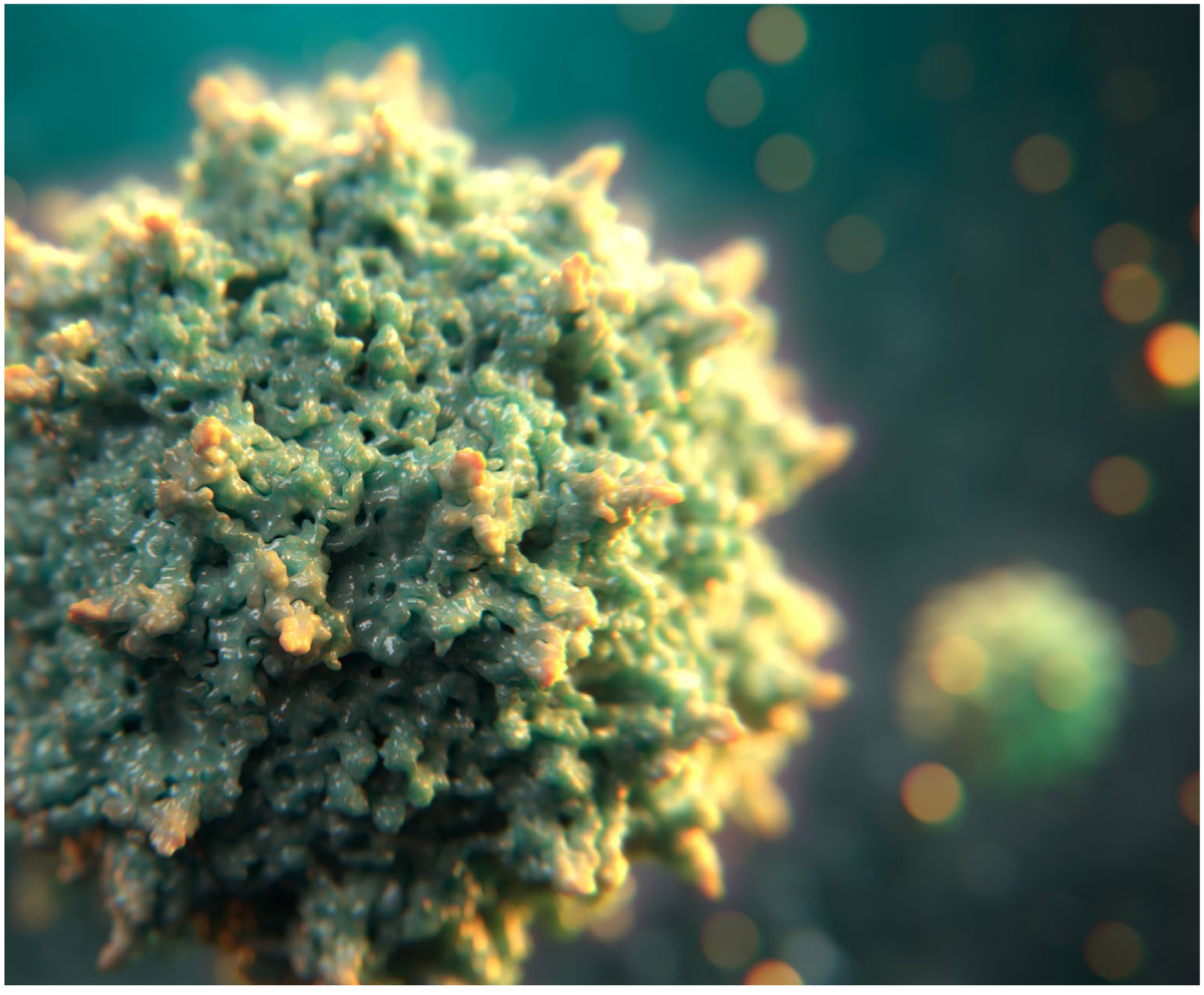
Advanced analytics for superior viral vectors



danaher

The power of precision

Contents

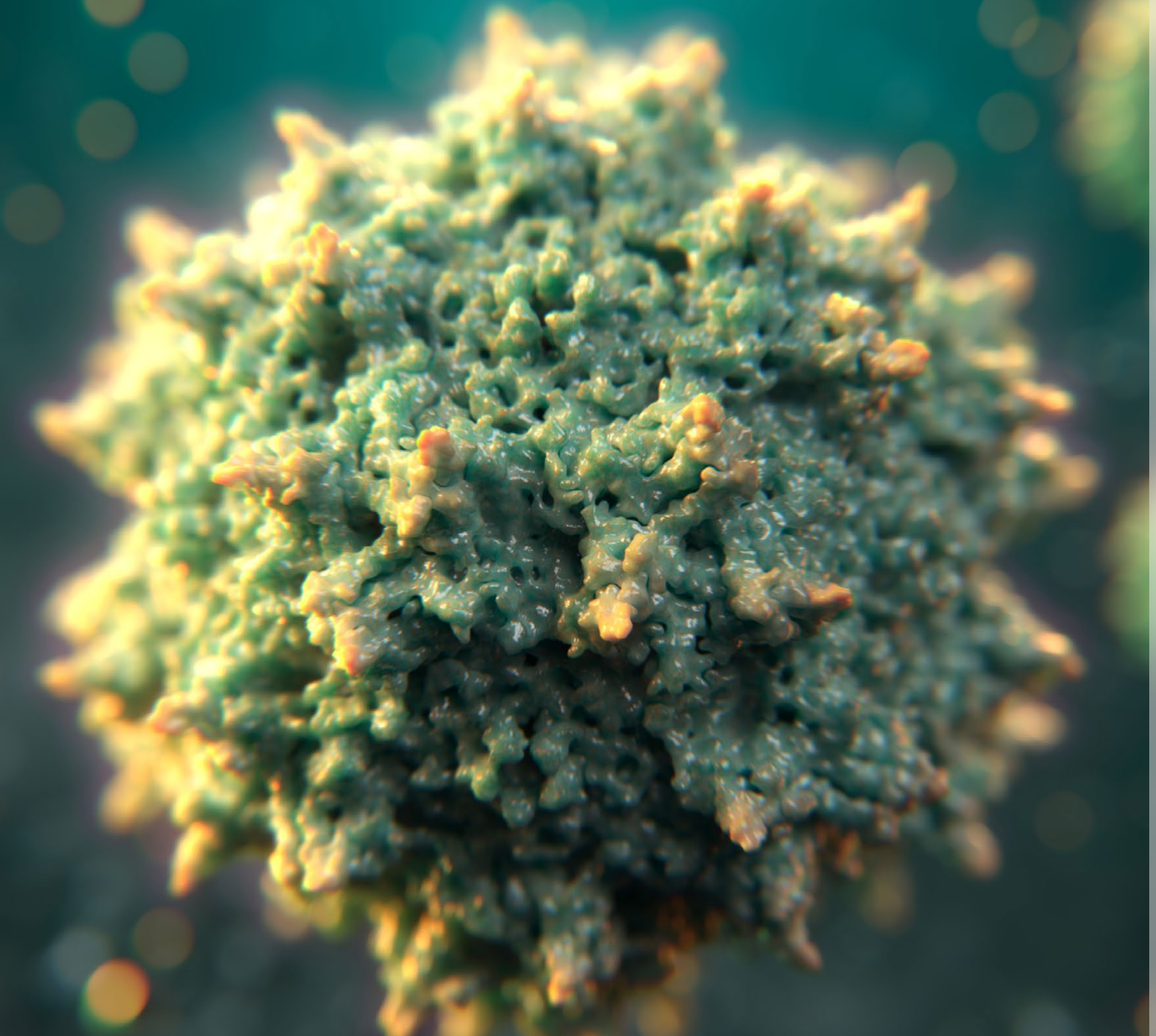


Introduction

Viral vectors are successfully used as gene therapy vehicles and vaccines.

Their development means overcoming many challenges. From plasmids to complex viruses, intuitive, innovative, and informative analytical solutions let you stay focused on the science.

Work with an evolving partner to realize the full potential of your viral vector-based drugs and discover how you can break through analytical boundaries with streamlined technology.



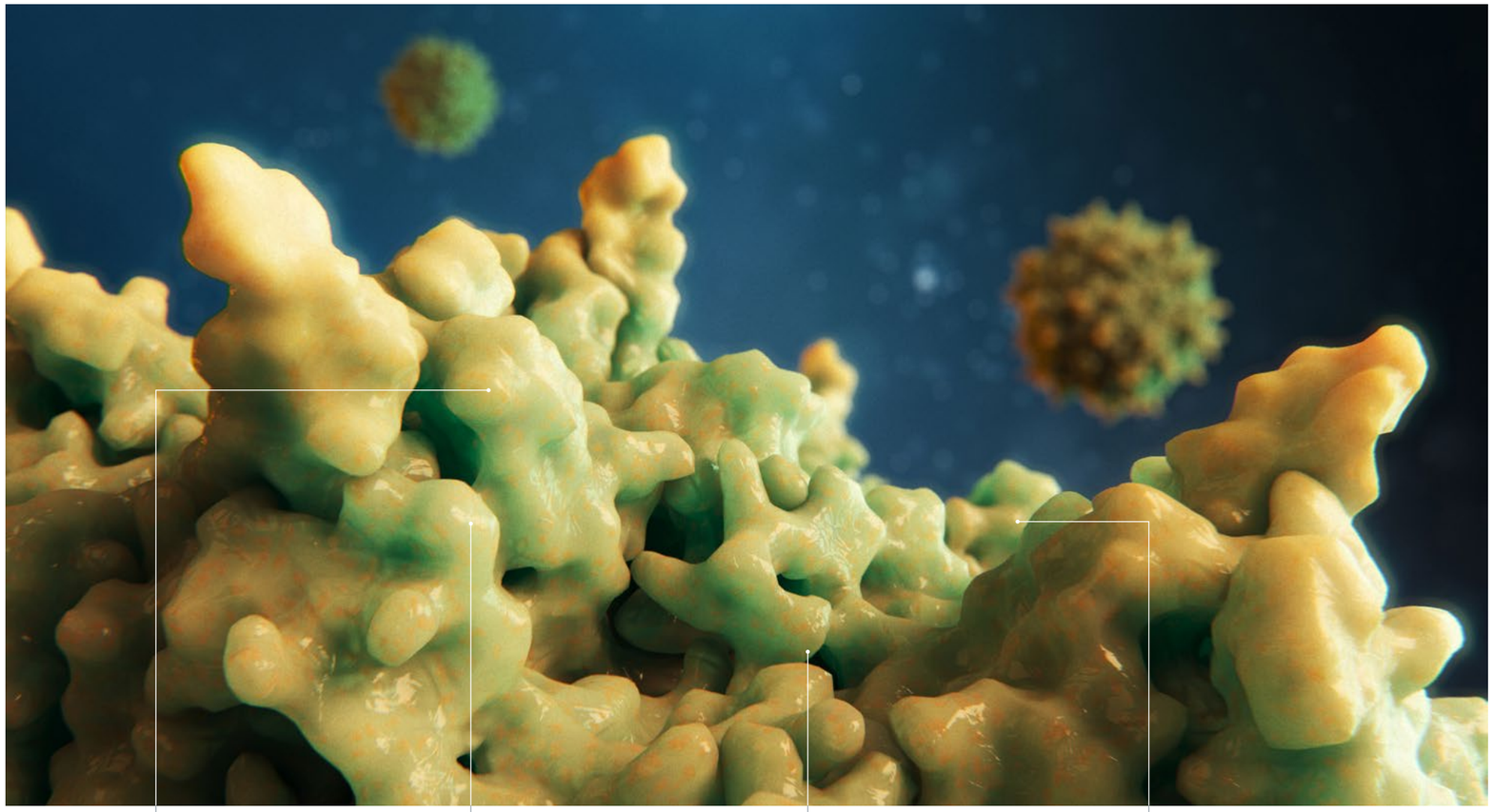
01

Viral vectors

Viral vectors

Viruses are believed to have been around for several billion years already. It is nature's optimized way to deliver genetic material into cells.

The outlook of curing genetic diseases through virus-enabled gene therapy, rather than treating symptoms, sparked a mind shift in (bio)pharmaceutical research. In addition, the ability for transient transgene expression has been investigated for vaccine usage, with the first viral vector vaccine for human use being approved in 2010. Apart from adenoviruses (AVs), adeno-associated viruses (AAVs), lentiviruses (LVs), and more, engineered viruses and synthetic virus-like particles are studied for their potential. Fully understanding viral vector drugs is of utmost importance to ensuring the quality and safety of future medicines.



"Understanding AAV critical quality attributes is imperative for the development of AAV particles usable in gene therapy. CE plays a crucial role in monitoring the safety and efficacy of AAV particles and can be adopted from early-stage discovery to manufacturing. The PA 800 Plus system from SCIEX equipped with the laser-induced fluorescence (LIF) detector provides reproducibility while offering the sensitivity and resolution required for analyzing AAV assembly stoichiometry and integrity of the genome cargo."

Andrea Martorana (PhD)
Lead Scientist Analytical Development, AviadoBio Ltd

Intact capsid protein characterization

Determine protein integrity and purity of capsid proteins and achieve high-level information on post-translational modifications (PTMs).

Capsid protein peptide mapping

Assess protein sequences in depth and fully understand PTMs of viral vector proteins.

Full-and-empty capsid ratios

Understand the quantity of viral vectors with intact genomes to assess product quality.

Viral protein purity

Assess protein integrity and purity of your viral vectors to help ensure vector potency.

Proteome profiling

See beyond the expected and determine effects on the entire proteome in an unbiased manner when modifying gene expression.

Viral genome

Achieve definite answers on the integrity and purity of your viral genomes to enable desired vector potency, immunogenicity and transduction efficiency.

Residual host cell nucleic acids

Determine quantities and lengths of process-related impurities to assess the impact on vector potency and the risk of undesired immunogenicity.

Host cell protein (HCP) ID

Perform identification (ID) and quantitation of proteins derived from packaging and producer cell lines regardless of the cell line and availability of antibodies against host cell protein targets.

HCP monitoring

Robustly monitor hundreds of impurities from various packaging and producer cell lines.

Expert Q&A:

Comprehensive AAV analysis with CE

AAVs are complex drugs, consisting of a protein shell—the capsid—and a single-stranded desoxyribonucleic acid (DNA) genome, including the desired transgene. Here, Dr. Jane Luo, an expert in molecular biology, answers pressing questions on the usage of capillary gel electrophoresis (CGE) for the characterization of critical quality attributes (CQAs) of AAVs.

How much sample do you need to perform the comprehensive AAV analysis with CE?

For the comprehensive AAV analysis, we performed a capsid protein analysis with CE sodium dodecyl sulfate (CE-SDS) and the genome analysis with CGE using the BioPhase 8800 system. We then determined the full-and-empty ratio based on genome and protein titers. For both assays, we used 30 µL to 40 µL of the sample with a titer in the range of ~1x1011 to ~1x1013 gene copies (GC)/mL.

What is the linear dynamic range for the genomic titer determination by CE?

The linear range for the method I worked on ranged from 1×1010 GC/mL to 2×1013 GC/mL. This is a linear dynamic range of 3.3 orders of magnitude and was suitable for the samples we analyzed.

Do you need eight points for the calibration curve to determine sample titers?

The short answer is no; you do not need to have eight points. From my perspective, there are two aspects associated with this question. One is linearity and the other one is detection range. For establishing linearity, the ICH guidelines from the 'International Council for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use' (ICH) recommend using a minimum of five concentration endpoints. For the dynamic range, the ICH guidelines recommend the range to cover the test concentration and up to 30% above and below the test concentration. The method I presented in my webinar covers about three orders of magnitude, which is suitable for most AAV samples.

You mentioned a viral protein (VP) variant peak. Can you explain what this peak is?

Absolutely. The VP3 variant peak, sometimes also called VP3 prime (VP3'), is a shorter version of the VP3 protein that is derived from an alternative translation initiation site. For further information on the identification of the VP3 prime variant, I suggest the following publication from a research group in Japan [Human Gene Therapy. 32(21–22):1403-1416]. This paper explains that the translation initiation site at methionine (M) 203 can be skipped, and an initiation at M211 led to an eight amino acid shorter protein. As you can see from my results, the VP3 variant is very well separated from VP3, demonstrating the great resolution of the CE method. In addition, the detection of a VP3 fragment with liquid chromatography coupled to ultraviolet detection and MS (LC-UV-MS) was described. This fragment was linked to the hydrolysis of the VP3 at the C-terminus, caused by the low pH of the mobile phase and the high temperature used for the column oven during the liquid chromatography analysis, which are standard settings needed for LC-UV-MS analysis. Scientists using liquid chromatography (LC) for analyzing capsid proteins should be aware of this and might want to consider complementary evaluation with CE.

Which signal-to-noise ratio did you use for determining the lower limit of quantitation (LLOQ)?

We follow the ICH guidelines. For the determination of the LLOQ, the signal-to-noise ratio was ten or slightly above ten.

Do you need specific software for calculating the full and empty ratio?

You do not need special software to calculate the full and empty ratio. It is a simple division of the genome titer by the capsid titer. A standard calculator or Microsoft Excel will be fine.

Are you aware of full-and empty capsid assessments using ratios based on results from enzyme-linked immunosorbent assays (ELISAs) and polymerase chain reaction (PCR)? Can you comment on the differences between that method and yours?

Yes, there are publications for which a ratio calculation based on capsid titer from size exclusion chromatography (SEC) or ELISA and the genome titer from qPCR was used for determining full and empty ratios. These ratios rely on two vastly different methodologies, and therefore, data will have compounded variability. That is one downside to consider. In addition, the genome titer from qPCR methods often targets only the regions of inverted terminal repeat (ITR) sequences. This can lead to overestimating the genome titer since capsids with partial sequences or partial genomes, which contain the ITR but not the gene of interest, will be considered. With the presented method, we clearly separate the intact genome from the partial genome and the small size impurities and, therefore, avoid overestimation of the genome titer.

Certain PCR workflows use DNAse or benzonase treatments before samples are analyzed. Do samples need any pre-processing steps prior to CE analysis?

My recommendation is to do a simple extraction of the nucleic acid using commercially available kits and heat the sample to avoid secondary structure formation prior to CE analysis. Since we do not need to rely on amplification for CE-based genome integrity analysis while achieving high sensitivity with fluorescent dye and laser-induced fluorescence detection, a pre-processing step is not required. You can add a benzonase treatment step and subsequent inactivation and removal of benzonase before nucleic acid extraction. Comparing the results of benzonase-treated to non-treated samples helps to decipher the amount and size range of nucleic acid impurities present outside of the AAV capsid.

Can you elaborate on the time needed for different analytical techniques in comparison to your method?

The exact time requirements for techniques, such as PCR/ELISA, electron microscopy (EM), analytical ultracentrifugation (AUC), etc., will depend on the specific setup being used. I presented estimates for each technique in my webinar, which are based on published literature. From the comparison, you can see that it will take 2-3 workdays to perform comprehensive CQA analysis using a combination of techniques and instrumentation, while these parameters can be assessed within a typical workday using a single CE platform instead.

Read Q&A blog

Dr. Jane Luo
Senior scientist for Cell and Gene Therapy Applications in the Strategic Technical Marketing team at SCIEX.

She earned her PhD in Biochemistry from the City University of New York, received postdoctoral training in Molecular Biology and Cell Biology at Weill Cornell Medical College and Harvard Medical School and conducted cancer research as an assistant adjunct professor at UC Irvine. In 2002, she moved to industry to develop capillary electrophoresis-based products and applications.

Intact viral protein characterization

With AAVs, 3 viral proteins (VPs) build the capsid. In the case of non-enveloped viruses, the capsid is directly interacting with the host cells. Hence, integrity and PTM profiles of VPs are important quality criteria that can affect viral uptake. Chromatographic separation of VPs can be difficult to achieve, however, due to their similar physical properties. In addition, low-abundance protein impurities can be missed.

- Ensure the integrity of your capsid proteins with high-quality accurate mass data and intuitive acquisition software
- Set new frontiers for intact protein and impurity characterization with 3D visualization options for deconvoluted data
- Obtain relevant information on identities and quantities of proteoforms based on time-resolved deconvolution

Obtain more information with increased ease using 3D deconvoluted data

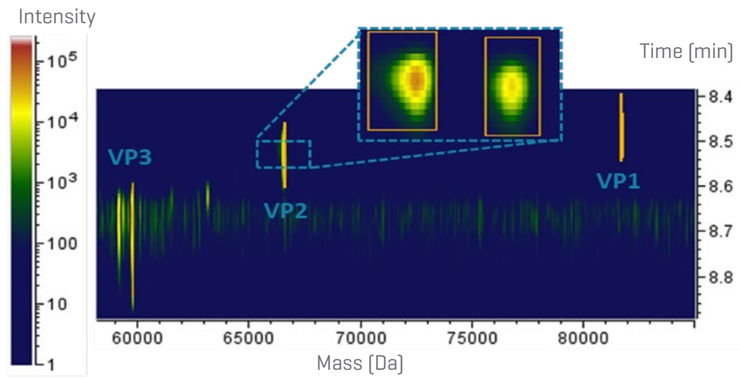


Figure 6: 3D heatmap of AAV8 capsid proteins cells showing the intensity, the retention time (RT) and the molecular weight (MW) using scan-by-scan, time-resolved deconvolution in Biologics Explorer software.

Quantities

Protein	Avg. Mass	RT	Volume	Volume [%]
V1 + Acetyl	81666.5	8.46052	1152.98	0.191584
V1 + Acetyl + Phospho	81746.8	8.46571	147.446	0.0245004
V2	66517.2	8.51561	24419.6	4.05768
V2 + Phospho	66597.3	8.52536	15914.9	2.6445
V3 + Acetyl	59804	8.66032	560177	93.0817

Figure 7: Results table of identified AAV8 capsid proteins with PTMs. Average MW, RT, and calculated volume based on 3D deconvolution is shown using Biologics Explorer software.

Discover more details in the technical notes about AAV analysis using the ZenoTOF 7600 system and the X500B QTOF system

Read technical note **ZenoTOF 7600 system**

Read technical note **X500B QTOF system**

Viral protein peptide mapping

Sequence confirmation and identification of low-abundance PTMs require a deeper look into the viral proteins. A peptide-mapping approach can provide information on product quality attributes (PQAs) and CQAs. Low sample amounts, however, are a challenge for analytical assays. Additional challenges include the identification of deamidation-derived isomers and fragile PTMs that can affect the charge heterogeneity and, as a result, viral uptake.

- Obtain high protein sequence and fragment coverage despite limited sample quantities with highly sensitive, accurate mass data acquisition
- Identify PTMs and their locations—including glycosylations, sulfations, and phosphorylations—with excellent spectral quality
- Differentiate amino acid isomers and localize fragile PTMs with an intuitive alternative fragmentation technique

Achieve high MS/MS sequence coverage and fully understand challenging PTMs

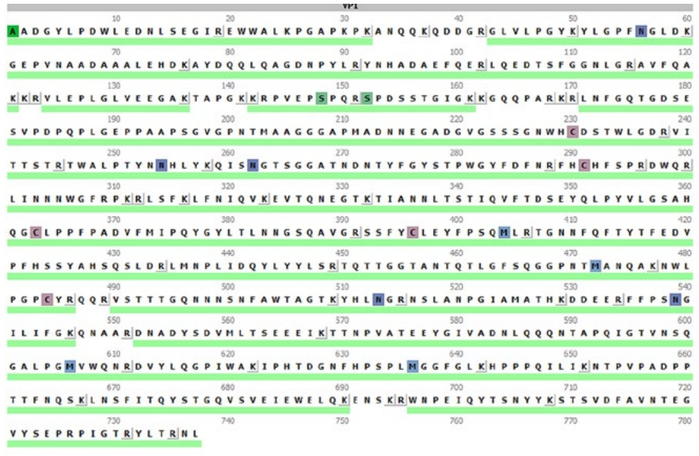


Figure 8: Sequence coverage map of AAV8 VP1. A nearly complete sequence coverage (94.7%) was obtained from a single injection of a tryptic digest of AAV8 using data-dependent acquisition (DDA) and processing with Biologics Explorer software.

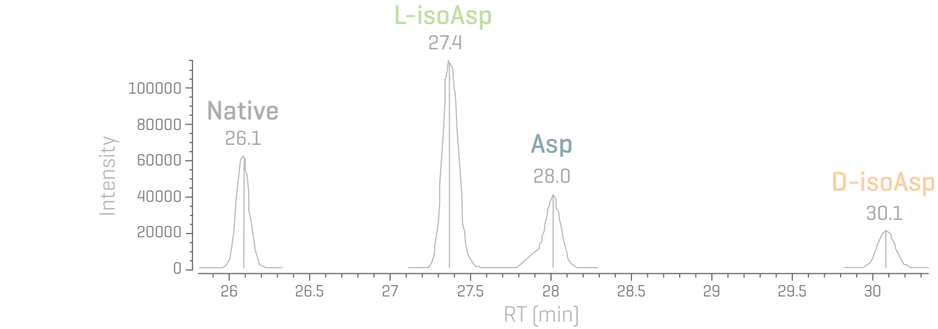


Figure 9: Identification of 3 deamidated species at N57 of the peptide YLGPFNGLDK (z = 2). Top: Extracted ion chromatograms (XICs) of the different deamidated species and the native peptide. Bottom: Zoom-in to EAD MS/MS data showing signature fragment ions for differentiation of aspartic acid (Asp) and isoaspartic acid (isoAsp) deamidated forms of N57, for example z5 - 57 for isoAsp and z5 - 44 for Asp.

Learn more about AAV PTM analysis in this technical note

Read technical note

Protein purity

Viral vector characterization for vaccine and therapeutic drug development includes assessing the viral proteins. While information on purity and protein ratios can be obtained with liquid chromatography-based methods, an orthogonal approach with CE is advantageous to avoid missing VP' forms. High resolving power, throughput capabilities, and reproducibility are important factors for protein purity assays.

- Determine protein-based titer with confidence and understand protein profiles and impurities using high separation power
- Reclaim your time with faster method development and the ability to run larger sample batches
- Avoid lengthy assay adjustments with a kit-based protein profiling workflow suitable across serotypes and viral vectors

Leverage excellent resolution and sensitivity for the characterization of VP proteins

Understand your viral proteins independently of the serotype

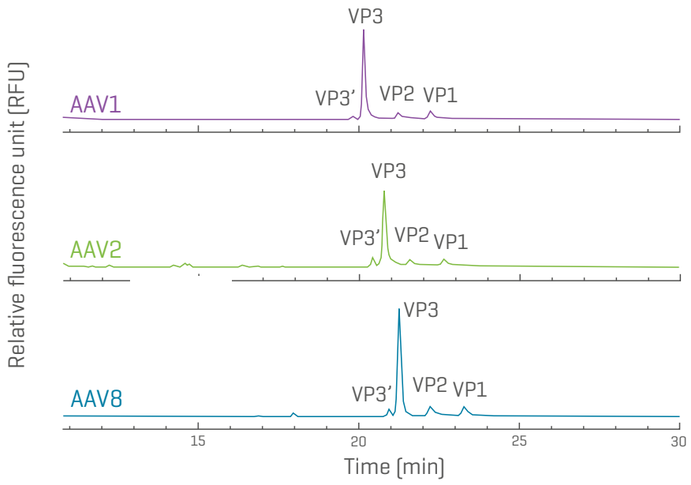


Figure 10: Separation of VP proteins from different AAV serotypes. Excellent resolution of VP proteins labeled with fluorescence dye was achieved with CE-SDS-LIF.

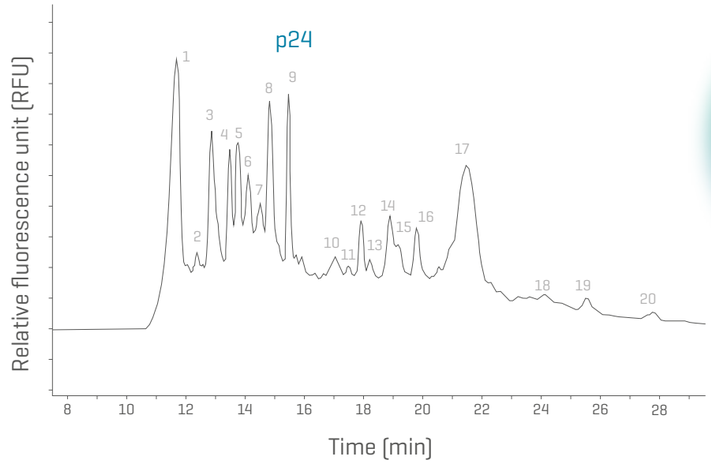
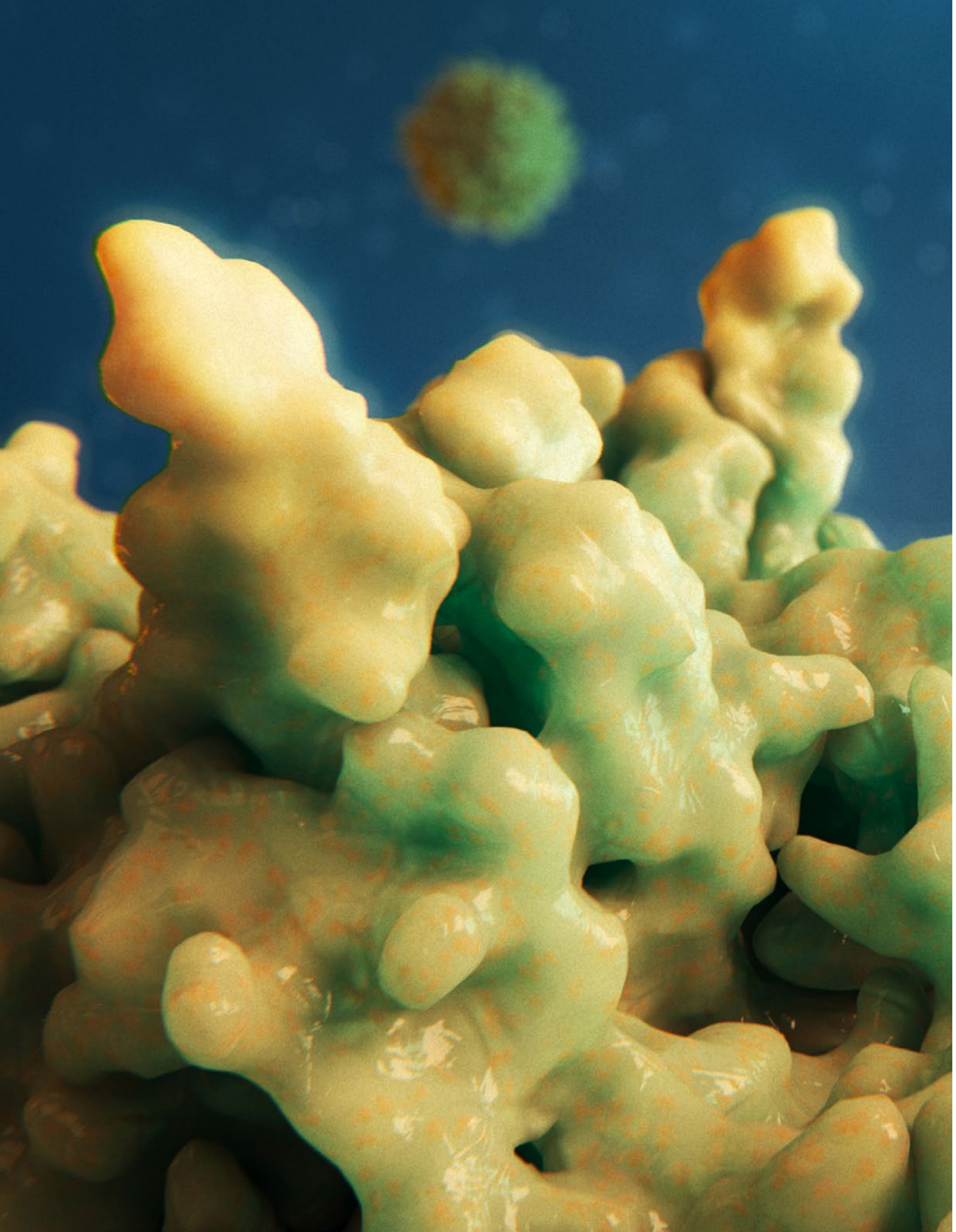


Figure 11: CE-SDS-LIF of Lentivirus proteome. 20 peaks were associated with the lentivirus sample. Peak 9 was identified as the p24 proteins based on spike-in experiments (not shown).

Discover more details in the technical notes about AAV capsid purity assessment and lentivirus protein analysis

Read technical note **AAV**

Read technical note **LENTIVIRUS**



Genome integrity and purity

The integrity and purity of the viral genome are CQAs that impact vector potency, immunogenicity, and transduction efficiency. However, the limited sizing capabilities of some analytical methods can pose challenges for assessing the entire viral genome, especially for viruses with larger genetic cargo. Furthermore, distinguishing between product-related impurities, such as degraded genomes and intact genomes, and ensuring their accurate quantitation can be challenging.

- Confidently determine genome integrity, genome titer, and impurities using high separation power
- Simplify viral vector genome analysis by avoiding lengthy assay adjustments with a workflow suitable across serotypes and viral vectors
- Run high-quality analyses smoothly and reproducibly with a kit-based turnkey solution
- Streamline data management through compatibility with data management systems

Assess genome integrity and nucleic acid impurities on one platform

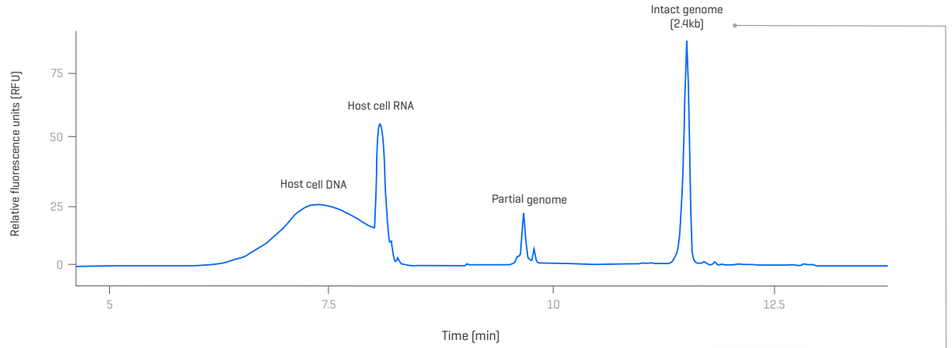


Figure 12: High-resolution genome integrity analysis of AAV8 with CGE and LIF detection. The intact genome of 2.4 kb was well separated from potential partial genomes and host cell ribonucleic acid (RNA), and DNA impurities.

Take a deeper dive in the technical notes about AAV genome analysis and lentivirus genome analysis

Read technical note **AAV**

Read technical note **LENTIVIRUS**

2.4 kb
genome fully separated from impurities

Full-and-empty capsid ratios

In addition to characterizing the genome and viral proteins, assessing the ratio of capsids with an intact genome (full capsids) vs. partial or empty capsids is necessary for comprehensive viral vector characterization. A variety of methods exist to determine this CQA, but the assays can have limitations. For example, some assays are cumbersome, must be adjusted for each serotype, or require high levels of expertise, or provide limited understanding of partially filled capsids.

- Take back your time by assessing multiple CQAs with high-quality data on a single platform with kit-based assays
- Determine genome integrity, capsid proteins, and full-and-empty ratios, including partial capsids with serotype-independent workflows
- Cover your compliance needs through compatibility with common data management systems

Take back your time by assessing multiple CQAs with high-quality data on a single platform with kit-based assays

Determine multiple CQAs, including full-and-empty ratios, on one platform

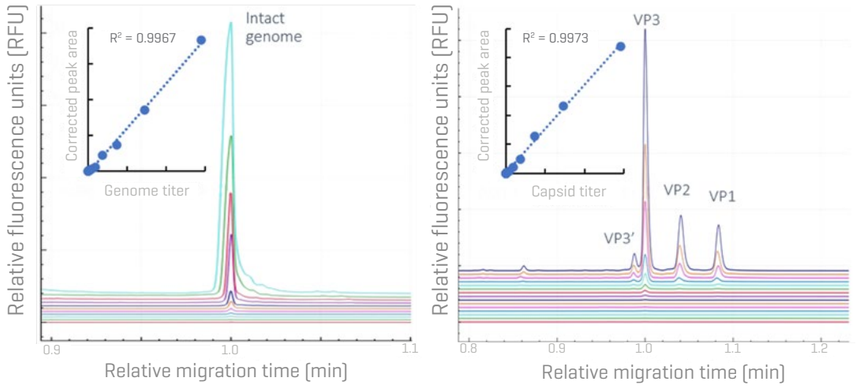


Figure 13: Standard curves for full-and-empty capsid determination. Left: AAV genome titer determination with CGE. The linear dynamic range (LDR) was determined from 2.56x1010 GC/mL-2.62x1013 GC/mL with R^2 = 0.9967. Right: AAV capsid titer determination with LDR from 6.41x109 GC/mL-2.62x1013 GC/mL with R2 = 0.9973.

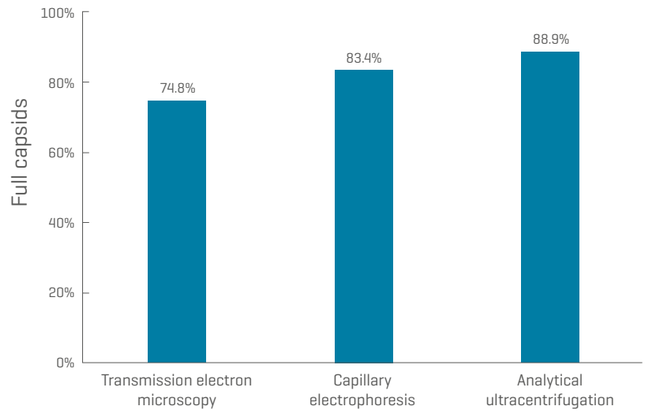


Figure 14: Comparison of the percentages of full capsids determined by different technologies. A good correlation between transmission electron microscopy and analytical ultracentrifugation was observed in comparison to CE.

<11%
difference between methods

Discover more details in the technical note about full-and-empty AAV assessment

Read technical note

Residual host cell DNA

Host cell DNA (HCD) is a process-related impurity that can be present in cell culture-derived products. Due to shearing during production, varying sizes of residual DNA might be present in a product. Since DNA with >200 base pairs (bp) could encode for undesired proteins, reliable size determination, and simultaneous quantitation are crucial for product safety. Challenges arise for risk assessment if only DNA quantity is determined without information on sizes.

- Determine quantities and sizes of residual host cell DNA in your therapeutic or vaccine with high resolving power and customizable size ranges
- Achieve the highest sensitivity and quantitative performance when sample amounts are limited with LIF detection
- Cover your compliance needs through compatibility with common data management systems

Determine risks of residual DNA using accurate size information and abundance

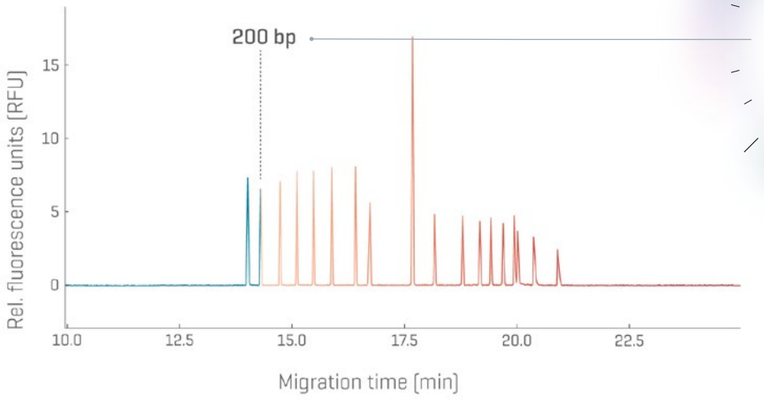


Figure 15: Electropherogram showing baseline separation of a linear dsDNA ladder from 100-15,000 bp.

200 bp
marker

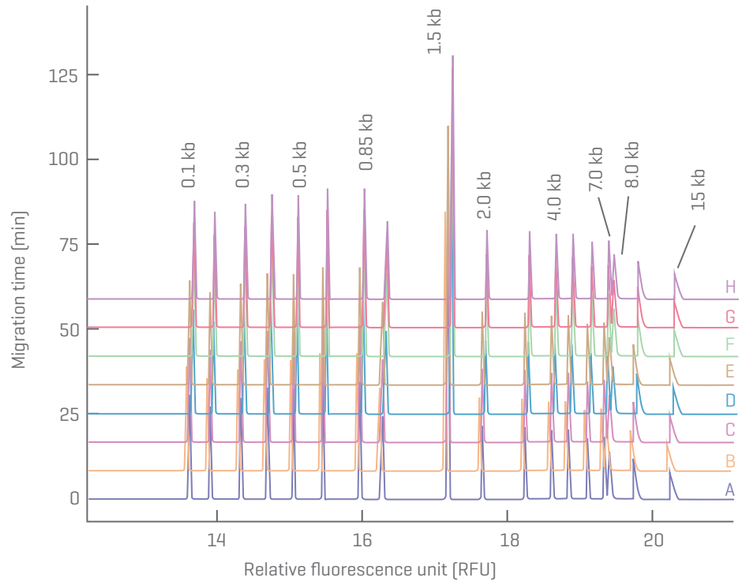


Figure 16: Representative electropherograms demonstrating the assay repeatability of the 1 kb Plus Linear DNA Ladder in single injections across eight capillaries in one cartridge using the BioPhase 8800 system.

Discover more details in the technical note about the intermediate precision study of DNA analysis

Read technical note

Host cell protein identification and quantitation

Another class of process-related impurities that can impact the safety and efficacy of viral vector products are proteins derived from packaging cells. The diverse landscape of packaging cell lines and the desire to deliver relevant medicines to patients more quickly drive the need for new strategies. Identifying process-related impurities with confidence requires highly adaptable workflows that do not need months of development time for different viral vector products.

- Streamline viral process development through relevant information on the identity and quantity of HCPs
- Avoid missing critical impurities using an unbiased data-independent acquisition (DIA) approach with excellent coverage and detection depth
- Take back your time for the identification and simultaneous quantitation of residual HCPs without the need for lengthy assay development

Identify HCPs with confidence using high-quality DIA

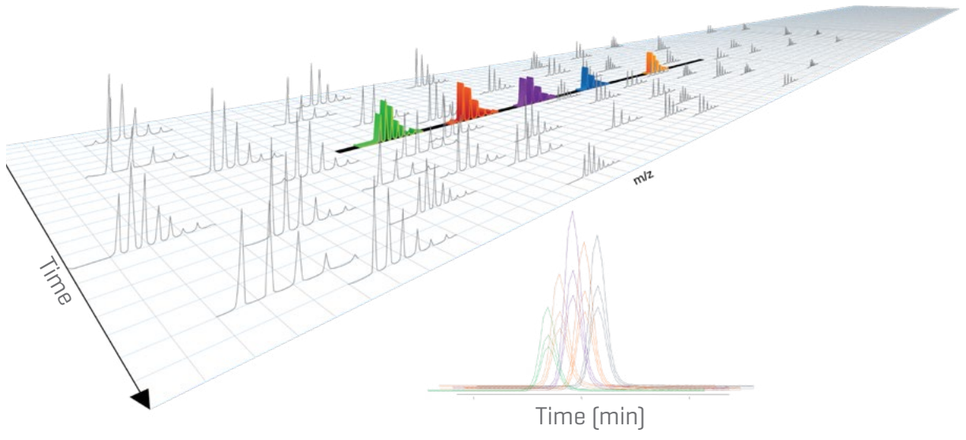
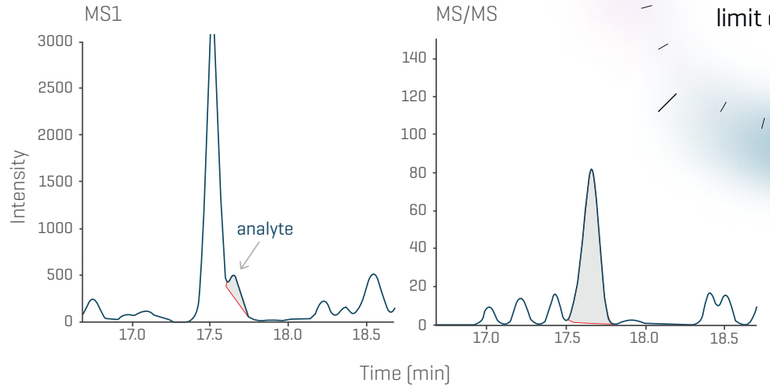


Figure 17: Schematic of a SWATH window. Top: Different precursors within a given m/z range (SWATH window) are present at a given time point (colored peaks) and selected for fragmentation. Bottom: The peak profiles of these precursors and related isotopes show slightly different elution times, which can be used for deconvolution.

Figure 18: Comparison of XICs of peptides in complex matrix. Left: XIC of precursor m/z of a peptide shows a high level of interference affecting the signal-to-noise and lower limit of detection. Right: XIC of a SWATH fragment m/z of the same peptide with reduced background results in better signal-to-noise and lower limit of detection.



Reduced background results in better signal-to-noise and lower limit of detection

Discover more details in the technical note for HCP analysis of lentivirus samples

Read technical note

Monitoring of host cell proteins

Monitoring hundreds of protein impurities can provide valuable information about product quality during process changes, such as upscaling, and reduce risks for the final product. While ligand-binding assays meet quantitation and throughput needs, obtaining actionable results can be a challenge. Understanding which protein impurities have changed can provide tremendous insight that can help streamline optimization of processes.

- Understand product changes on a protein-specific basis without the need for months of assay development
- Move past bottlenecks and maintain flexibility when there are changes to packaging cell lines and no ligand-binding assays are readily available
- Achieve highly sensitive analyte detection, accuracy and precision
- Confidently transfer assays to quality control (QC) with compliance-ready options and a proven track record of supporting quantitation for good practice (GxP) environments

Monitor and quantify hundreds of analytes with optimal quantitative performance

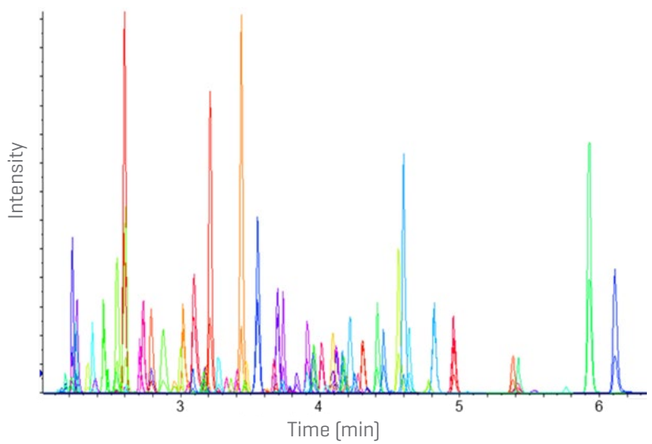
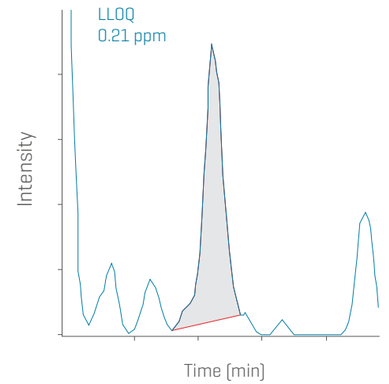
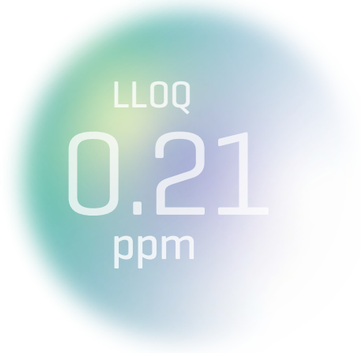


Figure 19: XICs of peptide surrogates for 48 target proteins in a biotherapeutic digest. More than 200 transitions were monitored in a scheduled MRM with a total run time of 8 min.

Figure 20: Quantitative data from one surrogate peptide transition in biotherapeutic digest. XIC shows LLOQ at 0.21 parts-per-million (ppm) for chosen MRM transition. Accuracy and precision values achieved for 3 replicate injections of different concentrations of the target protein are shown to the right.





Component Name	Actual Conc...	Num. Values	Percent CV	Accuracy
VNLLSAIK.+2y7.light	0.21	3 of 3	14.89	100.54
VNLLSAIK.+2y7.light	0.85	3 of 3	3.03	97.15
VNLLSAIK.+2y7.light	3.39	3 of 3	11.55	103.07
VNLLSAIK.+2y7.light	13.56	3 of 3	11.84	97.21
VNLLSAIK.+2y7.light	54.23	3 of 3	7.11	106.66
VNLLSAIK.+2y7.light	216.91	3 of 3	6.74	104.26
VNLLSAIK.+2y7.light	867.65	3 of 3	5.06	91.12

Discover more details in the technical note about quantitation of HCP peptides

Read technical note

Proteome profiling

Viral vectors bear the risk of idiosyncratic integration into the host genome. In addition, gene editing can affect the phenotype in various ways based on the complexity and interdependency of protein networks. Genomic readouts cannot provide sufficient insights into the potential disruption of gene regulators or detect changes to the proteome. Protein assays, such as Western blots, on the other hand, are limited by antibody availability and cannot detect unexpected proteome-wide changes.

- Break through the boundaries of gene editing by seeing and identifying the unexpected
- Unravel the effects of gene editing on the proteome level with DIA and dig deeper into changes despite limited sample amounts
- Achieve confident identification and simultaneous quantitation with excellent MS/MS data quality

Over
90%
of proteins quantified

Monitor and quantify hundreds of analytes with optimal quantitative performance

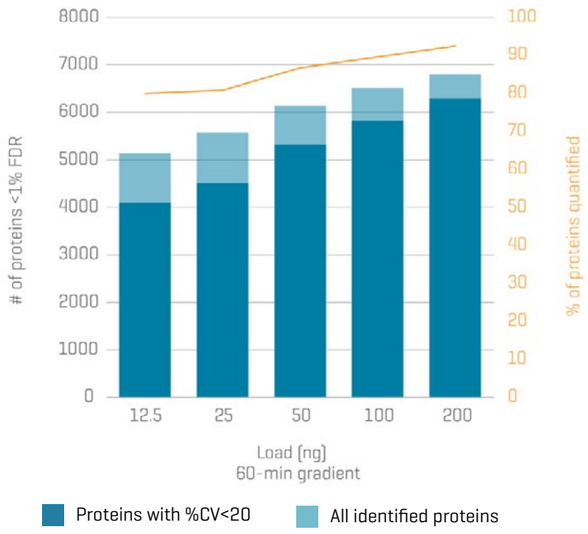
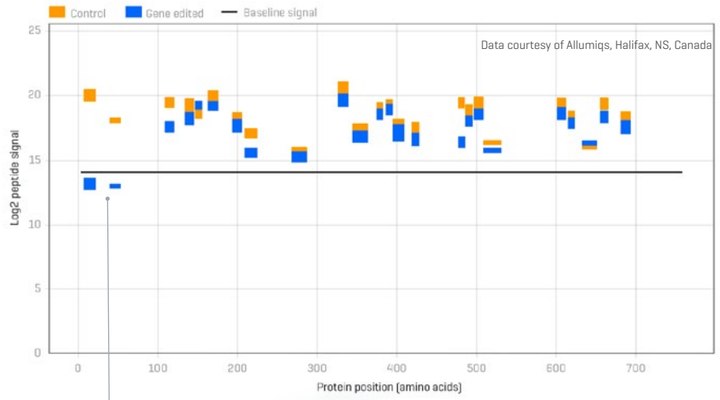
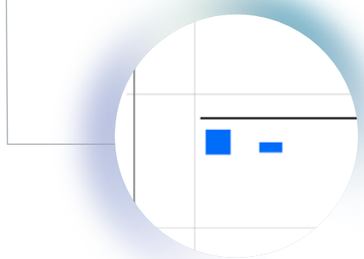


Figure 21: Identified and quantified proteins using SWATH DIA. Columns show a number of proteins identified with false discovery rate (FDR) <1% for different loading amounts of cell lysate digest using a 60 min gradient with 5 µL/min flow rate. The line shows the amount of proteins with %CV<20% used for quantitation as percentage of total amount of identified proteins with FDR<1%.

Figure 22: Insertion-deletion (INDEL) errors induced by gene editing detected by bottom-up proteomics using SWATH DIA. The expectation is that all peptides in the gene-edited samples (blue) are below the baseline signal. However, compared to the control (orange), only two peptides at the N-terminus were silenced based on a shift in the initiation of the protein transcription. Bar thickness represents standard deviation of the peptides.





Only the N-terminus of the protein was affected by gene editing based on a shift in the initiation site of the protein transcription

Discover more details in the technical note about SWATH DIA for biomarker ID and quantitation

Read technical note

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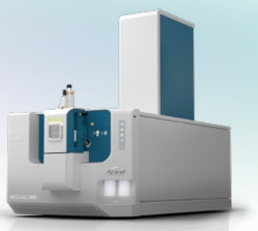

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Tips and tricks from our application experts:

AAV analysis with LC-MS

Zhengwei Chen [PhD], Staff Applications Scientist at SCIEX, US, shares his tips and tricks on AAV analysis using LC-MS/MS with the ZenoTOF 7600 system and EAD.

Dr. Zhengwei Chen serves as a Staff Application Scientist on the Biopharma Application Demo Team at SCIEX. Zhengwei is an MS expert across a broad spectrum of biopharmaceutical workflows, such as intact and native mass spectrometry, post-translational modifications, glycan analysis, proteomics and glycoproteomics. Zhengwei's expertise is built upon a strong academic background from Prof. Lingjun Li's distinguished lab and multiple years of experience at Regeneron, supporting all stages of drug development as part of an analytical chemistry group.

Tip 1: Sample handling

Utilize detergents at optimal concentrations, such as 6M urea or 8M guanidine hydrochloride, to ensure your AAV capsids are thoroughly denatured. This step is crucial for enabling effective enzymatic digestion. My recommendation is to use ~1 µg of the digested sample to assess the initial response when using an analytical flow setup. Adjust the injection volume, aiming at a total ion current (TIC) signal of high E7 cps using a ZenoTOF 7600 system.

Tip 2: Differentiation of amino acid isomers

EAD allows for effective differentiation between amino acid isomers, for instance between aspartic acid (D) and isoaspartate (isoD), through diagnostic side chain fragments. When asparagine (N) in the capsid proteins of AAVs undergo deamidation, the isomers D and isoD can be formed, resulting in protein charge variants, which can alter the physical and functional stability. I recommend employing a DDA experiment with the EAD kinetic energy set to 7 eV. This approach will ensure extensive sequence coverage and enable the precise identification of amino acid isomers.

Tip 3: Detailed characterization of phosphorylation

Surface-exposed tyrosine (Y), serine (S), and threonine (T) residues on AAV capsids can be phosphorylated, followed by ubiquitination and degradation by the cell proteasome. Phosphorylation is, therefore, directly linked to transduction efficiency and is an important PTM for AAV studies. EAD preserves the attachment of the labile phosphate groups to the peptide backbone fragments and enables the precise pinpointing of phosphorylation sites. This is particularly valuable in intricate scenarios where multiple phosphorylation sites may exist on a single peptide. I suggest starting your analysis with a tryptic digestion followed by EAD analysis. For more complex cases, employing enzymes like Asp-N before proceeding with EAD analysis may enhance the confidence of identification.

Tip 4: Comprehensive glycosylation analysis

Glycosylation in AAVs is likely to impact gene delivery and expression by affecting viral tropism, entry, and infectivity. Leveraging EAD, complex glycosylation patterns can be unraveled, providing detailed insights into the peptide backbone and pinpointing glycosylation sites precisely. Simultaneously, EAD can distinguish glycan isomers, such as α2,3 and α2,6 sialic acids. This dual capability reveals intricate glycan structures and enhances our understanding of the functional implications of glycosylation in viral vector biology. I suggest starting your analysis by employing the intact glycopeptide method with the settings outlined here.

More questions?

Contact us

Tips and tricks from our application experts:

AAV analysis with CE

Peter Holper, Staff Applications Scientist at SCIEX, US, shares his tips and tricks on AAV analysis using CE with the BioPhase 8800 system and the PA 800 Plus system.

Tip 1: Leverage the flexibility in injection modes

When starting out with a new viral vector product, my recommendation is to compare three different modes of injection using UV detection. First, start with a standard electrokinetic injection, which allows for the highest theoretical resolution. Next, use a pressure/hydrodynamic injection, which will inject the same plug regardless of sample ionic strength and provide a quick estimate of the titer. Finally, use a field-amplified sample stacking (FASS) injection to achieve the highest sensitivity, while understanding it is the most sensitive injection method to the ionic strength of the matrix. Comparing these three peak profiles can give significant insight into the optimal separation conditions for each molecule analyzed.

Tip 2: Deal with low sample amounts

During early-stage development of AAV vectors, oftentimes only a few micrograms of proteins or less are available for analytics. However, most analytical technology is not practical for applications with low protein concentrations or small sample volumes. To improve the sensitivity of CE-SDS, my recommendation is to use laser-induced fluorescence (LIF) detection instead of UV absorbance. Comparing the results from the different injection types (tip 1) will help you determine if additional sensitivity and transition to LIF detection is needed.

Tip 3: Optimize fluorescence dye labelling

Labeling procedure can pose challenges and require optimization for each product. Currently, the most common

fluorescent dye used in CE-SDS-LIF is Chromeo P503, which has a low quantum yield when not bound to a protein and thus does not require additional cleanup after conjugation. When optimizing the labeling procedure with Chromeo P503, I find the dye-to-protein ratio to be the most important factor. If this ratio is not optimal, low signal or high peak tailing is often observed. I find that estimating the protein titer by referring to the peak area achieved with pressure injection (tip 1) can be highly beneficial since only the genome titer may be known at this point.

Peter Holper has over 15 years of experience in biopharma, including his role as an analytical chemist at Eli Lilly and Company where he was responsible for developing the analytical control strategy for bioproducts. He has extensive experience in analytical method development for biologics and held various positions with increasing responsibility. Peter currently works at SCIEX as a Staff Applications Scientist in Redwood City, California, where he is responsible for developing and optimizing CE applications and providing customer demo sample analysis support.

More questions?

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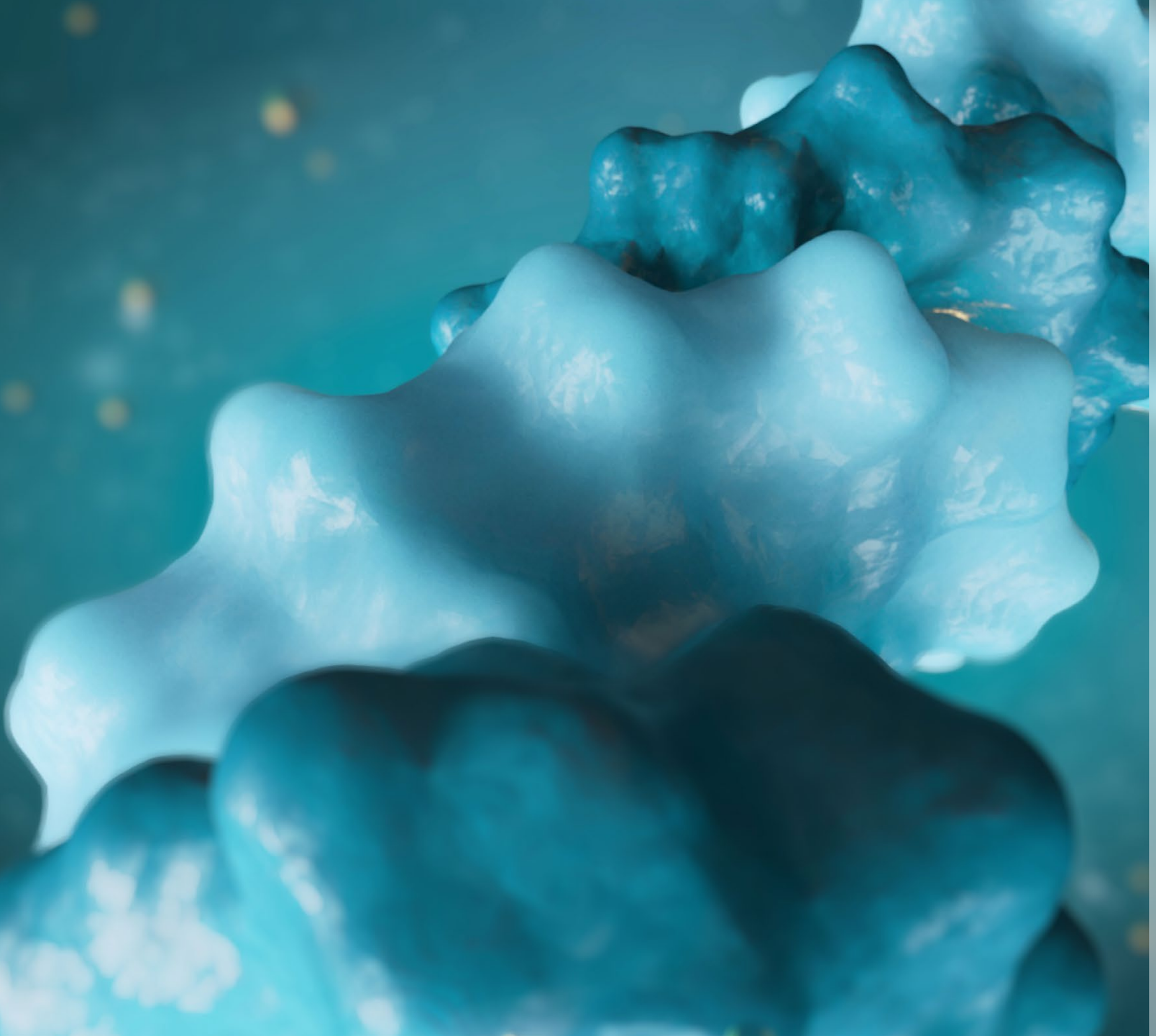


02

Plasmid DNA

Plasmid DNA

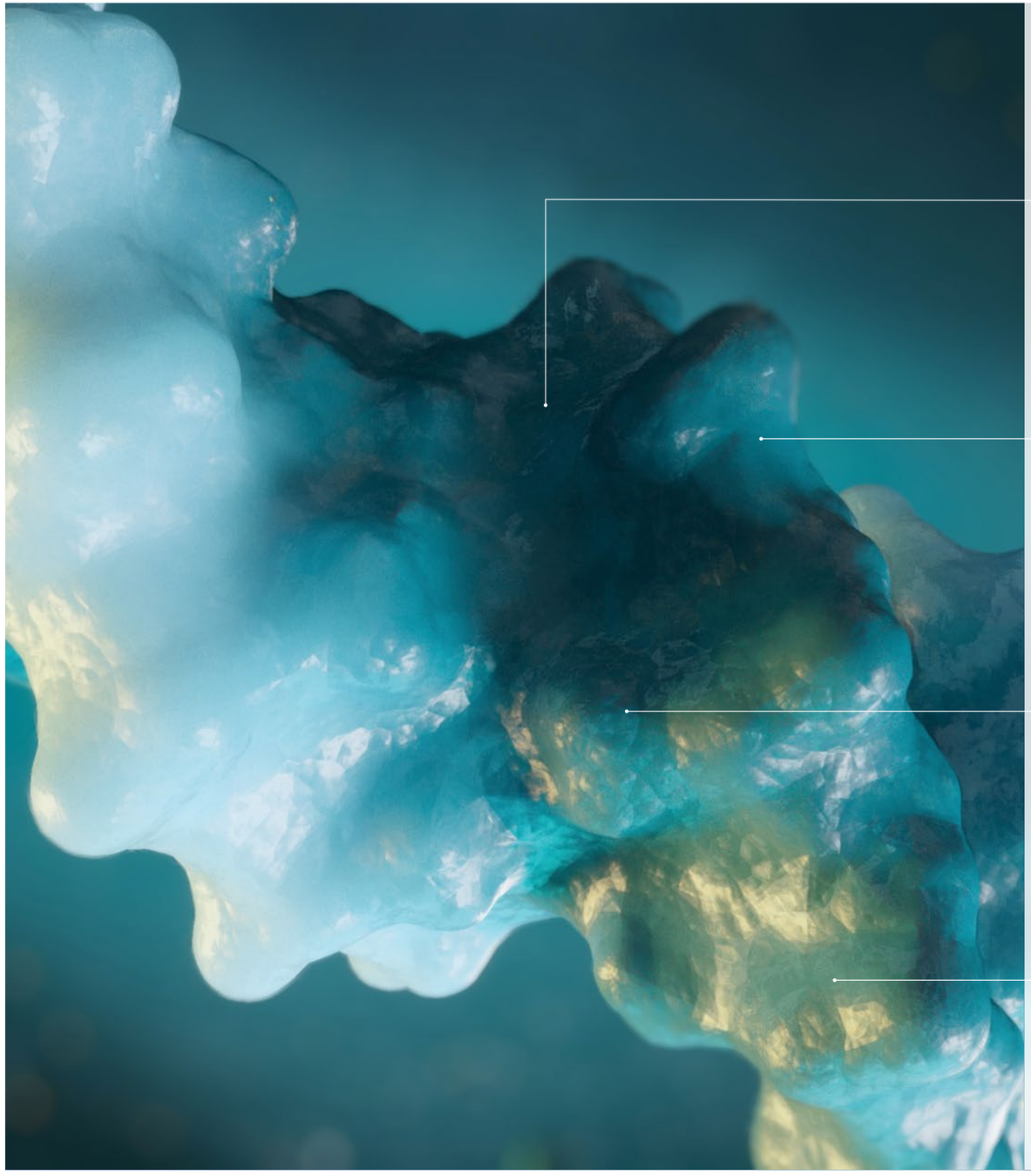
Double-stranded DNA plasmids are an extremely versatile tool frequently used for genetic engineering in biotechnology applications.

In a medical context, plasmid DNA (pDNA) can be used directly—as vaccine or for ex vivo cell therapy for instance—but also serve as raw or critical starting material for the manufacturing of protein drugs, viral vectors, and mRNA.

Generally, pDNA contains several regions to enable its function within drug manufacturing: An origin for the replication in bacteria, the gene of interest (GOI), a promoter to enable the expression of the GOI, antibiotic resistance genes for selection as needed, and in case of viral vector production, long terminal repeats (LTRs). The pDNA quality directly impacts the quality of subsequent protein, nucleic acid, or viral vector products and must therefore be ensured.

"Plasmid DNA (pDNA) is a widely used starting material in the manufacturing process of mRNA-based vaccines or viral vectors. Consequently, a high pDNA quality must be ensured. Capillary gel electrophoresis with laser-induced fluorescence (CGE-LIF) on a PA 800 Plus system from SCIEX offers accurate and highly sensitive pDNA analyses, enabling a reliable assessment of pDNA quality prior to further processing."

Roman Herzog (PhD)
Group Leader Bioanalytics (R&D), WACKER Chemie AG



pDNA restriction fragments

Achieve excellent resolution over a large size range for fragment-based ID of dsDNA.

Residual nucleic acids

Understand the sizes and amounts of residual host cell nucleic acids.

pDNA purity

Separate plasmid isoforms with high resolution and assess the purity and stability of your pDNA.

Linear DNA size

Determine accurately the size of your linearized pDNAs over a wide size range.

Expert Q&A:

Plasmid manufacturing

Plasmid DNA serves a variety of purposes—from critical starting material for proteins, mRNA or viral vectors to drug substance. Here, Dr. Emma Bjorgum, an expert in plasmid manufacturing provides insights into the process and an outlook on the future.

Learn how to set up your program for success with Aldevron

Learn more

What applications does Aldevron manufacture plasmid DNA for?

Aldevron manufactures plasmid DNA for a variety of end applications. Much of our experience and expertise is comprised of manufacturing plasmid DNA for cell and gene therapy applications. We also manufacture for all phases and stages of pipeline development from early discovery to commercial applications. Aldevron is among the first to offer plasmid DNA at full current Good Manufacturing Practice (cGMP) or clinical grade and has pioneered a mid-grade between research grade and full cGMP, called GMP-Source. This has allowed us to support an estimated 1500 clinical trials run by over 1000 clients. We thrive on supporting clients from the early stages of their clinical programs through commercialization.

How does your support vary by application?

We can provide plasmid DNA for various applications. Two specific examples include support of mRNA and AAV gene therapies and vaccines. For mRNA applications, we provide plasmid DNA as a linearized product and can perform the linearization with a client-designated enzyme. We also screen the plasmid construct prior to manufacturing to optimize conditions for both yield and stability of the poly(A) tail (if encoded). For AAV drugs, we optimize conditions for scale up by evaluating different host cell lines and temperature combinations for inverted terminal repeat (ITR) retention. A third example is our investment in next-generation plasmid technology, Nanoplasmid vectors. Nanoplasmids are comprised of very small, efficient backbones (~500 bases). Removal of bacterial and antibiotic resistance genes improves both safety and performance. One area where Nanoplasmids are showing particularly strong performance is as a homology-directed repair (HDR) donor template for CRISPR knock-in applications.

How do you ensure the quality of your plasmids at the different quality levels you offer?

Aldevron offers a comprehensive quality control testing panel of assays for the release of plasmid DNA. Assays include various methods for identity, safety, bacterial host components, and bioburden/sterility. Almost all our assays are conducted in-house, and methodologies are closely aligned for testing and release of RUO, GMP-Source and GMP methods.

How have the requirements for plasmids changed over the past 5-10 years?

In the earlier days of cell and gene therapy, there were hardly any references to plasmid manufacturing recommendations where plasmid DNA is utilized as a critical starting material or raw material. As cell and gene therapy has continued to see additional approvals, we have seen more recent considerations from the agency for CAR-T therapies with a recommendation to remove any unnecessary transgene in the vector, such as antibiotic resistance markers. Aldevron's Nanoplasmid technology ameliorates this concern as it utilizes a sucrose selection technology negating the need for any antibiotics in the manufacturing process.

What changes do you anticipate moving forward?

Moving forward, we are likely to see additional scrutiny on vector backbones and the removal of any extraneous sequences. We are also likely to see increased specificity on scale and how manufacturers can deliver exactly what is needed at the point in time of clinical development. Aldevron is focused on providing the 'right sized' scale for manufacturing and can meet both exact quantity and batch deliverables.

What innovation is helping to drive the industry forward and how will analytics need to evolve?

Newer vector technologies, such as nanoplasmid, can help address concerns with extraneous sequences in the plasmid backbone size since it consists of only 200 bp. Another innovation area is next-generation microbial cell lines to improve the yield and stability of plasmid DNA, such as the REVIVER cell line. Additionally, non-viral delivery systems are tackling challenges in the industry for payload delivery by lowering costs and delivering products without the constraints of a viral system. Additionally, innovation around the client experience is a key focus for us. Over the past 2 years we have been intensely focused on the client experience and have made incredible progress streamlining the new program on-boarding process, reducing lead times, and eliminating deviations. For example, in 2023, we were able to reduce our lead time by up to 80% from construct selection through product release.

mRNA is driving industry growth. How does Aldevron support the mRNA modality from a plasmid perspective?

Aldevron can provide linear plasmid DNA at any scale and quality level (RUO, GMP-Source and GMP). Our processes allow for linearization with the client-selected enzyme, including a purification step post-linearization to ensure the product is free from any remaining enzyme. We can provide analytical testing for the final linearized product to confirm the percentage of linearized plasmid in addition to poly(A) tail length. Several of our clients get linear plasmid DNA from us and do the IVT and other reactions internally. Increasingly, clients are taking advantage of Aldevron's broader RNA services, including linear plasmid, IVT and capping reactions, lipid nanoparticle encapsulation and sterile fill-finish services. That includes all the associated analytics, such as CGE, for instance.

What additional services are popular with those manufacturing plasmid for clinical applications?

Additional services often required to support plasmid DNA for clinical services include stability testing of both final plasmid DNA product and master cell banks. Commercialization support services such as process characterization and process validation are also often required in the late phases of clinical development. Additionally, regulatory services are often utilized to support Chemistry, Manufacturing and Control (CMC) sections of Investigational New Drug (IND) filings or Biologics License Applications (BLAs).

Emma Bjorgum is the Vice President of Client Services of the DNA Business Unit at Aldevron with a focus on product strategy and portfolio management. She has been employed in the cell and gene therapy industry for over a decade with 9 years of experience at Aldevron. Before Aldevron, Emma worked for Millipore Sigma as a Business Development Manager for the Viral and Gene Therapy Manufacturing business unit. She also worked for Be The Match Biotherapies as a Business and Market Analyst. Emma obtained her BA in Biology with minors in Chemistry and Psychology from Concordia College in Moorhead, MN.

pDNA topology and purity

Plasmids can exist in three primary topological forms: covalently closed circular (ccc) often referred to as supercoiled (sc), open-circular (oc) and linear. The sc form is desirable during plasmid manufacturing and for subsequent protein expression, viral vector manufacturing, or DNA vaccines. Differentiating conformational isoforms and assessing the purity and stability of pDNA is crucial for ensuring product quality, whether it is the critical starting material or drug substance.

- Rely on excellent resolution for different topological variants of pDNA
- Achieve high sensitivity for early-stage development samples with LIF detection
- Confidently transfer assays from development to QC with excellent precision and streamline data management through compatibility with data management systems

Differentiate different topological variants and determine purity with ease

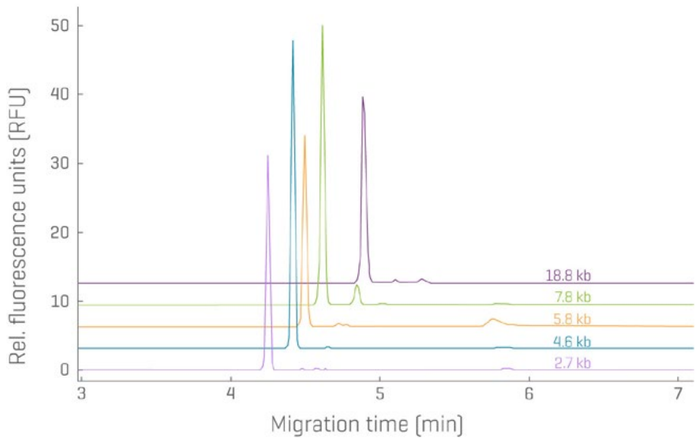


Figure 23: Separation of topological isoforms of 5 plasmids (2.7–18.9 kb) using the DNA 20 kb Plasmid and Linear kit.

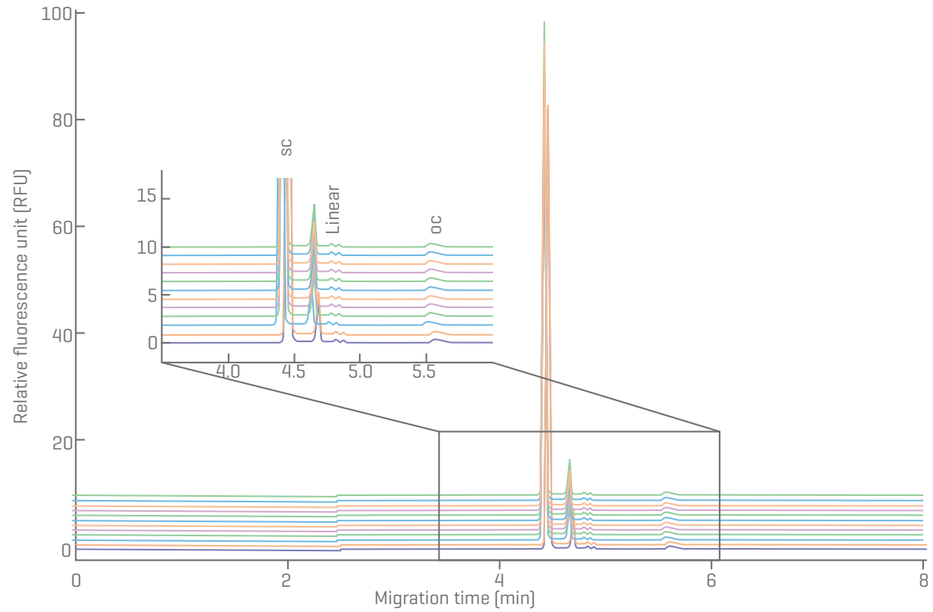


Figure 24: Assay repeatability of a 7.9 kb plasmid. The sample was injected from the same well for 12 consecutive injections and analyzed on the BioPhase 8800 system with a BioPhase BFS capillary cartridge - 8 x 30 cm using the DNA 20 kb Plasmid and Linear kit.

Discover more details in the technical note about plasmid purity monitoring

Read technical note

pDNA linearization efficiency and sizing

Linearized DNA serves as a template for mRNA and other IVT RNAs and minimizes off-target or elongated mRNA transcripts due to read-through transcription. The linearization efficiency of pDNA is, therefore, an important quality attribute of DNA starting material. Furthermore, sizing of the linearized plasmid and assessment of its purity can help determine the quality of linearized pDNA.

- Determine linearization efficiency with excellent separation for different topological variants of pDNA
- Assess linear DNA sizes and purity confidently with ultra-high resolution over a wide size range
- Confidently transfer assays from development to QC and streamline data management through compatibility with data management systems

Assess linear DNA sizes and purity confidently with ultra-high resolution over a wide size range

Understand linearization efficiency and linear DNA sizes

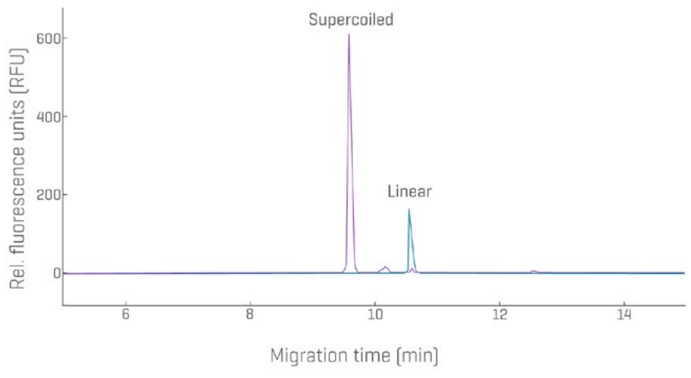


Figure 25: Linearized plasmid purity analysis and size estimation of a 7.9 kb plasmid sample. The electropherogram shows the sample prior to linearization, containing mainly the supercoiled isoform and after linearization.

Baseline separation between 7000 and 8000 bp

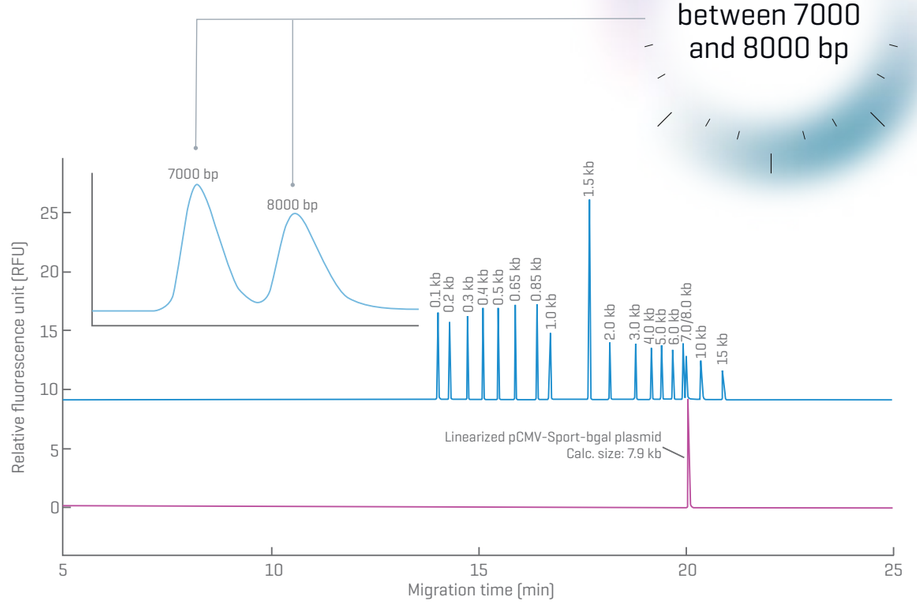


Figure 26: Size determination of the linearized 7.9 kb with a BioPhase BFS capillary cartridge - 8 x 50 cm. Top: The 1 kb Plus DNA Ladder with the inset showing the resolution between the 7,000 bp and 8,000 bp fragments. Bottom: The linearized plasmid sample with calculated size.

Discover more details in the technical note about plasmid purity and linear DNA sizing

Read technical note

pDNA restriction fragment analysis

Several analytical techniques for plasmid identity testing exist. However, homologous regions, such as poly[A] tails, LTR and ITR, present a challenge for sequencing-based methods. The repetitive nature of these regions makes it difficult to obtain accurate information on their length and composition. Tailored restriction fragment analysis with high resolving CGE provide an alternative that is not affected by long, homologous pDNA regions.

- Achieve identity testing with excellent resolution of DNA restriction fragments over a wide size range
- Rely on results with excellent accuracy and precision
- Confidently transfer assays from development to QC and streamline data management through compatibility with data management systems

Determine pDNA fragment sizes across a wide range

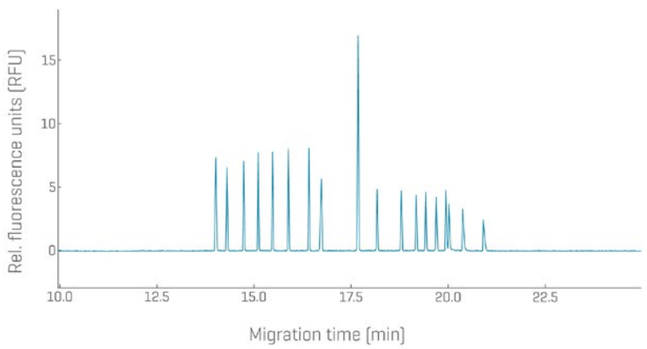


Figure 27: Electropherogram showing baseline separation of a linear dsDNA ladder from 100-15,000 bp.

More questions?

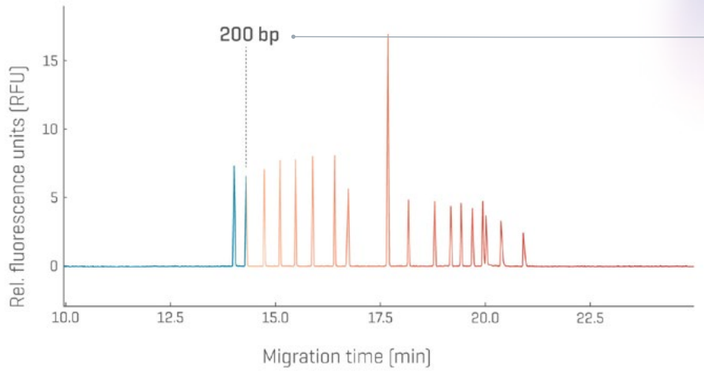
Contact us

Residual host cell nucleic acids

After transforming bacteria with the desired plasmid and selection of a clone, fermentation is used for the expansion of pDNA. Extracted pDNA from the lysed host cells is concentrated and cleaned up. Quality assessment, therefore includes analytical testing for residual host nucleic acids as a process-related impurity. Size estimation and simultaneous quantitation of residual DNA and RNA are crucial to enable risk assessment and ensure product safety.

- Estimate size and amounts of residual host cell nucleic acids
- Rely on results with excellent accuracy and precision
- Achieve high sensitivity for early-stage development samples with LIF detection

Assess residual nucleic acid sizes and quantities simultaneously



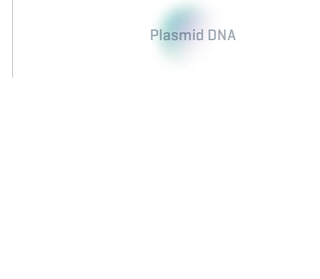


Figure 28: Electropherogram showing baseline separation of a linear dsDNA ladder from 100-15,000 bp.

More questions?

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Analytical solutions for plasmid DNA

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- Residual nucleic acid analysis
- Larger sample sets

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