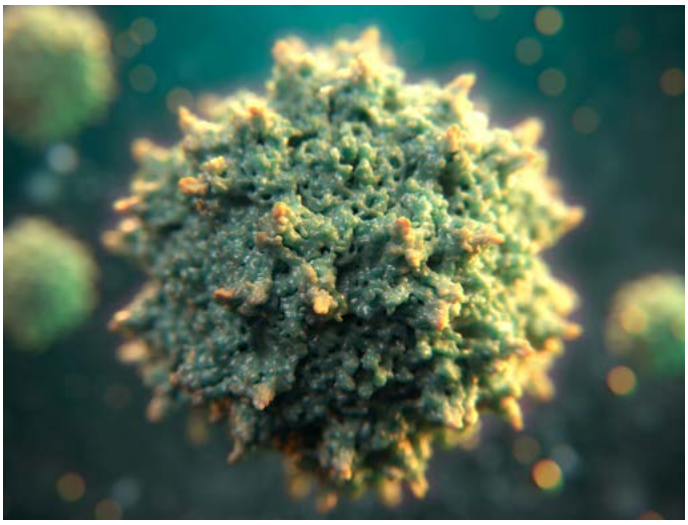
SCIEX

Cell and Gene Therapy Compendium

Breaking through analytical boundaries

Development of next-generation gene therapies and vaccines



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The power of precision

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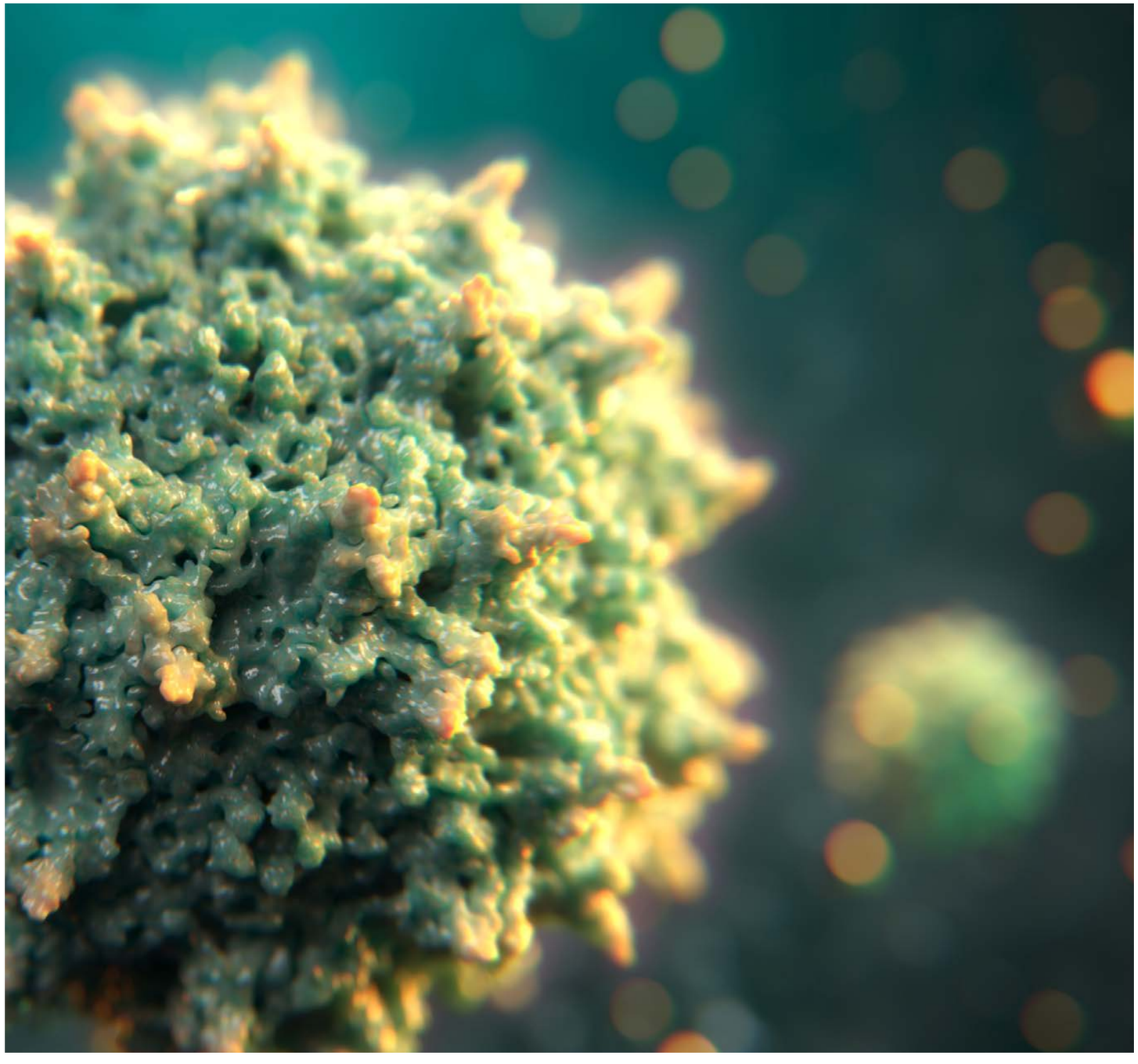


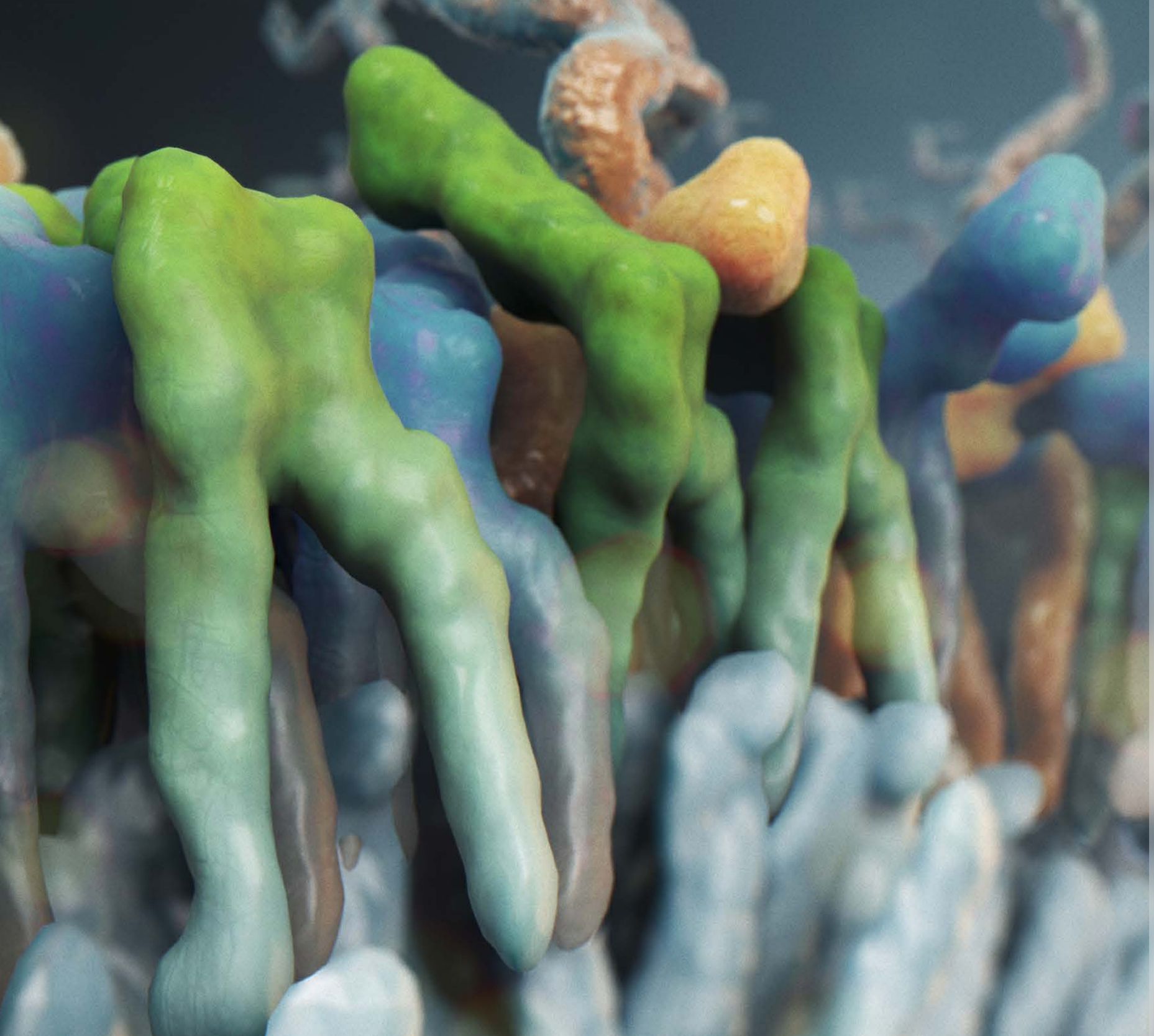
We can help you break through analytical boundaries and revolutionize medicine

Advancing human health has never been charged with so many possibilities before. Cell and gene therapy (CGT) products transform lives by going beyond treating symptoms, often with the goal of curing rare and lethal diseases.

Apart from gene therapy, oligonucleotide-based products are also used to treat diseases by modulating endogenous messenger RNA (mRNA) expression or by activating the immune response to fight malignant tumor cells. Furthermore, the platform capabilities of mRNA packaged in lipid nanoparticles (LNPs) create opportunities in preventative care. This has the potential to enable more rapid adaptation of vaccines against infectious diseases and germ mutations and allows for more cost-effective manufacturing. These factors are key to enabling wider global access of medicine and better preparation for future health crises.

Despite all the excitement new drug candidates can bring, no short cuts can be taken when ensuring their quality and proving their safety and efficacy. The complexity and novelty of these modalities require continuous learning and adaptation, out-of-the-box thinking and evolving analytical technologies to get answers to pressing questions.

Here at SCIEX, we are a team dedicated to analytical science. We can help you break through analytical boundaries and revolutionize medicine by providing capillary electrophoresis (CE) and mass spectrometry (MS) solutions. Together, we can reach new heights as we move towards the future of CGT.



01

Non-viral delivery

Non-viral delivery

Lipid nanoparticles (LNPs), are widely used for the delivery of vaccines and therapeutics such as in vitro transcribed (IVT) RNA, small interfering RNA (siRNA), antisense oligonucleotides (ASOs) and more.

4 different classes of lipids are used within LNPs: ionizable cationic lipids or cationic lipids, sterol lipids, helper lipids and PEG-lipids. The flexibility of LNPs regarding the type and size of the cargo, limited adverse effects and easier scale-up compared to viral vectors, are factors that contribute to the increased use of LNPs as delivery vehicles. As a result, it is important to analyze lipid raw material quality, characterize the lipids in the drug products and perform bioanalysis studies to help mitigate risks to patient safety and drug efficacy.

"LNPs pose unique analytical challenges, in part due to the complexity of their lipid excipients. Consequently, the detailed structure elucidation capabilities afforded by electron activated dissociation (EAD) in the ZenoTOF 7600 system from SCIEX provide the analytical chemist unparalleled capacity to identify problematic oxidative impurities inside ionizable lipids, and thereby de-risk the LNP therapeutic development process and expedite paths to the clinic."

Adam Crowe (PhD)
Sr. Manager Analytical Development, Cytiva

Raw material and LNP characterization

Explore how to perform comprehensive structural elucidation of lipid raw materials and lipids from LNPs.

MetID and bioanalysis

Overcome matrix complexity to perform identification of metabolites (metID) from lipids and bioanalysis studies.

Lipid quantitation

Streamline the quality control of raw material and the bioanalysis of LNPs with efficient and sensitive analytical technology.

Expert Q&A:

Lipid impurity analysis with LC-MS/MS and EAD

Characterizing the lipid raw material is a crucial step towards the successful development of LNP-based drugs. Dr. Adam Crowe explains why and provides insights into the learnings from his team based on many years of research on a variety of ionizable lipids.

In your opinion, what are the best analytical techniques for assessing the purity of ionizable lipids?

At Cytiva, my team and I use at least three different methodologies to look at quality. We use charged aerosol detection (CAD) for the overall profile, the liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) with EAD method described in my webinar, and a fluorescence-based assay. The assessment should not be taken lightly, in my opinion. The detailed analysis of the ionizable lipid is paramount for the success of a project. In my experience, one of the most common ways that clinical programs based on LNPs fail is a lack of careful assessment of the raw material.

Can you detect N-oxides with CAD?

Yes and no. You will run into two problems. N-oxides tend to elute very close to the main peak of the ionizable lipid. While you can chromatographically separate them, the gradients required are quite long and you will need prior expertise in what you are trying to separate. The other issue is the relative abundance. Because the N-oxides are an intermediate product that degrades further, you never form huge amounts of it. At ~0.1% relative abundance is when I start to get concerned about N-oxide formation. This makes it difficult for CAD to detect N-oxides because of the method's limited dynamic range, adding to the challenge of having to know what to look for.

At which levels do N-oxides impact mRNA efficacy?

This is an interesting question. It seems in very, very low abundance. We've had the luxury of looking at the adduct formation of 20 to 30 different ionizable lipids. Since the N-oxide itself is not reacting with the mRNA, but presumably an aldehyde—a degradation product of the N-oxide as described by Packer et al. in 2021—predictions are challenging. As a summary, I can say that when N-oxides are present in a significant quantity, meaning ≥1% abundance, we see very significant adduct formation.

Do you have any thoughts on acceptable levels of N-oxides or adducts?

As I mentioned before, N-oxide levels above a 0.1% threshold is where we start to consider adduct formation a problem. However, it is the lipidation event itself that you will need to monitor and do rate calculations on to assess the severity. This is because the N-oxides are diagnostic, but not necessarily predictive of the rate of adduct formation. There are cases where you can see relatively low N-oxide amounts, but the rate of adduct formation on the RNA is quite fast.

In such cases, it is likely that the N-oxides have already degraded to another reactive species.

Do you use MS/MS with EAD only for raw materials, or do you also monitor N-oxides in formulated LNPs?

You can absolutely use the LC-MS/MS with EAD method I presented in my webinar for formulated LNPs. It's obviously less complex to investigate a particular raw material compared to a formulated LNP because you have less species in a sample. However, it can absolutely be done. I recommend reaching out to your SCIEX representative as they might have further information on that topic.

Can you elaborate on how much MS method optimization is typically required and how much time you need to process the data? Can you efficiently transfer methods to new lipids?

It's not a whole lot. Although EAD is very tunable, there are very discrete ranges for the type of fragmentation we are seeking for ionizable lipids. Generally, lipids require high-energy fragmentation for achieving relevant bond breakage—we used around 15 electron volt (eV). If you want to determine the behavior of your specific lipids, you can set up a method with different energies within one injection. The data obtained by EAD are fragment-rich and manual analysis can take some time. However, SCIEX provides Molecule Profiler software as a solution, which can process lipid EAD data and does a lot of the interpretation for you. Historically, we would spend almost a week peering through the data and manually assigning the species that are there. Now, this is done in a ~10-minute computational run through the software followed by a manual check, so it's quite convenient.

Could you give some more detailed information about the MS method setup of the ZenoTOF 7600 system? Did you use targeted, data-dependent or data-independent analysis?

The method used was data-dependent acquisition (DDA)—or information-dependent acquisition (IDA), as some people call it in the industry—for fragmenting the top five candidates, combined with an inclusion list. The inclusion list contained the m/z of expected impurities of the ionizable lipid MC3, such as the addition of oxygen, demethylation, water loss, etc. More information on the method settings can be found in this technical note. Depending on your needs, you can increase the candidate ions and adjust the inclusion list.

Do you have recommendations for how to mitigate adduct formation between the ionizable lipid N-oxide and the RNA?

It really comes down to the quality of your ionizable lipid. Ensuring that the amount of oxygen is minimized and that the ionizable lipid is not heated or exposed to oxidizing agents will help reduce the amount of N-oxides. Ensuring that the purification after synthesis is robust will help as well. Generally speaking, you want to carefully consider your manufacturing synthesis, mechanism, route and purification of your ionizable lipid to mitigate lipidation.

Read Q&A blog

Adam Crowe (PhD)
Sr. Manager Analytical Development, Cytiva

Dr. Adam Crowe manages a multi-discipline team at Cytiva, tasked with developing novel analytical assays related to LNPs and nanomaterials for drug delivery. During his tenure, Adam has garnered broad expertise in analyzing particle payloads, physiochemical characteristics, excipients, and in vitro potency using a variety of analytical methodologies. Notably, he leveraged cutting-edge LC-MS technology for LNP characterization. Additionally, Adam is the technical lead for the American Society for Testing and Materials (ASTM) guide document, outlining best analytical practices for the LNP field.

Characterization of lipids and related impurities

Ionizable lipids are key components of LNPs, complexing the negatively charged cargo and facilitating the cellular uptake. Their quality is critical for a stable and efficient product. Even very low abundance N-oxide impurities can lead to a loss of function. Their structural identification and differentiation from other impurities is an analytical challenge. Furthermore, saturation of double bonds of the lipids could impact the structure of LNPs and affect the final product.

- Fully understand the structures of your cationic or ionizable lipid components using EAD
- Differentiate between oxidated species and accurately localize double bonds or saturations with EAD
- Avoid missing relevant product excipients by leveraging a linear dynamic range >5 orders of magnitude and signal-to-noise enhancement with the Zeno trap

Stop the guess work – Determine exact locations of oxygen double bounds, saturations and more

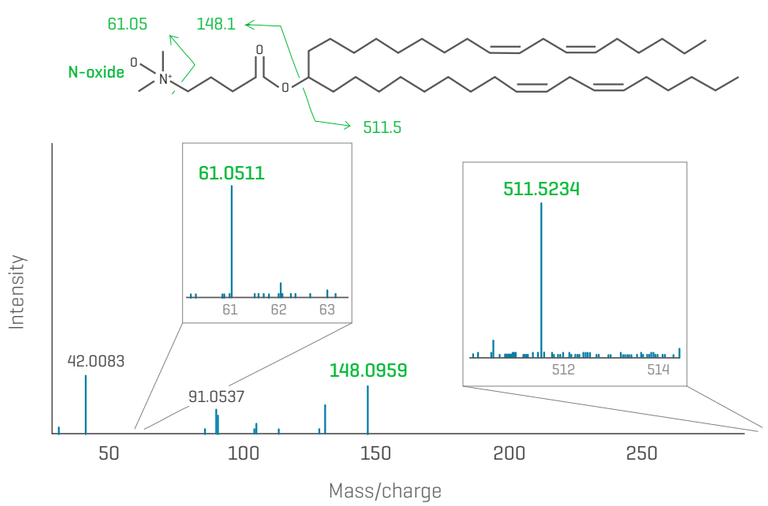
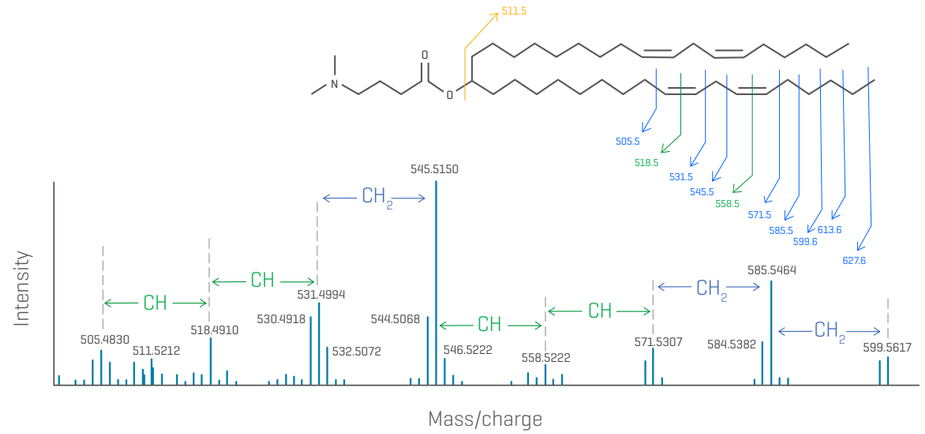


Figure 1: MS/MS EAD data of D-Lin-MC3-DMA (MC3) impurity. EAD-derived fragments can be used to pinpoint an oxygen incorporation to the tertiary amine headgroup of MC3. Encircled m/z show diagnostic fragment ions for the identification of an N-oxide impurity derived from MC3.

Figure 2: MS/MS EAD data of MC3. Figure shows a zoom in to the fragmentation data of C-C bonds for structural elucidation and specific localization of double bonds, and saturations with EAD.



Discover more details in the technical notes for MC3 and for ALC-0315

Read technical note **MC3**

Read technical note **ALC-0315**

Streamlined lipid quality control

Commonly, 4 lipid classes (ionizable cationic lipids or cationic lipids, sterol lipids, helper lipids and polyethylene glycol (PEG) lipids are mixed in defined ratios to form LNPs with desired physical-chemical properties. To ensure quality criteria are met, lipid raw materials and LNP batches need to be monitored. Following lipid characterization and impurity identification, quantitative monitoring can be streamlined.

- Leverage excellent sensitivity for quantitation of lipids and breakdown products in raw materials and formulated LNPs
- Rely on robustness and low %CVs with best-in-class triple quadrupole technology
- Streamline data acquisition and data processing with intuitive software

Detect and quantify different lipid species with high sensitivity and precision

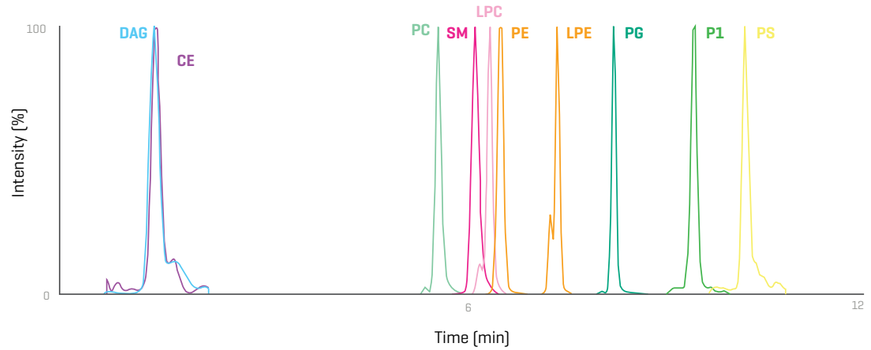


Figure 3: Extracted ion chromatograms for different lipid classes of a liposome with chromatographic separation. Using a normal phase scheduled multiple reaction monitoring (MRM) approach, very good class separation was achieved to avoid isobaric interferences and improve confidence in lipid species identification. Diacylglycerol (DAG), cholesteryl ester (CE), posphatidylcholine (PC), sphingomyelin (SM), lysoposphatidylcholine (LPC), phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS).

Discover more details in the technical note on lipid quantitation from liposomes

Read technical note

Lipid MetID and bioanalysis

Following administration, LNPs can travel to different parts of the body and undergo metabolic changes. Frequently, the non-endogenous cationic or ionizable cationic lipids are used as surrogates for quantitative analyses of LNPs in in vivo samples. Multiple bioanalytical end points from a single administration with small sample volumes require analytical assays with high sensitivity. In addition, matrix interferences and structural elucidation of metabolites need to be overcome.

- Elucidate the structures of your cationic or ionizable lipid components and related metabolites using EAD
- Overcome matrix interferences and achieve outstanding quantitative results for difficult-to-fragment lipids with accurate mass spectrometry and the Zeno trap
- Achieve identification and quantitation of different lipid species and metabolites in parallel

Leverage powerful structural elucidation and quantitation capabilities in one experiment

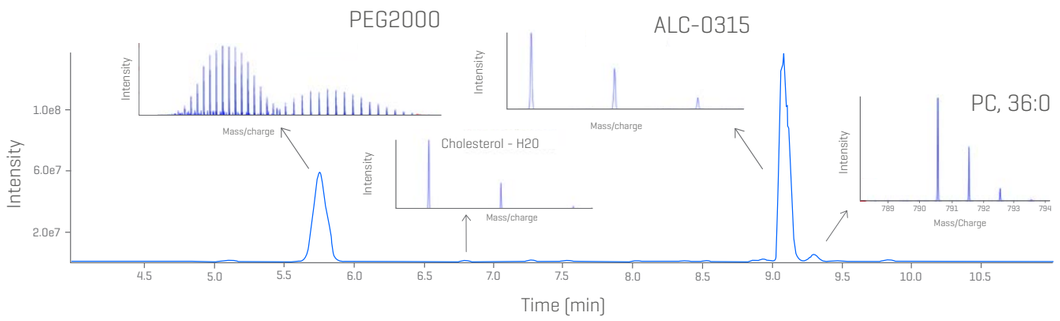
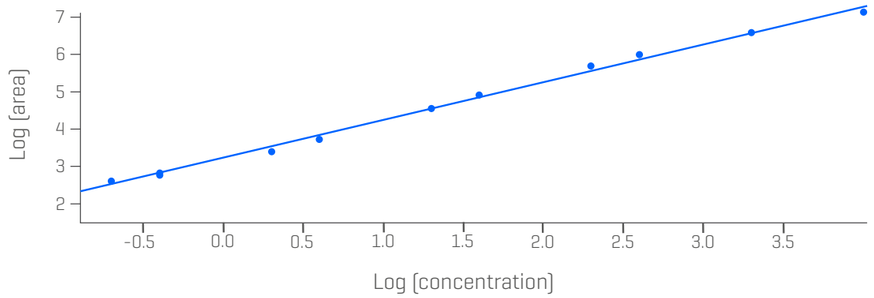


Figure 4: Identification of all four lipid species from an LNP. Total ion chromatogram showing the pegylated lipid 2-[[polyethylene glycol]-2000]-N,N-ditetradecylacetamide, PEG2000, the sterol lipid cholesterol, the ionizable lipid 6-[[2-hexyldecanoyl]oxy]-N-[6-[[2-hexyldecanoyl]oxy]hexyl]-N-[4-hydroxybutyl]hexan-1-aminium (ALC-0315) and PC without unsaturated carbon bonds as helper lipid with related time-of-flight (TOF) MS data.



Row	Component Name	Actual Con...	Num....	Mean	Standard Dev...	Percent CV	Average Accura...	Value #1	Value #2	Value #3
5	ALC-0315 766	0.2000	3 of 3	2.139e-1	3.124e-2	14.61	106.94	2.255e-1	1.785e-1	2.377e-1
6	ALC-0315 766	0.4000	3 of 3	3.467e-1	1.295e-1	37.33	86.69	4.904e-1	3.106e-1	2.392e-1
7	ALC-0315 766	2.0000	3 of 3	1.910e0	2.902e-1	15.19	95.50	1.800e0	2.239e0	1.691e0
8	ALC-0315 766	4.0000	3 of 3	4.084e0	3.449e-1	8.44	102.10	4.451e0	4.035e0	3.766e0
9	ALC-0315 766	20.0000	3 of 3	2.258e1	7.906e-1	3.50	112.90	2.210e1	2.349e1	2.215e1
10	ALC-0315 766	40.0000	3 of 3	3.550e1	9.135e-1	2.57	88.74	3.475e1	3.522e1	3.652e1
11	ALC-0315 766	200.0000	3 of 3	1.739e2	7.365e0	4.23	86.95	1.822e2	1.681e2	1.714e2
12	ALC-0315 766	400.0000	3 of 3	4.255e2	2.031e1	4.77	106.38	4.469e2	4.232e2	4.065e2
13	ALC-0315 766	2000.0000	3 of 3	2.257e3	3.894e1	1.73	112.85	2.275e3	2.284e3	2.212e3
14	ALC-0315 766	10000.0000	3 of 3	1.010e4	9.605e1	0.95	100.95	9.990e3	1.012e4	1.018e4

Figure 5: Calibration curve for ALC-0315. LNPs were spiked into plasma and extracted using solid phase extraction. Calibration curve is based on extracted ion chromatograms run in triplicates.

More questions?

Contact us

Analytical solutions for lipids and LNPs

Suitable for:

- In-depth structural elucidation
- Simultaneous relative quantitation
- High flexibility to perform a range of additional workflows

ExionLC AE system

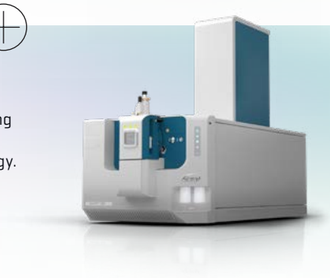
An LC system that seamlessly integrates with your SCIEX MS for reproducible data, batch after batch.

Learn more

ZenoTOF 7600 system

A high-resolution solution, combining powerful MS/MS sensitivity and alternative fragmentation technology.

Learn more



Molecule Profiler software

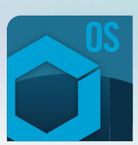
A software solution to identify impurities and biotransformations for a wide variety of modalities.

Learn more

SCIEX OS software

The next generation of operating and processing solution for an enhanced LC-MS/MS analysis experience.

Learn more



Suitable for:

- Targeted analysis and monitoring
- Excellent quantitative performance

ExionLC AE system

An LC system that seamlessly integrates with your SCIEX MS for reproducible data, batch after batch.

Learn more

SCIEX 7500+ system

A new standard for resilience and robustness, engineered to maintain sensitivity for longer.

Learn more

SCIEX OS software

The next generation of operating and processing solution for an enhanced LC-MS/MS analysis experience.

Learn more

Tips and tricks from our application experts:

Lipid impurity analysis with EAD

Paul Norris (PhD), Sr. Application Support, US at SCIEX, shares his tips and tricks on lipid analysis using LC-MS/MS with EAD.

Tip 1: Establish ideal sample concentrations

Start with a concentration of 20-200 ng/mL in 15:85 (v:v) water:acetonitrile for the ionizable lipid as raw material or as part of a nanoparticle formulation. I recommend targeting a TOF MS signal of >1E5 counts per second (cps) for the extracted ion chromatogram of the ionizable lipid on a ZenoTOF 7600 system and adjusting the concentration accordingly. This will result in high quality EAD spectra. For identification of impurities, I suggest preparing a 100x higher concentration. Since impurities are typically present in much lower abundance, a higher concentration is required to produce ideal EAD spectra. Typically, 2 µg/mL works well, however, concentration may need to be adjusted depending on the signal observed.

Tip 2: Resolve ionizable lipids and impurities

Chromatographic separation of ionizable lipids, their impurities and degradation products works particularly well with reversed phase C18 columns with a larger pore size (e.g., 300 Å). Start with a mobile phase consisting of 15% water and 85% (v:v) organic solvents like acetonitrile or methanol (or a mixture of both) with 10 mM ammonium acetate and a 10 min gradient ending in 100% organic solvent. This should resolve most ionizable lipid impurities and enable clean EAD spectra for characterization. Make sure to include a 5–10-minute wash at 100% organic before re-equilibrating the column.

Tip 3: Attain comprehensive ionizable lipid fragmentation

Comprehensive EAD spectra can be obtained by using optimized parameters based on the lipid structure. From my experience, an electron beam current at 5000 nA and an electron kinetic energy from 12—16 eV work best for most ionizable lipids. These settings also work well for most natural structural lipids with a similar size and structural composition (e.g., phosphatidylcholines). Great spectral quality with high signal-to-noise can be achieved with a reaction time of 30—35 ms and an accumulation time of approximately 100 ms.

Tip 4: Set up method for impurities

Typical impurities observed for a range of ionizable lipids include N-oxides, epoxides and hydroxyl functional groups at desaturated carbons relative to the parent structure. Additionally, saturation, desaturation, methylation and demethylation of the parent structure can be observed. I recommend designing MS/MS methods that incorporate these putative impurities in an inclusion list within a data-dependent experiment. With that setup, you can achieve detailed MS/MS information of expected impurities and of unknown impurities in your sample.

More questions?

Contact us

Paul Norris (PhD)
Sr. Application Support, US at SCIEX

Paul Norris specializes in the profiling and characterization of bioactive lipid mediators in the context of physiological and pathophysiological processes. He has a wealth of experience maximizing the capabilities of triple quadrupole and Q-TOF solutions for omics discovery and life sciences. Paul's extensive lipidomics experience started in the lab of Edward Dennis at UCSD where he contributed to studies as part of the LIPID MAPS consortium before joining Brigham and Women's Hospital to lead a lipidomics core facility, supporting numerous resolution pharmacology projects.

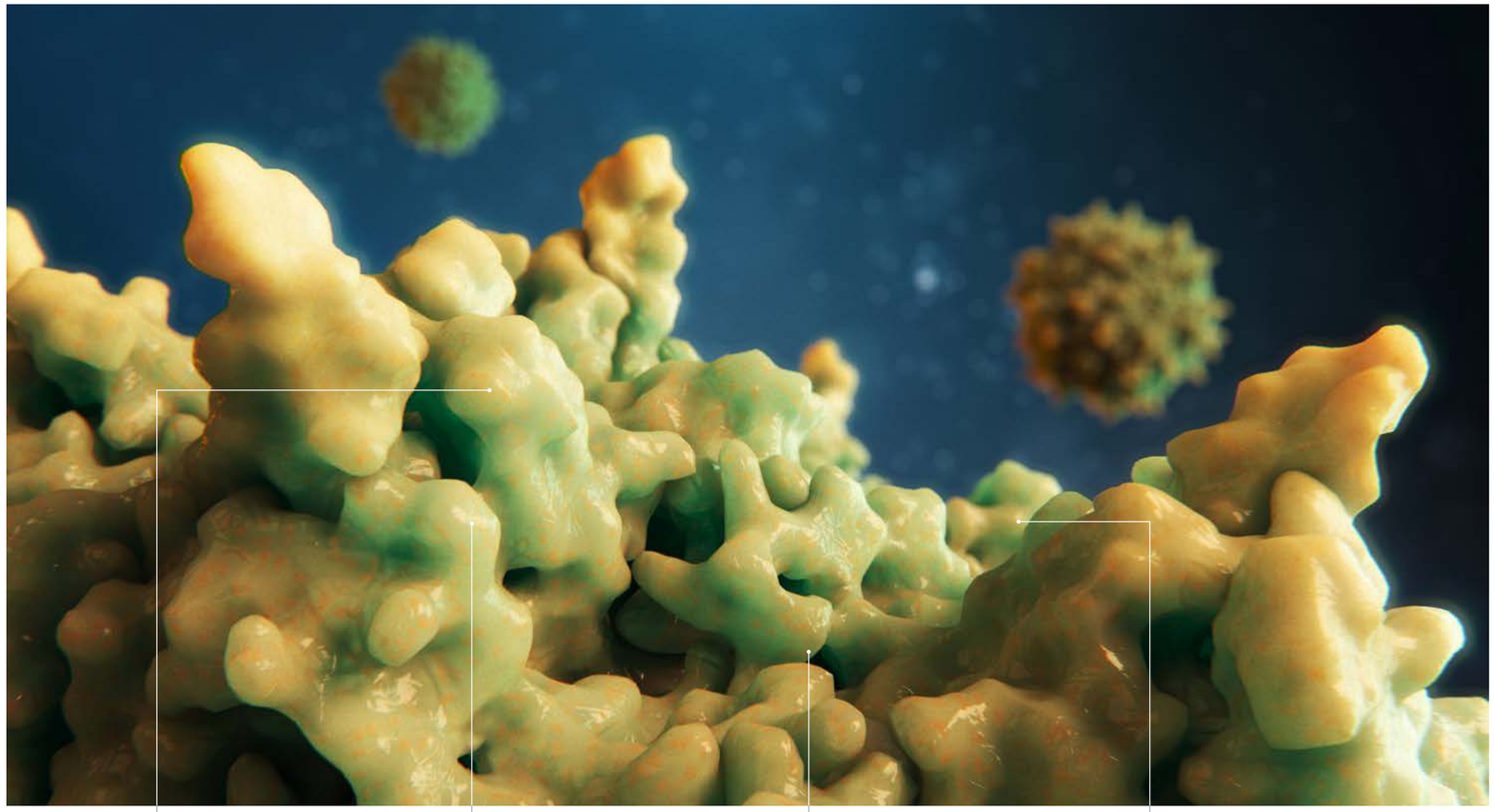
02

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 thesafety 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 range 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 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 on 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

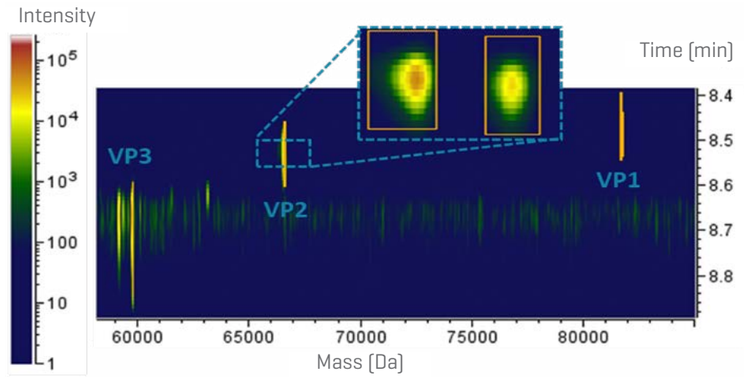


Figure 6: 3D heatmap of AAV8 capsid proteins cells showing the intensity, the retention time (RT) and the molecular weight (MW) using scan-by-scan, time-resolved deconvolution in Biologics Explorer software.

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 localizing fragile PTMs with an intuitive alternative fragmentation technique

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

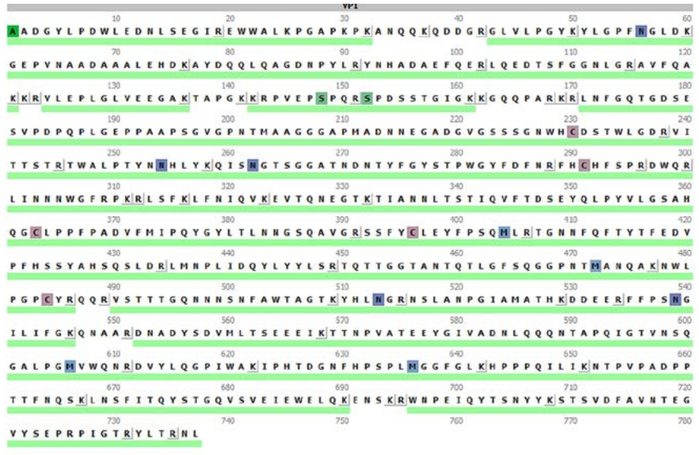


Figure 8: Sequence coverage map of AAV8 VP1. A nearly complete sequence coverage (94.7%) was obtained from a single injection of a tryptic digest of AAV8 using data-dependent acquisition (DDA) and processing with Biologics Explorer software.

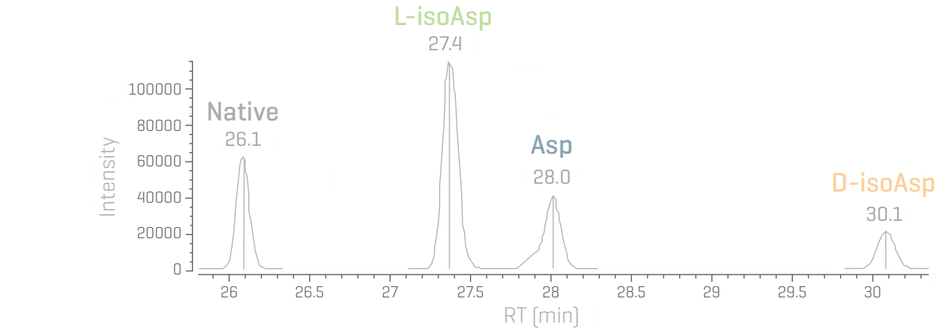


Figure 9: Identification of 3 deamidated species at N57 of the peptide YLGPFNGLDK (z = 2). Top: Extracted ion chromatograms (XICs) of the different deamidated species and the native peptide. Bottom: Zoom-in to EAD MS/MS data showing signature fragment ions for differentiation of aspartic acid (Asp) and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

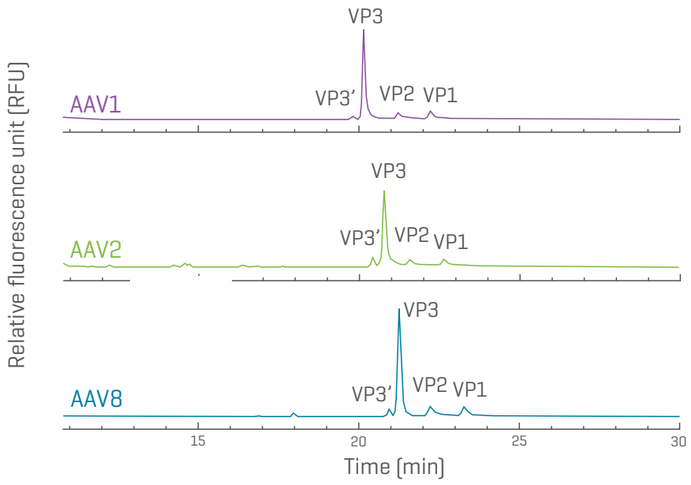
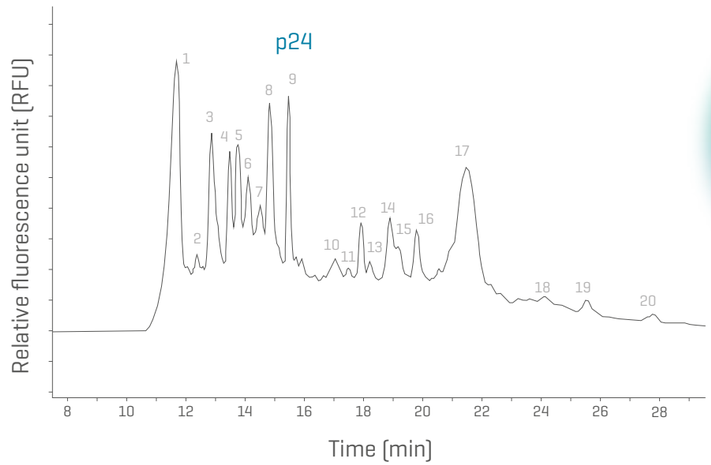


Figure 10: Separation of VP proteins from different AAV serotypes. Excellent resolution of VP proteins labelled with fluorescence dye was achieved with CE-SDS-LIF.



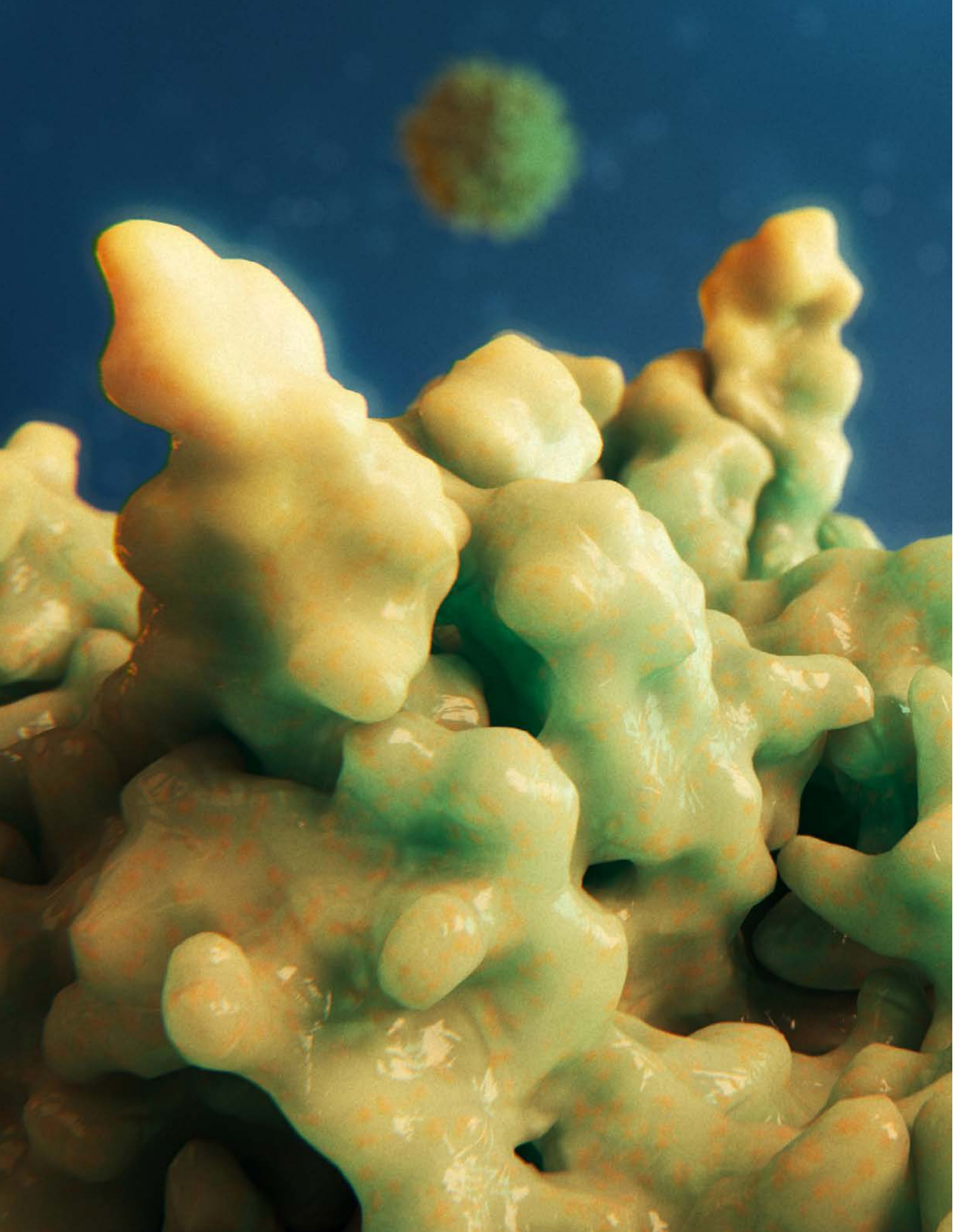
p24

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 **AVV**

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

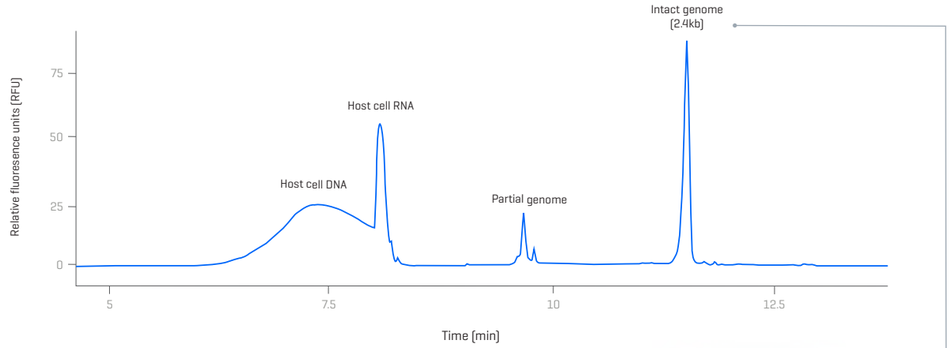


Figure 12: High-resolution genome integrity analysis of AAV8 with CGE and LIF detection. The intact genome of 2.4 kb was well separated from potential partial genomes and host cell ribonucleic acid (RNA), and DNA impurities.

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

Read technical note **AVV**

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

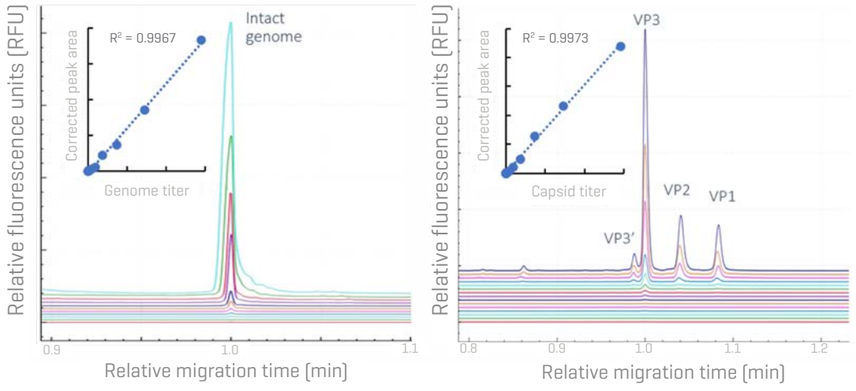


Figure 13: Standard curves for full-and-empty capsid determination. Left: AAV genome titer determination with CGE. The linear dynamic range (LDR) was determined from 2.56x1010 GC/mL-2.62x1013 GC/mL with R^2 = 0.9967. Right: AAV capsid titer determination with LDR from 6.41x109 GC/mL-2.62x1013 GC/mL with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

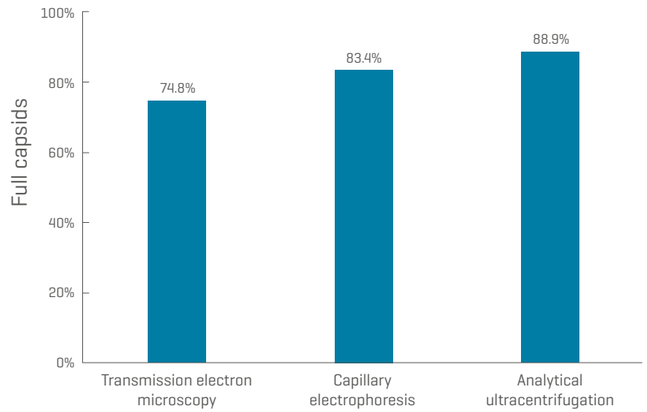


Figure 14: Comparison of the percentages of full capsids determined by different technologies. A good correlation between transmission electron microscopy and analytical ultracentrifugation was observed in comparison to CE.

<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

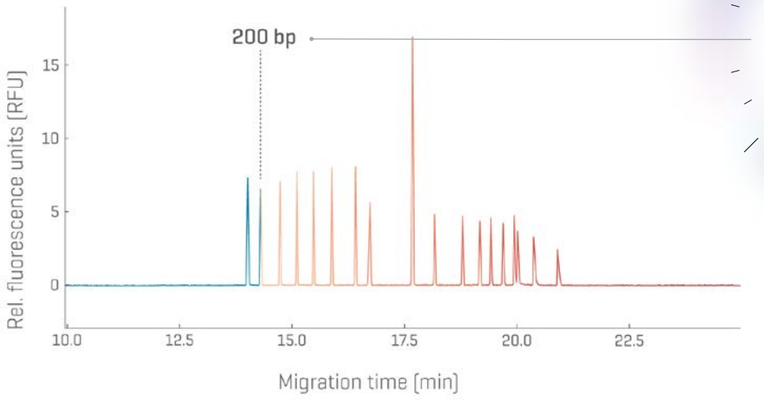


Figure 15: Electropherogram showing baseline separation of a linear dsDNA ladder from 100-15,000 bp.

200 bp
marker

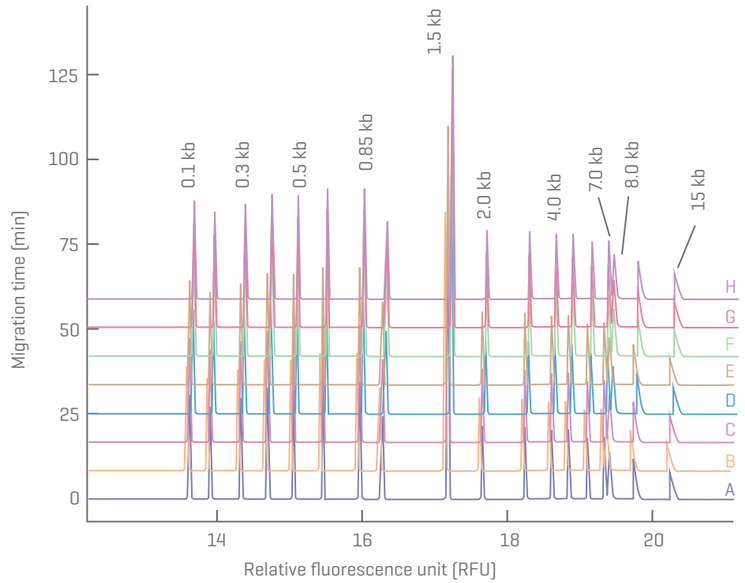


Figure 16: Representative electropherograms demonstrating the assay repeatability of the 1 kb Plus Linear DNA Ladder in single injections across eight capillaries in one cartridge using the BioPhase 8800 system.

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

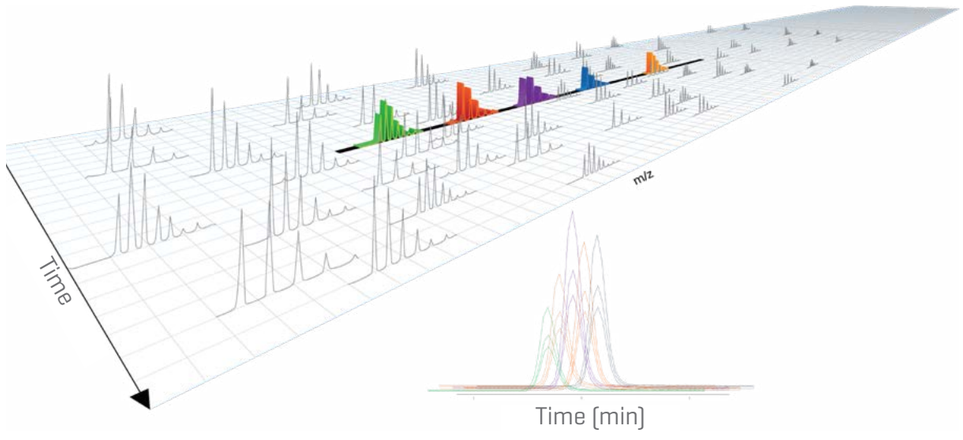
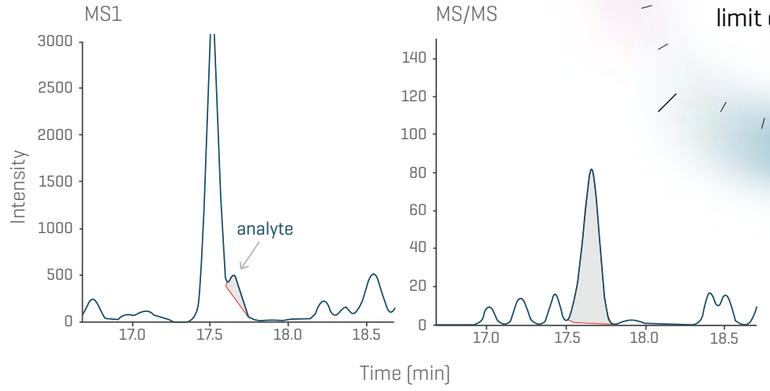


Figure 17: Schematic of a SWATH window. Top: Different precursors within a given m/z range (SWATH window) are present at a given time point (colored peaks) and selected for fragmentation. Bottom: The peak profiles of these precursors and related isotopes show slightly different elution times, which can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

Figure 18: Comparison of XICs of peptides in complex matrix. Left: XIC of precursor m/z of a peptide shows 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

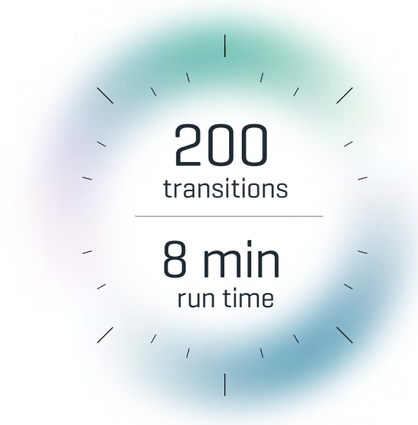
Discover more details in the technical note for HCP analysis of lentivirus samples

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

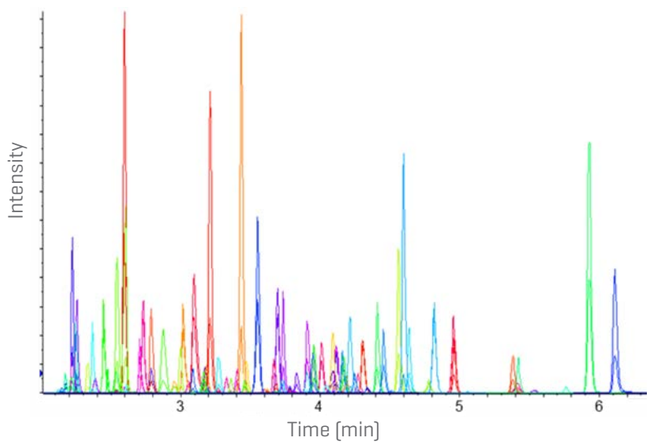
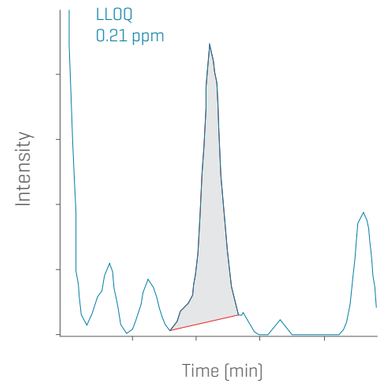
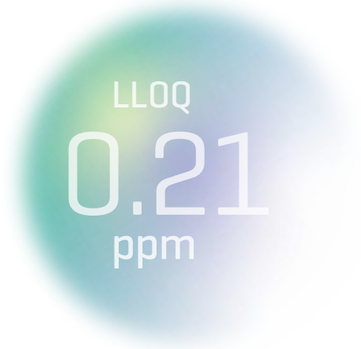


Figure 19: XICs of peptide surrogates for 48 target proteins in a biotherapeutic digest. More than 200 transitions were monitored in a scheduled MRM with a total run time of 8 min.

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

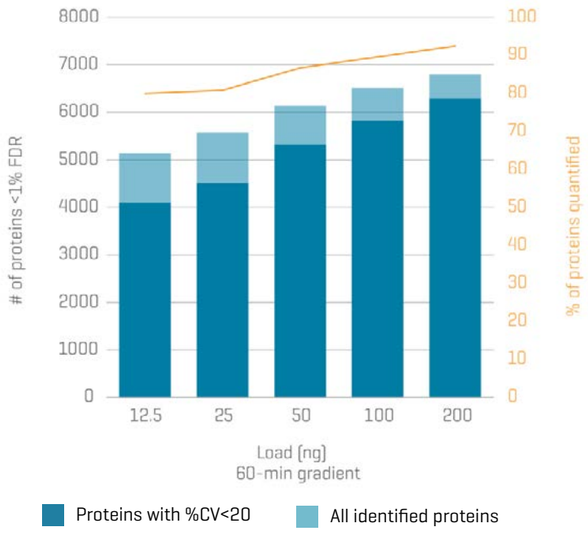
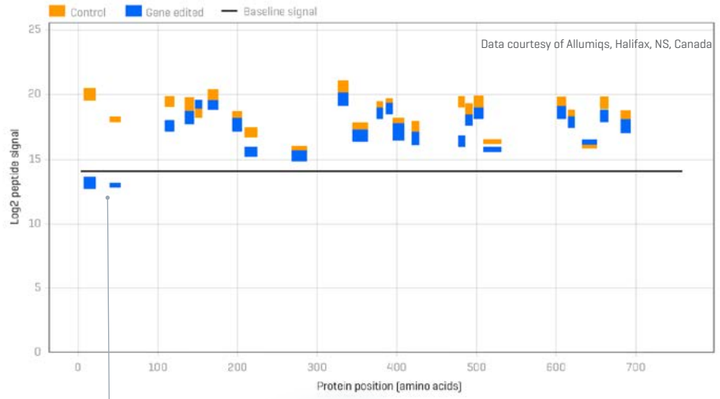


Figure 21: Identified and quantified proteins using SWATH DIA. Columns show number of proteins identified with false discovery rate (FDR) <1% for different loading amounts of cell lysate digest using a 60 min gradient with 5 µL/min flow rate. Line shows amount of proteins with %CV<20% used for quantitation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

Analytical solutions for viral vectors

Suitable for:

- Assessment of multiple CQAs of viral vectors
- Rapid method development and larger sample sets
- Analysis of host cell nucleic acids

BioPhase 8800 system

Purpose-built for achieving high quality data efficiently across various analytical assays.

Learn more

RNA 9000 Purity & Integrity kit

An intuitive kit to assess RNA and ssDNA integrity, purity and size, compatible with BFS capillaries.

Learn more

DNA 20 kb Plasmid and Linear kit

A kit to perform reproducible pDNA purity assessment and size estimation of linear dsDNA with ease.

Learn more

CE-SDS Protein Analysis kit

A high-performance kit with a sieving gel matrix, enabling protein purity and integrity analysis.

Suitable for:

- Assessment of multiple CQAs of viral vectors
- Analysis of host cell nucleic acids
- Smaller sample sets

PA 800 Plus system

A solution enabling confident decision making and QC-readiness for your biopharmaceutical products.

Learn more

RNA 9000 Purity & Integrity kit

An intuitive kit to assess RNA and ssDNA integrity, purity and size, compatible with BFS capillaries.

Learn more

DNA 20 kb Plasmid and Linear kit

A kit to perform reproducible pDNA purity assessment and size estimation of linear dsDNA with ease.

Learn more

SDS-MW Analysis kit

A kit including BFS capillaries for protein analysis using a replaceable gel matrix.

Analytical solutions for viral vectors

Suitable for:

- Intact VP characterization
- Peptide mapping including isomer differentiation and ID of challenging PTMs
- High flexibility to perform a range of additional workflows

ExionLC AE system

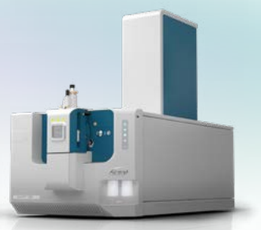
An LC system that seamlessly integrates with your SCIEX MS for reproducible data, batch after batch.

Learn more

ZenoTOF 7600 system

A high-resolution solution, combining powerful MS/MS sensitivity and alternative fragmentation technology.

Learn more



Biologics Explorer software

A powerful software tool to support challenging LC-MS/MS protein characterization assays.

Learn more

SCIEX OS software

The next generation of operating and processing solution for an enhanced LC-MS/MS analysis experience.

Learn more

Suitable for:

- Intact VP characterization
- Peptide mapping including ID of PTMs
- Intuitive operation

ExionLC AE system

An LC system that seamlessly integrates with your SCIEX MS for reproducible data, batch after batch.

Learn more

X500B QTOF system

An intuitive QTOF system, designed to facilitate everyday biologic characterization assays.

Learn more

Biologics Explorer software

A powerful software tool to support challenging LC-MS/MS protein characterization assays.

Learn more

SCIEX OS software

The next generation of operating and processing solution for an enhanced LC-MS/MS analysis experience.

Learn more

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 undergoes 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 concentration 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

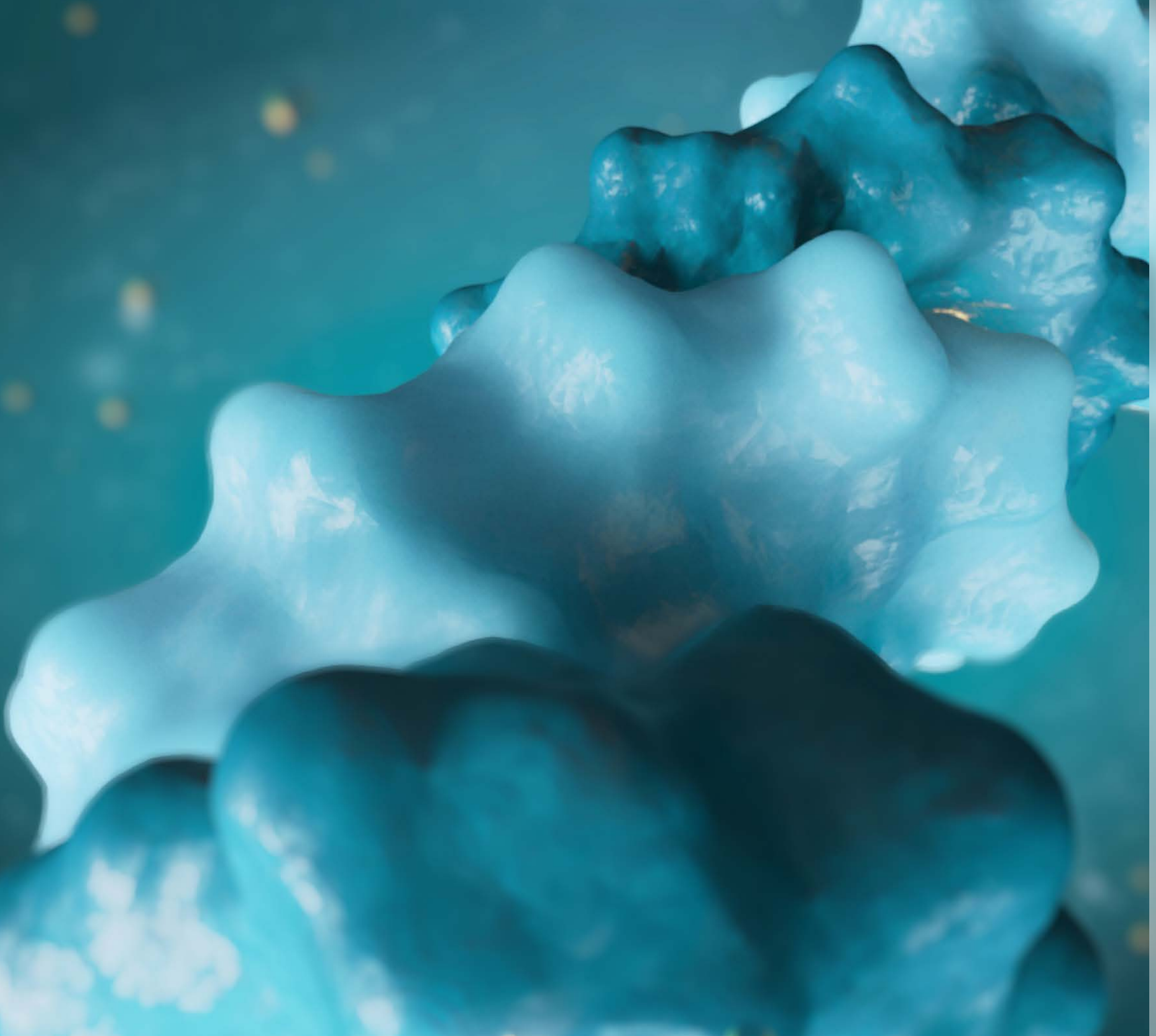
Labeling procedure can pose challenges and require optimization for each product. Currently, the most common

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?

Contact us



03

Plasmid DNA

Plasmid DNA

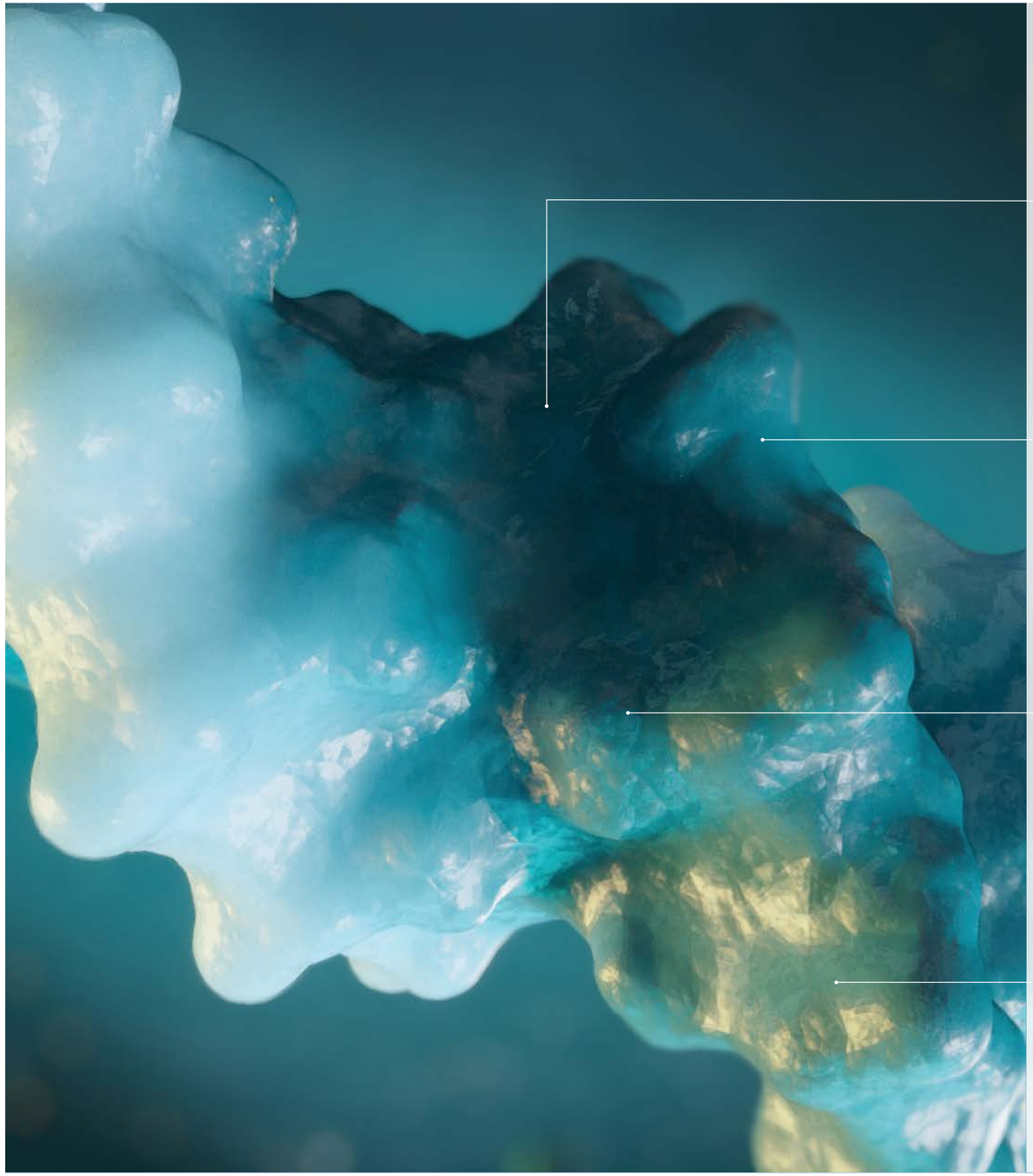
Double-stranded DNA plasmids are an extremely versatile tool frequently used for genetic engineering in biotechnology applications.

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 improve 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 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

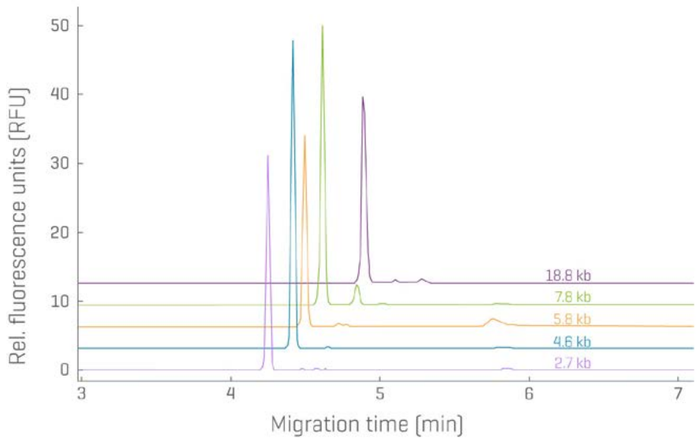


Figure 23: Separation of topological isoforms of 5 plasmids (2.7–18.9 kb) using the DNA 20 kb Plasmid and Linear kit.

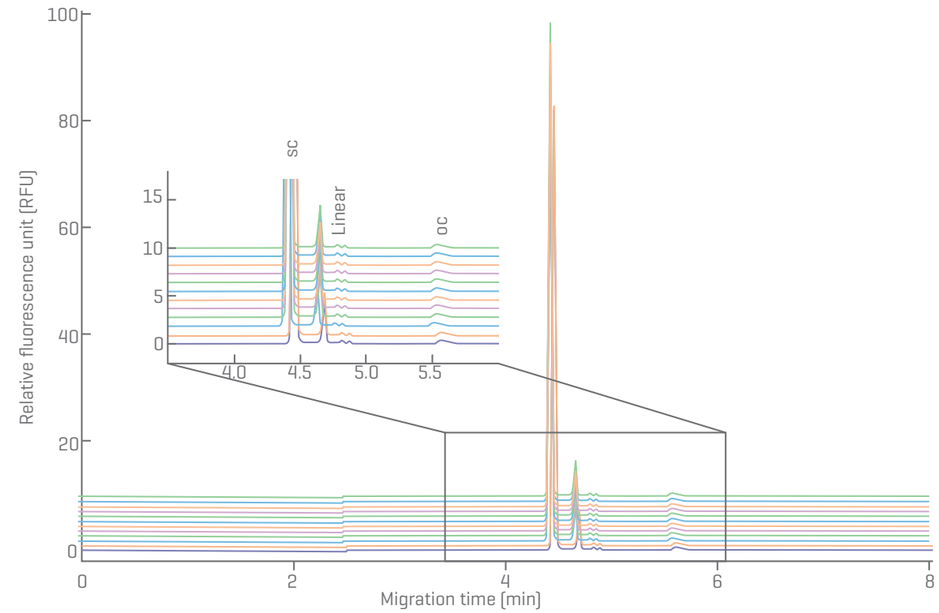


Figure 24: Assay repeatability of a 7.9 kb plasmid. The sample was injected from the same well for 12 consecutive injections and analyzed on the BioPhase 8800 system with a BioPhase BFS capillary cartridge - 8 x 30 cm using the DNA 20 kb Plasmid and Linear kit.

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

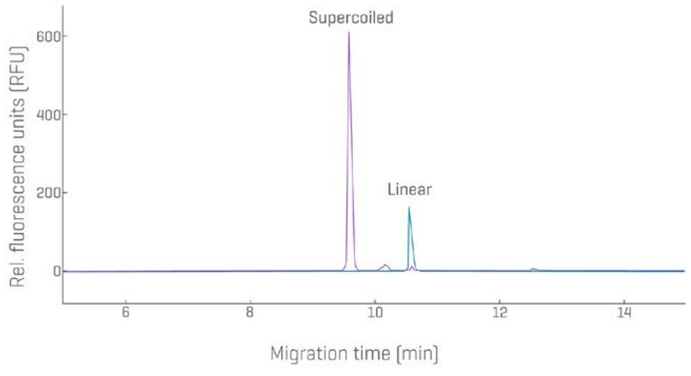


Figure 25: Linearized plasmid purity analysis and size estimation of a 7.9 kb plasmid sample. The electropherogram shows the sample prior to linearization, containing mainly the supercoiled isoform and after linearization.

Baseline separation between 7000 and 8000 bp

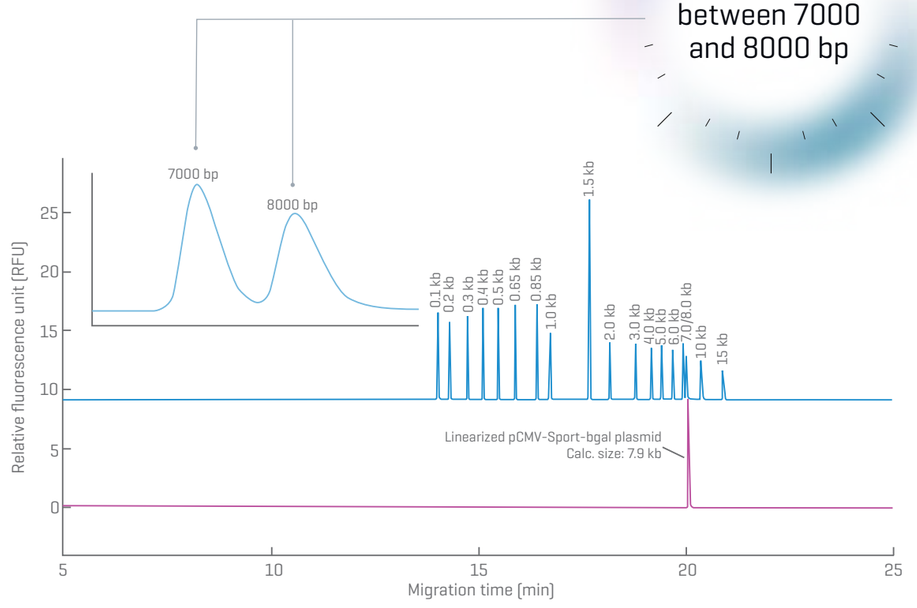


Figure 26: Size determination of the linearized 7.9 kb with a BioPhase BFS capillary cartridge - 8 x 50 cm. Top: The 1 kb Plus DNA Ladder with the inset showing the resolution between the 7,000 bp and 8,000 bp fragments. Bottom: The linearized plasmid sample with calculated size.

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

Read technical note

pDNA restriction map

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

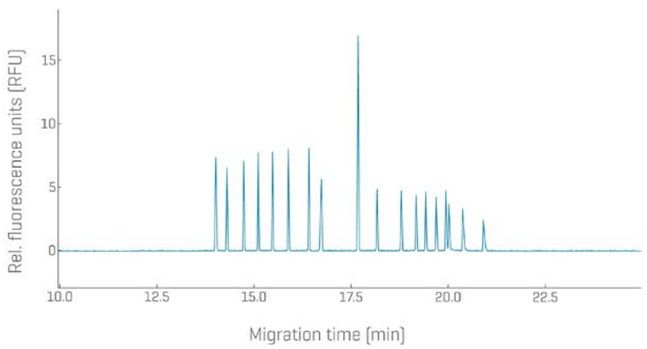


Figure 27: Electropherogram showing baseline separation of a linear dsDNA ladder from 100-15,000 bp.

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

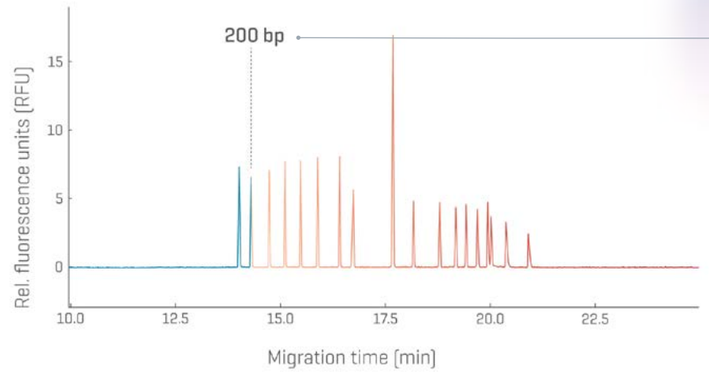


Figure 28: Electropherogram showing baseline separation of a linear dsDNA ladder from 100-15,000 bp.

200 bp marker

More questions?

Contact us

Analytical solutions for plasmid DNA

Suitable for:

- pDNA purity analysis
- Linear DNA sizing and fragment analysis
- Residual nucleic acid analysis
- Larger sample sets

BioPhase 8800 system

Purpose-built for achieving high quality data efficiently across various analytical assays.

Learn more

DNA 20 kb Plasmid and Linear kit

A kit to perform reproducible pDNA purity assessment and size estimation of linear dsDNA with ease.

Learn more

BFS capillary cartridge

A pre-assembled bare-fused silica 8-capillary cartridge available in 30 and 50 cm total length.

Suitable for:

- pDNA purity analysis
- Linear DNA sizing and fragment assessment
- Residual nucleic acid analysis

PA 800 Plus system

A solution enabling confident decision making and QC-readiness for your biopharmaceutical products.

Learn more

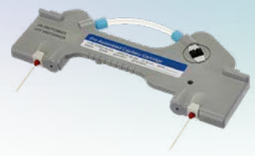
DNA 20 kb Plasmid and Linear kit

A kit to perform reproducible pDNA purity assessment and size estimation of linear dsDNA with ease.

Learn more

BFS capillary cartridge for the PA 800 Plus system

A pre-assembled bare-fused silica single capillary cartridge available in 30 cm total length.



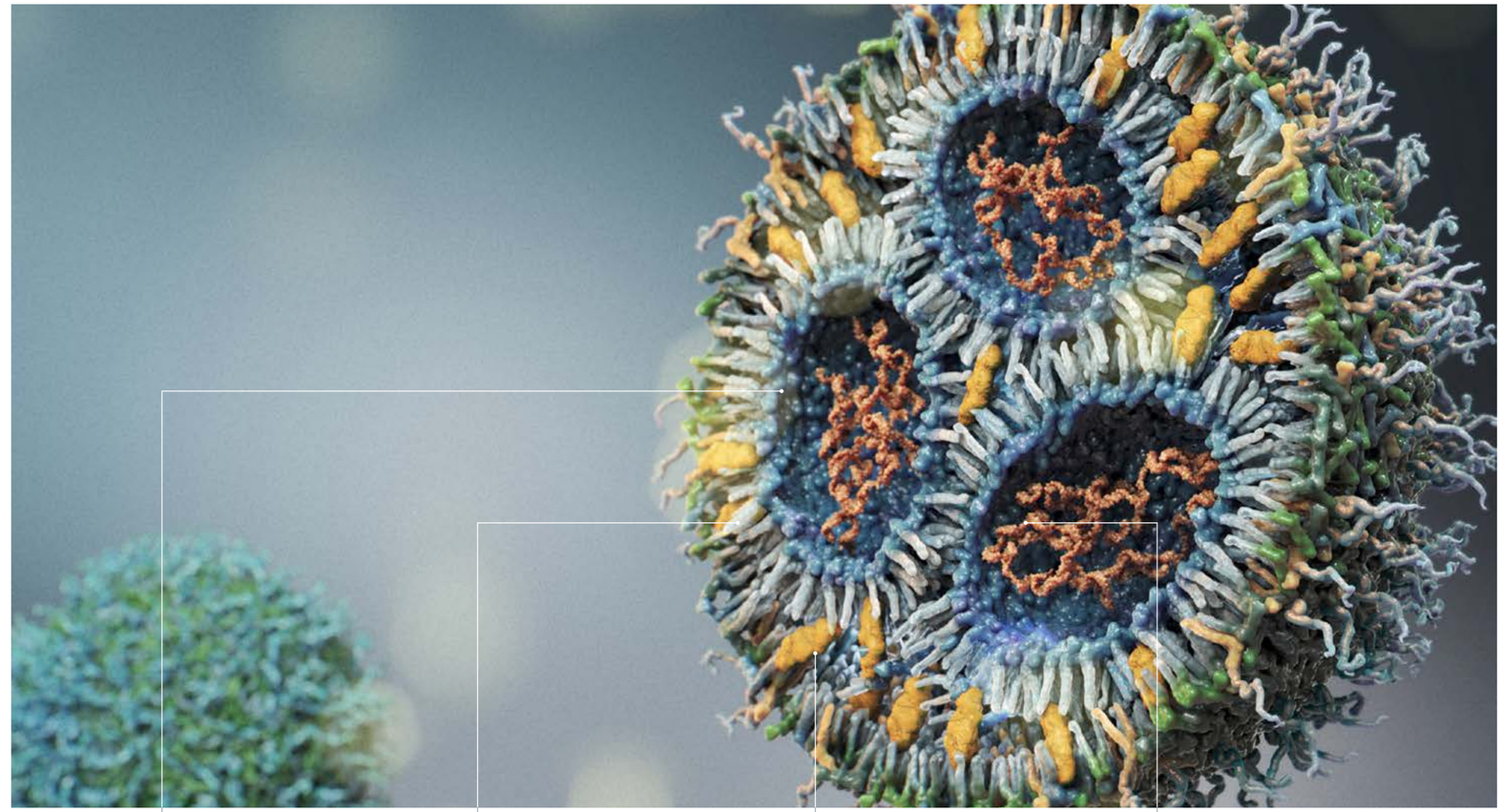
04

IVT RNA

IVT RNA

Discovered in the 1960s, mRNA has had a long lead time, approved regulatorily for the first time as a vaccine in 2021.

For the large and fragile cargo to enter cells effectively, the delivery mechanisms needed to evolve amongst other factors. Today, different in vitro transcribed RNA (IVT RNA) types, such as linear mRNA, large self-amplifying RNA (saRNA) or self-replicating RNA (srRNA), and circular RNA without 5' and 3' ends are approved for drugs or are in clinical phases. Their usage in medicine is highly diverse as seen by their application in vaccines, gene editing, replacement therapy and neoantigen expression. For any drug application, the comprehensive characterization of the large and often heterogeneous IVT RNA is critical.



"Given the "one break no effect" with mRNA, the integrity of mRNA drugs must be assessed to ensure its quality. Capillary gel electrophoresis (CGE) is a key tool in this field. CGE systems, such as the BioPhase 8800 system from SCIEX, offers high resolution and can efficiently characterize RNA profiles, enabling accurate quality control. Ensuring the integrity and purity of mRNA formulations is essential for the development of effective and safe therapeutics."

Jérémie Parot (PhD)
Research Scientist, SINTEF

mRNA integrity
Separate impurities from your IVT RNA and assess the integrity and purity of your product.

Circular RNA
Ensure separation of circular products from linear precursors and assess product purities.

poly(A) assessment with MS
Leverage LC-MS for the characterization of your mRNA 3' ends.

Encapsulation
Determine encapsulation efficiencies of mRNA in LNPs and simultaneously assess mRNA integrity.

saRNA and srRNA integrity
Break through boundaries for assessing purity and integrity of large RNA products beyond 9 kb.

5' end cap
Achieve identification and quantitation of capping structures and intermediates.

poly(A) assessment with CE
Characterize the poly(A) tail length and distribution of your IVT RNA with CE.

Protein expression
Identify and quantify expressed proteins and characterize PTMs of proteins.

Expert Q&A:

Adressing stability challenges of mRNA-LNPs

mRNAs are fragile molecules that are not meant to be very stable. Increasing their stability requires careful assessment of multiple factors. Here, Dr. Jingtao Zhang (Catalent® Pharma Solutions) provides his insights.

Can you provide details for the conditions of your pre-injection rinses?

Prior to starting a sample sequence, we perform an acid wash, a water wash and then fill the capillaries with gel. As part of our optimized method, we use 70 psi for 2 minutes for the acid wash, followed by a water wash at 70 psi for 2 minutes. To fill the capillaries with gel, we use 50 psi for 5 minutes. Once the capillaries are filled with gel, we run the separation method briefly—for 2 minutes—to pull out any small impurities before moving ahead to any samples. In addition to pre-injection rinses, we found it extremely important to use sample loading solution (P/N 608082) to dilute samples prior to injection. It helped us improve the peak shape and resolution of our mRNA samples and the RNA ladder.

Can you elaborate on your settings for the pressure injection of mRNA?

We used 1 psi for 5 seconds. However, you can adjust the duration depending on your needs. We have also used electrokinetic injection in the past, which applied -1.0 kV for 6 seconds to load samples into the capillaries. This method also worked well and required less sample, but we decided to stick with pressure injection since it provides higher reproducibility in our experience.

Is there a size limit for mRNA analysis on the BioPhase 8800 system?

I'm not aware of a specific size limit on the BioPhase 8800 system. The ladder from the RNA 9000 Purity & Integrity kit covers a range from 500 nt to 9,000 nt. While most mRNA we work with is within this range, larger mRNA can be analyzed when extending the run time, with the caveat of working outside of the calibration curve range which would impact sizing accuracy. This would be something interesting to investigate.

Which capillaries do you use for mRNA analysis?

The capillaries we used for the BioPhase 8800 system are bare-fused silica (BFS) capillaries as part of the BioPhase 8800 BFS capillary cartridge (P/N 5080121). The cartridge contains 8 pre-installed capillaries, each 30 cm long, and a detection window 20 cm from the inlet. Liquid-based temperature control of the separation temperature is incorporated with these cartridges.

Can you comment on the transferability of your extraction method with the Triton X-100 and temperature settings for CGE analysis to other mRNA products?

In general, the method can be transferred to different mRNA products. However, I suggest optimizing the surfactants and the temperature settings since different mRNA products can be more susceptible to secondary structure formation, aggregation or formation of multimers, and may have different sensitivity towards temperature. You really want to make sure that your surfactant concentration is suitable for your mRNA product. In case you are getting poor recovery, you would want to investigate surfactant types or concentration. Since different mRNA products exhibit different sensitivities to temperature, I recommend optimizing sample incubation temperatures. For instance, a test range of 40 to 70 degrees Celsius, including different incubation times for a given temperature, is a good starting point. I also recommend optimizing your cartridge temperature as well to reduce the formation of secondary structures.

Did you find that the recovery of mRNA from your LNPs is dependent on the type of ionizable lipid used?

We tried several types of ionized lipids and different compositions of LNPs. For the ones we tried, we have not experienced significant issues related to recovery using an optimized extraction method. We did observe that non-optimized extraction methods, including sample preparation such as surfactants level and denaturants, could affect recovery.

Are you able to comment on other approaches regarding stability, such as lyophilization?

The approach we currently take is focused on preserving the formulation through freezing. This adds inconvenience and increases costs. During lyophilization, water is removed from the LNP system, and a product can be stored in refrigerated conditions, potentially even at room temperature. The requirements for upholding a suitable cold chain would be dramatically reduced. Alternative solutions for storage of mRNA products, like lyophilization, are therefore very interesting. A lot of work still needs to be done, particularly regarding lyophilization of LNPs.

Can you comment on how excipients affect mRNA integrity?

Various excipients—lipid and non-lipid excipients—play an especially important role in stabilizing the overall product and can also affect the active ingredient, the mRNA. For instance, it is known that secondary structures of mRNA are pH dependent. Excipients, such as buffer salts, that modulate the pH can therefore affect the mRNA structure. Lipid excipients are crucial to the drug's efficacy and tolerability. As a result of this and stability concerns, we need to pay a lot of attention to their quality. Impurities in excipients can lead to degradation of mRNA. A now well-known example is the reaction of mRNA with aldehydes, which can exist as impurities in lipids. When evaluating excipients, my suggestion is to decouple the effects of the excipients from those related to the excipients' quality.

Is there any carrier system being used for mRNA other than LNPs?

Up until now, LNPs leveraging ionizable lipids are the most clinically validated system for mRNA delivery. LNPs come in different flavors, usually using ionizable or cationic lipids as a key component, and can complex the negatively charged mRNA cargo. Some LNP research focuses on the usage of biodegradable lipids to improve the biocompatibilities while others have focused on targeted systems for enhanced efficiencies. Other systems can be used for non-viral delivery, such as polymers or cell-penetrating peptides (CPP). In some cases, polymeric systems can be coupled with LNPs to gain the best of both worlds. It's an exciting field with a lot of ongoing research.

Read Q&A blog

Dr. Jingtao Zhang is the Scientific Director in the Biologics Group at Catalent® Pharma Solutions. He is responsible for developing new technical capabilities and product solutions to solve clients' pressing pharmaceutical problems with a special focus on expanding mRNA-LNP within Catalent Pharma Solutions. He has more than 16 years of experience in R&D and commercialization of small molecules, peptides, oligonucleotides, biologics and mRNA-LNP drug products in pharmaceutical and CDMO settings. Jingtao received his PhD in Chemical Engineering from the University of Wisconsin-Madison and authored/co-authored more than 30 articles in peer-reviewed journals.

mRNA integrity and purity

The stability of fragile RNA cargo requires careful testing during development because mRNA-based drugs can lose their efficacy when truncated. In addition, process-related nucleic acid impurities may pose safety concerns. The integrity and purity of mRNA constructs are therefore important aspects for product quality. High resolution separation with excellent reproducibility enables the assessment of these product quality parameters accurately and reliably.

- Break through analytical boundaries with ultra-high resolution and excellent reproducibility
- Determine the integrity and purity of your nucleic acid products from 50 up to 9,000 nucleotides (nt) and beyond
- Confidently transfer assays from development to QC and streamline data management through compatibility with data management systems

Confirm integrity and determine purity with excellent resolution and reproducibility

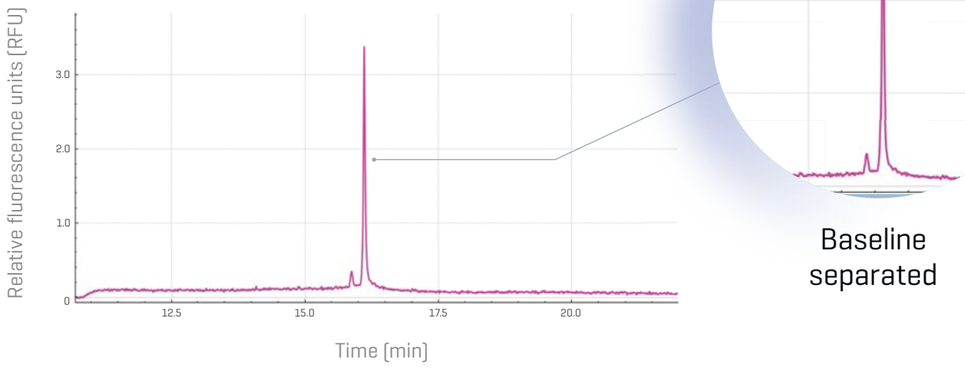


Figure 29: Electropherogram of mRNA extracted from an LNP analyzed using the BioPhase 8800 system. The mRNA of 1.929 kb was encapsulated in an LNP with MC3 as the ionizable lipid.

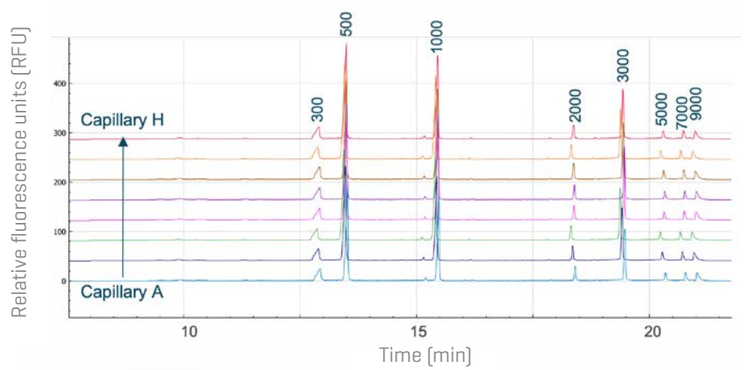


Figure 30: Reproducibility across all 8 capillaries of the BioPhase 8800 system. The electropherograms of the single-stranded ladder, spanning from 150-9,000 nt, show full separation and excellent reproducibility for the entire size range of the ladder from the RNA 9000 Purity & Integrity kit.

RNA 9000 Purity & Integrity kit

An intuitive kit to assess RNA and ssDNA integrity, purity and size, compatible with BFS capillaries.

Learn more

Discover more details in the technical note about mRNA integrity and purity assessment

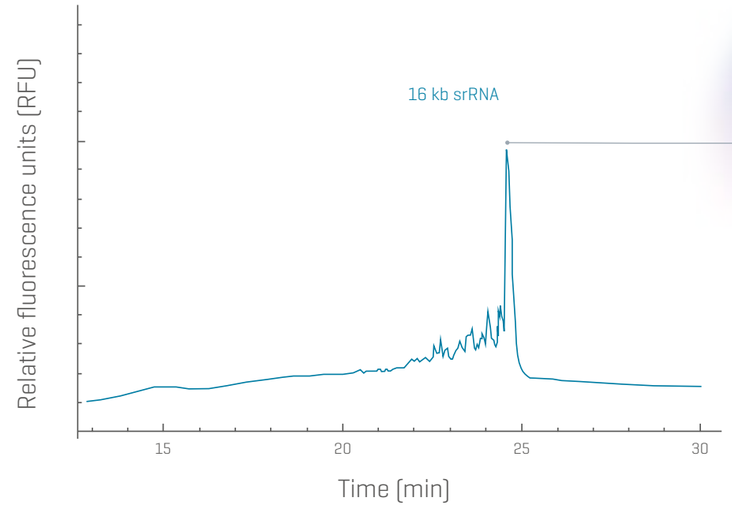
Read technical note

srRNA integrity and purity

Conventional mRNA and base-modified mRNA (bmRNA), which incorporates chemically modified nucleotides, are non-replicating IVT RNAs. SrRNA is an emerging third type that is based on an engineered viral genome, devoid of viral structural protein genes. The self-replicating ability makes srRNA a promising tool for new therapeutic drugs, despite challenges with its length.

- Determine the integrity and purity of your srRNA even beyond 9,000 nt
- Break through analytical boundaries with ultra-high resolution and excellent reproducibility
- Confidently transfer assays from development to QC and streamline data management through compatibility with data management systems

Confirm integrity and monitor impurity profiles with high resolution and reproducibility



16 kb
well resolved

Figure 31: Electropherogram of research-grade srRNA material (16 kb) obtained from Replicate Bioscience, San Diego, CA, USA.

Discover more details in the technical note about srRNA integrity and purity assessment

Read technical note

Circular RNA assessment

Circular RNAs are next-generation IVT RNAs that provide the benefit of high resistance towards exonucleases—without 5′ caps or poly(A) tails. To achieve circular RNAs, linear precursors are chemically or enzymatically ligated. Understanding the purity of the circular product requires a high-resolution separation workflow, which can separate linear precursors, degradation and high molecular weight products from the desired circular RNA.

- Take charge of your product quality and determine the efficiency of your circulation processes
- Break through analytical boundaries with ultra-high resolution and excellent reproducibility
- Confidently transfer assays from development to QC and streamline data management through compatibility with data management systems

Full separation of linear precursor and circular RNA

Fully separate linear precursors from circular RNAs with the highest reproducibility

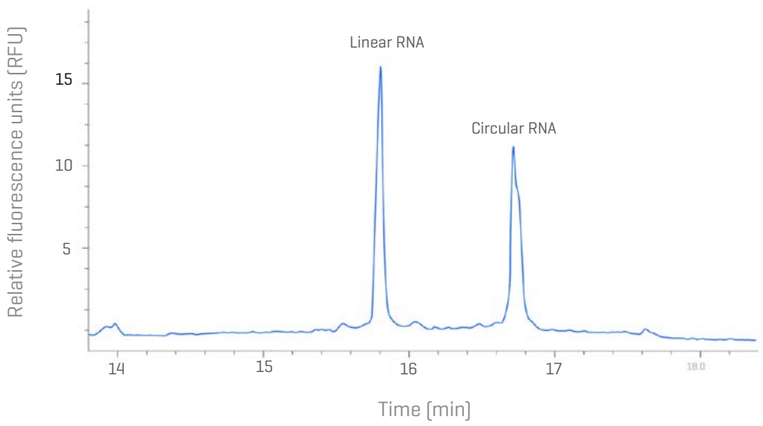


Figure 32: Separation of circular from linear precursor RNA with CGE-LIF. The linear RNA product migrates faster through the gel matrix than the circular RNA product due to a smaller effective cross-section.

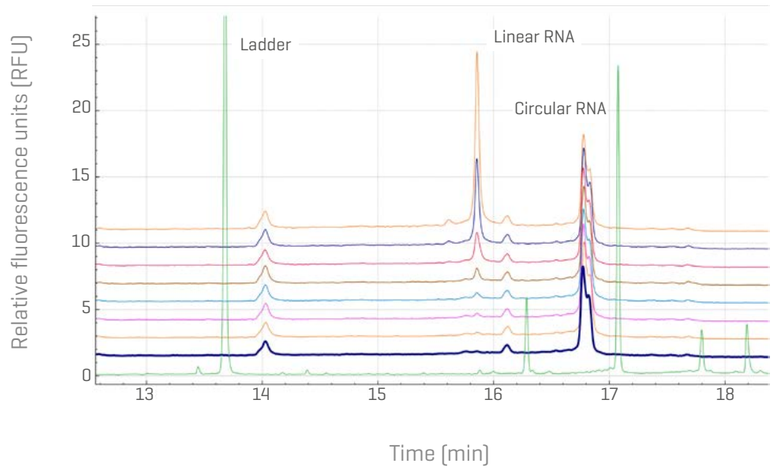


Figure 33: Sensitivity assessment for linear RNA impurity. The circular RNA product was spiked with a serial dilution of linear precursor. A detection limit of 0.1% relative to the circular product was determined. Green trace: ssRNA ladder from RNA 9000 Purity & Integrity kit.

Break through analytical boundaries with ultra-high resolution and excellent reproducibility

Discover more details in the technical note about circular RNA assessment

Read technical note

Encapsulation efficiency of mRNA

LNPs are designed to keep the fragile genetic cargo safe. Degradation and loss of function of nucleic acids is accelerated if they are not sufficiently encapsulated. In addition, the cellular uptake of the drug can be impeded. The encapsulation efficiency of the genetic cargo is therefore an important quality criteria to be optimized and monitored during the development of LNP-based drugs.

- Take charge of development decisions by understanding encapsulation efficiencies of drug substances with a reliable kit-based CE workflow
- Determine free and encapsulated mRNA amounts with excellent repeatability and sensitivity
- Simultaneously monitor degradation products in your samples leveraging exceptional resolving power

Achieve it all:

- Excellent resolution
- Sensitivity
- Linearity with low %CVs

Achieve it all: Excellent resolution, sensitivity and linearity with low %CVs

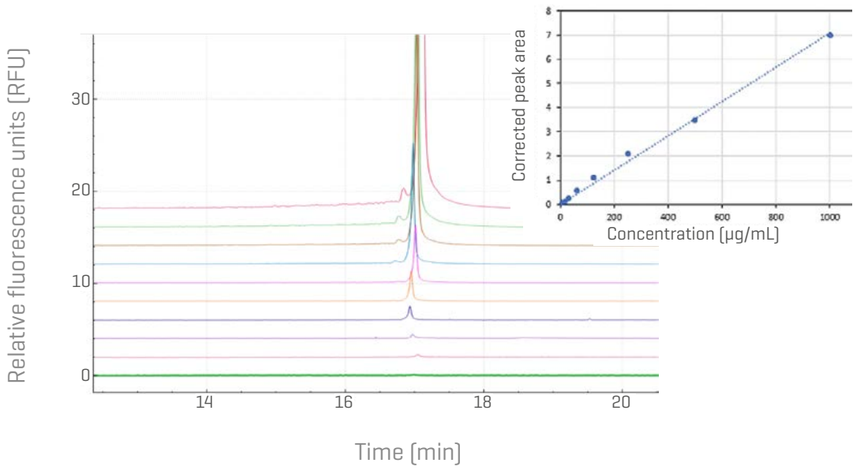


Figure 34: Serial dilution of mRNA standard. Electropherograms show excellent migration time reproducibility. Corrected peak area vs. mRNA concentration showed a linear correlation with $R^2 = 0.9989$.

Nominal (µg/mL)	Measured (µg/mL)				%CV	Accuracy (%)
	#1	#2	#3	Mean		
400	387	390	382	386	1.1	97
500	494	489	480	489	1.7	98
600	574	580	570	575	0.9	96

Figure 35: Results of replicate injections of different mRNA concentrations. Reproducible peak areas with very low %CV and very high accuracy were determined based on triplicates.

Reproducible peak areas with very low %CV and very high accuracy were determined based on triplicates.

Discover more details in the technical note about mRNA encapsulation efficiency analysis with CE

Read technical note

5’ capping analysis

The 5’ cap of IVT mRNA has a direct impact on its stability and translation efficiency and is therefore considered a CQA. Since G cap, cap 0 and the mature cap 1 are linked to different pharmacological efficacies, detailed characterization and simultaneous relative quantitation is needed to ensure product quality. The differences between the different capping structures are only 1-2 methyl groups, which requires high resolving power to be distinguished.

- Characterize 5’ caps and intermediate products reliably using excellent time-of-flight (TOF) MS data quality
- Obtain relative quantitative information automatically or tailor quantitative calculations specifically to your needs
- Take back your time with intuitive acquisition and processing software

Take back your time with intuitive acquisition and processing software

Understand your product quality with ease using high-quality data

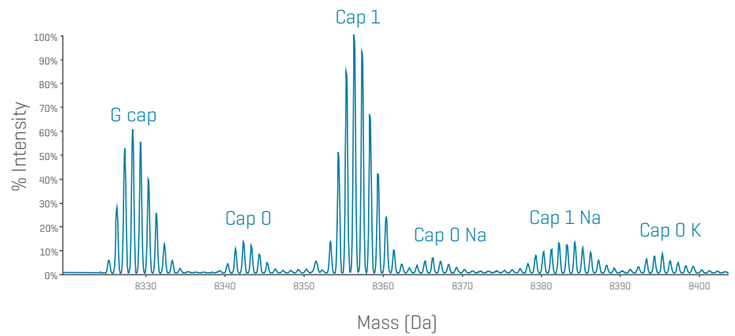


Figure 36: Deconvoluted data for mRNA 5’ end with isotopic resolution. Different capping intermediates and mature cap 1 species as well as sodium (Na) and potassium (K) adducts were identified.

	Name	Neutral Mass	Peak Area	% Area
1	Capped - ppUncap	7980.17	8.84E+05	34.57
2	Gcap	8325.24	5.62E+05	21.96
3	Cap1	8353.27	4.02E+05	15.73
4	Capped - pUncap	7900.21	3.21E+05	12.53
5	ppp Uncap K+ adduct	8098.06	1.38E+05	5.41
6	ppp Uncap	8060.13	1.22E+05	4.79
7	Cap0	8339.27	7.12E+04	2.78
8	ppp Uncap Na+ adduct	8082.13	3.32E+04	1.30
9	Capped - ppUncap Na+ adduct	8002.17	2.12E+04	0.83
10	Capped - ppUncap K+ adduct	8018.13	2.24E+03	0.09

Figure 37: Results of identified mRNA capping moieties. Table shows identified species, associated molecular weight and relative quantitative information using Molecule Profiler software.

Learn more

Read technical note

3’ end poly(A) tail of mRNA with LC-MS

Polyadenylation is needed to enable product stability and translation efficiency of mature mRNA products. As a result, the length and distribution profile of the poly(A) tail during development are highly relevant concerns, whether the tail is template-encoded, enzymatically added or applied through a combination of these approaches. The detailed characterization of poly(A) tails requires accurate data with high resolving power.

- Leverage excellent data quality through exceptional negative ionization efficiency and declustering of adducts with state-of-the-art source design
- Uncover relevant information on poly(A) tails, such as molecular weight and distribution profiles
- Trust in your high-resolution results with great mass accuracy

Dig deeper into your product quality with high-quality data

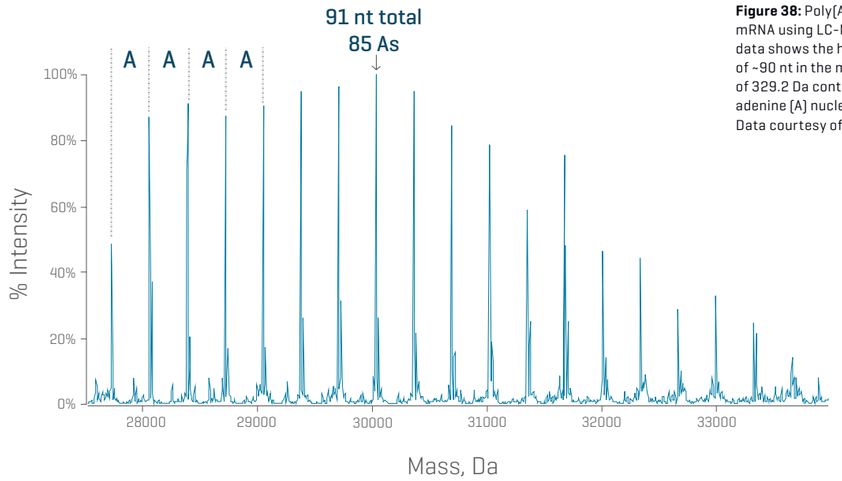


Figure 38: Poly(A) tail analysis from digested mRNA using LC-MS. Deconvoluted TOF-MS data shows the heterogeneity of the tail length of ~90 nt in the mRNA sample. Each Δ amu of 329.2 Da contributes to the addition of 1 adenine (A) nucleotide in the polynucleotide. Data courtesy of Phenomenex, CA, USA.

More questions?

Contact us

3’ end poly(A) tail of mRNA with CGE-UV

Since the 3’-end poly(A) tail is a CQA affecting product stability and translation efficiency, its optimization and monitoring throughout development and manufacturing is critical. The determination of length and distribution profiles require accurate assays with high resolving power and repeatability. To enable implementation in QC environments, intuitive and robust assays are needed.

- Take control of your IVT RNA 3’ CQA with reproducible, high-quality CE data
- Dig deeper than ever before into the dispersity of your 3’ poly(A) tails with single-nucleotide resolution
- Confidently transfer assays from development to QC and streamline data management through compatibility with data management systems

Enable yourself with single-base resolution and excellent reproducibility

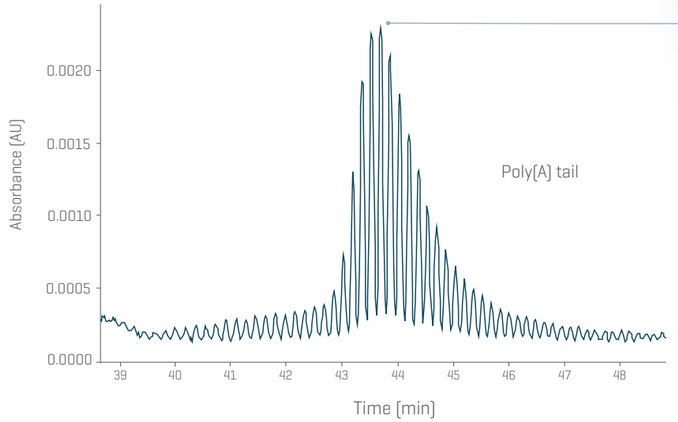
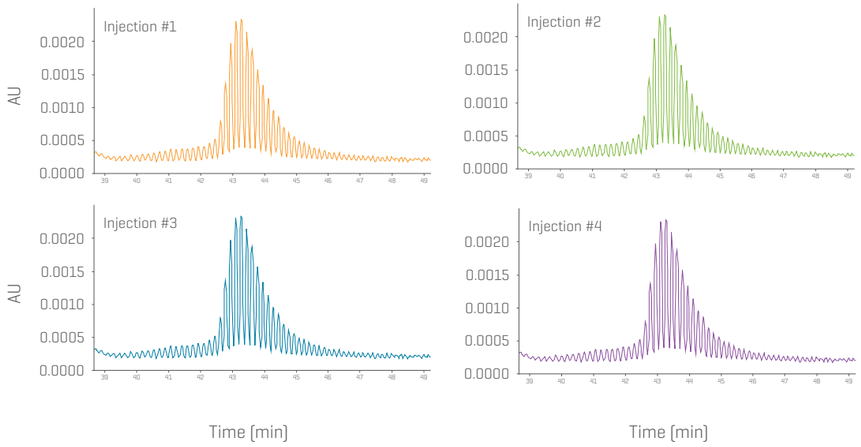


Figure 39: Poly(A) tail analysis from mRNA with CGE-UV. Electropherogram shows single-base resolution of mRNA poly(A) tails with most abundant species of 121 nt in length.

Figure 40: mRNA poly(A) tail reproducibility analysis with CGE-UV. The 4 replicate injections show high reproducibility in terms of migration time and peak profiles.

Dig deeper than ever before into the dispersity of your 3’ poly(A) tails with single-nucleotide resolution



Discover more details in the technical note about poly(A) tail analysis with CGE

Read technical note

Protein expression analysis

Upon successful delivery of the genetic cargo, drug substances such as IVT RNA are supposed to induce protein expression. While ELISAs and western blots are widely used to determine functional potency, these assays are limited by the availability of antibodies with high specificity for the target protein. Flexible approaches to determine protein expression that do not rely on antibodies can help with adhering to timelines and fast-paced changes in development pipelines.

- Break through the boundaries of complex matrices and achieve reliable identification and excellent quantitation simultaneously
- Leverage impeccable quantitative performance for decision making with high linear dynamic range and low limits of detection and quantitation
- Streamline your quantitative data processing with state-of-the art software

Don’t let matrices hold you back. Gain signal-to-noise with high resolution quantitation.

Don’t let matrices hold you back—level up your MS/MS sensitivity

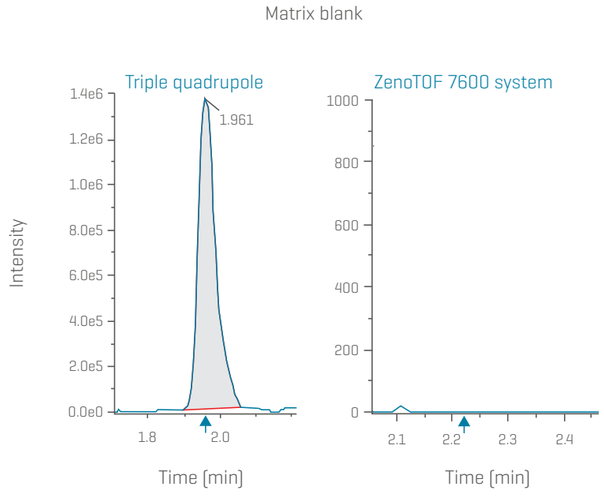


Figure 41: Comparison of matrix blanks. Same matrix blank was injected onto a triple quad system and the ZenoTOF 7600 system. The high resolution on the TOF system resulted in a clean blank and therefore lower limits of quantitation for a peptide analyte compared to the triple quad data obtained.

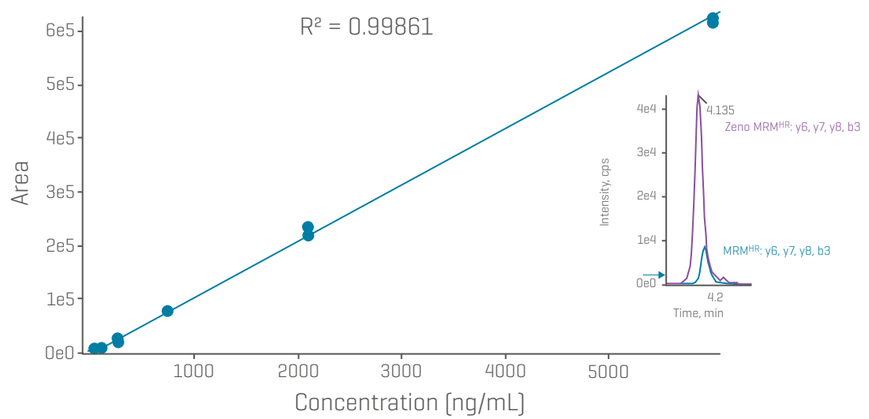


Figure 42: Peptide quantitation in complex matrix. Calibration curve obtained for a peptide in complex matrix using the sum of multiple fragment ions enhanced by the Zeno trap. Inset shows a comparison of peak intensities obtained when summing the same peptide fragment ions with the Zeno trap enabled (pink) and without (blue).

Increase fragment intensities with the Zeno trap

Discover more details in the technical note about peptide quantitation in matrix

Read technical note

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- High-quality separation
- Rapid method development and sample analysis

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BFS capillary cartridge

A pre-assembled bare-fused silica 8-capillary cartridge available in 30 and 50 cm total length.

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- Single nucleotide resolution for poly(A) 3’–ends
- Large srRNA assessment

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RNA 9000 Purity & Integrity kit

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BFS capillary cartridge

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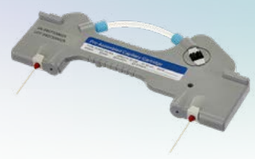

ssDNA 100-R kit

Designed for ultra-high resolution analysis of single-stranded nucleic acids using coated capillaries.

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- Robust, analytical flow setup

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- Protein expression analysis
- High flexibility to perform a range of additional workflows

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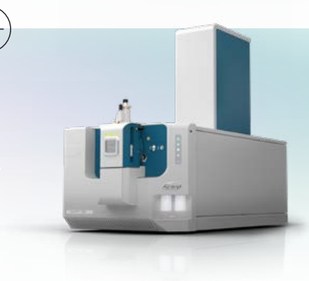

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05

Oligonucleotide therapeutics

Oligonucleotide therapeutics

In 1998, the US Food and Drug Administration (FDA) approved fomivirsen as the first oligonucleotide therapeutic. This approval marked a revolution of action mechanism discovered decades ago before finally coming to fruition.

Since then, the landscape of chemical modifications of oligonucleotides, conjugations and formulations has evolved tremendously, contributing to improvements in stability, efficacy and safety. Today, more than a dozen ASO drugs, including siRNA and aptamers are on the market, most of which are designated as orphan drugs for treating rare genetic diseases. Alongside the oligonucleotides, analytical solutions are required to keep up with the ever-decreasing concentrations of analytes of more potent drugs, pushing the boundaries of sensitivity.

"You can theorize all you want, but until you actually step into the lab and do the work, you don't know what the outcomes are going to be."

Shane Karnik
Sr. Laboratory Director, Aliri Bioanalysis

Characterization and impurity ID

Confirm sequences, understand molecular weights and achieve identification of impurities.

Oligonucleotide MetID

Achieve a better understanding of your ASOs by structural elucidation of their metabolites.

Bioanalysis and DMPK

Overcome quantitation challenges of oligonucleotides with highly sensitive and robust solutions.

Expert Q&A:

Bioanalysis of oligonucleotide therapeutics

The bioanalysis of oligonucleotides is particularly challenging, from sample preparation to method optimization and data analysis for multiply charged analytes. Here, Shane Karnik and Dr. Troy Voelker, both experts in the bioanalysis of oligonucleotides, share their experiences.

Dr. Troy Voelker is the Director of Laboratory Operations at Aliri Bioanalysis in Salt Lake City, Utah, leading the lab divisions including method development, method validation, instrument operations, and production groups. He obtained his PhD in synthetic organic chemistry and a post doctorate in medicinal drug development and drug metabolism. His bioanalytical industry experience spans over 18 years, including triple quadrupoles and high-resolution accurate mass platforms for large and small molecules. As Aliri's subject matter expert for oligonucleotide bioanalysis, he has led method development and validation for the past 10 years.

What type of column do you use for the bioanalysis of oligonucleotides to optimize peak shape, retention and carryover effects?

Troy Voelker: This is a common question, and I hate to give a general answer to it: I do not have one column that I go to every single time. I use the best column for the oligonucleotide that we are analyzing. Multiple vendors have specialty columns for this type of work, and I recommend going through that suite of options. There is a general consideration when looking for a column in terms of chemistry. You want a column that is going to be stable above 65°C, especially when you are analyzing siRNA, since you must elevate the temperature to melt the double strands. The high temperature can cause degradation over time for some column chemistries, which you want to avoid picking. We look at columns from different vendors and decide based on peak shape and carryover. You will have to do those experiments upfront to figure out which column is best for your oligonucleotide.

What is your recommendation for oligonucleotide quantitation on the ZenoTOF 7600 system? Do you usually use TOF-MS or MS/MS?

Troy Voelker: Both modes work for quantitation. Whether one is better suited for your project is something you want to explore in your method development stage. There are 2 main factors to keep in mind: intensity and signal to noise. Your MS2 data is not going to have the same intensity as your MS1 data. The main reason for that is because you are breaking the precursor apart into multiple fragments and therefore spreading the intensity across those fragments. On the other hand, you may achieve a better signal-to-noise ratio for a fragment compared to the precursor. That's often the case because of filtering out a lot of the background when selecting a particular precursor for fragmentation. When working with samples that contain metabolites that may start creating interferences by overlapping charge state envelopes, you may want to pick specific fragments that would be unique to the metabolite and the parent, respectively. The fast acquisition rates of the ZenoTOF 7600 system allow for collection of both full scan MS1 and multiple full scan MS2, while achieving the desired amount of data points across the peak for accurate quantitation. Since you can get both sets of data, you could still decide later which data set to use.

What internal standard are you using for LC-MS? Can you share general considerations for selecting internal standards for oligonucleotide quantitation?

Shane Karnik: For the work I presented, a structural analog internal standard with 2 more bases than the analyte oligonucleotide was used. Personally, I have never come across a project for which we got a stable isotope labeled internal standard created. Usually, the internal standards were based on alternating bases, additions or eliminations of bases, resulting in a different m/z than the analyte. As a general consideration, I recommend picking an internal standard that very much acts the same way as your compound. You want a standard that has the same extraction efficiency and the same ionization. Since you are going to look at peak area ratios between your analyte and standard, changes in responses like enhancements or ion suppression should be consistent between analyte and standard. We therefore do not use a universal standard. When we work on a client project with several drug lead candidates, we can take another candidate and use that as the internal standard.

Troy Voelker: I agree with Shane. A stable labeled internal standard is rare. The standard is almost always an analog. To add onto Shane's recommendations, if you are dealing with an oligonucleotide drug candidate with special chemistry, you may want to keep that consistent for your analog selection. In addition, I recommend using internal standards that are slightly higher in mass. With that potential, interferences with metabolites can be avoided. Metabolites such as N-1, N-2 may cause interferences with an internal standard that is lower in mass. We therefore do tend to go for a higher mass internal standard.you can get both sets of data, you could still decide later which data set to use.

When you quantify metabolites, do you typically quantify against reference material or use the parent?

Shane Karnik: There are 2 answers to this question. When doing regulated GLP work, you must have a well-characterized reference material for that metabolite. However, for early discovery work, when we are just looking for metabolites and want to get a general idea of the concentration of metabolites, we do not need a reference material. We can use the TOF-MS data, look for expected metabolites and extract those m/z from the data. For quantitation, we then assume a similar response as the parent or the internal standard and use the parent quantitation curve for the metabolites. This would be only for non-regulated discovery work.

Troy Voelker: I can add that there are likely going to be differences in the ionization between the parent and the metabolite. It is something that we generally observe. As Shane stated, the approach without reference material gives you an idea of the concentrations, but you are certainly not going to get an absolute concentration.

Does the ZenoTOF 7600 system allow for characterization of the oligonucleotides by analyzing the fragments? Is there oligonucleotide sequencing software available to process the data?

Shane Karnik: Yes. You can use the MS/MS data from the ZenoTOF 7600 system and process it with Molecule Profiler software to achieve fragment matching and confirm sequences of synthetic oligonucleotides. If you are trying to identify N-1, N-2, etc. metabolites, you can also use that approach to figure out from which end you are losing nucleotides to form the metabolites. We do that kind of work for reference material. Sometimes we only get UV spectra for these materials, which is not enough detail when we start development. The software helps us with that.

Read Q&A blog

Shane Karnik serves as the Senior Laboratory Director at Aliri Bioanalysis in Colorado Springs, Colorado. His responsibilities include leading a team of bioanalytical scientists supporting pharmaceutical drug development for non-GLP and GLP preclinical and clinical studies. Shane earned a Master of Science in chemistry from the University of Colorado while working full-time in R&D at a medical device company. He spent several more years in the pharmaceutical industry, developing and validating analytical methods for new drug submissions.

Characterization and impurity identification

ASOs are approximately 18–28 nt in length and are either single stranded or exist as duplexes, such as for siRNA. Chemical modifications are common to increase target binding and stability, while conjugations to N-acetylgalactosamine (GalNAc) or lipids are used for delivery and to increase specificity. Shortmers—impurities derived from the stepwise synthesis of ASOs—increase the complexity of synthetic oligonucleotide samples. The characterization of ASOs therefore requires a reliable and high-resolution solution.

- Trust your results with excellent raw data quality based on exceptional negative ionization efficiency and declustering of adducts
- Confidently confirm the sequence of your oligonucleotides and obtain relative quantitative information of full-length product (FLP) and impurities
- See more with less sample using the Zeno trap increasing sensitivities across the entire mass

Break through boundaries of duty cycles and obtain superior MS/MS data quality for low abundance metabolites with the Zeno trap

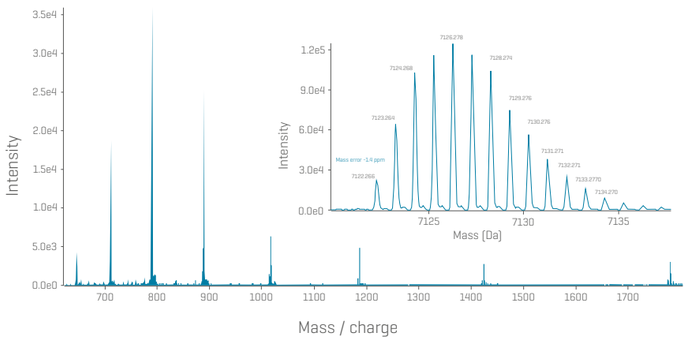
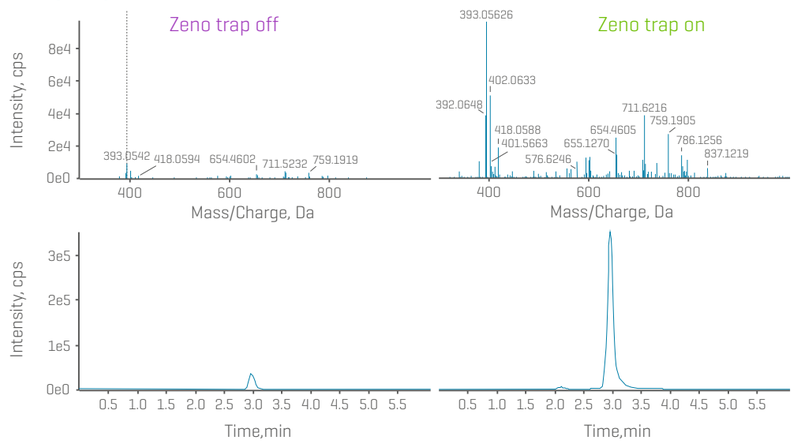


Figure 43: Data from an 18 nt phosphorothioated ASO with 2'-O-methoxyethyl (MOE) modifications. TOF-MS data shows different charge states with inset showing reconstructed data and calculated mass error.

Figure 44: Data comparison obtained from modified ASO (18 nt) without the Zeno trap (left) and with the Zeno trap (right). MS/MS spectra from precursor m/z = 711.62 (top). XIC from MS/MS fragment with m/z = 393.05 (bottom).

Up to **10x** higher intensities with the Zeno trap



Discover more details in the technical note about oligonucleotide characterization

Read technical note

Oligonucleotide MetID

Modifications to the Phospho-backbone and ribose sugars, which are often introduced to increase the stability of ASOs, also impact the metabolism profile. Depending on stabilizing modifications, endonucleases or exonucleases can hydrolyze ASOs within the sequence or result in shortmers from 5' and/or 3' ends. To determine the impact of metabolites on efficacy, toxicity and drug-drug interactions, structural identification is of high relevance.

- Achieve excellent raw data quality through exceptional negative ionization efficiency and declustering of adducts
- Confidently confirm oligonucleotide sequences of FLP and metabolites
- Break through boundaries of duty cycles and obtain superior MS/MS data quality for low abundance metabolites with the Zeno trap

Reliable metabolite identification

Identify and quantify metabolites reliably

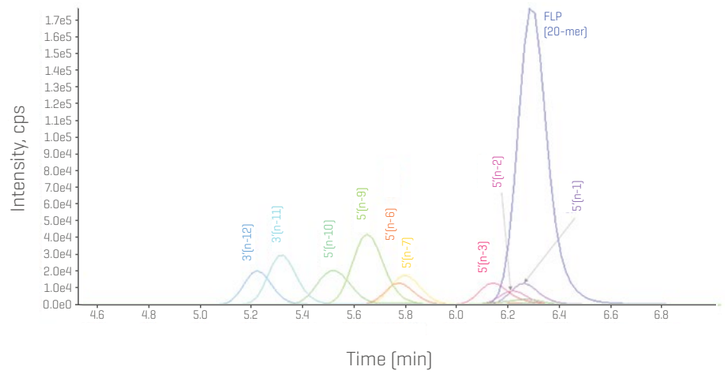
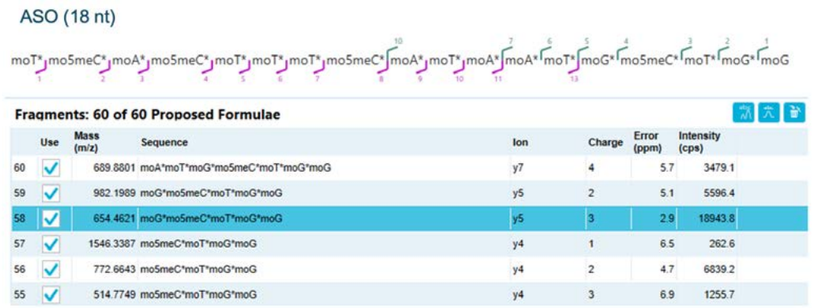


Figure 45: Chromatograms of a 20-mer ASO and spiked in metabolites. Representative XICs for the FLP with 2'-O-methoxyethyl and 5-methyl modifications and related metabolites (shortmers of 5' and 3'-ends) are shown.

Break through boundaries of duty cycles and obtain superior MS/MS data quality for low abundance metabolites with the Zeno trap



Use	Mass (m/z)	Sequence	Ion	Charge	Error (ppm)	Intensity (cps)
60	689.8801	moA*moT*moG*mo5meC*moT*moG*moG	y7	4	5.7	3479.1
59	982.1989	moG*mo5meC*moT*moG*moG	y5	2	5.1	5596.4
58	654.4621	moG*mo5meC*moT*moG*moG	y5	3	2.9	18943.8
57	1546.3387	mo5meC*moT*moG*moG	y4	1	6.5	262.6
56	772.6643	mo5meC*moT*moG*moG	y4	2	4.7	6839.2
55	514.7749	mo5meC*moT*moG*moG	y4	3	6.9	1255.7

Figure 46: Matching MS/MS fragments of an 18-mer ASO. Suggested sequence matching of detected MS/MS fragments and associated sequence with error calculation from Molecule Profiler software.

Discover more details in the technical note about metabolite identification

Read technical note

Bioanalysis and DMPK

Improved stability and specificity are factors contributing to an increase in potency of oligonucleotide therapeutics. Quantitative studies to support drug metabolism and pharmacokinetics (DMPK) require analytical assays with high sensitivity to detect the ever-decreasing concentrations of the active pharmaceutical ingredient (API) and its metabolites. Additionally, ion pairing agents, frequently used to enable chromatographic separation, result in a need for high robustness.

- Break through quantitation boundaries with high signal-to-noise and best-in-class robustness
- Achieve excellent negative ionization efficiency and declustering of adducts with state-of- the-art source design
- Cover your GxP needs with compliance-ready solutions

LDR 2.3

Don't choose between robustness and high sensitivity

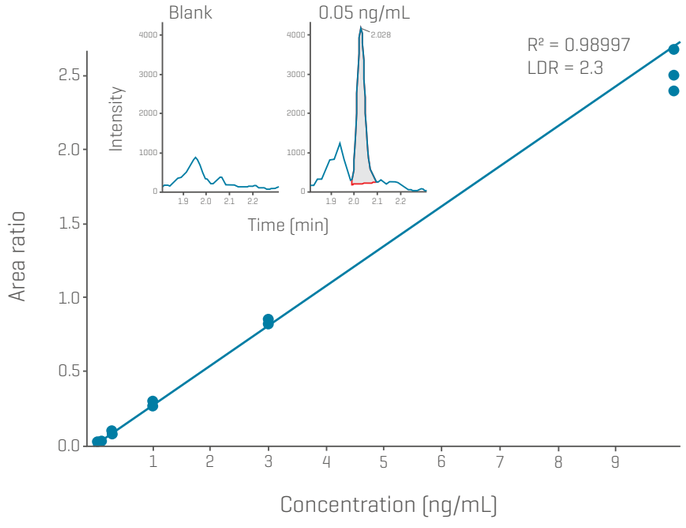


Figure 47: Quantitative performance of a 21-mer antisense strand from an siRNA-lipid conjugate in development using a SCIEX 7500 system. Calibration curve based on XIC adjusted using internal standard shows LDR of 2.3 (*n* = 3). Inset shows XIC of transition for blank sample and LLOQ at 0.05 ng/mL.

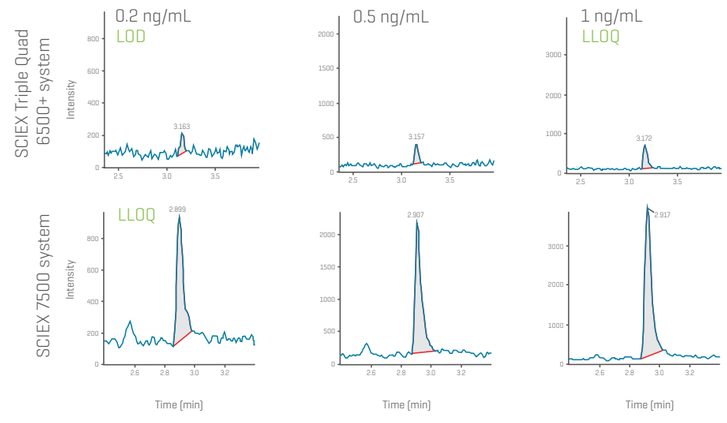


Figure 48: Comparison of XICs from a 20-mer ASO at different concentrations in extracted plasma. Top: Data from the SCIEX Triple Quad 6500+ system with LLOQ of 1 ng/mL. Bottom: Data obtained from the SCIEX 7500 system with LLOQ of 0.2 ng/mL.

LLOQ 0.2 ng/mL

Discover more details in the technical note about the quantitation of siRNA conjugates

Read technical note

Analytical solutions for oligonucleotide therapeutics

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- Molecular weight determination and sequence confirmation
- Robust, analytical flow setup

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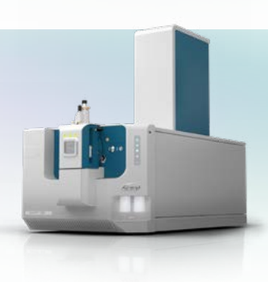

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Tips and tricks from our application experts:

LC-MS setup part I

DilipKumar Reddy Kandula (PhD), Staff Applications Scientist at SCIEX, US, shares his tips and tricks on how to prepare your system for oligonucleotide LC-MS analysis.

Dr. Dilip Reddy has 10 years of experience as a research scientist in different pharma and biopharma companies, where he focused on using MS for the analysis of biologics and small molecules. His work included the characterization of complex molecules, bioanalysis and MetID studies with triple quad and high-resolution LC-MS instrumentation. For the past 7 years, he applied his extensive knowledge within various functions at SCIEX. Dilip holds a PhD in protein characterization using mass spectrometry from the Shri JJT University in Rajasthan, India.

The background on formation of metal adducts

It is very common for oligonucleotides to form adducts with alkali metal ions during LC-MS analysis. Adduct formation occurs due to the electrostatic attraction between the negatively charged phosphate backbone of the oligonucleotide and the positively charged alkali metal ions. The formation of alkali metal adducts can negatively impact the data quality. Signal intensities of analytes are reduced due to spreading the signal across different adducts. Additionally, identification and accurate quantitation of species is further complicated. I summarized my tips below to help improve your oligonucleotide LC-MS analysis.

Tip 1: Choose the right consumables

Glass bottles tend to leach sodium ions, hence plastic bottles are recommended as containers for mobile phases. Before the first use, it is recommended to soak the plastic bottles overnight in isopropanol (IPA) containing 10% acetic acid and rinse them 10–15 times with milliQ water. Using high-quality LC-MS grade additives (acetic acid, ion pairing agents) and solvents can help minimize adduct formation. Ensure that a set of tubing and columns is set aside for oligonucleotide analysis usage only, such as the Phenomenex BioZen Oligo LC column.

Tip 2: Prepare your LC system

Before starting analysis, ensure your LC system is prepared for oligonucleotide analysis with ion pairing reversed phase liquid chromatography (IP-RP-LC). My recommendation is to flush the solvent and autosampler lines with LC-MS grade IPA for 10 to 15 minutes. Include your tubing and electrode for all flushing steps but use a connector piece instead of your column. Then, switch to 10% acetic acid for 1-2 hours. Doing “dummy” injections of a 10 % acetic acid solution is beneficial for cleaning the injection parts. As a next step, switch to LC-MS grade or MilliQ water for 1-2 hours to remove the acetic acid. Then install your oligonucleotide column and equilibrate system with mobile phases for analysis.

Tip 3: Determine an MS cleaning schedule

It is important to clean the ion source regularly to prevent contamination, reduce the adduct formation and maintain optimal sensitivity. Follow the SCIEX guidelines for cleaning procedures and recommended cleaning solutions and keep a specific cleaning schedule. A regular cleaning schedule of your source and MS front-end is recommended to help preventing any contamination travelling further into the system, especially when working with ion pairing agents for IP-RP-LC analysis.

More questions?

Contact us

Tips and tricks from our application experts:

LC-MS setup part II

Kaoru Karasawa, Professional Specialist, Application Support at SCIEX, Japan, shares her tips and tricks on oligonucleotides analysis with LC-MS

Tip 1: Sample handling

Oligonucleotides tend to bind to pipette tips and vials. In addition, oligonucleotides can be sensitive to the degradation by nucleases. I therefore recommend using low-binding LC vials and pipette tips and recommend wearing gloves and using nuclease-free water for sample preparation to minimize the risk of sample loss due to adsorption and/or degradation. Furthermore, the use of an internal standard is beneficial. It can help with minimizing the adsorption of the target analyte and can increase quantitative accuracy for quantitative studies.

Tip 2: Replace LC solvents

To facilitate chromatographic separation while enabling MS sensitivity, alkylamines and hexafluoro-2-propanol [HFIP] are commonly used for IP-RP-LC-MS analysis. However, the additives in the solvents can evaporate quickly. The change in concentration and pH can lead to changes in chromatographic performance and decreased MS signal intensities. I recommend checking in advance how long your solvent can be used and design experiments accordingly. If you notice a decrease in expected MS peak intensity, prepare fresh solvents.

Tip 3: Select suitable columns

In many cases, the separation of analytes from impurities or metabolites requires high column temperatures, and in some cases, can go up to 90°C. The analysis of double stranded analytes, such as siRNA, require high temperatures to separate the sense from the complementary antisense strand. In addition, the solvents used for IP-RP-LC are high in pH. I recommend carefully choosing a column that can withstand these conditions, such as the Phenomenex BioZen Oligo LC column.

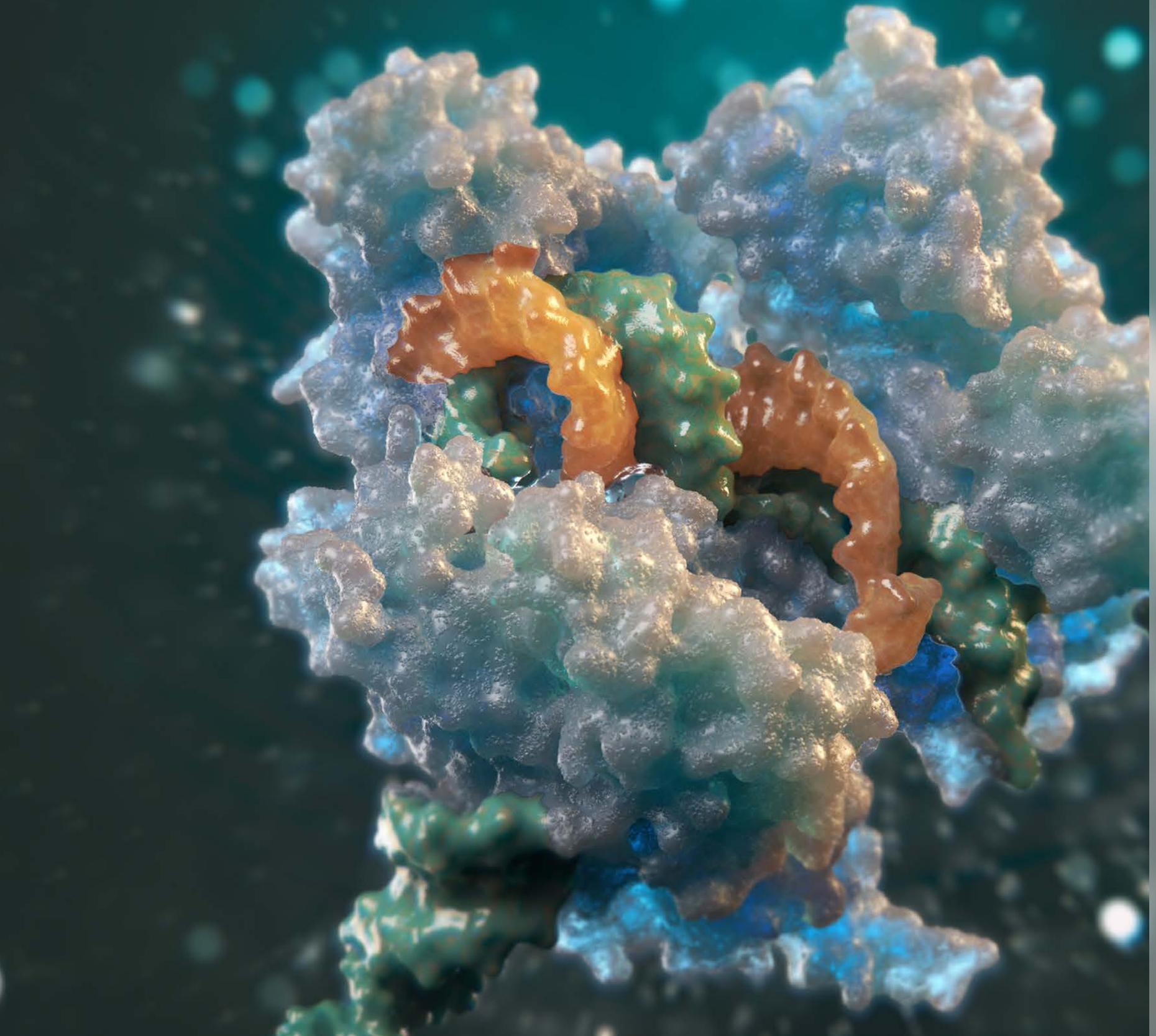
Tip 4: Optimize MS methods

The MS signal for therapeutic oligonucleotides is usually distributed across multiple charge states—mainly -2 to -10—and the distribution varies based on LC and MS conditions, sequence composition and length. For quantitative analyses, I recommend optimizing the collision energy for several charge states, since the most intense charge state does not necessarily provide the most intense fragment ion with high specificity. For qualitative analyses, such as sequencing, it is beneficial to combine the MS/MS information from different charge states for best results.

Kaoru Karasawa has more than 25 years of mass spectrometry experience, primarily in high-resolution MS. She leverages from extensive hands-on experience in impurity analysis, MetID and quantitation of small molecules and oligonucleotides for pharmaceutical and omics applications. Prior to joining SCIEX, Kaoru worked as a natural product chemist for the Roche Group, Japan. Currently, she is participating in a project for oligonucleotide development in Japan and contributing to the development of analytical techniques. Kaoru holds a Master's Degree in engineering.

More questions?

Contact us



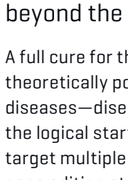
06

Gene editing

Gene editing

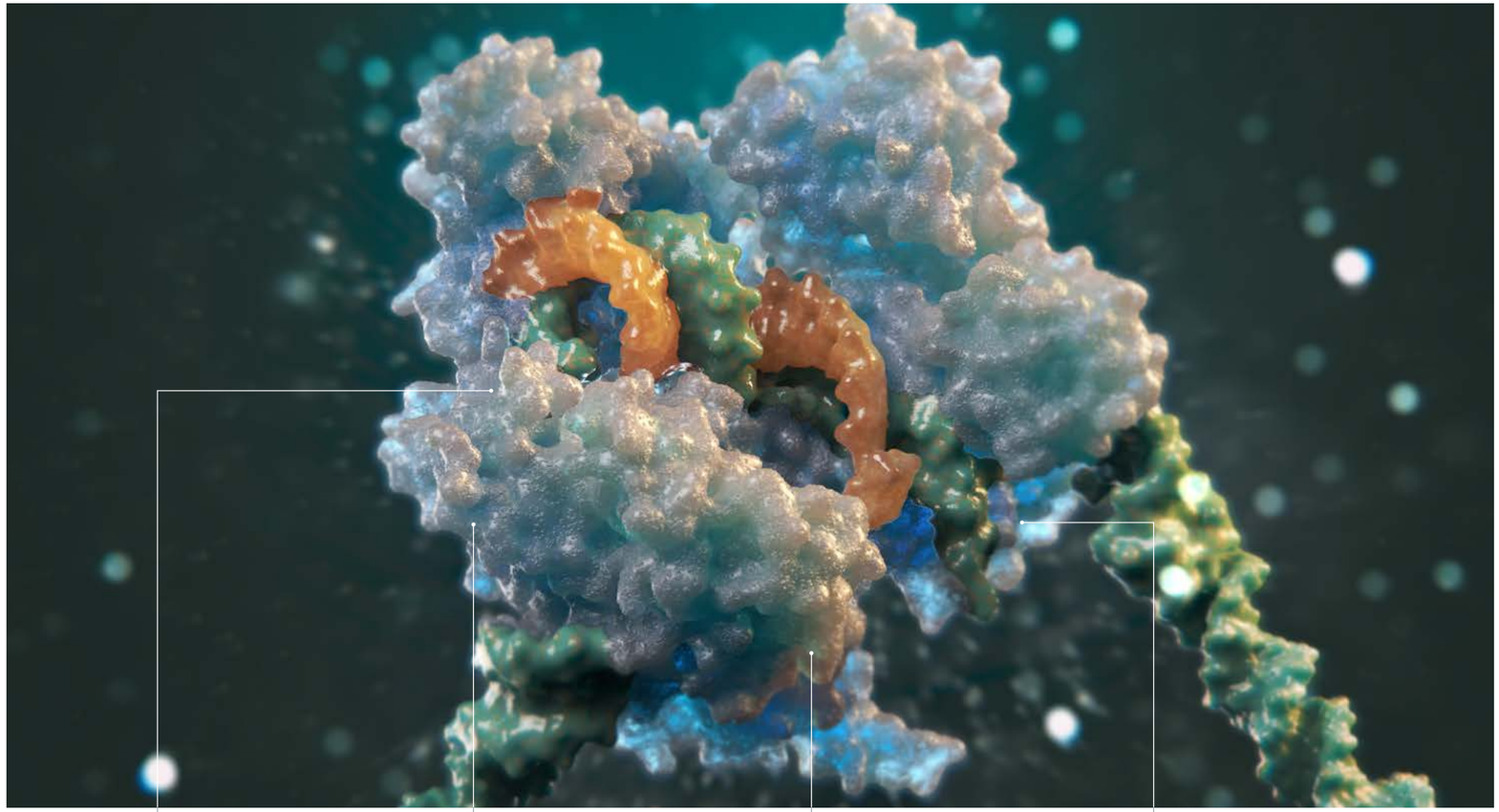
Gene editing approaches, such as CRISPR/Cas9, have tremendous potential to go beyond the treatment of genetic diseases.

A full cure for the majority of known human genetic diseases is theoretically possible by leveraging gene editing. Monogenetic diseases—diseases caused by mutations in one gene—are the logical starting point. However, research on how to target multiple gene locations using one treatment with gene editing stacking is still ongoing. In addition, CRISPR approaches bear the potential to target other diseases, such as cancer. Challenges around delivery to target cells, manufacturing costs and analytical assessments to ensure the safety of these new approaches, need to be overcome.



"What's the outcome in the cell? Is it on target? Is it off target? What are those undesired effects? Is there some percentage of misintegration? Answering these questions is what we focus on with our ZenoTOF 7600 system."

Hugo Gagnon (PhD)
Chief Scientific Officer, Allumiqs



sgRNA and Cas9 mRNA purity

Separate impurities and determine the integrity and purity of sgRNA and Cas9 mRNA in one experiment. simultaneously.

pegRNA purity

Move past secondary structures and assess the purity of guide RNAs despite high sequence complementary.

Intact mass characterization

Achieve accurate intact molecular weight information of guide RNAs and related impurities.

Cas9 protein characterization

Confirm protein sequences and understand PTMs and amino acid isomers in depth.

Proteome profiling

Understand gene editing effects better with an unbiased proteome-wide approach.

Expert Q&A:

Overcoming challenges to assess pegRNA purity

Prime editing using prime editing guide RNAs (pegRNAs) is one of the latest approaches for gene editing. Here, Ashley Jacobi (Integrated DNA Technologies, IDT) and Tingting Li (SCIEX) share their experiences on overcoming analytical challenges linked to pegRNAs.

Can you comment on the typical lifespan of the capillaries used for pegRNA analysis?

Tingting Li: The capillary used for this assay is the coated DNA capillary for the PA 800 Plus system available in the ssDNA 100-R kit (PN 477480). It is validated for 100 runs. However, my personal experience is that more runs can be performed. For the pegRNA analyses, I conducted >150 runs and the capillary can still be used for further analyses. I used the same capillary to develop the method presented in the webinar and to analyze the pegRNAs of different lengths, from different lots on different instruments and on different days. The key to achieving a long capillary lifetime is to avoid exposing the capillary ends to air for more than 3 minutes. Additionally, it is important to store the capillary appropriately after usage. When not in use, always rinse and store the capillary in unused gel buffer at 2°C to 8°C with both capillary ends submerged in Tris-Borate-Urea buffer.

Can you provide further insights into your strategy for addressing the challenge of pegRNA peak shape?

Tingting Li: Optimizing the peak shape of pegRNA was indeed a challenging task. Our hypothesis is that highly complementary primer binding sites (PBS) and protospacer sequences in pegRNA molecules caused the formation of hairpin and secondary structures, resulting in a very broad peak when analyzed with CE under standard conditions. To confirm this, we synthesized 4 pegRNAs with varying lengths and their corresponding non-complementary RNAs (NC RNAs) with the same PBS sequence but with a non-targeting protospacer. We analyzed all 8 samples and found that the NC RNAs exhibited a singular sharp peak while we observed broad, unresolved peaks for all pegRNAs. This further indicated that the broad, undesired peak shape observed for pegRNAs was likely due to the formation of high-order structures caused by their highly complementary sequence design. For the next step of method development, we focused on a robust denaturing technique for CGE purity analysis of pegRNAs with high levels of secondary structure. We explored various denaturation methods during the sample preparation. However, we did not achieve significant improvements of the peak shape using these different sample preparation procedures. We then focused on optimizing the conditions during the separation process. The breakthrough came with heat-based denaturation during separation in the CE capillary. We maintained a capillary temperature of 50°C throughout the separation process. Based on the peak profiles achieved, we concluded that this step prevented the formation of inter- and/or intramolecular hydrogen bonds of pegRNA molecules. With the temperature-controlled capillary at 50°C, we can achieve a single sharp peak for each of the pegRNA samples.

Could you please elaborate on the temperature control feature of your CE system and its role in improving the accuracy of pegRNA purity analysis?

Tingting Li: Certainly. The unique capillary temperature control feature of the PA 800 Plus system is a critical aspect that ensures precise and stable temperature control during the separation process. This is achieved by an inert liquid circulating through the cartridge around the separation capillary. Compared to air used in other systems, the inert liquid can maintain temperature more precisely and consistently throughout the analysis. For the analysis of oligonucleotides like pegRNA, the stable temperature control is vital as it minimizes the variations in migration time and peak shape, resulting in highly reproducible results for purity analyses.

How can the denaturing technique and gel matrix be adapted for other analyses with high levels of secondary structures? Can you provide any advice?

Tingting Li: The denaturing technique presented in the webinar and the gel matrix can be adapted for the analysis of other oligonucleotides with high levels of secondary structures. I recommend testing different denaturation agents and temperatures for sample preparation and during the CE separation process while considering the characteristics of your oligonucleotides, such as heat and pH stability for example. In addition, the gel matrix, separation voltage and separation time can be optimized to achieve the needed resolution and peak shape. I believe the flexibility of this method and system can open possibilities for investigating various oligonucleotides and their functions in different research areas.

How do you foresee the CGE purity workflow impacting future research and advances in CRISPR technologies?

Ashley Jacobi: High quality synthetic guide RNAs with high purity is very important for the efficiency of your gene editing experiments. This holds true not only for prime editing but for any CRISPR technology. As these technologies move from research towards therapeutic use, the purity of the starting material is of the utmost importance. Having analytical technology that provides accurate purity determination is a necessity to achieve desired outcomes and bring safe gene editing technologies to market.

Can you comment on modalities beyond pegRNAs in your pipeline?

Ashley Jacobi: We are always looking for improvement and innovation within IDT. The data shown in the webinar was up to about 200 nucleotides. However, we are investigating even longer guide RNAs. Another avenue we are exploring is different chemical modifications. How do those modifications affect editing efficiency and/or the stability of these compounds? It is something we are actively looking into.

Read Q&A blog

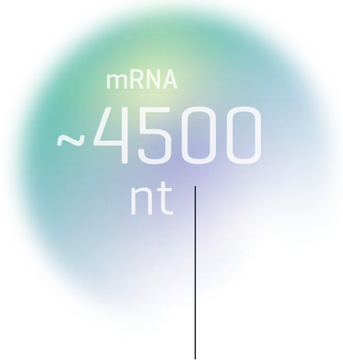
Ashley Jacobi is the Director of Application and Market Development at IDT. She received her Molecular Genetics & Biochemistry degree from Cornell College. Ashley has spent the last 18 years holding several roles in research and product development at IDT, focusing on RNAi and antisense oligo technologies, and CRISPR gene editing. Her work on CRISPR includes optimizing the composition and delivery of synthetic RNAs complexed to recombinant CRISPR nucleases and understanding the genetic outcomes following CRISPR gene editing.

Tingting Li is the Manager of Cell and Gene Therapy at SCIEX. Tingting has more than 10 years of experience in CE holding different roles in Shanghai, China and Brea, US, focusing on scientific and specialist product support. Her focus includes new workflow development and characterization of cell and gene therapies. Prior to SCIEX, Tingting served as an analytical scientist and project leader in the pharma industry for analytical method development and method validation for several years. Tingting holds a Master's degree in Chemistry from Rutgers University New Brunswick.

sgRNA and Cas9 mRNA integrity and purity

For therapeutic purposes, the naturally occurring CRISPR RNA (crRNA) and the trans-activating CRISPR RNA (tracrRNA) are commonly engineered into a single-guide RNA (sgRNA). While sgRNAs consist of approximately 100 nt, Cas9-encoding mRNAs are >4000 nt in size. Confirming the integrity and assessing the purity quantitively for both RNAs—sgRNA and Cas9 mRNA—are key for successful gene editing.

- Assess the quality of product with various sizes in one analysis without compromising on data quality
- Understand related nucleic acid impurities and determine sizes and quantities, simultaneously
- Cover your compliance needs through compatibility with common data management systems



Break through limitations based on secondary structure formations

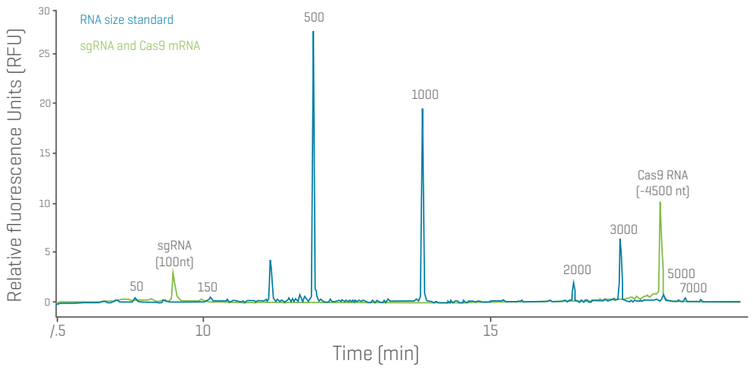


Figure 49: Overlay of electropherograms from a mixture of sgRNA and Cas9 mRNA and an RNA ladder using the BioPhase 8800 system. The mRNA of ~4500 nt and the sgRNA of 100 nt can be assessed within the same analysis (green trace). The RNA ladder is shown in blue.

Cas9-encoding mRNAs are >4000 nt in size

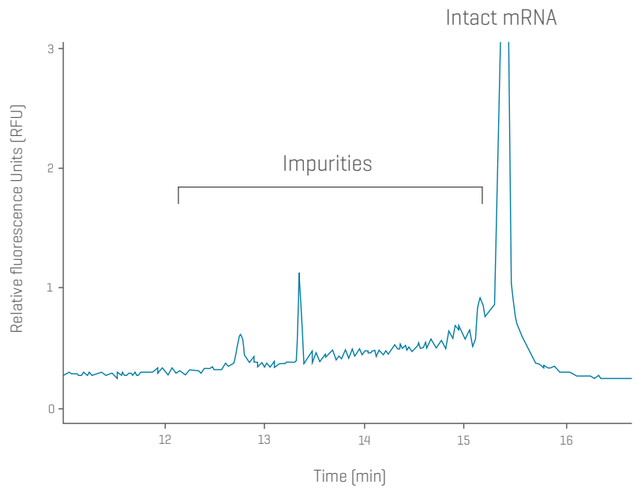


Figure 50: Zoomed-in electropherogram of Cas9 mRNA. The resolution of the CGE method allows for separation of impurities from the main product and quantitation of Cas9 mRNA purity.

Discover more details in the technical note about sgRNA and CRISPR Cas9 mRNA analysis

Read technical note

pegRNA purity for prime editing

PE is a promising approach with increased specificity and efficiency. It consists of an sgRNA with a reverse transcriptase template sequence and a primer binding site. With a length of ~120-250 nt, synthetic pegRNAs are prone to impurities derived from their stepwise synthesis. In addition, their complementary bases can lead to secondary structure formation that is resistant to common denaturation strategies, posing an additional analytical challenge to overcome.

- Achieve superior resolution and repeatability for purity assessments of intermediate products
- Break through the boundaries of secondary structure with liquid-based temperature control
- Streamline data management and cover your compliance needs through compatibility with common data management systems

Break through limitations based on secondary structure formations

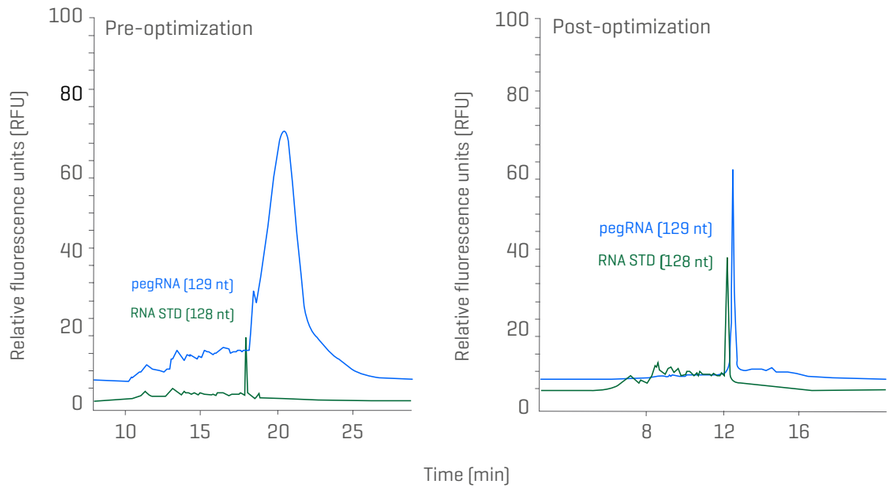


Figure 51: CGE analysis of pegRNA (blue trace) and non-complementary RNA standard (STD, green trace) with similar length. Left: Before method optimization, the pegRNA (blue trace) shows extensive tailing compared to the non-complementary STD (green trace). Right: After method optimization, the pegRNA sample shows a single sharp peak with comparable peak width as the non-complementary STD.

Sharp peaks
with optimized protocol

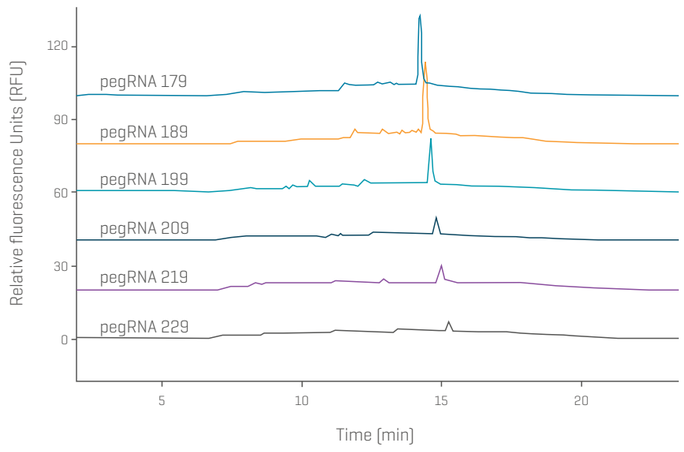


Figure 52: CGE analysis of pegRNAs with different lengths using the optimized method on a PA 800 Plus system. pegRNAs from 179 nt to 229 nt were tested with the optimized protocol, all resulting in sharp peaks.

Discover more details in the technical note about pegRNA purity analysis

Read technical note

Intact mass characterization of guide RNAs

Guide RNAs can vary in size from ~17 nt and ~65 nt in length for crRNA and tracrRNA respectively, to ~100 nt for sgRNA and up to 250 nt for newer approaches, such as pegRNA. While the specificity and efficiency can be increased with the latest sgRNAs, the likelihood of introducing impurities increases during the stepwise synthesis of larger RNAs. The quality of sgRNAs is crucial for achieving high gene editing efficiencies. An error in 1 nucleotide can result in significantly reduced efficiency.

- Leverage excellent raw data quality through exceptional negative ionization efficiency and decluster adducts with state-of-the-art source design
- Confidently confirm identities of your guide RNAs based on deconvoluted molecular weights with great mass accuracy
- Uncover relevant information on impurities and obtain relative quantitative information

pegRA 122nt

Assess mass information of guide RNAs and impurities with ease

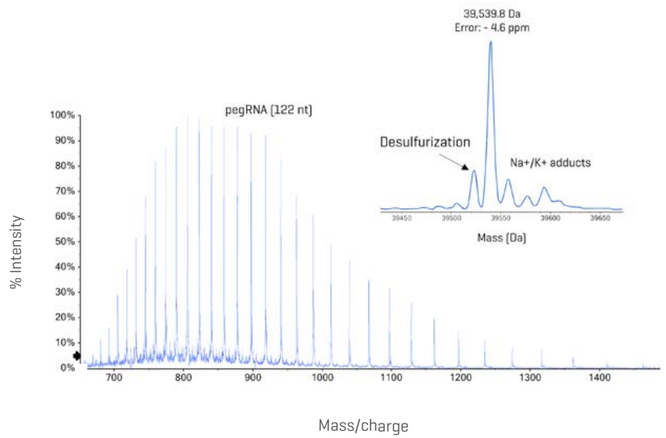


Figure 53: TOF-MS data from pegRNA with 122 nt. Raw TOF-MS data shows charge state envelope of pegRNA. Inset shows deconvoluted mass with excellent mass accuracy. Additionally, lower intensity peaks could be attributed to desulfurization and metal adduct formations.

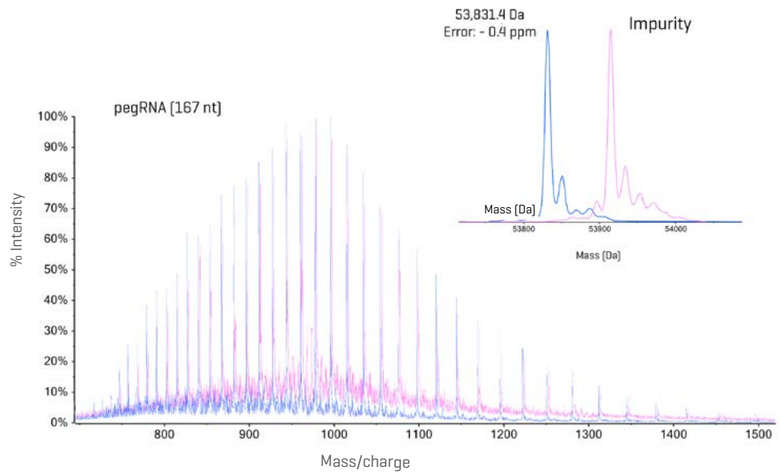


Figure 54: TOF-MS data from pegRNA sample with 167 nt. Blue trace shows TOF-MS raw data and deconvoluted mass (inset) of desired product. Pink trace shows TOF-MS raw data and deconvoluted mass (inset) of unexpected impurity with higher mass.

pegRA 167nt

Discover more details in the technical note about large oligonucleotide LC-MS analysis

Read technical note

Cas9 protein characterization

To increase target specificity further, Cas9 fusion proteins are being studied. A deeper look into these engineered proteins is required to confirm the target amino acid sequence and identify low-abundance PTMs to ensure product quality. While a peptide-mapping approach can provide relevant information, it can be challenging to achieve high enough sequence coverage and full elucidation of PTMs to characterize PQAs and CQAs.

- Obtain high protein sequence and fragment coverage with limited sample amounts through highly sensitive data acquisition
- Differentiate amino acid isomers and determine relative quantities and exact locations of PTMs including highly fragile PTMs with EAD
- Take back your time with accurate and streamlined data processing

Know your sequence and fully understand challenging PTMs

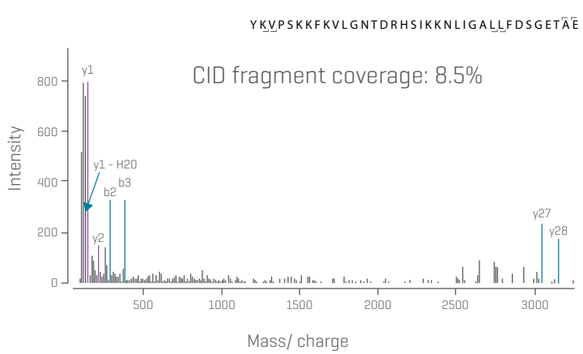


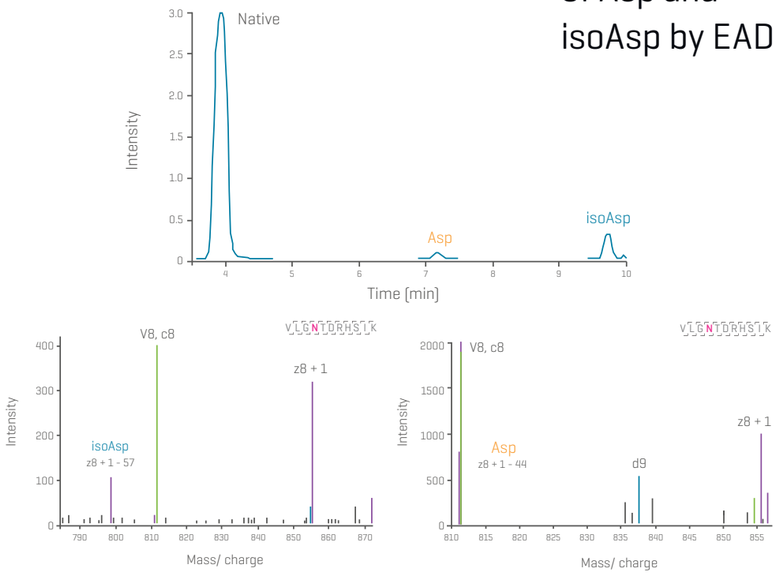
Figure 55: Fragment coverage comparison for long peptide from the Cas9 protein. EAD shows extensive fragmentation compared to CID, providing higher confidence in the identity of the peptide.

>5x

more fragments detected with EAD than CID

Figure 56: Differentiation of Asp and isoAsp by EAD. Top: XICs of the native and deamidated versions of the peptide VLGNTDRHSIK from the Cas9 protein. Bottom: EAD MS/MS spectra providing differentiation between Asp and isoAsp.

Differentiation of Asp and isoAsp by EAD



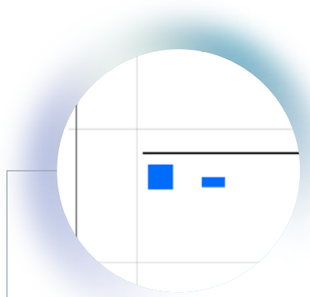
Discover more details in the technical note about Cas9 protein analysis with EAD

Read technical note

Proteome profiling for gene editing

In addition to specificity challenges resulting in off-target effects, 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.

- Understand the effects of gene editing on the proteome level in an unbiased way with Zeno SWATH DIA
- Dig deeper into changes despite limited sample amounts with increased sensitivity using the Zeno trap
- Achieve confident identification and simultaneous quantitation with excellent MS/MS data quality



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

Dig deeper into the proteome and understand changes with amino acid resolution

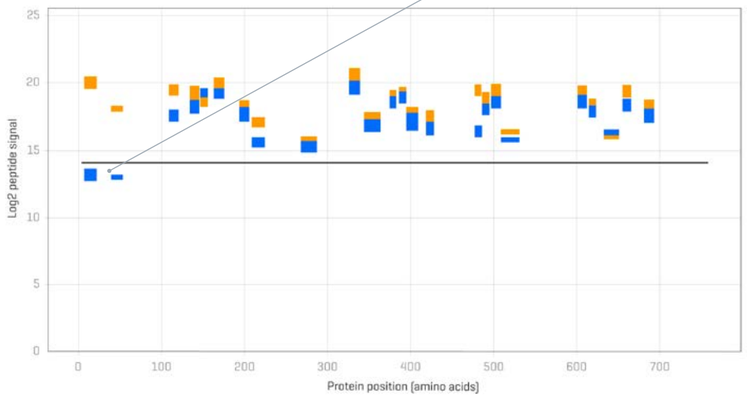
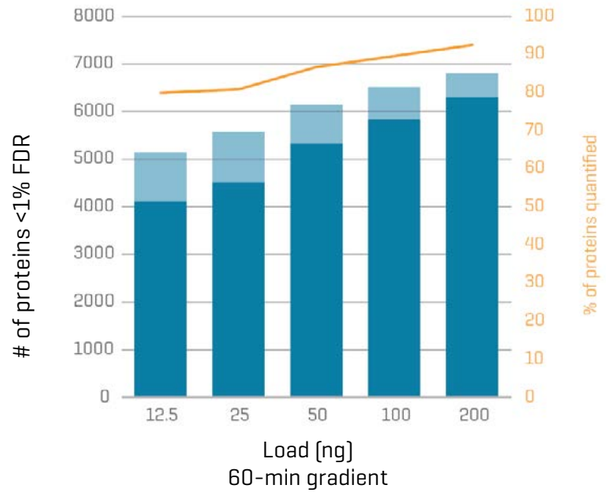


Figure 57: Insertion-deletion (INDEL) errors induced by gene editing detected by bottom-up proteomics using SWATH DIA. The expectation is that all peptides in the gene-edited samples (blue) are below the baseline signal. However, compared to the control (orange), only two peptides at the N-terminus of the protein were silenced based on a shift in the initiation of the protein transcription. Bar thickness represents standard deviation of the peptides.



Over
90%
of proteins quantified

Figure 58: Identified and quantified proteins using SWATH DIA. Columns show number of proteins identified with a false discovery rate (FDR)<1% for different loading amounts of cell lysate digest using a 60 min gradient. Line shows amount of proteins with %CV<20% used for quantitation as percentage of total amount of identified proteins with FDR<1%.

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

Read technical note

Analytical solutions for CRISPR/Cas9

Suitable for:

- High-quality separation of sgRNA and Cas9 mRNA
- Rapid method development and sample analysis

BioPhase 8800 system

Purpose-built for achieving high quality data efficiently across various analytical assays.

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RNA 9000 Purity & Integrity kit

An intuitive kit to assess RNA and ssDNA integrity, purity and size, compatible with BFS capillaries.

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BFS capillary cartridge

A pre-assembled bare-fused silica 8-capillary cartridge available in 30 and 50 cm total length.

Suitable for:

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- High-resolution pegRNA analysis
- Smaller sample sets

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RNA 9000 Purity & Integrity kit

An intuitive kit to assess RNA and ssDNA integrity, purity and size, compatible with BFS capillaries.

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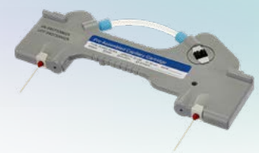
BFS capillary cartridge

A pre-assembled bare-fused silica single capillary cartridge available in 30 cm total length.



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Analytical solutions for CRISPR/Cas9

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- Cas9 protein characterization
- Robust analytical flow setup

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An LC system that seamlessly integrates with your SCIEX MS for reproducible data, batch after batch.

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X500B QTOF system

An intuitive QTOF system, designed to facilitate everyday biologic characterization assays.

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Biologics Explorer software

A powerful software tool to support challenging LC-MS/MS protein characterization assays.

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SCIEX OS software

The next generation of operating and processing solution for an enhanced LC-MS/MS analysis experience.

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Suitable for:

- Molecular weight determination of gRNAs, sgRNAs and pegRNAs
- Cas9 protein characterization including isomer differentiation
- High flexibility to perform a range of additional workflows

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Concluding remarks

"We are witnessing unprecedented advancements in genetic medicines, capable of treating once-incurable diseases. This progress is closely linked to the continuous evolution of analytical technologies. At SCIEX, we are devoted to precise science that enables our customers to solve their most impactful challenges through ground-breaking innovations and outstanding reliability and support."

Dr. Joe Fox
President at SCIEX

"Analytical chromatographic solutions are vital for generating the data needed to make crucial therapeutic drug decisions. With the rapid evolution of science and the emergence of personalized medicines, drug discovery researchers rely on high quality chromatographic consumables to accelerate the development timeline for new therapeutics. Phenomenex provides a diverse range of solutions designed specifically for this purpose. Our offerings include sample preparation, column chemistry, and round-the-clock technical support, all of which instill confidence in the data required to bring life-saving and life--enhancing therapies to market faster."

Dr. Kaveh Kahen
President at Phenomenex

"At Aldevron, our vision—a world where every cure is possible—reflects our 25 years of experience and our commitment to the future of cell and gene therapy. We are driven by a relentless pursuit to better serve therapeutic developers working on life-altering treatments for patient populations. To make this a reality, we will continue to see improvements in the speed, cost and safety profile of new therapeutics, enabled by platform processes and disruptive new technological advancements."

Jennifer Meade
President at Aldevron

"Advancing the next generation of therapeutics isn't based on scientific advancement alone. Future biomanufacturing will increase in its complexity, as new therapeutic modalities emerge and the demand for personalized medicine grows. Collaboration between industry, academia and government bodies must work together to create accelerated, efficient pathways for drug developers."

Emmanuel Abate
President at Cytiva

The power of precision

SCIEX Now support network

SCIEX Now

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- Access online training courses and articles.
- Manage software licenses linked to your registered instruments.
- View and report critical instrument statistics when connected to StatusScope remote monitoring service.
- Be a part of the SCIEX community by submitting questions and comments.
- Receive notifications from SCIEX with content based on your preferences.

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SCIEX Now learning hub

- SCIEX Now learning hub success programs provide LC-MS and CE training customized to meet your exact needs.
- With a selection of training methods and certifications available, you can build a mass spectrometry program that is most suited to your lab and users.
- Starting with a clear understanding of your desired learning outcomes, we aim to help you improve lab productivity and consistency by designing and delivering a program that is focused on knowledge advancement and retention.

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