毛细管电泳 (CE)

—— 细胞与基因治疗及小核酸应用文集



SCIEX

The power of precision

毛细管电泳 (Capillary Electrophoresis, CE) 作为一种高分辨率、高灵敏度的分离分析技术，近年来在生物药分析领域得到广泛应用。随着生物制药行业的快速发展，特别是单克隆抗体 (mAb)、抗体偶联药物 (ADC)、疫苗产品以及细胞与基因治疗 (CGT) 等创新药物的不断涌现，对质量控制技术提出了更高要求。

生物药具有结构复杂、修饰类型多样、质量属性高度关联等特点，传统分析手段在部分关键质量属性 (CQA) 的表征与监控中仍面临分辨率、通量或方法适配性的挑战。毛细管电泳技术凭借高分辨率、高灵敏度、自动化程度高及方法重复性良好等优势，已在生物药研发、工艺表征和质量控制中发挥重要作用，尤其适用于以下分析方向：

- 纯度与片段分析 (CGE / CE-SDS)，用于完整性、降解片段及杂质水平评估；
- 电荷异质性分析 (cIEF)，用于主峰、酸性峰和碱性峰等电荷变异体的分离与定量；
- 分子大小与聚集体相关分析 (CZE 等)，用于分离不同组分并辅助评估样品均一性；
- 核酸完整性及片段分析，用于 mRNA、寡核苷酸及其他核酸类样品的长度分布和完整性评价；
- 病毒载体结构分析 (如 AAV、慢病毒)，用于衣壳蛋白组成、核酸载荷及相关质量属性研究。

SCIEX 提供的毛细管电泳平台 (如 PA800 Plus、BioPhase 8800 及 Intabio ZT) 已在研发与质量控制 (QC) 环节广泛应用。

本应用文集基于近年行业发展趋势，筛选生物药领域中高市场需求、高技术价值的热点应用场景，按照应用类型进行系统整理，旨在为用户提供全面的应用参考及解决方案思路。

细胞与基因治疗产品

细胞与基因治疗 (CGT) 产品涵盖 CAR-T 等细胞治疗产品、AAV 与慢病毒等病毒载体，以及 mRNA、质粒和寡核苷酸等核酸相关产品，其质量控制需重点关注病毒载体结构与纯度、核酸完整性、片段分布、蛋白组成及残留杂质等关键质量属性。毛细管电泳 (CE) 凭借高分辨率、高灵敏度和样品消耗量低等优势，可为病毒载体衣壳蛋白分析、核酸片段与完整性评价以及相关杂质监控提供高效、可靠的分析手段，是 CGT 产品研发、工艺表征和质量控制中的重要技术平台。

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小核酸药物

小核酸药物主要包括反义寡核苷酸 (ASO)、小干扰 RNA(siRNA)、微小 RNA(miRNA) 及适配体等寡核苷酸类分子，其质量控制需重点关注序列准确性、链长与片段分布、纯度与有关物质、修饰一致性、降解产物、残留盐类和工艺相关杂质等关键质量属性。毛细管电泳 (CE) 凭借高分辨率、微量进样、快速分离和多模式检测能力，可用于小核酸药物的纯度分析、长度分布评价、杂质谱研究及方法开发，为小核酸药物的研发表征、工艺优化和质量控制提供高效、稳定且可平台化的分析手段。

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Comprehensive CQA analysis for AAV drug production by multiple workflows on a single CE platform

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This technical note highlights the versatility of the BioPhase 8800 system for quality assessment of adeno-associated virus (AAV). The same capillary electrophoresis (CE) system can be used for plasmid purity and isoform identification analysis, viral protein (VP) characterization, AAV purity, and genome integrity analyses. AAVs are the most commonly used viral vectors for gene therapies because they offer a safe and effective way to deliver therapeutic genes to patients, allowing for long-term expression and potentially curative treatments for various genetic diseases.[1,2] The complexity of AAV vector production requires monitoring multiple critical quality attributes (CQAs) throughout the process to ensure the safety and efficacy of AAV drugs.[3] This technical note demonstrates the capability of the BioPhase 8800 system to provide multiple workflows for assessing a range of CQAs across the AAV production process.

Key features

- **One CE platform for the entire AAV production process:** Quality assessments of starting materials, in-process samples and the final product using the BioPhase 8800 system and ready-to-use, intuitive reagent kits
- **Plasmid purity monitoring throughout the plasmid production process by CGE-LIF:** Accurate and reproducible purity analysis for purified plasmids of various sizes and in-process samples
- **High-resolution AAV purity and VP ratio analysis by CE-SDS-LIF:** High precision for AAV purity analysis with the %RSD of < 0.5% across 8 capillaries and 5 injections
- **High-resolution AAV genome integrity analysis by CGE-LIF with excellent assay reproducibility:** High-resolution separation of the intact genome peak from impurities

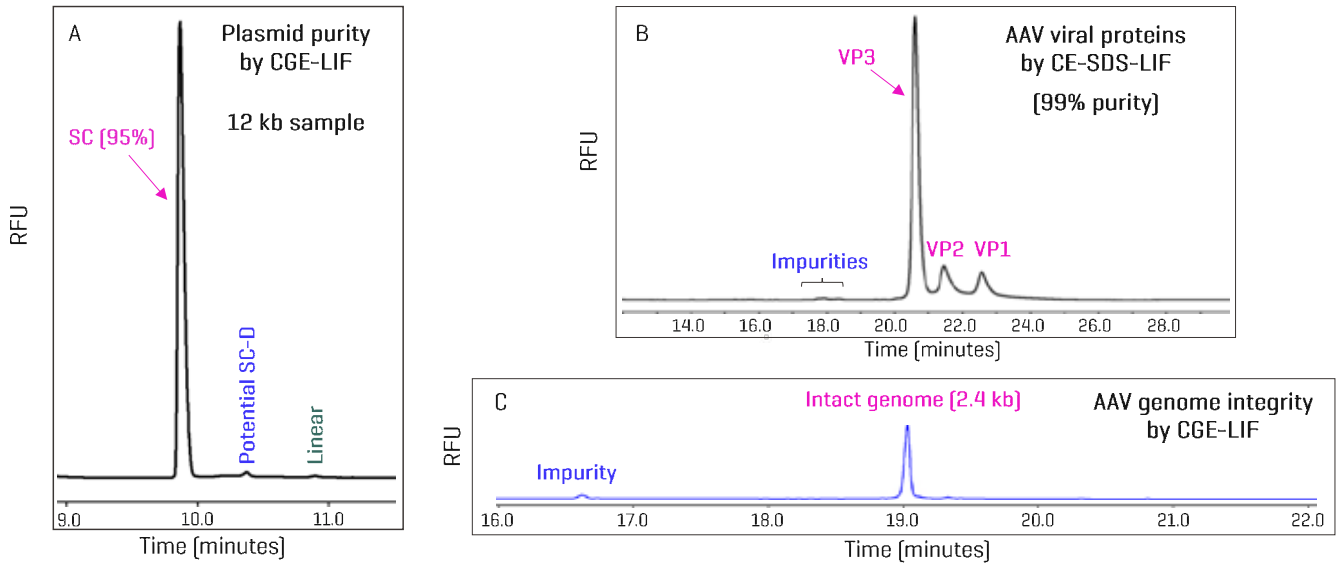


Figure 1. Analyses of plasmid purity, AAV viral proteins, and genome integrity on a single platform: the BioPhase 8800 system. A 12 kb plasmid for AAV production was analysed using the DNA 20 kb Plasmid and Linear kit (Panel A). The supercoiled (SC), the potential SC dimer (SC-D), and the linear isoforms were well resolved. The purity of this sample was determined to be 95% based on the corrected peak area percentage (%CPA) of the SC isoform. Results of viral proteins and genome integrity analyses of an AAV sample using the BioPhase CE-SDS Protein Analysis kit or the RNA 9000 Purity & Integrity kit are shown in panels B and C. Three VP proteins were resolved from each other and separated from impurities. The capsid protein purity was 99%. The intact genome of 2.4 kb in size was well-separated from the impurity peak. CGE-LIF: capillary gel electrophoresis with laser-induced fluorescence detection. RFU: relative fluorescence units.

Introduction

AAV is a small virus with a non-enveloped icosahedral protein shell and a single-stranded DNA genome of about 4.7 kilobases. Due to their excellent safety profile and high efficiency in transducing various target tissues, AAV vectors have become a popular choice for gene therapy.[1] So far, the FDA has approved 8 AAV-based drugs, with numerous others currently in clinical trials.[3,4]

The triple-transfection method is one of the most widely used techniques for producing AAV vectors.[5] This involves co-transfecting permissive cells, such as HEK293 cells, with three plasmids: a packaging plasmid with the Rep and Cap genes, a gene of interest plasmid containing the transgene flanked by AAV inverted terminal repeats (ITRs), and a helper plasmid encoding adenoviral helper genes. Chromatography and/or gradient ultracentrifugation are typically used to purify packaged AAV vectors (full capsids) from contaminants such as cellular debris, host cell DNA and RNA, and empty AAV capsids. However, some contaminants may remain in the purified viral vector product. Additionally, errors during genome encapsidation in AAV production can result in heterogeneous populations, including intact and partial genomes.[3] To ensure the safety and efficacy of the AAV drugs, assessments of multiple CQAs must be performed throughout the entire AAV production process, from the plasmid starting materials to in-process samples to the final product. Traditionally, multiple platforms have been used to accomplish this task. This technical note demonstrates streamlined quality assessments throughout the AAV production process using a single CE platform.

Methods

Materials: The DNA 20 kb Plasmid and Linear kit (P/N: 5311708), the RNA 9000 Purity & Integrity kit (P/N: C48231), the BioPhase CE-SDS Protein Analysis kit (P/N: C30085), the BioPhase BFS capillary cartridge - 8 x 30 cm (P/N: 5080121), the BioPhase Plate Pack starter kit (P/N: 5080311), and the sample loading solution (SLS) (P/N: 608082) were from SCIEX (Framingham, MA). Rainin LTS filter tips were from Mettler Toledo (Oakland, CA). Nuclease-free water (NFW) (P/N: AM9932) and N-Ethylmaleimide (NEM) (P/N: 040526) were obtained from Thermo Fisher Scientific (Cambridge, UK). The Chromeo P503 dye (P/N: 15106) was from Active Motif (Waterloo, Belgium). Benzonase (P/N: E1014-5KU) was purchased from Millipore Sigma (Dorset, UK). The QIAquick PCR purification kit (P/N: 28104) and Proteinase K (P/N: 19131) were from Qiagen (Manchester, UK).

A Rep/Cap plasmid, a gene of interest plasmid, a helper plasmid sample (ranging from 7-12 Kb), packaged AAV of two different serotypes, and an empty AAV sample were provided by Pharmaron Biologics (Liverpool, UK) or commercially sourced. The XbaI (P/N: R0145S) restriction enzyme and the Nt.AlwI nicking enzyme (P/N: R0627S) kits containing their corresponding 10x reaction buffer were from New England Biolabs (NEB) (Hertfordshire, UK).

Addition of the SYBR™ Gold Nucleic Acid gel stain to the DNA 20 kb Plasmid and Linear gel: To analyse a full plate of 96 samples, 20 mL of the gel was removed from the bottle, pre-warmed to room temperature, and transferred to a 50 mL conical tube. Then, 80 µL of SYBR™ Gold Nucleic Acid gel stain (pre-thawed) was added. The tube was capped tightly and gently inverted 20 times to mix well, avoiding air bubbles. Finally, the tube was wrapped in aluminum foil to prevent photobleaching before use.

Sample preparation for the plasmid analysis: The plasmid DNA samples at the various stages of the production process were buffer exchanged to the DNA 20 kb Plasmid and Linear sample buffer using an Amicon Ultra-0.5 Centrifugal Filter Unit (10 kDa, P/N: UFC501024) and diluted to a final concentration of 5 ng/µL. The diluted plasmid sample was then transferred at 50 µL per well to the sample plate for analysis on the BioPhase 8800 system. The purified plasmid samples and one of the in-process samples were not buffer-exchanged. Instead, they were diluted with the DNA 20 kb Plasmid and Linear sample buffer directly before CE analysis. To prepare the linearized and open circular (OC) isoforms, the 7 kb plasmid sample was digested with the XbaI restriction enzyme or the Nt.AlwI nicking enzyme following the manufacturer's instructions.

Sample preparation for AAV capsid protein analysis by CE-SDS-LIF on the BioPhase 8800 system: Sample preparation was carried out as described in a previous SCIEX technical note[6] except that the dithiothreitol was replaced with NEM. 50 µL of the prepared sample was transferred to the sample plate and analysed on the BioPhase 8800 system. After the CE separation,

the ratio of the corrected peak areas of VP1:VP2:VP3 was calculated and normalized by the lysine contents of each VP using the Empower 3 Chromatography Data System (CDS).

Sample preparation for AAV genome integrity analysis by CGE-LIF on the BioPhase 8800 system: Filter tips were used for all steps to minimize unintended nucleic acid degradation. Sample preparation was carried out as described in a previous SCIEX technical note[7] except the AAV sample at 1 x 10^{12} vg was treated with Benzonase and Proteinase K before genome extraction with the QIAquick PCR purification kit. Then, 50 µL of the sample was transferred into each well on the sample plates before CE analysis using the Nucleic Acid Extended Range Gel and the SYBR™ Green II RNA gel stain.

Instrument and software: The BioPhase 8800 system with UV/LIF (P/N: 5089278)—equipped with a laser-induced fluorescence (LIF) detector utilizing an excitation wavelength of 488 nm and an emission wavelength of 520 nm—was from SCIEX. A 600 nm bandpass emission filter (PN 5085177) from SCIEX was used for CE-LIF analysis of AAV capsid proteins. The BioPhase 8800 driver for Empower (P/N: 5306620, from SCIEX) was used to operate and acquire data from the BioPhase 8800 system utilizing the LIF detector while interfacing with Waters Empower 3 CDS. The BioPhase 8800 driver for Empower enables direct control of the BioPhase 8800 system hardware, sample setup, method design, data collection, and storage of multichannel data in the Empower 3 CDS database alongside linked sample information (sample identifier, sample set, method set, and instrument method). Post-acquisition, data processing, and analysis were performed using the Empower 3 CDS.

Instrument setup: Buffer plates and sample plates were prepared based on the experimental design and plate map generated by the BioPhase software, as described in the DNA 20 kb Plasmid and Linear kit application guide[8], the BioPhase CE-SDS Protein Analysis kit application guide[9], and the RNA 9000 Purity & Integrity kit application guide.[10]

Results and discussion

Streamlined platform assays for quality assessment throughout the AAV production process using a single CE system: Figure 1 illustrates how analyses of plasmid purity, AAV viral proteins, and genome integrity can all be performed on a single platform: the BioPhase 8800 system. In Panel A, a 12 kb plasmid that was used for AAV production was analysed using the DNA 20 kb Plasmid and Linear kit. The SC, the potential SC-D, and the linear isoforms were well resolved. The OC isoform was not detected for this particular batch of plasmid. The purity of this sample was determined to be 95% based on the %CPA of the SC isoform. Panels B and C show the results of viral proteins and genome integrity analyses of the same AAV sample using the BioPhase CE-SDS Protein Analysis kit or the RNA 9000 Purity & Integrity kit. Three VP proteins were resolved from each other and separated from impurities for an accurate determination of purity and VP ratio. The capsid protein purity of this sample was 99%. The ratio of corrected peak areas of VP1, VP2, and VP3 was calculated and normalized by the lysine contents of each VP using the custom settings in the Empower 3 CDS. The VP ratio was 1.0:2.7:17.2 for VP1:VP2:VP3 (Figure 4). The intact genome of 2.4 kb in size was well separated from the impurity peak. These platform CE assays are readily available to analyse a range of plasmid sizes and AAV serotypes, enabling efficient quality assessment throughout the AAV production process.

Plasmid purity analysis and isoform identification: Plasmid is a critical starting material for AAV drug production. Plasmid DNA has several topological isoforms, including SC, OC, linear, and multimeric. Degradation during processing and storage can convert SC to OC or linear forms. Plasmid purity is assessed by measuring the percentage of the SC isoform due to its high transfection efficiency.[11] To achieve a high AAV yield, plasmids with %SC above 90% are desired. Therefore, it is critical to achieve high-resolution separation of different plasmid isoforms. In addition, the main isoforms need to be clearly identified. Three plasmids used for AAV production with sizes of 7 kb, 10 kb, and 12 kb were analysed using the DNA 20 kb Plasmid and Linear kit and the pre-assembled bare-fused silica (BFS) cartridge on the BioPhase 8800 system. Results in panels A to F of Figure 2 demonstrate a high-resolution separation of the SC isoform from the potential SC-D, the linear, and the OC isoforms for all 3 plasmids. The purity was determined as 98% for the 7 kb plasmid, 95% for the 10 kb plasmid, and 94% for the 12 kb plasmid based on the %CPA of the SC isoform. In addition, electropherograms obtained with the 3 plasmids (panels G to I) illustrate the positive correlation between the migration time of the SC isoform and the size of the plasmids. This capability can be beneficial for fast screening of the crude plasmid samples during plasmid production.

As an example of isoform identification, panels J, K, and M of Figure 2 present a stacked view of electropherograms obtained with the undigested 7 kb plasmid sample (red trace), the linearized 7 kb plasmid (blue trace), and the nicked 7 kb plasmid (black), highlighting the identification of peaks corresponding to the SC, the linear and OC isoforms. These platform methods for plasmid purity and peak identification were also developed for plasmids ranging from 6 to 15 kb (data not shown) and can be readily applied to analysing various plasmids for AAV production.

Successful purity monitoring throughout the plasmid production process: Previous work has shown that plasmid analysis by CGE-LIF with the DNA 20 kb Plasmid and Linear kit has the potential to analyse in-process plasmid samples due to an excellent limit of detection (LOD) of < 5 pg/µL.[12] Monitoring the plasmid quality across the production process is beneficial for process control and improvement. A 12 kb plasmid was purified by ion exchange chromatography. Samples from the "Chromatography 1 Load", "Chromatography 1 Eluate", and "Purified Plasmid" stages of the plasmid production process were buffer exchanged and analysed using the DNA 20 kb Plasmid and Linear kit. As shown in Figure 3, panels A to C, the purity for the "Chromatography 1 Load" sample was 90% with the detection of the linear, OC, potential SC-D isoforms, and the unknown impurities. The purity of the "Chromatography 1 Eluate" sample was 93%, with a relatively lower amount of linear, OC, potential SC-D, and unknown impurities than in the

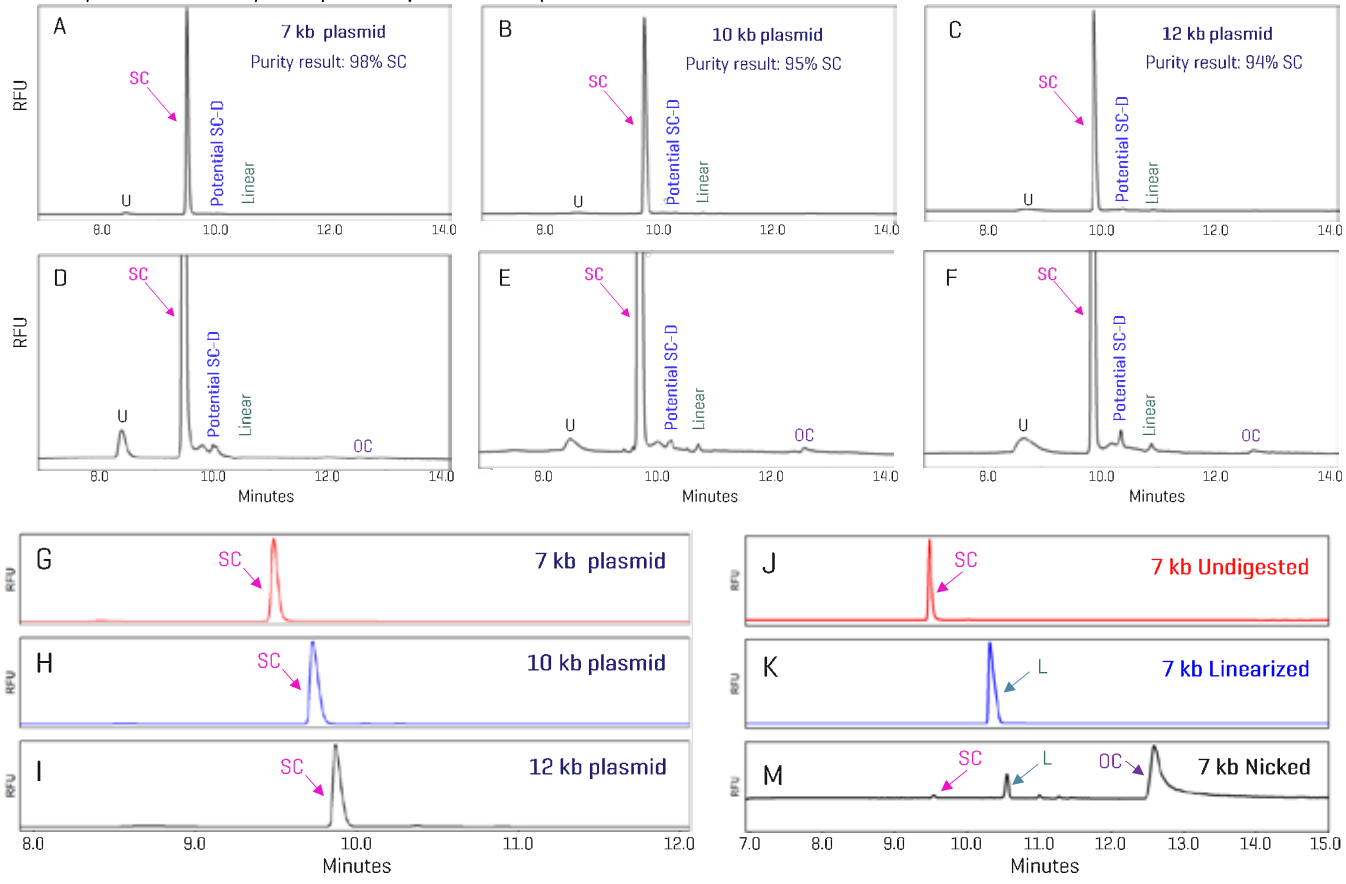


Figure 2. Plasmid purity analysis and peak identification using the DNA 20 kb Plasmid and Linear kit. Three AAV starting material plasmids: a 7 kb plasmid (panel A), a 10 kb plasmid (panel B), and a 12 kb plasmid (panel C) were analysed on the BioPhase 8800 system. Panels D, E, and F are expanded views of panels A, B, and C, showing a high-resolution separation of the SC isoform from the potential SC-D, the linear, and the OC isoforms. The purity was determined as 98% for the 7 kb plasmid, 95% for the 10 kb plasmid, and 94% for the 12 kb plasmid based on the %CPA of the SC isoform. U: unknown impurities. Panels G, H, and I show a stacked view of the electropherograms obtained with the 3 plasmids, demonstrating the positive correlation between the migration time of the SC isoform and the size of the plasmids. Panels J, K, and M show a stacked view of electropherograms obtained with the undigested 7 kb plasmid sample (red), the linearized 7 kb plasmid (blue), and the nicked 7 kb plasmid (black), identifying the peaks for the linear and OC isoforms.

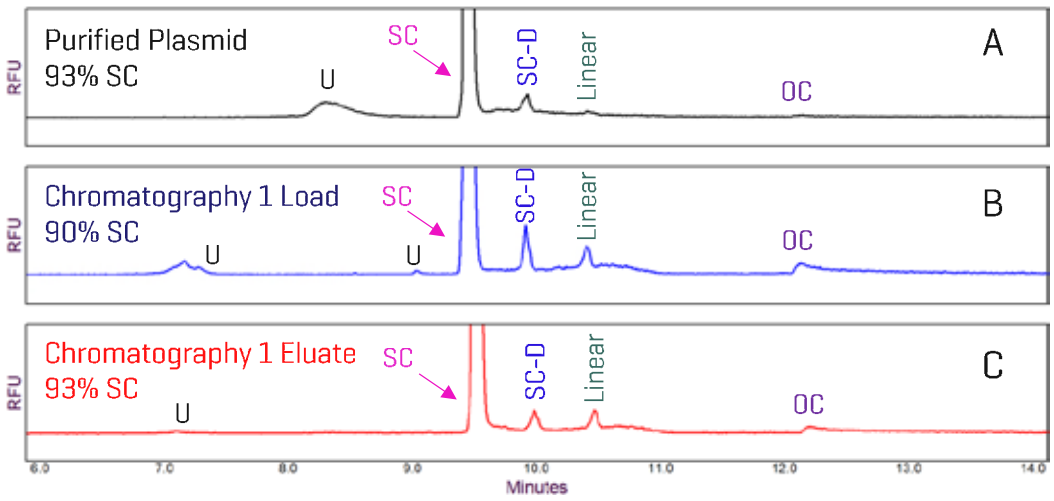


Figure 3. Successful purity monitoring throughout the plasmid production process. A 12 kb plasmid used for AAV production was purified by ion exchange chromatography. Samples from the "Chromatography 1 Load", "Chromatography 1 Eluate," and "Purified Plasmid" stages of the plasmid production process were buffer exchanged and analysed using the DNA 20 kb Plasmid and Linear kit. The purity was determined and indicated in panels A, B, and C. U: unknown impurity.

"Chromatography 1 Load" sample, indicating the improvement in purity by the chromatography process. The purified plasmid sample had a 93% purity with very low linear and no detectable OC isoform, indicating further removal of linear and OC isoforms by post-chromatography steps in the production process. Similar results were obtained with a 7 kb plasmid and a 10 kb plasmid (data not shown). These results demonstrate that CGE-LIF can successfully monitor the plasmid purity throughout the plasmid production process. Moreover, plasmid purity analysis of in-process samples without buffer exchange yielded results comparable to those with buffer exchange (data not shown), further enhancing the efficiency of the workflow.

Excellent assay reproducibility for plasmid purity analysis on the BioPhase 8800 system: Since the BioPhase 8800 system is a multi-capillary system that separates 8 samples simultaneously and can analyse up to 96 samples in a sequence, it is essential to assess the inter-capillary and the intra-capillary variations. The purified 7 kb and 12 kb plasmids were diluted in the DNA 20 kb Plasmid and Linear sample buffer to 5 ng/µL and analysed to evaluate the inter- and intra-capillary repeatability using the DNA 20 kb Plasmid and Linear kit. The results, including mean and %RSD values of the plasmid purity, are summarized in Tables 1 and 2. For the 7 kb plasmid, %RSD values of plasmid purity were within 1.5% across 8 different capillaries or between 4 different days in the same capillary. The overall average purity value of 32 injections was 98% for this sample, with an overall %RSD of 0.8%. For the 12 kb plasmid, %RSD values of plasmid purity were within 4.0% across 8 different capillaries or between 4 different days in the same capillary. The overall average purity value of 32 injections was 94% for this sample, with an overall %RSD of 2.3%. These results demonstrate high assay reproducibility for plasmid purity analysis on the BioPhase 8800 system using the DNA 20 kb Plasmid and Linear kit.

Successful AAV purity analysis through characterization of viral proteins: The AAV capsid is composed of 3 main proteins, which are approximately 87kD (VP1), 73kD (VP2), and 61kD (VP3) in size.[13] AAV purity analysis is a CQA for AAV products. In addition, the VP ratio is crucial for AAV integrity and directly impacts effective genetic payload delivery.[14] The CE-SDS-LIF workflow was used to assess AAV purity and VP ratio through the analysis of VPs. Figure 4 shows results obtained with two serotypes of AAV (panels A and B) using the BioPhase CE-SDS Protein Analysis kit and the Chromeo P503 dye on the BioPhase 8800 system. With both AAV samples, the 3 VPs were resolved from each other and well separated from the impurities. In addition, the VP3' was also detected with the AAV serotype A sample. The VP3' and VP3 peaks differ by 8 amino acids due to their difference in translation start site, as reported previously.[15] The resolution between VP3 and VP3' peaks demonstrates the outstanding resolving power of CE on the BioPhase 8800 system. The VP3' was not detected in the AAV serotype B sample, which is consistent with the published literature that covered both serotypes.[15] The purity of the two AAV samples

was determined to be 90% and 99%, respectively, indicating the high quality of the AAV samples. Furthermore, the normalized VP ratios [VP1:VP2:VP3] for the AAV serotypes A and B samples were determined as 1.0:3.4:19.0 and 1.0:2.7:17.2, respectively. The VP ratio values were previously shown to be characteristics of different AAV serotypes,[16] and of different production processes of the same serotype.[14] Therefore, assessment of the VP ratio can be useful for process development of a range of AAV serotypes. The same workflow was applied to two additional AAV serotypes with minor modifications based on their physical properties (data not shown), supporting the idea that the CE-SDS-LIF can be used as a platform method for AAV purity and VP ratio analyses.

Table 1. Assay reproducibility for purity analysis of a 7 kb plasmid using the DNA 20 kb Plasmid and Linear kit on the BioPhase 8800 system. Purified plasmid samples were diluted with the DNA 20 kb Plasmid and Linear sample buffer and transferred to the BioPhase sample plate for plasmid purity analysis. Mean and percent relative standard deviation (%RSD) values of plasmid purity were calculated across the 8 capillaries or between 4 sequences, each on a different day. The %RSD values were within 1.5% across 8 different capillaries or between 4 different days. The overall average purity value from all 32 injections was 98%, with an overall %RSD of 0.8%.

Sample sequence	Cap A	Cap B	Cap C	Cap D	Cap E	Cap F	Cap G	Cap H	Mean	% RSD (Inter-capillary)
1	98.69	97.14	98.28	97.54	97.81	97.81	98.53	98.66	98.06	0.6
2	98.63	98.22	98.79	98.14	98.36	98.34	98.36	98.42	98.41	0.2
3	97.53	97.60	98.47	96.46	97.20	97.85	98.26	98.34	97.71	0.7
4	98.52	98.13	98.67	98.21	95.39	99.23	96.68	98.43	97.91	1.3
Mean	98.34	97.77	98.55	97.59	97.19	98.31	97.96	98.46	98.02	
% RSD (Intra-capillary)	0.6	0.5	0.2	0.8	1.3	0.7	0.9	0.1		0.8

Table 2. Assay reproducibility for purity analysis of a 12 kb plasmid using the DNA 20 kb Plasmid and Linear kit on the BioPhase 8800 system. Purified 12 kb plasmid was diluted with the DNA 20 kb Plasmid and Linear sample buffer and transferred to the BioPhase sample plate for plasmid purity analysis. Mean %RSD values of plasmid purity were calculated across the 8 capillaries or between 4 sequences, each on a different day. The %RSD values were within 4.0% across 8 different capillaries or between 4 different days. The overall average purity value from all 32 injections was 94%, with an overall %RSD of 2.3%.

Sample sequence	Cap A	Cap B	Cap C	Cap D	Cap E	Cap F	Cap G	Cap H	Mean	% RSD (Inter-capillary)
1	95.42	95.29	95.55	95.11	95.10	94.89	94.76	94.91	95.13	0.3
2	95.32	94.42	95.21	95.29	95.70	93.85	94.79	94.66	94.91	0.6
3	93.64	92.94	94.59	92.40	92.33	91.91	94.09	93.64	93.19	1.0
4	96.18	88.96	87.95	88.92	95.19	89.86	94.89	95.08	92.13	3.8
Mean	95.14	92.90	93.33	92.93	94.58	92.63	94.63	94.57	93.84	
% RSD (Intra-capillary)	1.1	3.0	3.9	3.2	1.6	2.4	0.4	0.7		2.3

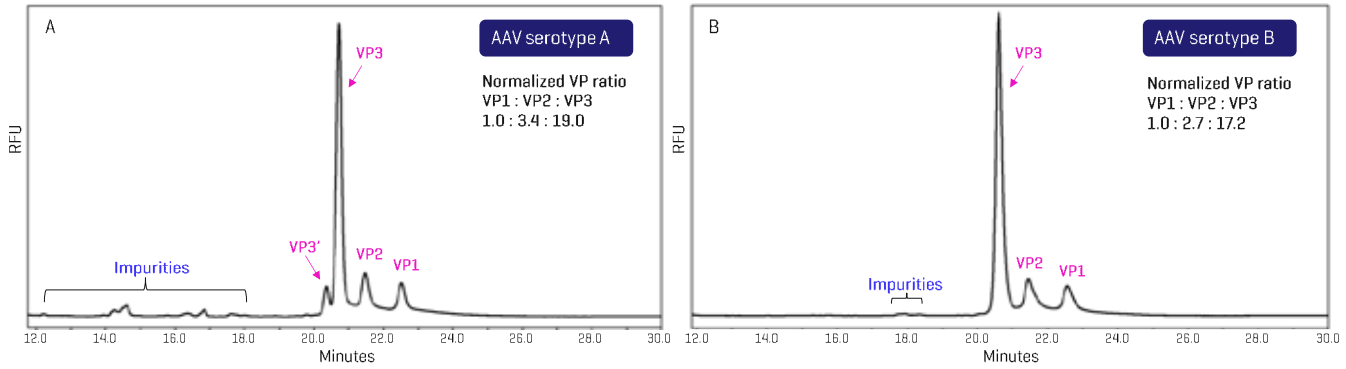


Figure 4. AAV purity and VP ratio analyses of two samples with different serotypes. Purified AAV serotype A and AAV serotype B samples were denatured and reduced in the presence of SDS and then labelled with a fluorescent dye Chromeo P503 before CE separation on the BioPhase 8800 system using the BioPhase CE-SDS Protein Analysis kit. The purity of the AAV serotype A and AAV serotype B was determined to be 90% and 99%, respectively. The normalized VP ratios (VP1:VP2:VP3) for the AAV serotype A and AAV serotype B samples were 1.0:3.4:19.0 and 1.0:2.7:17.2, respectively. VP3' is a variant of VP3 and was grouped with VP3 as a natural VP species for the purity calculation of the serotype A sample.

High precision AAV purity analysis by CE-SDS-LIF on the BioPhase 8800 system: To assess assay reproducibility for AAV purity analysis, an AAV serotype B sample was added to the BioPhase plate and injected five times. Table 3 summarizes the results obtained. The mean purity value was 99% in each column across eight capillaries or within each capillary for five injections. The mean purity value from all 40 injections was also 99%, indicating high consistency in purity measurements. The %RSD values were less than 0.5% across eight capillaries or between five injections within each capillary, demonstrating excellent assay repeatability and high precision.

Table 3. High assay repeatability of AAV purity analysis. The AAV serotype B sample was transferred to the BioPhase sample plate and injected 5 times for AAV purity analysis. The results are summarized in the table below. Mean and %RSD values for AAV purity were calculated across the 8 capillaries or between 5 injections. The %RSD was less than 0.5% across 8 different capillaries or between 5 injections.

Sample Injection	Cap A	Cap B	Cap C	Cap D	Cap E	Cap F	Cap G	Cap H	Mean	% RSD Across Capillaries
1	99	99	99	99	99	99	99	99	99	0.3
2	99	99	99	99	99	99	99	99	99	0.2
3	99	99	99	99	99	99	100	99	99	0.3
4	99	99	99	99	98	99	99	98	99	0.4
5	98	99	99	99	98	99	99	99	99	0.3
Mean	99	99	99	99	99	99	99	99	99	
% RSD Across injections	0.4	0.1	0.2	0.1	0.4	0.2	0.2	0.3		0.3

High-resolution AAV genome integrity analysis by CGE-LIF with excellent assay reproducibility: Genome integrity is a CQA for AAV drugs. Errors during the encapsidation could lead to AAV capsids containing a partial or truncated genome and negatively impact the efficacy and safety of AAV drugs.[3] Therefore, assessing the encapsidated genome's quality and size is necessary. Two different AAV serotypes consisting of full capsids were tested for genome integrity, along with an empty capsid AAV sample. The same sample-preparation procedure was used to extract AAV genomes from all samples before analysing them on the BioPhase 8800 system using the RNA 9000 Purity & Integrity kit as described in the methods section. Results are shown in Figure 5A. No peak correlating to the intact genome was observed in the empty AAV serotype A capsid, and peaks correlating with the size of the expected intact genome were observed in the full 4.6 kb AAV serotype A and 2.4 kb AAV

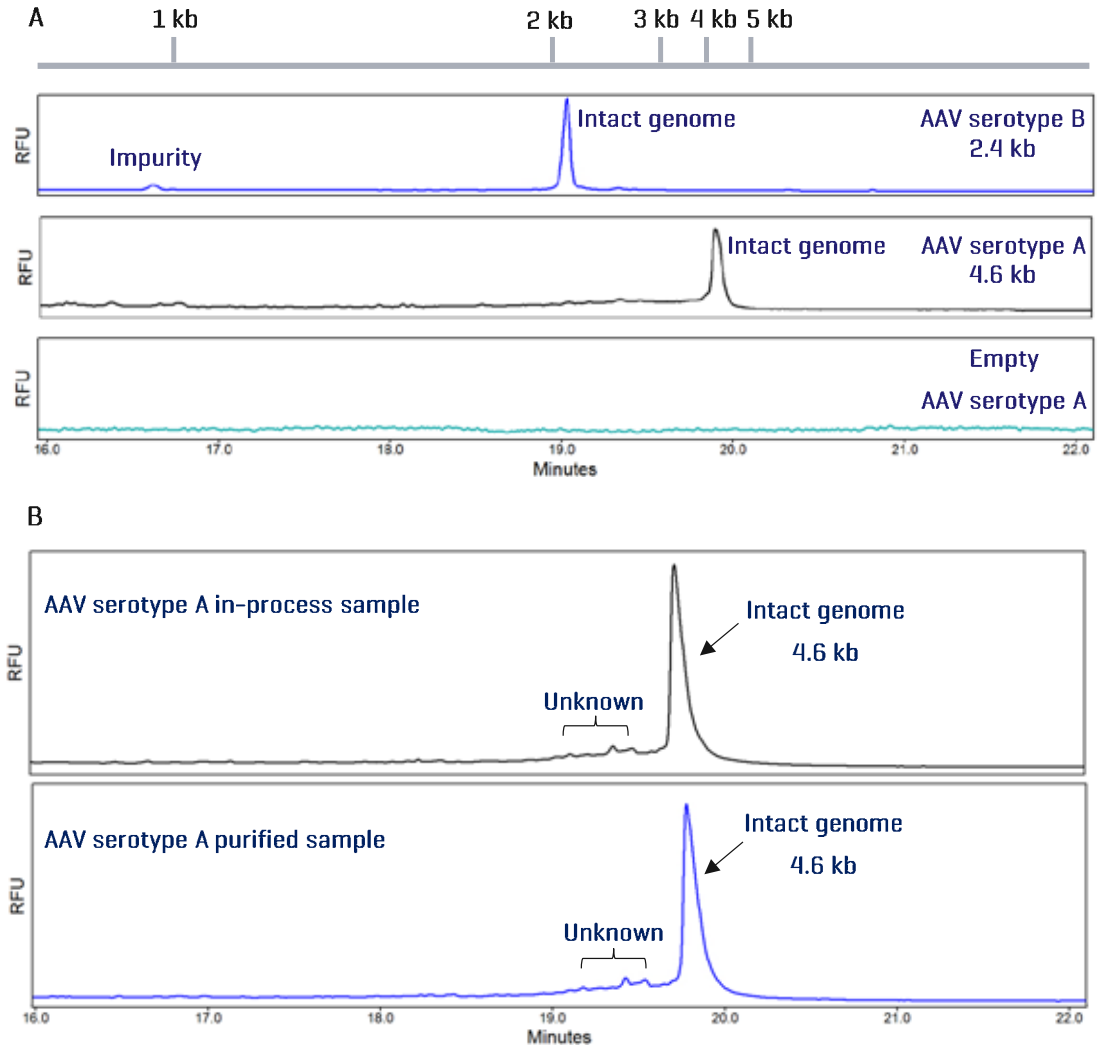


Figure 5. Genome integrity analysis of purified full capsids, empty capsids, and in-process AAV samples. The AAV genomes were extracted from the AAV samples and analysed on the BioPhase 8800 system using the RNA 9000 Purity & Integrity kit as described in the methods section. In panel A, purified AAV serotype B (blue trace) and AAV serotype A (black trace) full capsids and empty AAV serotype A (green trace) capsids were analysed. The intact genomes were well separated from impurities. The top bar shows the sizes of standard fragments from an ssDNA ladder. Panel B shows a comparison of the results obtained from an in-process (black trace) and a purified (blue trace) sample of the AAV serotype A. The AAV serotype A purified sample in panel B was a different sample from the one used in panel A.

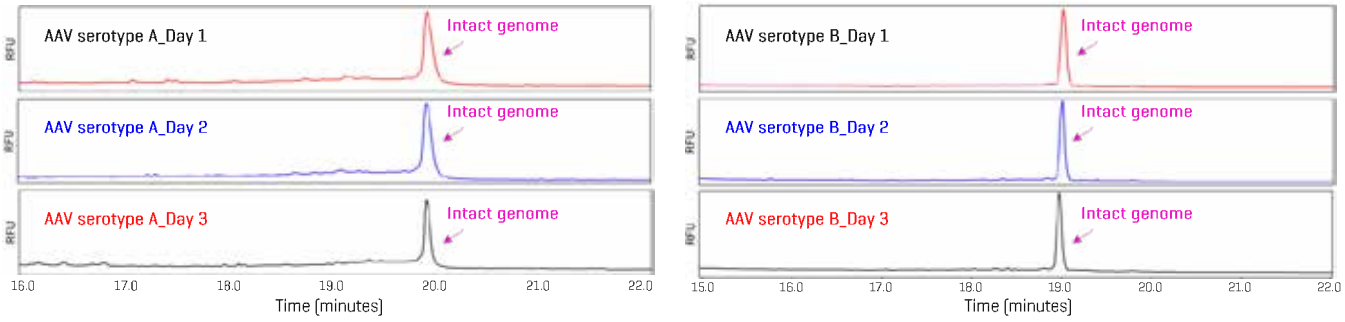


Figure 6. Excellent assay reproducibility of the genome integrity analysis. The purified genomes of the AAV serotype A and serotype B samples were analysed on three different days on the BioPhase 8800 system using the RNA 9000 Purity & Integrity kit. Profiles obtained on 3 different days were consistent.

serotype B samples. In addition, the intact genome peak was well-separated from the impurity peak for the full capsid AAV serotype B samples. The sizes of the intact genomes were verified using an ssDNA ladder. Figure 5B shows results obtained with an in-process and a purified AAV serotype A sample. A comparison of the two electropherograms indicates that consistent peak profiles were obtained. In addition, the relative migration times and sizes of the intact genomes were comparable between the two samples. These results demonstrate that the CGE-LIF method can be used as a platform method for genome integrity analysis for multiple AAV serotypes and both purified and in-process samples.

To assess the assay reproducibility of the genome integrity analysis, the purified genomes from an AAV serotype A sample and an AAV serotype B sample were analysed on three different days on the BioPhase 8800 system using the RNA 9000 Purity & Integrity kit. As shown in Figure 6, profiles in electropherograms obtained on 3 different days were consistent for both samples. With both serotypes, the relative migration times of the intact genome peaks were consistent among the runs on 3 different days, indicating excellent assay reproducibility. The profile of the AAV serotype B in Figure 6 looked slightly different from the one in Figure 5A. This is because the AAV serotype B samples in Figure 5A and in Figure 6 were from two different batches, both of which were commercially sourced. This observation indicates that the genome integrity workflow can be used to monitor batch-to-batch variations.

Conclusions

- Platform CE workflows for plasmid and AAV purity and genome integrity assessments were developed across the AAV production process
- ALL workflows were performed on one single instrument with pre-assembled cartridges and ready-to-use, user-friendly reagent kits
- The CGE-LIF workflow was validated for plasmids of various sizes with excellent repeatability and was successfully utilized to monitor plasmid purity quantitatively throughout the production process, offering a clear advantage over slab gels
- The CE-SDS-LIF workflow was successfully employed for high-precision AAV purity analysis and VP ratio assessment.
- High-resolution AAV genome integrity analysis by CGE-LIF workflow was demonstrated with excellent assay reproducibility.

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A new method for monitoring plasmid purity: seamless method transfer and consistent results across single and multi-capillary systems

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This technical note provides insight into a versatile and valuable new tool for plasmid purity analysis. The DNA 20 kb Plasmid and Linear kit is compatible with pre-built capillary cartridges, enhancing efficiency and robustness. It enables high-quality separation with purity percentages (purity%) and consistent plasmid isoform profiles similar to those obtained with the dsDNA 1000 kit on the PA 800 Plus system, ensuring reliable assay transfer between kits. It also supports method transfer between the single-capillary PA 800 Plus system and the multi-capillary BioPhase 8800 system, providing consistent peak profiles, migration times (MTs) and purity% across both platforms while accommodating various throughput requirements. The kit offers easy-to-use, high-resolution plasmid DNA topology analysis across a wide size range (2.7–18.9 kb), efficiently separating supercoiled (SC) isoforms from other topological isoforms and impurities for comprehensive and efficient characterization of plasmids.

Key plasmid analysis features of the DNA 20 kb Plasmid and Linear kit

- **Results comparable to those obtained with the dsDNA 1000 kit:** achieves purity% and consistent plasmid isoform distributions similar to those obtained with the dsDNA 1000 kit on the PA 800 Plus system, validating its effectiveness and ensuring seamless assay transfer
- **Seamless method transfer between single-capillary (PA 800 Plus system) and multi-capillary (BioPhase 8800 system) systems:** demonstrates consistent peak profiles, MTs and purity% on both platforms
- **Ease of use and versatility for high-resolution plasmid DNA topology analysis**: enables high-resolution analysis of plasmids across a wide size range (2.7–18.9 kb), effectively separating topological isoforms with ready-to-use gel and pre-built capillary cartridges (Figure 1)

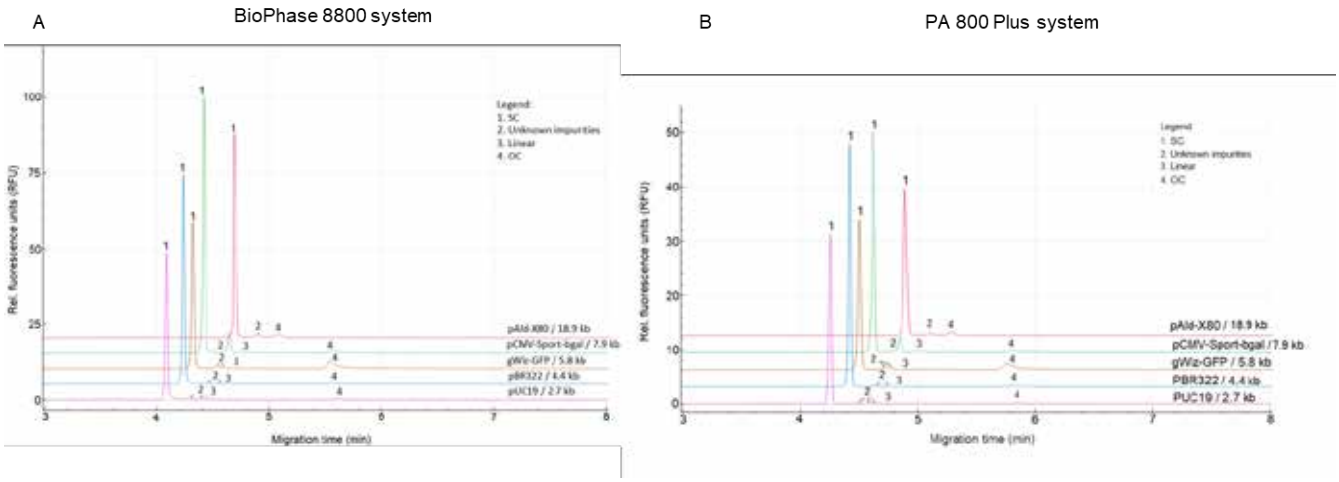


Figure 1. High-resolution separation of topological isoforms in plasmids across a wide size range (2.7–18.9 kb). Five plasmid samples were analyzed using the DNA 20 kb Plasmid and Linear kit on the BioPhase 8800 system (A) and the PA 800 Plus system (B).

Introduction

Plasmid DNA is a fundamental starting material in cell and gene therapy manufacturing processes, including messenger RNA (mRNA) production and the generation of viral vectors or gene-of-interest (GOI) plasmids.[1] Additionally, plasmid DNA is used as an active pharmaceutical ingredient (API) for protein production. Analyzing plasmid topology and purity is essential for establishing a robust plasmid DNA quality control strategy.[2]

Plasmid purity analysis involves assessing the presence of various topological forms, such as SC, open circular (OC), linear and others.[3] It is critical to monitor the percentage of total SC plasmid DNA relative to lower amounts of other forms. High-resolution techniques are necessary to accurately distinguish these forms and ensure that plasmid preparations meet the required quality standards. Advanced analytical methods, such as capillary electrophoresis (CE), offer automated rapid and precise separation of plasmid isoforms.

The dsDNA 1000 kit is a commonly used method for plasmid topology analysis on CE systems from SCIEX. It involves reconstituting lyophilized gel powder to prepare the gel buffer, manually diluting the gel for optimized separation of plasmid samples and assembling the cartridge with the coated DNA capillary. These steps can be time-consuming, delay analysis and time to result and are prone to user errors.

This technical note introduces the DNA 20 kb Plasmid and Linear kit, a new plasmid analysis kit that features ready-to-use reagents and compatibility with pre-built bare-fused silica (BFS) capillary cartridges, providing rapid and high-resolution analysis of plasmids across a wide size range (2.7–18.9 kb). In the analysis of 5 plasmids 2.7–18.9 kb in size, the new kit achieved purity results similar to those obtained with the dsDNA 1000 kit, validating the effectiveness of the new method and streamlining the transition to the new DNA 20 kb Plasmid and Linear kit.

The performance of the new kit was evaluated on both the single-capillary PA 800 Plus system and the multi-capillary BioPhase 8800 system and compared. The kit demonstrated consistent peak profiles, MTs and purity% across both platforms, enabling smooth method transfer and the ability to meet varying throughput requirements. Additionally, a titration experiment was conducted to determine the optimal plasmid concentration for topological isoform analysis using the new kit, ensuring accurate and reliable results. The 8-capillary BioPhase 8800 system facilitates rapid titration curve optimization by simultaneously analyzing samples at different dilution points in a single run. The reliability of the new DNA 20 kb Plasmid and Linear kit makes it suitable for use at various stages of biotechnological and biopharmaceutical processes to help ensure the integrity and quality of plasmid DNA.

Methods

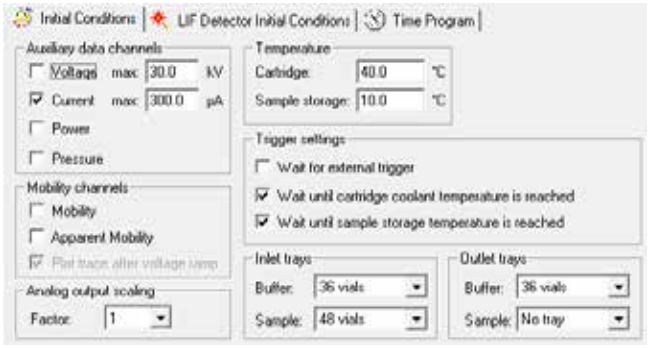
Materials: The DNA 20 kb Plasmid and Linear kit (P/N: 5311708) was from SCIEX (Framingham, MA) and contained DNA 20 kb Plasmid and Linear gel, DNA 20 kb Plasmid and Linear sample buffer, DNA 20 kb Plasmid test mix, SYBR™ Gold Nucleic Acid gel stain*, DNA 20 kb Plasmid and Linear conditioning solution, 00.1 N HCl and CE Grade water. The BioPhase BFS capillary cartridge - 8 x 30 cm (P/N: 5080121) and BioPhase sample and reagent plates (4,4,8) (P/N: 5080311) were from SCIEX. Rainin LTS filter tips were from Mettler Toledo (Oakland, CA). Nuclease-free water (NFW) (P/N: AM9932) was obtained from Thermo Fisher Scientific (Waltham, MA). The dsDNA 1000 kit (P/N: 477410) and the pre-assembled capillary cartridge (P/N: A55625) were from SCIEX. A 10x concentrated Tris Borate EDTA stock solution (10x TBE) (P/N: T4415) was from Sigma-Aldrich (St. Louis, MO).

The 2.7 kb pUC19 Vector plasmid DNA (P/N: N3041S) at 1,000 µg/mL was from New England Biolabs (NEB) (Boston, MA). The 4.4 kb pBR322 plasmid DNA (P/N: SD0041) at 0.5 µg/µL and the 7.9 kb plasmid pCMV·SPORT-βgal (P/N: 10586014) was from Thermo Fisher Scientific (Carlsbad, CA). The 5.8 kb gWiz-GFP plasmid DNA (P/N: 5006) and the 18.9 kb pALD-X80 AAV Helper plasmid (P/N: 5017-10) were from Aldevron (South Fargo, ND).

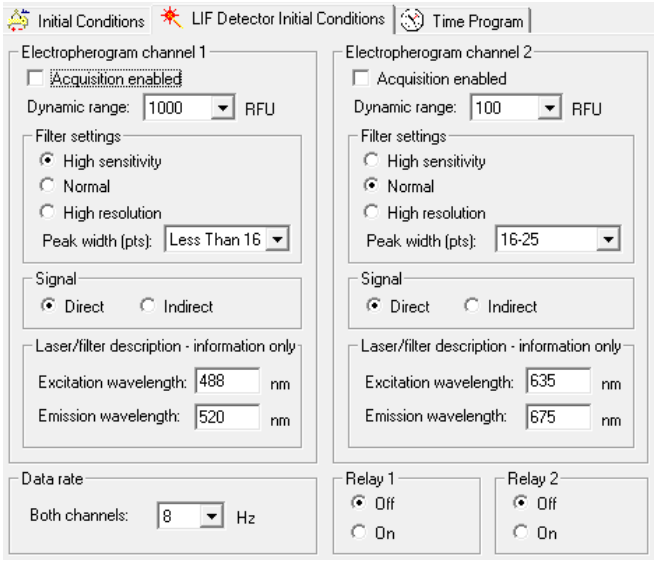
Sample preparation for analysis using the DNA 20 kb Plasmid and Linear kit: The plasmid DNA sample was thawed on ice for about 20 minutes and diluted in the DNA 20 kb Plasmid and Linear sample buffer to a final concentration of 1 ng/µL. The diluted plasmid sample was then transferred at 100 µL per well to the sample plate for analysis on the BioPhase 8800 system or 100 µL per sample vial for analysis on the PA 800 Plus system.

Sample preparation for analysis using the dsDNA 1000 kit: Plasmid samples were diluted with 1x TBE buffer to 8 ng/µL for analysis, and then 100 µL of samples were transferred to a sample microvial for analysis on the PA 800 Plus system.

A



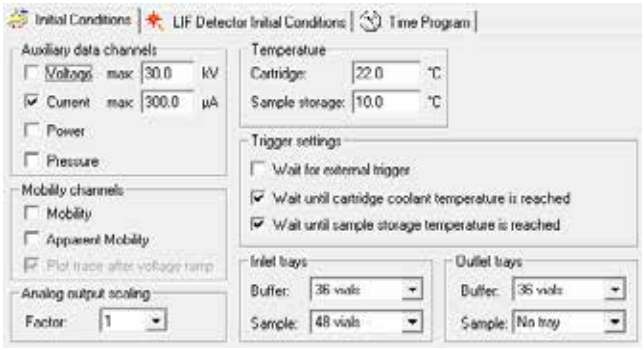
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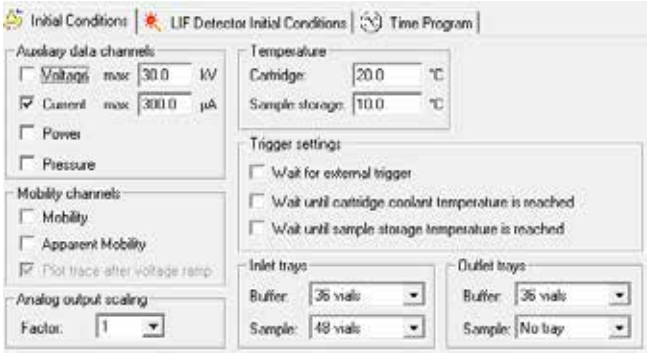
D



E



F



G



Figure 2. Parameter settings for PA 800 Plus system methods using the DNA 20 kb Plasmid and Linear kit. Different initial conditions (A, D and F) and time programs (C, E and G) for conditioning, separation and shutdown are provided. LIF detector initial conditions (B) were the same for all 3 methods.

Gel buffer preparation using the dsDNA 1000 kit: To rehydrate the gel buffer, 20 mL of 0.2 µm filtered deionized water was added to the gel buffer vial. The mixture was gently stirred with a small stirring bar for up to 24 hours or until the dried gel was completely dissolved. The hydrated gel is stable for 1 month if stored at 4°C. Then, the gel solution was diluted 1:10 with 1x TBE (90mM tris, 90mM borate and 2mM EDTA at pH 8.3). The diluted gel was filtered through a 0.45 µm filter. The 1x TBE was prepared by diluting the 10x TBE buffer stock solution 10 times with deionized water. The resulting buffer solution was filtered with a 0.2 µm filter. SYBR™ Gold Nucleic Acid gel stain was added to the gel at 100 µL in 10 mL gel.

Cartridge assembly using the dsDNA 1000 kit on the PA 800 Plus system: The DNA capillary was installed according to the instructions (P/N: 726412) in the dsDNA 1000 kit. The total capillary length was 40.2 cm, with 30 cm as the length to the detection window.

Instrument and software: The BioPhase 8800 system—equipped with laser-induced fluorescence (LIF) detection utilizing an excitation wavelength of 488 nm and an emission wavelength of 520 nm—was from SCIEX. Data acquisition and analysis were performed using BioPhase 8800 software 1.2 from SCIEX. A PA 800 Plus system—equipped with LIF detection and solid-state laser with excitation wavelength at 488 nm and a 520 nm bandpass emission filter—was from SCIEX. Data acquisition and analysis were performed using 32 Karat software 10.2.

Instrument analysis methods: Samples prepared for analysis using the DNA 20 kb Plasmid and Linear kit were analyzed on the BioPhase 8800 system and the PA 800 Plus system to assess the comparability between these 2 platforms. The methods used on the BioPhase 8800 system were provided in the DNA 20 kb Plasmid and Linear kit application guide,[4] while the methods for the PA 800 Plus system are shown in Figure 2. The BioPhase BFS capillary cartridge - 8 x 30 cm was used for analysis on the BioPhase 8800 system, while the pre-assembled capillary cartridge was used for analysis on the PA 800 Plus system.

Samples prepared for analysis using the dsDNA 1000 kit were analyzed on the PA 800 Plus system. The methods used were described in a previously published technical note.[5] A coated DNA capillary at 40 cm total length (30 cm effective length) was used for analysis using the dsDNA 1000 kit on the PA 800 Plus system.

Results and discussion

Comparison of plasmid purity analysis using the dsDNA 1000 kit and the DNA 20 kb Plasmid and Linear kit: The dsDNA 1000 kit has been used for plasmid purity analysis on the PA 800 Plus system for over 10 years and is a gold standard analytical method. However, with this method, the gel must be reconstituted and the gel dilution factor must be optimized based on the size of the plasmid to analyze. Additionally, the assembly of the cartridge with the coated DNA capillary must be done manually, which can be time-consuming and prone to user errors. The DNA 20 kb Plasmid and Linear kit, on the other hand, provides a ready-to-use, pre-optimized gel that works for plasmids across a wide size range. This kit also includes pre-built capillary cartridges, which significantly enhances efficiency, reduces variation and minimizes manual preparation. The streamlined process of the new kit results in a notable reduction in method time, allowing for faster and more reliable plasmid analysis.

This study compared the purity analysis of 5 different plasmids in a size range of 2.7–18.9 kb using the DNA 20 kb Plasmid and Linear kit and the dsDNA 1000 kit on the same PA 800 Plus system. Figure 3A shows results obtained using the DNA 20 kb Plasmid and Linear kit. Figure 3B shows the separation of topological isoforms of the identical 5 plasmids using the dsDNA 1000 kit on the same system. The signal observed in Figure 3B differs from that in Figure 3A, which can be attributed to differences in sample concentration and dye concentration in the gel between the 2 kit methods. Additionally, the DNA 20 kb Plasmid and Linear kit employs a different capillary with a distinct internal diameter at the detection window compared to the dsDNA 1000 kit. These variations in experimental conditions contribute to the differences in signal intensity shown in Figure 3A and Figure 3B.

Despite these differences, the overall profiles and purity assessments remain comparable, demonstrating the robustness and reliability of both methods. Table 1 summarizes the average plasmid purity in the percentage of SC (SC%) from 3 replicate analyses of each of the 5 plasmids using the dsDNA 1000 kit and the DNA 20 kb Plasmid and Linear kit on the same PA 800 Plus system. Results in this table further demonstrate that comparable purity levels were obtained with both kits, indicating that each method effectively identifies and quantifies impurities in the plasmid samples. This ensures reliable and consistent results across different plasmid sizes.

The new DNA 20 kb Plasmid and Linear kit offers several advantages over the dsDNA 1000 kit. It features a ready-to-use, non-lyophilized gel and is compatible with pre-built BFS capillary cartridges, significantly simplifying the assay process and reducing setup time. In addition, the DNA 20 kb Plasmid and Linear kit achieves a faster separation time of approximately 10 minutes per sample compared to 25 minutes per sample with the dsDNA 1000 kit. This reduction in separation time enhances efficiency, making the DNA 20 kb Plasmid and Linear kit an ideal solution for high-quality plasmid purity analysis of larger sample batches. These benefits make it a superior choice for laboratories

seeking streamlined plasmid purity assessments without compromising data accuracy or reliability.

Overall, this work demonstrates the similarity and comparability of the profiles and purity assessments of plasmids using either the dsDNA 1000 kit or the DNA 20 kb Plasmid and Linear kit on the PA 800 Plus system. The remarkable consistency in plasmid isoform distributions ensures assay portability and ease of transfer. The DNA 20 kb Plasmid and Linear kit reduces lab work, making it an efficient option for plasmid analysis.

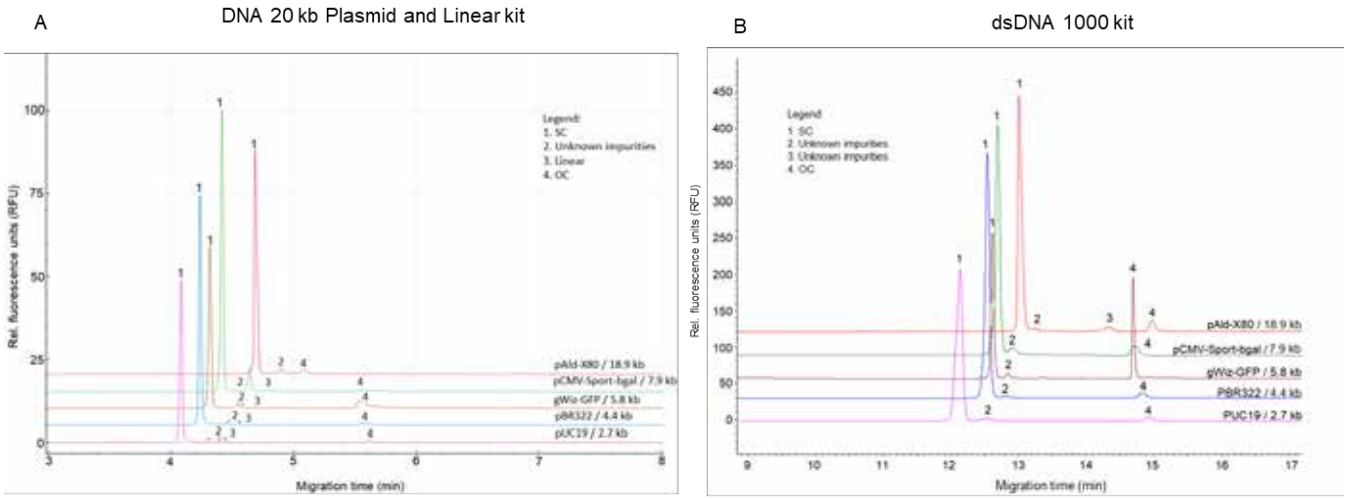


Figure 3. Separation of topological isoforms of 5 plasmids (2.7–18.9 kb) using the DNA 20 kb Plasmid and Linear kit (A) compared to the dsDNA 1000 kit (B) on the PA 800 Plus system. The comparison demonstrates that both the dsDNA 1000 kit and the DNA 20 kb Plasmid and Linear kit provide comparable plasmid purity and isoform distribution profiles, with the latter offering enhanced efficiency and reduced preparation time using the ready-to-use gel and pre-built capillary cartridge.

Table 1. Comparison of the average SC% in plasmid purity analysis using the DNA 20 kb Plasmid and Linear kit and the dsDNA 1000 kit.

Plasmid (size)	Average values of CPA% (N=3) analyzed on the PA 800 Plus system	
	DNA 20 kb Plasmid and Linear kit	dsDNA 1000 kit
pUC19 (2.7 kb)	96.25	95.57
pBR322 (4.4 kb)	98.36	95.83
gWiz-GFP (5.8 kb)	85.21	79.03
pCMV·SPORT-βgal (7.8 kb)	89.45	88.17
pALD-X80 (18.9 kb)	94.37	90.07

Table 2. Plasmid purity analysis using the DNA 20 kb Plasmid and Linear kit on the PA 800 Plus and the BioPhase 8800 systems.

Plasmid (size)	Average values PA 800 Plus system (N=3)		Average values BioPhase 8800 system (N=3)	
	SC		SC	
	MT (min)	CPA%	MT (min)	CPA%
pUC19 (2.7 kb)	4.25	96.25	4.09	94.88
pBR322 (4.4 kb)	4.42	98.36	4.23	96.63
gWiz-GFP (5.8 kb)	4.50	85.21	4.32	82.53
pCMV·SPORT-βgal (7.8 kb)	4.62	89.45	4.41	88.48
pALD-X80 (18.9 kb)	4.89	94.37	4.68	93.83

Method transfer between the single-capillary PA 800 Plus system and the multi-capillary BioPhase 8800 system using the DNA 20 kb Plasmid and Linear kit: Throughput needs often differ at different stages of drug development and manufacturing. Method transferability is crucial for laboratories needing to scale operations without compromising results. To assess the

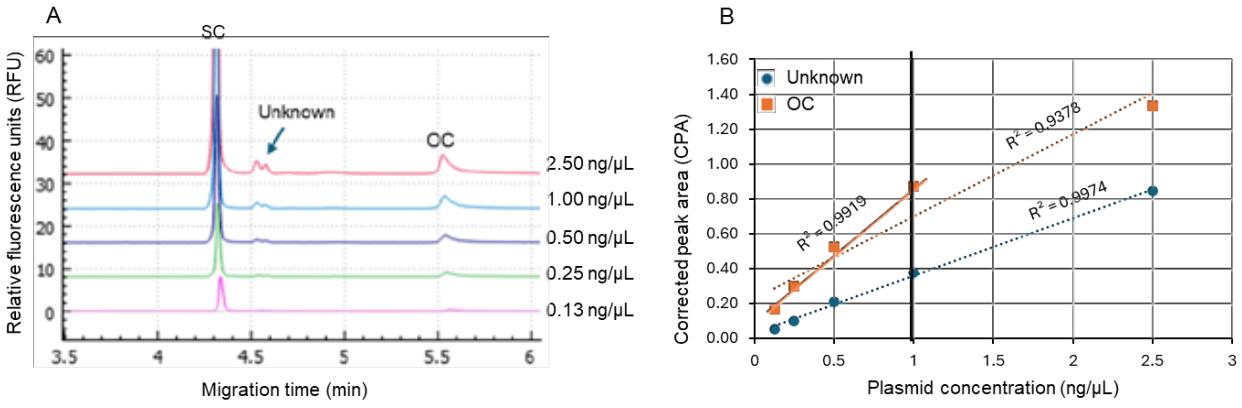


Figure 4. Determination of the optimal plasmid concentration for topological isoform analysis using the DNA 20 kb Plasmid and Linear kit on the BioPhase 8800 system. Panel A displays a zoomed-in view of the overlay of electropherograms of the gWiz-GFP (5.8 kb) plasmid serially diluted from 2.50 ng/µL to 0.13 ng/µL. Panel B shows the plots of CPAs of the OC isoform (orange dotted line) and the unknown impurity peak (blue dotted line) against the plasmid concentrations from 0.13 ng/µL to 2.50 ng/µL. The solid orange line illustrates the linear plot of CPAs of the OC isoform against the plasmid concentrations from 0.13 ng/µL to 1.00 ng/µL. The black vertical line indicates that 1.00 ng/µL was determined to be the optimal plasmid concentration.

transferability of plasmid purity analysis using the DNA 20 kb Plasmid and Linear kit between the 8-capillary BioPhase 8800 system and the single-capillary PA 800 Plus system, 5 plasmids were analyzed on both systems. The samples were analyzed in parallel on the BioPhase 8800 system (Figure 1A), while the same plasmids were analyzed sequentially in 5 different runs on the PA 800 Plus system (Figure 1B). Results in both panels show the baseline resolution of the main product, the SC isoform, from other isoforms and impurities for all 5 plasmids with sizes of 2.7–18.9 kb. In addition, the peak profiles for each plasmid were consistent between the 2 systems.

Table 2 summarizes the average MT and the plasmid purity in the corrected peak area percentages (CPA%) of the SC isoform from 3 replicate analyses. With both systems, the same trend of longer MTs for larger plasmid sizes was observed with similar time differences between different plasmids. The CPA% values of the SC isoform were highly similar between the 2 systems, demonstrating consistent purity assessments of the plasmid samples. This consistency is vital for reliable and reproducible results, allowing laboratories to switch between systems without compromising assay accuracy.

In summary, the DNA 20 kb Plasmid and Linear kit method can be seamlessly transferred between the PA 800 Plus system and the BioPhase 8800 system. This ensures consistent and reliable plasmid purity assessments between labs with varying throughput requirements. Consistent peak profiles, MTs and purity% across both systems demonstrate the robustness of the method and its ability to serve as a versatile and scalable solution for plasmid purity analysis in laboratories.

Determination of the optimal plasmid concentration for plasmid purity analysis using the DNA 20 kb Plasmid and Linear kit: Optimizing plasmid concentration is critical for accurate topological isoform analysis using the DNA 20 kb Plasmid and Linear kit. A titration experiment was performed using the multi-capillary BioPhase 8800 system to efficiently determine the optimal concentration. The system facilitates rapid screening, making it an ideal tool for initial optimization before transitioning to the PA 800 Plus system.

As an example, the gWiz-GFP (5.8 kb) plasmid was serially diluted from 2.50 ng/µL to 0.13 ng/µL with the DNA 20 kb Plasmid and Linear sample buffer, and the samples were analyzed simultaneously on the BioPhase 8800 system. Results were analyzed using the BioPhase software with the minimum S/N set at 10. As illustrated in Figure 4A, overlaid traces of the gWiz-GFP plasmid at different dilutions were obtained. The CPAs of unknown impurities and OC isoform were plotted against plasmid concentration (Figure 4B). The plot shows that the CPA of the unknown impurity has a good linear relationship with the plasmid concentration in the range of 0.13–2.50 ng/µL with an R2 value of 0.9974 (blue dotted line). In the same plasmid concentration range, the OC signal was linear between 0.13 ng/µL and 1 ng/µL but became saturated at 2.5 ng/µL. Therefore, the plasmid concentration range of 0.13–1.00 ng/µL was determined as a good concentration range for this plasmid

sample, with the 1.00 ng/µL concentration as the optimal concentration due to its strong signal. This rapid titration experiment, conducted within 10 minutes of separation time, efficiently determines the optimal plasmid concentration for topological isoform analysis, ensuring accurate and reliable results.

A similar titration experiment was performed to determine the optimal plasmid concentration for the remaining 4 plasmids (data not shown). The 1 ng/µL concentration provided a robust signal and accurate purity analysis for all 5 plasmids discussed in this technical note. This concentration was used to analyze the 5 plasmids using the DNA 20 kb Plasmid and Linear kit on the PA 800 Plus system and BioPhase 8800 system in this study.

Conclusions

- **Comparative analysis results:** the DNA 20 kb Plasmid and Linear kit obtains purity% results similar to those obtained with the dsDNA 1000 kit, validating the effectiveness and reliability of the new method
- **Consistent results across systems:** 5 different sizes of plasmids were analyzed on both single-capillary and multi-capillary systems, resulting in:
    - Similar peak profiles
    - Consistent migration times
    - Comparable purity%
- **Seamless method transfer:** facilitates efficient method transfer across different stages of the manufacturing process, accommodating varying throughput requirements
- **Versatile capabilities:** the ready-to-use DNA 20 kb Plasmid and Linear kit enables rapid and high-resolution analysis of plasmids ranging from 2.7 kb to 18.9 kb
- **Comprehensive solution:** the DNA 20 kb Plasmid and Linear kit offers a high-resolution, reliable, adaptable solution for plasmid analysis that is suitable for high-quality plasmid characterization and quality control

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Using the DNA 20 kb Plasmid and Linear kit on the PA 800 Plus system

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This technical note provides instructional details and a comprehensive assessment of assay repeatability using the DNA 20 kb Plasmid and Linear kit on the PA 800 Plus system from SCIEX. The DNA 20 kb Plasmid and Linear kit is engineered to analyze both plasmid and linear double-stranded DNA (dsDNA). As demonstrated in previous studies,[1–3] the single ready-to-use kit facilitates high-quality separation of plasmid topology, which is crucial for monitoring plasmid purity (supercoiled percentage), and delivers high-resolution separation of linear dsDNA, which enables size estimation and pattern identification (Figure 1).

For plasmid topology, tunable resolution can be achieved by adjusting the separation voltage. For linear dsDNA, ranging from 0.1 kb to 20 kb, tunable size resolution can be achieved by selecting different capillary lengths. The adjustable resolution and assay throughput for both plasmid and linear dsDNA, enabled by the flexibility of this workflow, helps ensure the precision and performance required for robust and reliable DNA analysis.

Key features

- **Streamlined operation with bare-fused silica (BFS) capillary:** Utilizing dynamic coating technology, the DNA 20 kb Plasmid and Linear kit simplifies plasmid and linear dsDNA analysis with a ready-to-use gel and <20 minutes of separation time, ensuring ease and efficiency
- **High-resolution separation of plasmid DNA topologies:** This technique distinguishes between different plasmid DNA topology—including supercoiled (SC), SC-multimer, linear and open circular (OC) species—with exceptional clarity
- **Tunable size resolution for wide-range linear dsDNA analysis:** The resolution for sizing linear dsDNA is 5%–10%, showing good repeatability across various capillary lengths

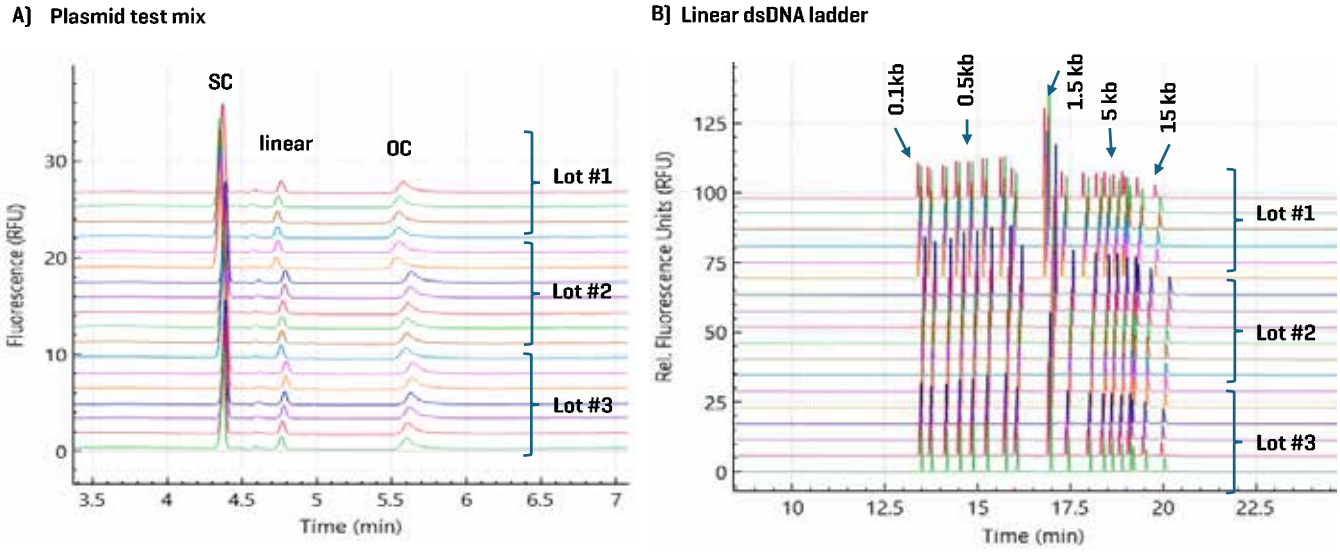


Figure 1. Demonstration of assay repeatability on a PA 800 Plus system with 3 different reagent lots. (A) Overlay of 18 electropherograms (6 injections x 3 reagent lots) for plasmid topological isoforms using the plasmid separation method with the DNA 20 kb Plasmid test mix in the kit as the sample. (B) Overlay of 18 electropherograms (6 injections x 3 reagent lots) for linear dsDNA size separation using the linear dsDNA high-resolution separation method with a linear dsDNA ladder (here, the 1 Kb Plus DNA Ladder from Thermo Fisher Scientific) as the sample.

Introduction

Plasmid DNA is a fundamental starting material in biopharmaceutical cell and gene therapy manufacturing processes, including the production of messenger RNA (mRNA) and the generation of viral vectors or gene-of-interest (GOI) plasmids. In addition, plasmid DNA is an indispensable molecular tool for supporting protein production.[4] Analyzing the presence of various topological forms—such as SC, linear, OC, and others—is critical for the quality control of plasmid DNA. Sizing and purity confirmation of linearized DNA intermediate before in-vitro transcription (IVT) mRNA production is also important in the production process.[5]

Confirmation of the gene mapping of the intended vector via endonuclease digestion at the pertinent restriction sites is recommended by the US Food and Drug Administration (FDA) as an important step during a plasmid identity check.[6–8] A confident comparison of the experimental restriction digestion pattern with the in-silico digestion pattern requires the assay to provide good size measurement and resolution over an extended size range. Both workflows can be achieved using the DNA 20 kb Plasmid and Linear kit on the PA 800 Plus system with good assay repeatability by following the detailed steps in this technical note. Additional information about the kit can be found in the application guide for the DNA 20 kb Plasmid and Linear kit (for the BioPhase 8800 system).[9] The information in this guide[9] also applies to using the kit on the PA 800 Plus system, except for instrument setup and operation, which will be detailed here.

Materials

Materials: All materials, unless listed otherwise, were from SCIEX. The DNA 20 kb Plasmid and Linear kit (P/N: 5311708) contains DNA 20 kb Plasmid and Linear gel, DNA 20 kb Plasmid and Linear sample buffer, DNA 20 kb Plasmid test mix, SYBR™ Gold Nucleic Acid gel stain,* DNA 20 kb Plasmid and Linear conditioning solution, acid wash/regenerating solution and CE Grade water. Universal vials (pack of 100, P/N: A62251), universal vial caps (blue, pack of 100, P/N: A62250) and PCR micro vials (200 µL, pack of 100, P/N: 144709) were used to contain reagents and/or samples on the PA 800 Plus system. For high-resolution linear dsDNA separation on a 50-cm capillary, a pre-cut BFS capillary (50 µm ID × 67 cm long, P/N: 338451) and a standard capillary cartridge assembly (P/N: 144738) were used to build the cartridge. For plasmid analysis and linear dsDNA on a 30-cm capillary, a pre-assembled capillary cartridge (P/N: A55625) was used to eliminate the need to cut the capillary and assemble the coolant tubes. Additional information can be found on the SCIEX website.[10]

Nuclease-free water (P/N: AM9932) and the 1 Kb Plus DNA Ladder (500 ng/µL, P/N: 10787018) were obtained from Thermo Fisher Scientific (Waltham, MA).

Storage of the DNA 20 kb Plasmid and Linear kit: Upon receipt, the acid wash/regenerating solution, CE Grade water and DNA 20 kb Plasmid and Linear sample buffer were stored at room temperature. The DNA 20 kb Plasmid and Linear conditioning solution and the DNA 20 kb Plasmid and Linear gel were refrigerated at 2°C to 8°C. The DNA 20 kb Plasmid test mix and the SYBR™ Gold Nucleic Acid gel stain* were kept at -35°C to -15°C.

Sample preparation

Sample preparation for the plasmid sample: The DNA 20 kb Plasmid test mix was thawed on ice for about 20 minutes, and 4 µL was mixed with 160 µL of DNA 20 kb Plasmid and Linear sample buffer. Then, 100–150 µL of the prepared sample was transferred into the universal vial with the insert.

For unknown plasmid DNA samples, a final concentration of 1 ng/µL is recommended for most samples. If needed, the optimal sample concentration can be confirmed using a concentration titration curve.[2] The diluted plasmid sample was then transferred at 100 µL per vial for analysis on the PA 800 Plus system.

Sample preparation for linear dsDNA sample: The 1 Kb Plus DNA Ladder was thawed on ice for about 20 minutes. A total of 2 µL of the linear DNA ladder was mixed with 320 µL of DNA 20 kb Plasmid and Linear sample buffer, and 100–150 µL of the prepared sample was then transferred into the PA 800 Plus sample vial with the insert.

For other linear DNA ladders or digested DNA mixture samples (not analyzed in this technical note), a final concentration of 0.5–1 ng/µL is recommended. For purified linear DNA samples, a concentration of around 0.1 ng/µL is recommended. All linear dsDNA samples should be diluted with DNA 20 kb Plasmid and Linear sample buffer. A total of 100 µL of the diluted sample

must be transferred into the universal vial with the insert for analysis on the PA 800 Plus system.

Instrument methods

General instrument operation details can be found in the methods development guide for controlling the PA 800 Plus system via 32 Karat software.[11] If using the PA 800 Plus Empower™ Driver as an instrument controller, refer to the user guide for system operation via Waters Empower™ software.[12] Detailed instrument methods using both 32 Karat software and the PA 800 Plus Empower™ Driver can be found at the end of this technical note in Figures 6–11, with Table 1 serving as reference indexes for these screenshots of instrument settings. With the PA 800 Plus Empower™ Driver, using the Next Inj. Delay feature enabled a 30-minute wait in the conditioning method without triggering an instrument error.

Results and discussion for plasmid analysis

The kit separates plasmids based on their topologies, resolving SC, linear, OC and multiple aggregates of the SC species in a single assay. The instrument methods for plasmid analysis separation can be found in Figures 6, 7 and 8. This allows for reporting on the purity of a given plasmid sample for process control. To demonstrate the repeatability of the kit for plasmid analysis, the plasmid test mix included in the kit was used as

Table 1. Index of screenshots showing the instrument settings for using both 32 Karat software and PA 800 Plus Empower™ Driver as control software for the PA 800 Plus system.

Capillary	Usage	Method	Figures
30 cm	Condition/coat new/used 30-cm capillary	Capillary condition	Figure 6A, 6B, 6C, 6D, 6E
30 cm	Clean 30-cm capillary if conditioning is not needed	Capillary rinse	Figure 7A, 7B, 7C, 7E
30 cm	Clean 30-cm capillary for storage	Capillary shutdown	Figure 7A, 7B, 7D, 7F
30 cm	Plasmid topology analysis	Plasmid separation	Figure 8A, 8B, 8C, 8E
30 cm	Plasmid topology of big (>3 kb) or complicated samples	Plasmid analysis high-resolution separation	Figure 8A, 8B, 8D, 8F
30 cm	Linear dsDNA with >20% size difference	Linear dsDNA high-speed separation	Figure 8A, 8B, 8D, 8F
50 cm	Condition/coat new/used 50-cm capillary	Capillary condition	Figure 9A, 9B, 9C, 9D, 9E
50 cm	Clean 50-cm capillary if conditioning not needed	Capillary rinse	Figure 10A, 10B, 10C, 10E
50 cm	Clean 50-cm capillary for storage	Capillary shutdown	Figure 10A, 10B, 10D, 10F
50 cm	High-resolution linear dsDNA separation	Linear dsDNA separation	Figure 11A, 11B, 11C, 11D

Table 2. Plasmid analysis repeatability using the plasmid separation method within a single test for intra- and inter-reagent lot variation. Intra-lot average and %RSD were calculated with results from N = 6 injections from each reagent lot. Inter-lot average and %RSD were calculated with results from N = 18 injections (6 injections × 3 reagent lots) on the same PA 800 Plus system with the same capillary.

	Average migration time, MT (min)			%RSD for MT			Average %SC	%RSD for %SC
Within lot	**SC**	**Linear**	**OC**	**SC**	**Linear**	**OC**	**SC**	**SC**
Lot #1	4.35	4.74	5.55	0.2	0.2	0.3	67.65	0.5
Lot #2	4.38	4.77	5.61	0.2	0.2	0.3	68.34	0.4
Lot #3	4.38	4.78	5.62	0.2	0.3	0.3	67.13	0.5
Inter-lot repeatability	***4.37***	***4.77***	***5.59***	***0.4***	***0.4***	***0.6***	***67.71***	***0.9***

a sample. The sample was separated with the plasmid separation method and analyzed using 3 different lots of reagents on a single system with the same capillary. For each reagent lot, 6 injections were performed. The overlay electropherograms of the 18 injections are shown in Figure 1A, and the quantitative analysis is summarized in Table 2. When using the same reagent lot, <1% RSD was achieved. Across 3 reagent lots, <1% RSD in peak migration time and SC% purity were also achieved, providing easy peak identification for automatic data processing. The purity reported for the sample was also consistent when different reagent lots were used.

Results and discussion for linear dsDNA analysis

The same DNA 20 kb Plasmid and Linear kit can be used for size-based linear dsDNA separation. The instrument methods can be found in Figures 9, 10 and 11. The linear dsDNA ladder—containing 18 markers of different sizes ranging from 0.1 kb to 15 kb—was used as the test sample. The resolving power at different size regions (defined by the marker size) with a 50-cm long capillary is shown in Table 3. The resolution was a theoretical extrapolation based on experimental results using the equation shown in Figure 2. The σ (deviation, minutes) values reflect the peak width for the markers, and the value used is the maximum value from 18 replicate injections. The k value used in both Δt and σ was the slope for the linear fitting between the log (bp, size) and the migration time of the existing marker. The results demonstrate 10% size resolving power with a resolution >1.5 in all size ranges and 5% size resolving power with a resolution >1.5 in the 0.3–7 kb size range.

$$USP\ resolution = 2 \times \frac{t_2 - t_1}{w_1 + w_2} = 2 \times \frac{t_2 - t_1}{4\sigma_1 + 4\sigma_2} = \frac{t_2 - t_1}{2\,(\sigma_1 + \sigma_2)} = \frac{\Delta t}{4 * \sigma}$$

$$\sigma = \frac{peak\ width\ at\ 50\%\ peak\ height}{k}$$

$$\Delta t = log\frac{1 + size\ difference/2}{1 - size\ difference/2} \times k$$

Figure 2. The formula used to calculate the resolution around the listed marker. The size difference can be 5% or 10%. The k in the formula is the slope for curve fitted between log (size) vs. peak migration time.

Table 3. Size resolution of linear dsDNA when using a 50-cm BFS capillary for analysis. Note that a 5% size resolution at 5,000 bp means the calculated resolution is 4,875–5,125 bp and a 10% size resolution at 5,000 bp means the calculated resolution is 4,750–5,250 bp.

Marker	Calculated resolution for a 5% size difference	Calculated resolution for 10% size difference
200	>1.0	>1.5
300	>1.5	>1.5
400	> 1.5	>1.5
500	>1.5	>1.5
650	>1.5	>1.5
850	>1.5	>1.5
1000	>1.5	>1.5
1500	>1.5	>1.5
2000	>1.5	>1.5
3000	>1.5	>1.5
4000	>1.5	>1.5
5000	>1.5	>1.5
6000	>1.5	>1.5
7000	>1.5	>1.5
8000	>1.0	>1.5
10000	>1.0	>1.5
15000	<1.0	>1.5

Table 4. Linear dsDNA repeatability within a single test for intra- and inter-capillary variation. The intra-lot average and %RSD were calculated with results from N = 6 injections with each reagent lot. The inter-lot average and %RSD were calculated with results from N = 18 injections (6 injections x 3 reagent lots).

	Average %CA		%RSD	
Intra-lot	LM	HM	LM	HM
Lot #1	52.03	47.98	0.9	1.0
Lot #2	52.42	47.59	0.5	0.6
Lot #3	51.85	48.15	0.3	0.4
Inter-lot repeatability	*52.10*	*47.90*	*0.8*	*0.8*

The assay repeatability for linear dsDNA analysis was also evaluated using 3 different reagent lots with 6 replicate injections from each lot on a single system with the same 50-cm long BFS capillary. The overlay electropherograms are shown in Figure 1B. High-quality linear dsDNA samples generally have a minimum purity of 60%.[13] In the sample, the % corrected area (%CA) for each marker is <10%, indicating that these values are closer to the number of impurity species in a sample than the main peak. To evaluate the reliability of the assay in reporting linear DNA purity, markers sized 0.1–1 kb are grouped and reported as lower markers (LM) and markers sized 1.5–15 kb are grouped and reported as higher markers (HM). The intra-lot and inter-lot average and %RSD for both groups are calculated and reported in Table 4. Four markers (at the lower and higher marker range) were selected to demonstrate the repeatability of linear DNA on the PA 800 Plus system. The results are shown in Table 5.

Table 5. Representative marker migration time (MT) repeatability within a single test for intra- and inter-capillary variation in linear DNA analysis with a 50-cm BFS capillary. The intra-lot average and %RSD were calculated with results from N = 6 injections on a 50-cm BFS capillary. The inter-lot average and %RSD were calculated with results from N = 18 injections (6 injections x 3 reagent lots).

	Average MT (min)				Max
Capillary	0.1 kb	1.5 kb	6 kb	15 kb	%RSD
Lot #1	13.44	16.86	18.73	19.84	0.3
Lot #2	13.54	17.05	18.97	20.11	0.2
Lot #3	13.49	16.97	18.88	20.02	0.2
Inter-lot repeatability	***13.49***	***16.96***	***18.86***	***19.99***	***0.6***

Results and discussion for method optimization

When analyzing plasmids, the previously discussed method (20 kV) provided baseline resolution for the 3 most known species (SC, linear, and OC). However, this method shows limitations when applied to more complex samples containing additional species, such as SC-multimer. To address this, a second method with reduced separation voltage (9 kV) was adopted, further improving the resolution for additional species in the sample (Figure 3). Although this second method was effective, the tradeoff was a longer runtime. Therefore, a fit-for-purpose choice between these methods depends on the complexity of the samples (lower voltage to resolve more species) and the time constraints of the project (higher voltage to reduce method cycle time). A comparison between the 2 methods for separating the plasmid test mix is shown in Figure 3, with the potential SC-dimer highlighted with a circle in each electropherogram. Another consideration when choosing the separation voltage for a plasmid sample is the size of the plasmid. When the plasmid is <3 kb, a higher separation voltage is recommended as it reduces the peak broadening due to diffusion (data not shown).

When analyzing linear dsDNA samples, if the impurity species and main peak have significant size differences, using a shorter capillary (30 cm) can be advantageous. A shorter capillary can reduce analysis time, enhancing throughput and efficiency.

Using a 30-cm capillary also allows plasmid analysis in the same testing sequence, providing the potential to estimate the size of the linear species in the sample. This approach can streamline the process, allowing for faster turnaround times. The reduced analysis time directly translates into quicker results, which is crucial in high-speed biopharmaceutical development where time is of the essence. A comparison between using the short (30-cm) and long (50-cm) capillary for linear dsDNA analysis is shown in Figure 4. The run time can be 10 minutes less when using the 30-cm capillary.

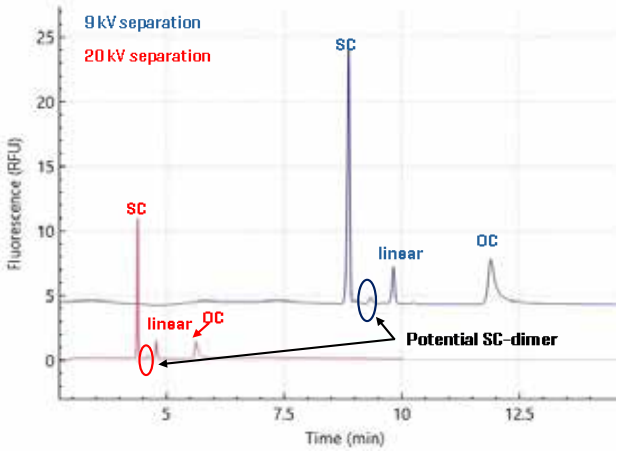


Figure 3. Impact of separation voltage on plasmid topology resolution with the same capillary. The same plasmid test mix was separated with a 9 kV plasmid high-resolution separation method (top trace) and a 20 kV plasmid separation method (bottom trace, default method).

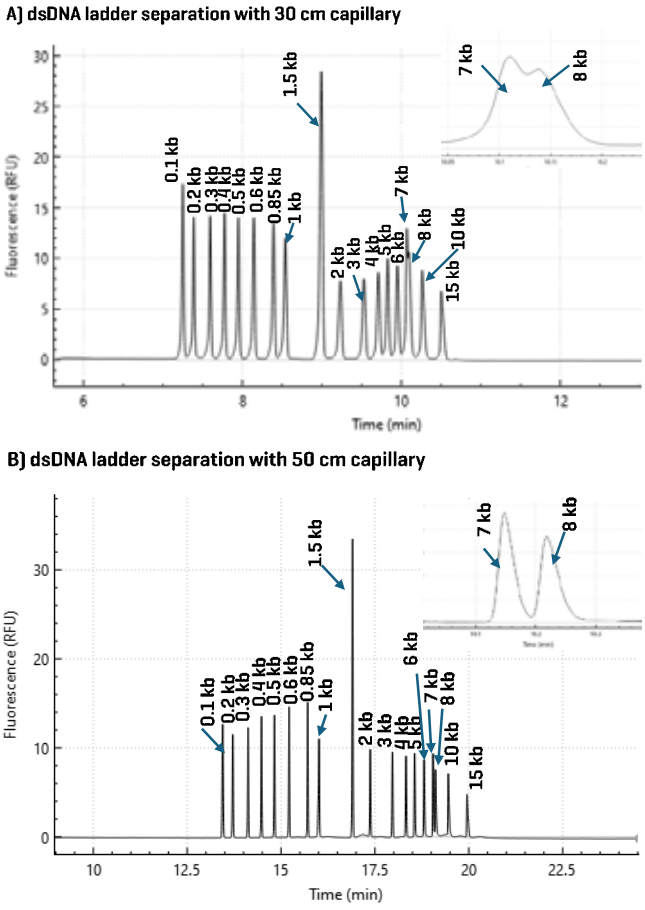


Figure 4. Impact of the separation capillary for linear dsDNA sizing resolution with the same field strength. [A] Linear dsDNA ladder separated on a 30-cm capillary with the resolution between the 7/8 kb marker highlighted in the insert. [B] Linear dsDNA ladder separated on a 50-cm capillary with the resolution between the 7/8 kb marker highlighted in the insert.

Running the samples with the PA 800 Plus Empower™ Driver

As mentioned previously, the PA 800 Plus system can be controlled using both 32 Karat software and the PA 800 Plus Empower™ Driver. The PA 800 Plus Empower™ Driver time programs for all the previously mentioned methods are listed in Table 1. An example electropherogram of the plasmid test mix and the linear DNA ladder is shown in Figure 5.

Conclusions

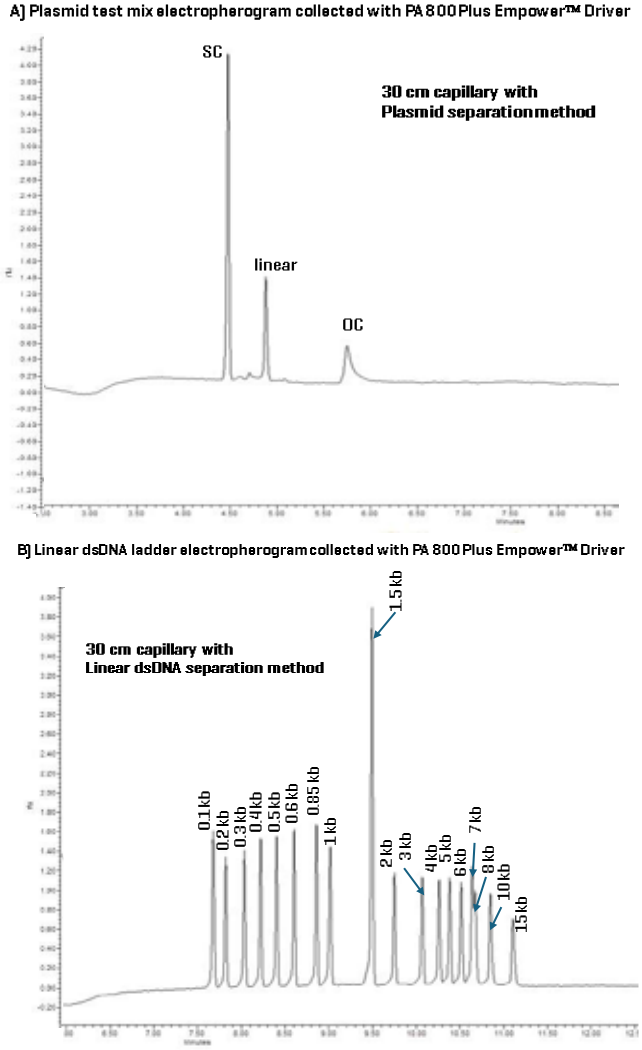


Figure 5. Example electropherograms with the PA 800 Plus Empower™ Driver. [A] Plasmid test mix separated on a 30-cm capillary with the plasmid separation method (20 kV separation voltage). [B] The linear dsDNA ladder separated on a 30-cm capillary with the linear dsDNA high-speed separation method (9 kV).

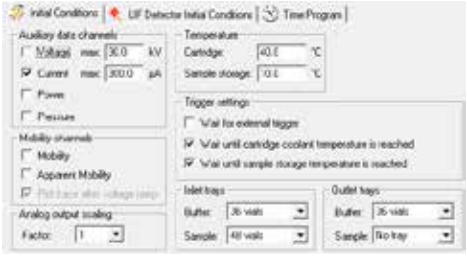
- **Easy-to-use kits and easy-to-set-up methods:** The DNA 20 kb Plasmid and Linear kit coupled with a BFS capillary provides an easy-to-use and flexible solution for both plasmid and linear dsDNA analysis. With customizable peak resolution and assay throughput, it allows for fit-for-purpose analyses with ease and precision.
- **Consistent results and good repeatability:** Both peak migration time and sample purity were shown to be <1% in %RSD for both plasmid and linear dsDNA analysis.
- **Flexibility in software control and data processing:** The new kit can be used on the single-capillary PA 800 Plus

system with both 32 Karat software and the PA 800 Empower™ Driver as control software. Data can be processed with the BioPhase 8800 system or the PA 800 Empower™ Driver.

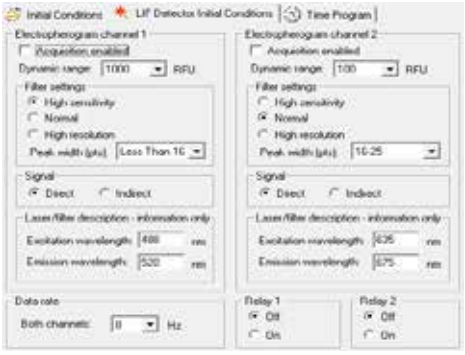
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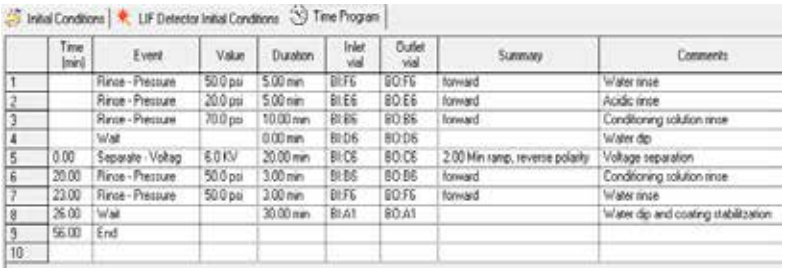
A) Initial Conditions for *capillary conditioning* method (30 cm)



B) LIF Detector Initial Conditions for *capillary conditioning* method (30 cm)

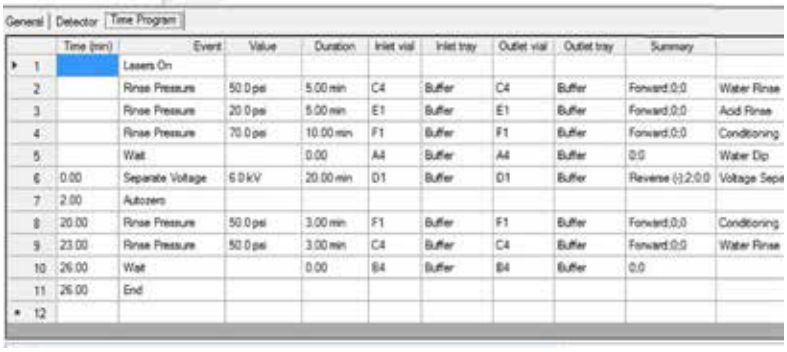


C) 32 Karat software Time Program for *capillary conditioning* method (30 cm)



	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1		Rinse - Pressure	50.0 psi	5.00 min	BI:F6	BO:F6	forward	Water rinse
2		Rinse - Pressure	20.0 psi	5.00 min	BI:E6	BO:E6	forward	Acidic rinse
3		Rinse - Pressure	70.0 psi	10.00 min	BI:B6	BO:B6	forward	Conditioning solution rinse
4		Wait		0.00 min	BI:D6	BO:D6		Water dip
5	0.00	Separate - Voltag	6.0 kV	20.00 min	BI:C6	BO:C6	2.00 Min ramp, reverse polarity	Voltage separation
6	20.00	Rinse - Pressure	50.0 psi	3.00 min	BI:B6	BO:B6	forward	Conditioning solution rinse
7	23.00	Rinse - Pressure	50.0 psi	3.00 min	BI:F6	BO:F6	forward	Water rinse
8	26.00	Wait		30.00 min	BI:A1	BO:A1		Water dip and coating stabilization
9	56.00	End						
10								

D) PA800 Plus Empower™ Driver time program for *capillary conditioning* method (30 cm)



	Time (min)	Event	Value	Duration	Inlet vial	Inlet tray	Outlet vial	Outlet tray	Summary	
1		Lasers On								
2		Rinse Pressure	50.0 psi	5.00 min	C4	Buffer	C4	Buffer	Forward:0:0	Water Rinse
3		Rinse Pressure	20.0 psi	5.00 min	E1	Buffer	E1	Buffer	Forward:0:0	Acid Rinse
4		Rinse Pressure	70.0 psi	10.00 min	F1	Buffer	F1	Buffer	Forward:0:0	Conditioning
5		Wait		0.00	A4	Buffer	A4	Buffer	0:0	Water Dip
6	0.00	Separate Voltage	6.0 kV	20.00 min	D1	Buffer	D1	Buffer	Reverse (-):2:0:0	Voltage Sepa
7	2.00	Autozero								
8	20.00	Rinse Pressure	50.0 psi	3.00 min	F1	Buffer	F1	Buffer	Forward:0:0	Conditioning
9	23.00	Rinse Pressure	50.0 psi	3.00 min	C4	Buffer	C4	Buffer	Forward:0:0	Water Rinse
10	26.00	Wait		0.00	B4	Buffer	B4	Buffer	0:0	
11	26.00	End								
12										

E) PA 800 Plus Empower™ Driver sequence set up with *Next Inj Delay (minutes)*

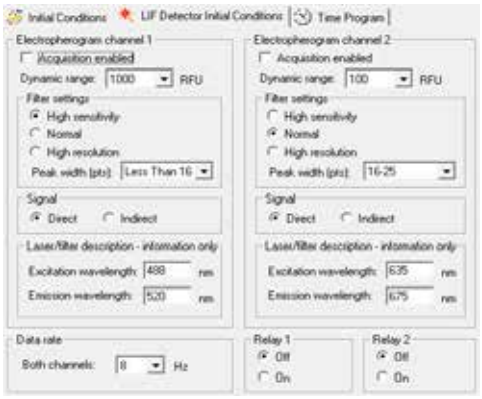
	Plate/Well	Inj Vol (uL)	# of Injs	Label	SampleName	Level	Sample Matrix	Function	Method Set / Report or Export Method	Label Reference	Processing	Run Time (Minutes)	Data Start (Minutes)	Next Inj. Delay (Minutes)
1	BI:A,1	10.0	1		Conditioning			Inject Samples	DNA BFS Conditioning		Normal	26.00	0.00	30.00
2	SI:A,1	10.0	1		Sample 1			Inject Samples	DNA BFS Separation		Normal	15.00	0.00	0.00
3	SI:A,2	10.0	1		Sample 2			Inject Samples	DNA BFS Separation		Normal	15.00	0.00	0.00
4	SI:A,3	10.0	1		Sample 3			Inject Samples	DNA BFS Separation		Normal	15.00	0.00	0.00
5	BI:E,1	10.0	1		Shutdown			Inject Samples	DNA BFS Shutdown		Normal	10.00	0.00	0.00

Figure 6. Instrument settings for the 30-cm capillary conditioning method. (A) Initial Conditions. (B) LIF Detector Initial Conditions. (C) 32 Karat software Time Program for the 30-cm capillary conditioning method. (D) PA 800 Plus Empower™ Driver Time Program for the 30-cm capillary conditioning method. (E) Waiting setup for the 30-cm capillary conditioning method using Next Inj. Delay (Minutes). **Note: When using the PA 800 Plus Empower™ Driver, the General tab should follow the settings shown in the Initial Conditions tab (Figure 6A), and the Detector tab should follow the settings shown in the LIF Detector Initial Conditions tab (Figure 6B).**

A) Initial Conditions for *capillary rinse and capillary shutdown* method (30 cm)



B) LIF Detector Initial Conditions for *capillary rinse and capillary shutdown* method (30 cm)



C) 32 Karat software time program for *capillary rinse* method (30 cm)

Initial Conditions | LIF Detector Initial Conditions | Time Program

	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	
1		Rinse - Pressure	50.0 psi	5.00 min	BI:E1	BO:E1	forward	Acidic Rinse
2		Rinse - Pressure	50.0 psi	5.00 min	BI:F1	BO:F1	forward	Water Rinse
3		Wait		0.00 min	BI:D1	BO:D1		water dip
4		Wait		0.00 min	BI:A1	BO:A1		Home
5		Laser - On						

D) 32 Karat software time program for *capillary shutdown* method (30 cm)

Initial Conditions | LIF Detector Initial Conditions | Time Program

	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comme
1		Rinse - Pressure	50.0 psi	5.00 min	BI:E1	BO:E1	forward	Acidic Rinse
2		Rinse - Pressure	50.0 psi	5.00 min	BI:F1	BO:F1	forward	Water Rinse
3		Wait		0.00 min	BI:D1	BO:D1		water dip
4		Wait		0.00 min	BI:A1	BO:A1		Home
5		Laser - Off						
6								

E) PA 800 Plus Empower™ Driver time program for *capillary rinse* method (30 cm)

General | Detector | Time Program

	Time (min)	Event	Value	Duration	Inlet vial	Inlet tray	Outlet vial	Outlet tray	Summary	Comments
1		Lasers On								
2	0.00	Separate Pressure	50.0 psi	5.00 min	E1	Buffer	E1	Buffer	Forward,0.0	Acid Rinse
3	5.00	Separate Pressure	50.0 psi	5.00 min	C4	Buffer	C4	Buffer	Forward,0.0	Water Rinse
4	10.00	Wait		0.00	A1	Buffer	A1	Buffer	0,0	Water Dip
5	10.00	End								
6										

F) PA 800 Plus Empower™ Driver time program for *capillary shutdown* method (30 cm)

General | Detector | Time Program

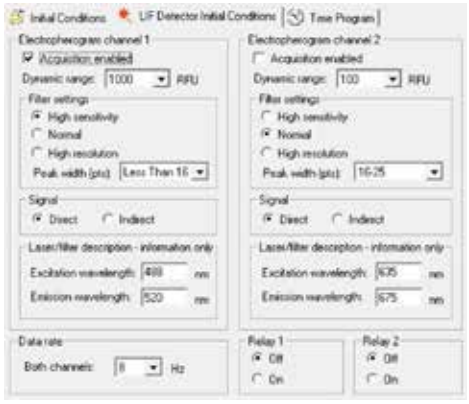
	Time (min)	Event	Value	Duration	Inlet vial	Inlet tray	Outlet vial	Outlet tray	Summary	Comments
1		Lasers Off								
2	0.00	Separate Pressure	50.0 psi	5.00 min	E1	Buffer	E1	Buffer	Forward,0.0	Acid Rinse
3	5.00	Separate Pressure	50.0 psi	5.00 min	C4	Buffer	C4	Buffer	Forward,0.0	Water Rinse
4	10.00	Wait		0.00	A1	Buffer	A1	Buffer	0,0	Water Dip
5	10.00	End								
6										

Figure 7. Instrument settings for the 30-cm capillary rinse and shutdown methods. [A] Initial Condition. [B] LIF Detector Initial Conditions. [C] 32 Karat software Time Program for the 30-cm capillary rinse method. [D] 32 Karat software Time Program for the 30-cm capillary shutdown method. [E] PA 800 Plus Empower™ Driver Time Program for the 30-cm capillary rinse method. [F] PA 800 Plus Empower™ Driver Time Program for the 30-cm capillary shutdown method. **Note: When using the PA 800 Plus Empower™ Driver, the General tab should follow the settings shown in the Initial Conditions tab (Figure 7A), and the Detector tab should follow the settings shown in the LIF Detector Initial Conditions tab (Figure 7B).**

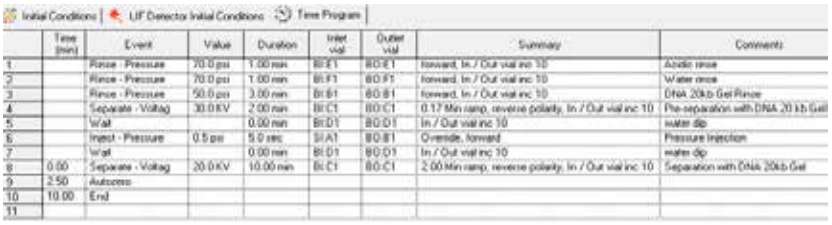
A) Initial Conditions for *plasmid separation* method (30 cm)



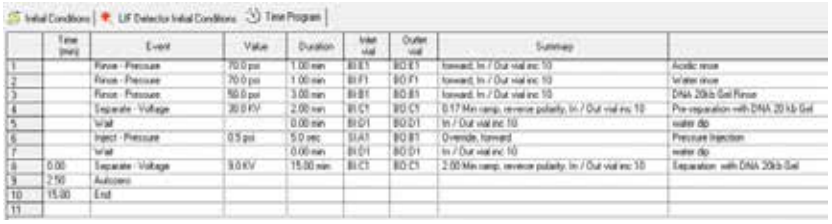
B) LIF Detector Initial Conditions for *plasmid separation* method (30 cm)



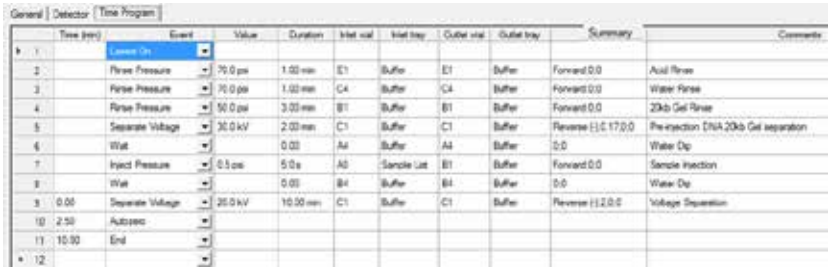
C) 32 Karat software time program for *plasmid separation* method (30 cm)



D) 32 Karat software time program for *plasmid high-resolution separation or Linear dsDNA high speed separation* method (30 cm)



E) PA 800 Plus Empower™ Driver time program for *plasmid separation* method (30 cm)



F) PA 800 Plus Empower™ Driver time program for *plasmid high-resolution separation or linear dsDNA high speed separation* method (30 cm)

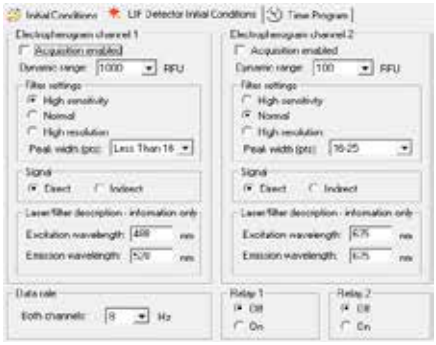


Figure 8. Instrument settings for the 30-cm capillary plasmid separation methods. (A) Initial Conditions. (B) LIF Detector Initial Conditions. (C) 32 Karat software Time Program for the 30-cm capillary plasmid separation method. (D) 32 Karat software Time Program for the 30-cm capillary plasmid high-resolution separation method or linear dsDNA high-speed separation method. (E) PA 800 Plus Empower™ Driver Time Program for the 30-cm capillary plasmid separation method or linear dsDNA high-speed separation method. (F) PA 800 Plus Empower™ Driver Time Program for the 30-cm capillary plasmid high-resolution separation method or linear dsDNA high-speed separation method. **Note: When using the PA 800 Plus Empower™ Driver, the General tab should follow the settings shown in the Initial Conditions tab (Figure 8A), and the Detector tab should follow the settings shown in the LIF Detector Initial Conditions (Figure 8B).**

A) Initial Conditions for capillary conditioning method (50 cm)



B) LIF Detector Initial Conditions for capillary conditioning method (50 cm)



C) 32 Karat software time program for capillary conditioning method (50 cm)

Initial Conditions | LIF Detector Initial Conditions | Time Program

	Time (min)	Event	Value	Duration	Inlet Vial	Outlet Vial	Summary	
1		Rinse - Pressure	50.0 psi	8.50 min	BI:F6	BO:F6	forward	Water rinse
2		Rinse - Pressure	20.0 psi	8.50 min	BI:E6	BO:E6	forward	Acid rinse
3		Rinse - Pressure	70.0 psi	17.00 min	BI:B6	BO:B6	forward	Conditioning Solution Rinse
4		Wait		0.00 min	BI:D6	BO:D6		water dip
5	0.00	Separate - Voltage	10.0 kV	20.00 min	BI:C6	BO:C6	2.00 kV/m ramp, reverse polarity	Conditioning solution separation /coating
6	20.00	Rinse - Pressure	50.0 psi	5.00 min	BI:B6	BO:B6	forward	conditioning solution Rinse
7	25.00	Rinse - Pressure	50.0 psi	5.00 min	BI:F6	BO:F6	forward	water rinse
8	30.00	Wait		30.00 min	BI:D6	BO:D6		
9	60.00	End						
10								

D] PA 800 Plus Empower™ Driver time program for capillary conditioning method (50 cm)

General | Detector | Time Program

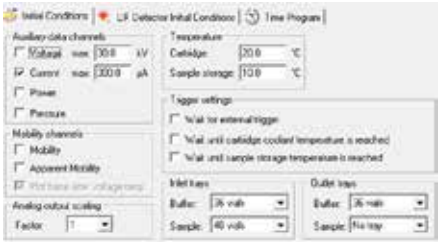
	Time (min)	Event	Value	Duration	Inlet Vial	Inlet Tray	Outlet Vial	Outlet Tray	Summary	Comments
1		Lasers On								
2		Rinse Pressure	50.0 psi	8.50 min	C4	Buffer	C4	Buffer	Forward:0:0	Water Rinse
3		Rinse Pressure	20.0 psi	8.50 min	E1	Buffer	E1	Buffer	Forward:0:0	Acid Rinse
4		Rinse Pressure	70.0 psi	17.00 min	F1	Buffer	F1	Buffer	Forward:0:0	Conditioning Solution Rinse
5		Wait		0.00	A4	Buffer	A4	Buffer	0:0	Water Dip
6	0.00	Separate Voltage	10.0 kV	20.00 min	D1	Buffer	D1	Buffer	Reverse (-):2:0:0	Voltage Separation
7	2.00	Autozero								
8	20.00	Rinse Pressure	50.0 psi	5.00 min	F1	Buffer	F1	Buffer	Forward:0:0	Conditioning Solution Rinse
9	25.00	Rinse Pressure	50.0 psi	5.00 min	C4	Buffer	C4	Buffer	Forward:0:0	Water Rinse
10	30.00	Wait		0.00	B4	Buffer	B4	Buffer	0:0	Water Dip
11	30.00	End								
12										

E) PA 800 Plus Empower™ Driver sequence set up with *Next Inj Delay (minutes)*

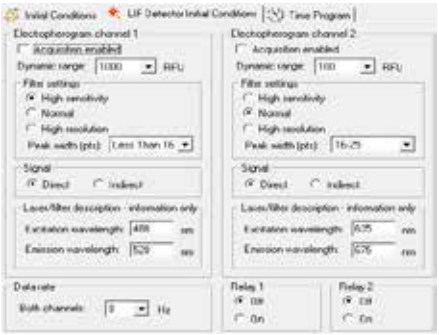
	Plate/Well	Inj Vol (uL)	# of Injs	Label	SampleName	Level	Sample Matrix	Function	Method Set / Report or Export Method	Label Reference	Processing	Run Time (Minutes)	Data Start (Minutes)	Next Inj. Delay (Minutes)
1	BI:A,1	10.0	1		Conditioning			Inject Samples	DNA BFS Conditioning		Normal	26.00	0.00	30.00
2	SI:A,1	10.0	1		Sample 1			Inject Samples	DNA BFS Separation		Normal	15.00	0.00	0.00
3	SI:A,2	10.0	1		Sample 2			Inject Samples	DNA BFS Separation		Normal	15.00	0.00	0.00
4	SI:A,3	10.0	1		Sample 3			Inject Samples	DNA BFS Separation		Normal	15.00	0.00	0.00
5	BI:E,1	10.0	1		Shutdown			Inject Samples	DNA BFS Shutdown		Normal	10.00	0.00	0.00

Figure 9. Instrument settings for the 50-cm capillary conditioning method. (A) Initial Conditions. (B) LIF Detector Initial Conditions. (C) 32 Karat software Time Program for the 50-cm capillary conditioning method. (D) PA 800 Plus Empower™ Driver Time Program for the 50-cm capillary conditioning method. (E) PA 800 Plus Empower™ Driver waiting setup for the 50-cm capillary conditioning method using Next Inj. Delay (Minutes). **Note: When using the PA 800 Plus Empower™ Driver, the General tab should follow the settings shown in the Initial Conditions tab (Figure 9A), and the Detector tab should follow the settings shown in the LIF Detector Initial Conditions tab (Figure 9B).**

A) Initial Conditions for *capillary* ***rinse*** *and capillary shutdown* method (50 cm)



B) LIF Detector Initial Conditions for capillary ***rinse*** *and capillary shutdown* method (50 cm)



C) 32 Karat software time program for *capillary rinse* method (50 cm)

Initial Conditions | LIF Detector Initial Conditions | Time Program

	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	
1		Rinse - Pressure	80.0 psi	5.00 min	BI:E1	BO:E1	forward	Acid Rinse
2		Rinse - Pressure	80.0 psi	5.00 min	BI:F1	BO:F1	forward	Water Rinse
3		Wait		0.00 min	BI:D1	BO:D1		water dip
4		Laser - On						
5								

D) 32 Karat software time program for *capillary shutdown* method (50 cm)

Initial Conditions | LIF Detector Initial Conditions | Time Program

	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	
1		Rinse - Pressure	80.0 psi	5.00 min	BI:E1	BO:E1	forward	Acid Rinse
2		Rinse - Pressure	80.0 psi	5.00 min	BI:F1	BO:F1	forward	Water Rinse
3		Wait		0.00 min	BI:D1	BO:D1		water dip
4		Wait		0.00 min	BI:A1	BO:A1		
5		Laser - Off						
6								

E) PA 800 Plus Empower™ Driver time program for *capillary rinse* method (50 cm)

General | Detector | Time Program

	Time (min)	Event	Value	Duration	Inlet vial	Inlet tray	Outlet vial	Outlet tray	Summary	Comments
1		Lasers On								
2	0.00	Separate Pressure	80.0 psi	5.00 min	E1	Buffer	E1	Buffer	Forward:0:0	Acid Rinse
3	5.00	Separate Pressure	80.0 psi	5.00 min	C4	Buffer	C4	Buffer	Forward:0:0	Water Rinse
4	10.00	Wait		0.00	A1	Buffer	A1	Buffer	0:0	Water Dip
5	10.00	End								
6										

F) PA 800 Plus Empower™ Driver time program for *capillary shutdown* method (50 cm)

General | Detector | Time Program

	Time (min)	Event	Value	Duration	Inlet vial	Inlet tray	Outlet vial	Outlet tray	Summary	Comments
1		Lasers Off								
2	0.00	Separate Pressure	80.0 psi	5.00 min	E1	Buffer	E1	Buffer	Forward:0:0	Acid Rinse
3	5.00	Separate Pressure	80.0 psi	5.00 min	C4	Buffer	C4	Buffer	Forward:0:0	Water Rinse
4	10.00	Wait		0.00	A1	Buffer	A1	Buffer	0:0	Water Dip
5	10.00	End								
6										

Figure 10. Instrument settings for the 50-cm capillary rinse and shutdown methods. (A) Initial Conditions. (B) LIF Detector Initial Conditions. (C) 32 Karat software Time Program for the 50-cm capillary rinse method. (D) 32 Karat software Time Program for the 50-cm capillary shutdown method. (E) PA 800 Plus Empower™ Driver Time Program for the 50-cm capillary rinse method. (F) PA 800 Plus Empower™ Driver Time Program for the 50-cm capillary shutdown method. **Note: When using the PA 800 Plus Empower™ Driver, the General tab should follow the settings shown in the Initial Conditions tab (Figure 10A), and the Detector tab should follow the settings shown in the LIF Detector Initial Conditions tab (Figure 10B).**

A) Initial Conditions *for linear dsDNA separation* method (50 cm)



B) LIF Detector Initial Conditions for *linear dsDNA separation* method (50 cm)
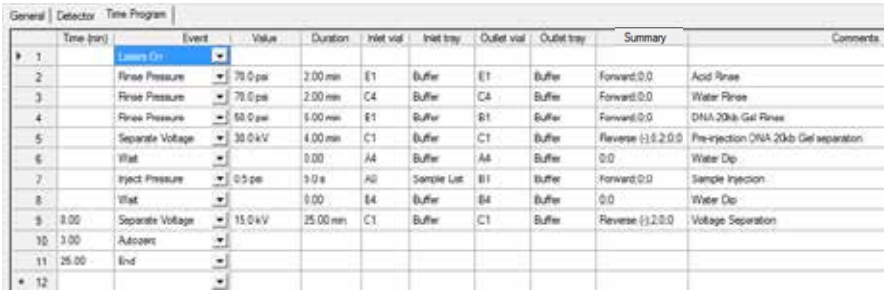


C) 32 Karat software time program *for linear dsDNA separation* method (50 cm)



	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	
1		Rinse - Pressure	70.0 psi	2.00 min	BI:E1	BO:E1	forward	Acid Rinse
2		Rinse - Pressure	70.0 psi	2.00 min	BI:F1	BO:F1	forward	Water Rinse
3		Rinse - Pressure	50.0 psi	5.00 min	BI:B1	BO:B1	forward	DNA 20 kb Gel Rinse
4		Separate - Voltage	30.0 kV	4.00 min	BI:C1	BO:C1	0.17 Min ramp, reverse polarity	Pre- separation with DNA 20kb Gel
5		Wait		0.00 min	BI:D1	BO:D1		Water dip
6		Inject - Pressure	0.5 psi	5.0 sec	SI:A1	BO:B1	Override, forward	Pressure injection
7		Wait		0.00 min	BI:D1	BO:D1		Water dip
8	0.00	Separate - Voltage	15.0 kV	25.00 min	BI:C1	BO:C1	2.00 Min ramp, reverse polarity	Separation with DNA 20kb Gel
9	3.00	Autozero						
10	25.00	End						
11								

D) PA 800 Plus Empower™ Driver time program *for linear dsDNA separation* method (50 cm)



	Time (min)	Event	Value	Duration	Inlet vial	Inlet tray	Outlet vial	Outlet tray	Summary	Comments
1		Lasers On								
2		Rinse Pressure	70.0 psi	2.00 min	E1	Buffer	E1	Buffer	Forward;0.0	Acid Rinse
3		Rinse Pressure	70.0 psi	2.00 min	C4	Buffer	C4	Buffer	Forward;0.0	Water Rinse
4		Rinse Pressure	50.0 psi	5.00 min	B1	Buffer	B1	Buffer	Forward;0.0	DNA 20kb Gel Rinse
5		Separate Voltage	30.0 kV	4.00 min	C1	Buffer	C1	Buffer	Reverse (-);0.2;0.0	Pre-injection DNA 20kb Gel separation
6		Wait		0.00	A4	Buffer	A4	Buffer	0.0	Water Dip
7		Inject Pressure	0.5 psi	5.0 s	A2	Sample List	B1	Buffer	Forward;0.0	Sample Injection
8		Wait		0.00	B4	Buffer	B4	Buffer	0.0	Water Dip
9	0.00	Separate Voltage	15.0 kV	25.00 min	C1	Buffer	C1	Buffer	Reverse (-);2.0;0	Voltage Separation
10	3.00	Autozero								
11	25.00	End								
12										

Figure 11. Instrument settings for the 50-cm capillary linear dsDNA analysis separation method. [A] Initial Conditions. [B] LIF Detector Initial Conditions. [C] 32 Karat software Time Program for the 50-cm capillary linear dsDNA analysis separation method. [D] PA 800 Plus Empower™ Driver Time Program for the 50-cm capillary linear dsDNA analysis separation method. **Note: When using the PA 800 Plus Empower™ Driver, the General tab should follow the settings shown in the Initial Conditions tab (Figure 11A), and the Detector tab should follow the settings shown in the LIF Detector Initial Conditions tab (Figure 11B).**

Using CE-SDS-LIF for profiling viral proteins of in-process control samples of adeno associated viral products

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[1]SK pharmteco Cell & Gene Europe – Yposkesi SAS, France; [2]SCIEX; Germany, [3]SCIEX; United Kingdom

This technical note highlights the capability of a capillary electrophoresis (CE)-based method for recombinant adeno-associated virus (rAAV) capsid protein characterization of in-process control (IPC) samples with a magnetic bead-based sample clean-up. The rAAVs are a common class of delivery vehicle for gene therapy due to their non-pathogenicity, low immunogenicity, and broad tropism. Each rAAV is composed of a protein shell called capsid and a single stranded DNA payload inside the capsid. The capsid is mainly made up of three viral proteins (VPs): VP1, VP2 and VP3. The capsid surface plays an important role in cell binding and transduction efficiency.[1] Good process controls are critical to ensure that rAAV-based therapies are safe, effective, and manufacturable at scale.[2] However, analyzing IPC samples face many challenges. IPC samples are often crude mixtures with low titer[3] and contain cell debris, plasmids, host nucleic acids and proteins that could interfere with analysis through matrix effects. IPC sample analysis also requires a quick turnaround time for fast, informed decision-making. This technical note describes the optimization of a high-resolution, high-sensitivity CE-SDS-LIF (laser-induced fluorescence detection) method for fast rAAV capsid protein analysis of IPC samples and viral titer estimation in final rAAV products on a multi-capillary CE platform.

Key features of CE-SDS-LIF for rAAV IPC samples

- Showcases the capability of CE-SDS-LIF, together with a magnetic bead clean-up, to assess the quality of AAV capsid proteins in IPC samples in 2 hours, enabling fast decision making during the rAAV manufacturing process.
- Demonstrates the capability of CE-SDS-LIF in viral titer estimation of final rAAV products.
- Highlights how the BioPhase 8800 system can be used to speed up method development for different AAV serotypes.
- Demonstrates the application of CE-SDS-LIF to various AAV serotype samples including AAV2, AAV5, AAV8 and AAV9.

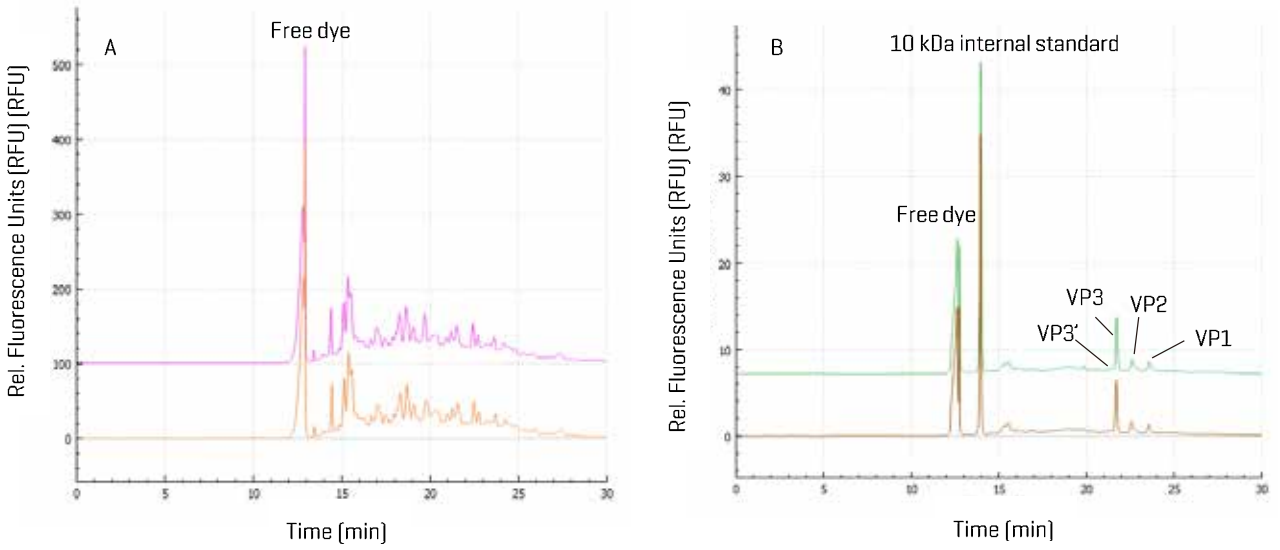


Figure 1. A comparison of the CE-SDS-LIF analysis of AAV8 capsid proteins in an IPC sample without sample cleanup (panel A) or with sample cleanup (panel B). In panel A: Pink trace: sample 1, preparation replicate 1; orange trace: sample 1, preparation replicate 2; In panel B: green trace: sample 1, preparation replicate 1; brown trace: sample 1, preparation replicate 2. VP3': a VP3 variant.

Introduction

The rAAV is one of the most widely used gene delivery vehicles for gene therapy. The AAV capsid is composed of 3 main proteins, which are approximately 87 kDa (VP1), 73 kDa (VP2), and 61 kDa (VP3) in size.[4] Good process controls in the manufacturing of rAAVs are critical due to the complex nature of gene therapy products and the stringent requirements for safety, efficacy, and consistency.[1] Monitoring the manufacturing process with quality assessment of IPC samples is crucial for maintaining process control and providing insights for process improvements.[2] However, the viral titer of IPC samples could be at 1 x 10^{10} VP (viral particles)/ML or even lower,[3] which is below the detection limit of UV absorbance assays and thus requires LIF detection. Over recent years, CE-SDS with LIF detection has been used to profile proteins by size in low-concentration protein samples.[5] Protein labelling using Chromeo P503 has enabled the viral protein ratio to be measured in low-titer AAV products in quality control (QC) samples, but has not been applied to in-process samples.[6]

In this study, a published CE-SDS-LIF method for rAAV capsid analysis[6] was implemented in a new lab after the sample preparation conditions for new rAAV serotypes were quickly optimized using the multi-capillary BioPhase 8800 system. The optimized method was used for viral titer estimation of the final rAAV products. Furthermore, the capability of the CE-SDS-LIF method, together with the use of magnetic bead cleanup, in analyzing the IPC samples at various production stages was demonstrated and provided insights for a better understanding of the rAAV manufacturing process.

Methods

Chemicals: The BioPhase CE-SDS Protein Analysis kit (P/N: C30085) with the CE-SDS Separation Buffer and SDS-MW Sample Buffer and the 10 kDa Internal Standard (P/N: A26487) were from SCIEX (Marlborough, MA). The Chromeo P503 dye (P/N: 30693-1MG) and dimethyl sulfoxide (DMSO) (P/N: 472301-100ML) were from Millipore Sigma (Burlington, MA); the Dynabeads CaptureSelect AAVX Magnetic Beads for cleanup (P/N: 2853522001) and dithiothreitol (DTT) (P/N: P2325) were from Thermo Fisher Scientific (St. Louis, MO).

Sample preparation: 5 µL of AAV sample, diluted to around 1 x 10^{13} GC/mL in nuclease-free water was mixed with 5 µL of SDS-MW Sample Buffer and 4 µL of 1M DTT in water. Then, 2 µL of 10 kDa Internal Standard pre-diluted 1:500 was added, and the sample was reduced. The labelling was done by the addition of 1 µL of Chromeo p503 dye working solution (prepared by a 1 to 100 dilution of the 1 mg/mL stock solution in DMSO) and incubated at 90°C for 10 minutes (min). Then, water was added to reach a final volume of 100 µL and incubated at 90°C for another 10 min. 100 µL of the diluted, prepared sample solution was transferred into a well of the sample plate for analysis on the BioPhase 8800 system. Samples with AAV2, AAV5, AAV8, and AAV9 serotypes were prepared following the same sample preparation procedure. When magnetic beads were utilized for sample clean-up, the procedure from the supplier of the beads was followed.

CE-SDS separation method: Separations were performed using the BioPhase 8800 system and a BioPhase BFS Capillary Cartridge – 8 x 30 cm (P/N: 5080121). Each 50 µm I.D. bare fused silica (BFS) capillary had a 20 cm effective length.

Capillary conditioning: 0.1M NaOH rinse for 2 minutes at 70 psi, 0.1M NaOH rinse for 8 minutes at 20 psi, 0.1M HCl rinse for 5 minutes at 20 psi, CE Grade Water rinse for 2 minutes at 20 psi, and CE-SDS Separation Buffer rinse for 10 minutes at 80 psi before each run. The applied electric field strength was 500 V/cm for all capillary electrophoresis analyses in reversed polarity mode (anode at the detection side). The samples were electrokinetically injected at 5 kV for 60 seconds.

CE detection: Dye-labelled AAV proteins were detected using a LIF detector, which employed a solid-state laser with an excitation wavelength of 488 nm and a 600 nm bandpass emission filter.

CE data processing: The BioPhase software version 1.2.22 was used for data acquisition and processing.

Results and discussion

High-resolution, high-sensitivity capsid protein analysis of IPC samples by CE-SDS-LIF with a sample cleanup by magnetic beads: An AAV8 IPC sample was analyzed for capsid proteins on the multi-capillary BioPhase 8800 system by CE-SDS-LIF with p503 dye labelling. In Figure 1, panel A shows the results obtained when the IPC sample was not purified with the AAVX magnetic beads. The sample was analyzed in two replicates. In both traces, although many protein peaks were present, the AAV viral capsid protein peaks were not detected. Panel B in Figure 1 illustrates the results obtained with the same IPC sample after the sample was cleaned up using the AAVX magnetic beads. The three VP proteins: VP1, VP2, and VP3, were detected and baseline-resolved. Furthermore, a VP3 variant, VP3', was also detected and well separated from the VP3 peak. The difference between VP3 and VP3' was 8 amino acids or about 900 Daltons due to an alternative translation starting site for this serotype as described in published literature.[7] These results demonstrated the high resolving power of the CE-SDS-LIF workflow on the

BioPhase 8800 system and the capability of high-sensitivity analysis of capsid proteins in IPC samples when used together with a magnetic bead-based sample cleanup.

The detailed description of the optimization of the CE-SDS-LIF workflow for AAV capsid protein analysis is provided below.

Optimization of sample preparation conditions: In the first stage of this project, the dye labelling conditions were optimized. The BioPhase 8800 system's ability to inject eight samples simultaneously allowed five labelling temperatures and two incubation times to be tested simultaneously, making method optimization efficient (Figure 2). The signal level for VP proteins was below the detection limit at the labelling temperature of 60°C. At a 70°C labelling temperature, only the VP3 peak was detected. At 80°C, all 3 VP peaks were detected. There was better consistency found at 10 minutes of incubation time than at 5 minutes. The signal of all 3 VP peaks was significantly higher at 90°C and plateaued at 100°C. At 80°C, 90°C, and 100°C of labelling temperature, the signal level was slightly higher at 10 minutes of incubation time than at 5 minutes. Therefore, the optimal labelling temperature was determined to be 90°C, and the optimal incubation time was identified as 10 minutes. Excellent resolution was obtained between VP1, VP2, VP3, and VP3' peaks. The resolution between VP3 and VP3' peaks demonstrates the outstanding resolving power of the CE-SDS-LIF workflow on the BioPhase 8800 system. Due to the small size difference between VP3 and VP3', VP3' was not well separated from VP3 by SDS-PAGE with silver staining. It only became well identified when CE-SDS was used for AAV capsid protein analysis.[7] Furthermore, a VP3 fragment, a separation condition-related artifact during liquid chromatography (LC) analysis of capsid proteins, was not present in the analysis by CE-SDS.[7] Therefore, CE-SDS provides improved performance for AAV capsid analysis compared to SDS-PAGE and LC.

In addition to the labelling temperature and time, the volume of DTT solution required (1, 2, 4, or 6 µL), the amount of SDS-MW Sample Buffer used to denature the sample (12, 18, and 24 µL), the concentration of dye solution (0.2 mg/mL and 0.1 mg/mL) were also optimized using an AAV8 sample (data not shown).

The optimized conditions used for sample preparation included adding Chromeo p503 dye (1 µL at 0.1 mg/mL), 10 kDa internal standard (2 µL, pre-diluted 1:500), SDS-MW Sample Buffer (12 µL), and 1M DTT (4 µL) to the AAV sample (5 µL).

Estimation of viral particle concentration in AAV samples by CE-SDS-LIF: After the sample preparation conditions were optimized, the linearity of the detector response to the AAV titer was evaluated. AAV8 samples were serially diluted from 4.38×10^{13} to 4.27×10^{10} capsids/mL, reduced, and labelled under the optimized conditions. The detector response was evaluated using the ratio of the corrected peak areas of the VP3 and the 10 KDa internal standard, which helped to normalize the effect of labelling efficiency and injection volume during the analysis. Figure 3 shows that with a detector PMT gain setting of 10, an excellent linear response was observed with an R^2 value of 0.998 when the peak area ratio of VP3 to 10 kDa internal standard was plotted against the AAV8 titer. The AAV titer values

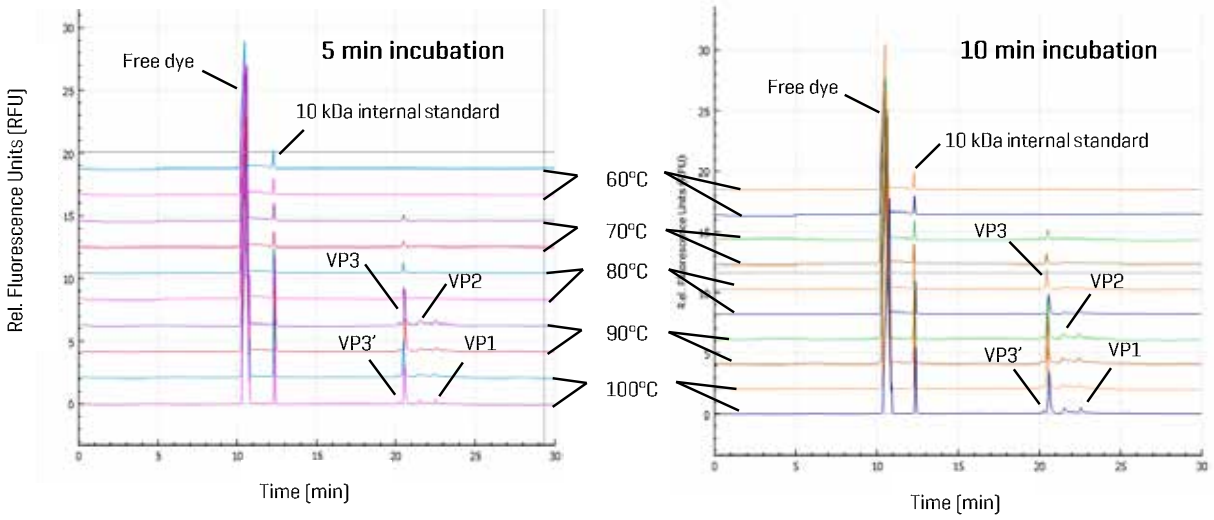


Figure 2. The effect of incubation time and the labelling temperature on the intensity of the viral proteins of the AAV8 samples. Each temperature was tested in duplicate.

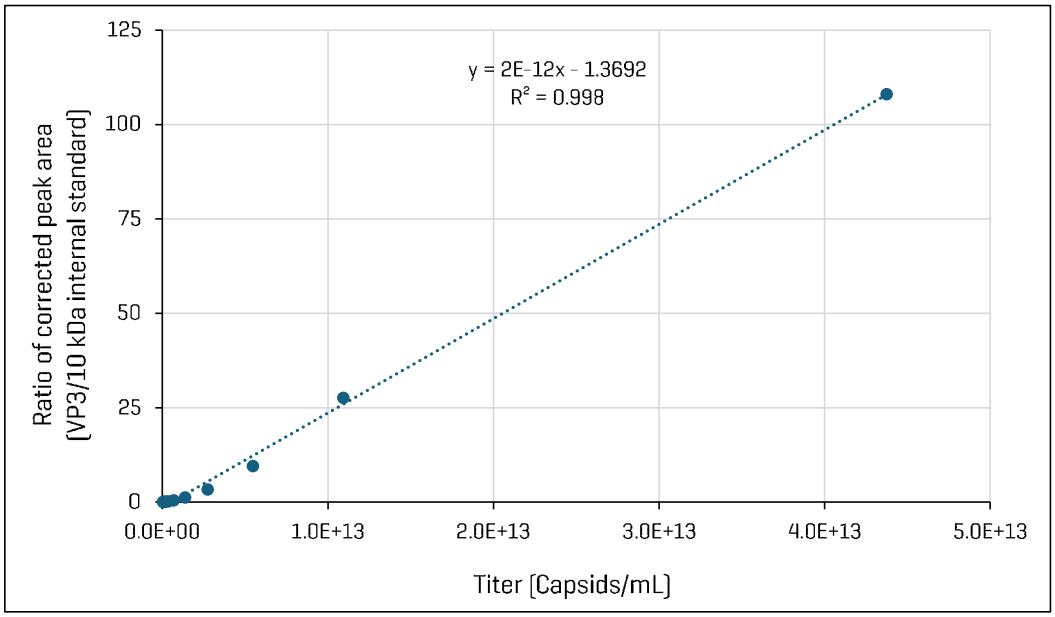


Figure 3. The standard curve was generated by plotting the VP3 /10 kDa corrected peak area (CPA) ratio against the AAV8 sample titer in the range of 4.27×10^{10} to 4.38×10^{13} capsids/mL. An outlier data point at 2.19×10^{13} capsids/mL was removed.

Table 1. Comparison of the CE-SDS-LIF viral titer method with an orthogonal method, which uses qPCR and AUC for commercial AAV8 samples. Recovery values between 80% and 120% were displayed in green font, while those below 80% or above 120% were shown in brown font.

	CE-SDS-LIF titer		qPCR & AUC titer			Recovery
	VP3 / 10 kDa CPA ratio	Titer (capsids/mL)	qPCR (VG/mL)	AUC (% Full)	Titer (capsids/mL)	CE-SDS-LIF titer / qPCR & AUC titer
Sample 1	1.5	1.5×10^{12}	4.4×10^{11}	42%	1.1×10^{12}	139%
	1.6	1.5×10^{12}				143%
Sample 2	166.1	8.4×10^{13}	4.5×10^{13}	53%	8.5×10^{13}	99%
	129.5	6.5×10^{13}				77%
Sample 3	42.7	2.2×10^{13}	1.9×10^{13}	59%	3.2×10^{13}	68%
	51.1	2.6×10^{13}				81%
Sample 4	196.5	9.9×10^{13}	4.6×10^{13}	52%	8.8×10^{13}	112%
	215.4	1.1×10^{14}				123%

of 4 AAV8 samples were determined using the standard curve displayed in Figure 3. Separately, the same 4 AAV8 samples were analyzed by an orthogonal method that measures the genome titer by real-time PCR (qPCR) and the percentage of full capsids by analytical ultracentrifugation (AUC). Since AAV samples contain both empty and full capsids, but only full capsids are detected by qPCR, when converting the genomic titer to capsid titer, it is necessary to take into account the empty particles in the AAV sample. Therefore, with this orthogonal method, the AAV capsid titers for these 4 samples were determined by dividing the genome titer by the percentage of full capsids from AUC. The AAV titers determined using the CE-SDS-LIF method were similar to those obtained with the orthogonal method (Table 1). The recovery was calculated by dividing the titer obtained by CE-

SDS-LIF by the titer obtained by the orthogonal method, the "qPCR & AUC titer". The recovery was in the range of 68% to 143%. This was in line with the consideration that the CE-SDS-LIF is a totally different methodology from the qPCR and AUC. In addition, the capsid titer determined using qPCR and AUC results may have a stack-up effect due to variations from both methods.

Analysis of IPC samples: AAV production is a long and complicated process. Monitoring the quality of IPC samples allows for real-time adjustments to the production process, improving yields and reducing waste. The CE-SDS-LIF approach with sample preparation conditions optimized for QC samples was then applied to IPC samples at different stages of AAV production. However, the number of AAV capsids in IPC samples is significantly lower than in the QC samples. Consequently, the AAV samples need to be enriched before capsid protein analysis. As shown in Figure 1A, when the unpurified, unlysed AAV8 harvest was analyzed by CE-SDS-LIF with Chromeo p503 dye, none of the VP proteins were detected due to high background noise from impurities. The viral proteins were hidden among the peaks of other proteins present in the unpurified samples. In contrast, as shown in Figure 1B, when the unlysed AAV8 harvest was cleaned up using magnetic beads, all the VP proteins were detected and well resolved consistently in two different experiments. Similar results were also obtained with lysed AAV8 harvest and lysed AAV9 harvest samples (data not shown). Since the unlysed and lysed harvests occur early in the AAV production process, the capability of CE-SDS-LIF, in combination with magnetic bead cleanup, enables the early detection of process changes that may affect the quality of the AAV. The total analysis time of IPC samples was 2 hours, from magnetic bead cleanup to labelling by p503 dye to finishing the separation on the BioPhase 8800 system. This is faster than the 4 to 6 hours analysis time by ELISA, enabling faster decision-making during process improvements of rAAV production.

Analysis of other AAV serotypes: To further investigate whether this approach could be applied to additional serotypes, unlysed harvest samples from serotypes AAV2 and AAV5 were also analyzed (Figure 4). In both samples, VP3, the dominant viral protein, could be detected, but the VP1 and VP2 peaks were not observed. This was likely due to the lower concentration of viral particles in these samples. Consistent results were obtained with duplicate sample preparations for AAV2 and AAV5 samples, supporting good assay reproducibility.

The ability to detect viral proteins in IPC samples using CE will enable scientists to better optimize process conditions to not only improve viral titer but also help optimize the capsid viral protein ratio to improve the quality of the final product. The viral protein ratio of IPC samples is currently unavailable when conventional techniques such as ELISA are used. Therefore, using the CE-SDS-LIF workflow on the BioPhase 8800 system for AAV capsid analysis is highly advantageous, as it offers the capability to analyze both QC and in-process samples effectively. Its versatility positions it as a promising analytical tool for AAV production.

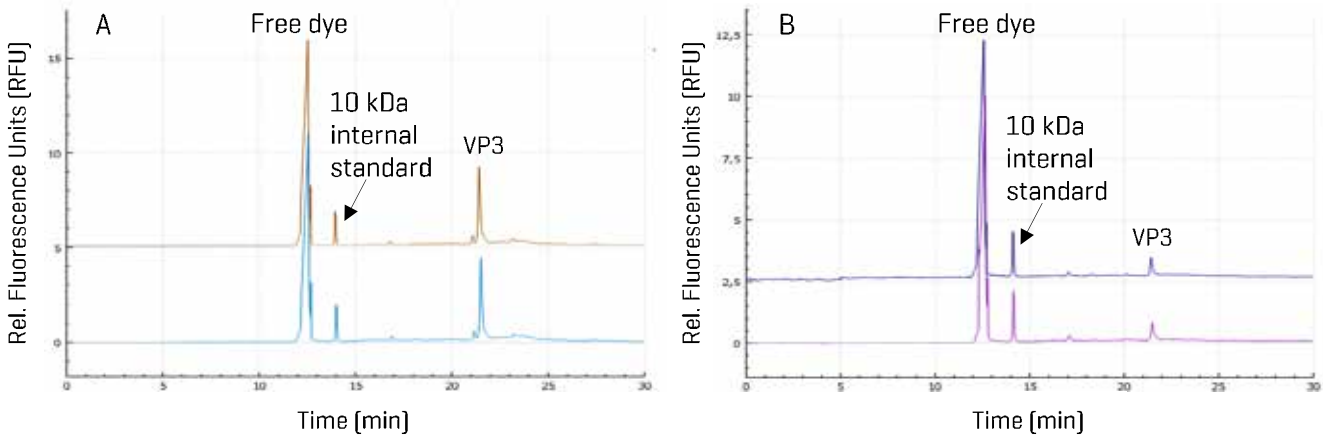


Figure 4. Analysis of unlysed harvest of (A) AAV2 sample (duplicate preparation) and (B) AAV5 sample (duplicate preparation).

Conclusions

- CE-SDS-LIF reference method was transferred to an analytical lab successfully. Once the sample preparation conditions were optimized, the method proved effective for all the different AAV serotypes tested.
- The method provided a linear detector response to viral proteins and produced results that closely match orthogonal techniques, making it suitable for quantifying viral particles in final products.
- When a magnetic bead sample cleanup was employed, this technique was able to detect viral proteins even from very early steps of the process, such as unlysed harvest. This enables the viral protein ratio to be measured earlier upstream, as well as the viral titer of process samples, enabling earlier detection of quality changes during production.

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Acceleration of method optimization for AAV capsid purity analysis using multi-capillary electrophoresis platform

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Introduction

The purity of AAV capsid proteins has important implications for efficacy of the viral vector.[1] A robust analytical method for assessment of the capsid purity for a range of serotypes that can provide reliable results in a timely fashion is desirable. Multiple factors such as temperature and buffer concentrations affect the capsid protein stability which impacts the assembly of the capsids. Optimization of these parameters are needed in order to perform accurate evaluations. However, the analysis time for optimization of each parameter can be time consuming. This technical note demonstrates how the SCIEX BioPhase 8800 system can be used to significantly reduce time for method optimization by a one multi-capillary electrophoresis system

CE-SDS (capillary electrophoresis sodium dodecyl sulfate) technology is popular for protein analysis in the biopharmaceutical industry because of its automated, quantitative protein separation capability. It is high resolution, reproducible and less laborious than traditional SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis).[2]

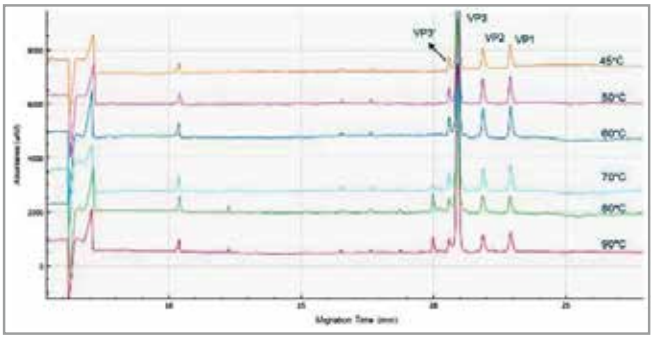


Figure 1. Electropherograms of the analysis at temperatures ranging from 45° C to 90° C to determine the optimized incubation temperature.



Figure 2. The BioPhase 8800 system equipped with LIF and UV detectors and consumable/reagent kits.

CE-SDS has been successfully used for separation of the various capsid proteins and the results are consistent across serotypes.[3,4] Method optimization for those studies was done using a single capillary format, which can limit throughput.

This technical note highlights the use of the BioPhase 8800 system for accelerating method development for AAV. The ability to perform 8 separations in parallel allows for faster processing of samples and overall increased throughput.

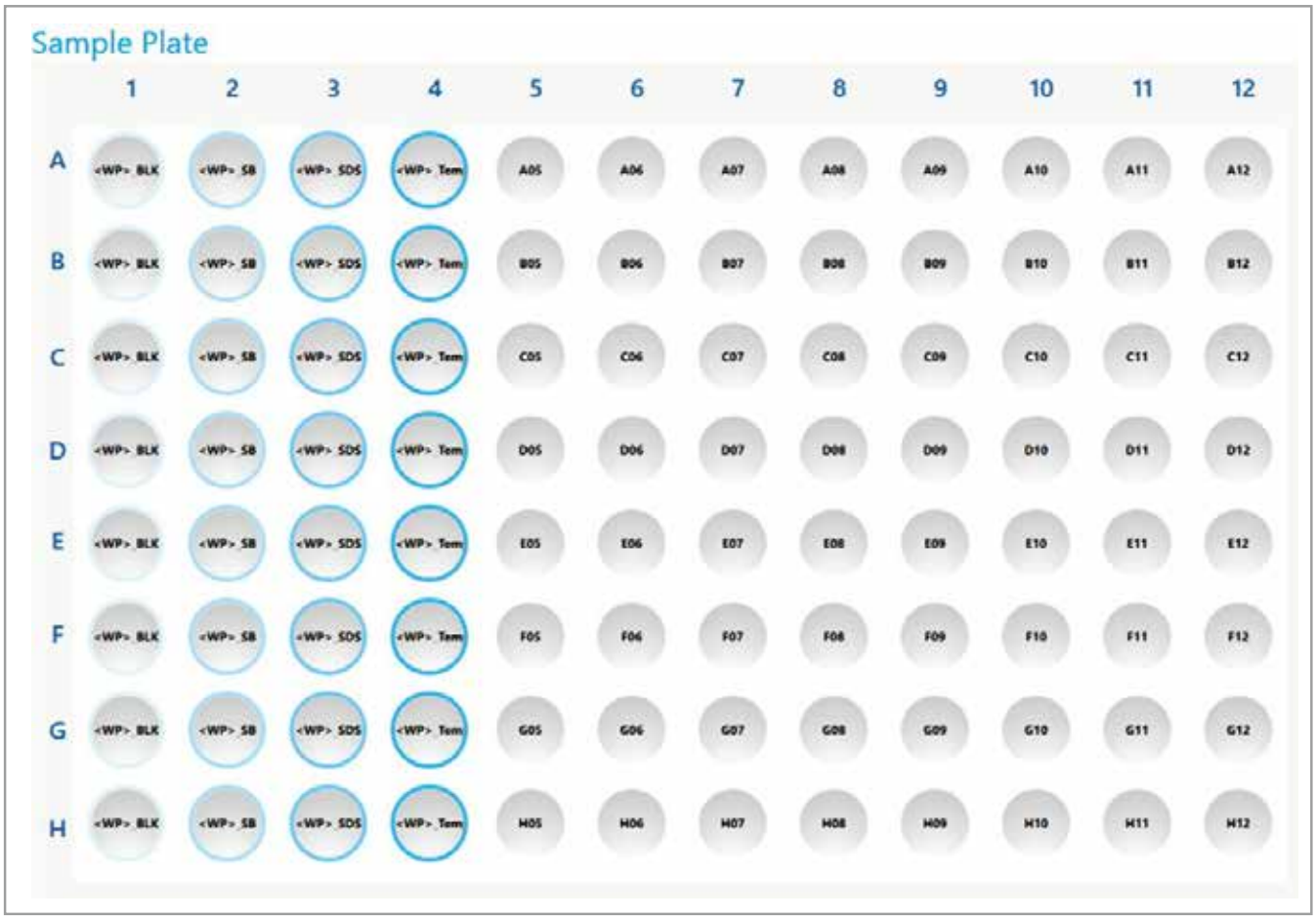
Key features

- Optimization of 3 separate parameters takes 4 hours to complete on the multi-capillary BioPhase 8800 system compared to 48 hours on the single capillary PA 800 Plus.
- Easy-to-use new processing software provides flexibility in viewing and reporting of data results.
- Optimized parameter settings obtained on the BioPhase 8800 system correlate well with values on the PA 800 Plus.
- Efficient labeling by Chromeo dye P503 and a simple sample preparation procedure completed in less than 1 hour.

Materials and methods

Materials

Chemicals: The SDS-MW Analysis Assay kit (Part # 390953, SCIEX, Brea, CA) with the SDS-MW gel buffer and sample buffer were from SCIEX (Framingham, MA, USA). The Chromeo P503 dye (PN 15106) was from ACTIVE MOTIF (Carlsbad, CA, USA). Sodium dodecyl sulfate (PN L4390-100G), and 2-mercaptoethanol (PN M3148-100ML), N-Ethylmaleimide (PN 04259), DL-Dithiothreitol (PN D9163), and all other chemicals were from Sigma Aldrich (St. Louis, MO, USA).



	1	2	3	4
A	Blank	1X SB	5% SDS	40° C
B	Blank	2X SB	4% SDS	45° C
C	Blank	4X SB	3% SDS	50° C
D	Blank	5X SB	2% SDS	60° C
E	Blank	8X SB	1.5% SDS	70° C
F	Blank	10X SB	1% SDS	80° C
G	Blank	20X SB	0.5% SDS	90° C
H	Blank	Blank	0.25% SDS	Blank

Figure 3. Configuration of the DOE samples in a 96 well plate. Blanks were placed in row 1, column a to H. Samples with various diluted sample buffer were placed in row 2, samples with different %SDS in row 3, and samples with different incubation temperatures in row 4. Analysis of 3 rows of samples were completed in 4 hours using the BioPhase 8000 system compared to 48 hours for single capillary platform.

p2

Samples: Packaged AAV8 of pAV-CMV-GFP with titer at 1.00 X 10^{13} GC/mL (titer as supplied by vendor) was purchased from Vigene Biosciences (Rockville, MD, U.S.A.). An AAV1-CMV-GFP (Cat # SL100803, Lot # AAV62058) sample and an AAV2-CMV-GFP (Cat # SL100812, Lot # AAV62099) were purchased from SignaGen Laboratories. All the samples with different sample preparations were loaded onto a 96 well plate for analysis as shown in figure 3.

Sample preparation for AAV CE-SDS-UV

The method is developed and optimized using AAV8 samples. For the optimization of sample preparation procedure, 5 µL of AAV8 sample solution was mixed with 5 µL of incubation buffer and 1.5 µL of 2-mercaptoethanol in a 0.65 mL micro-centrifuge tube at a constant temperature for 10min. Samples were then allowed to return to room temperature before DI water was added to the mixture. The diluted mixture was transferred into

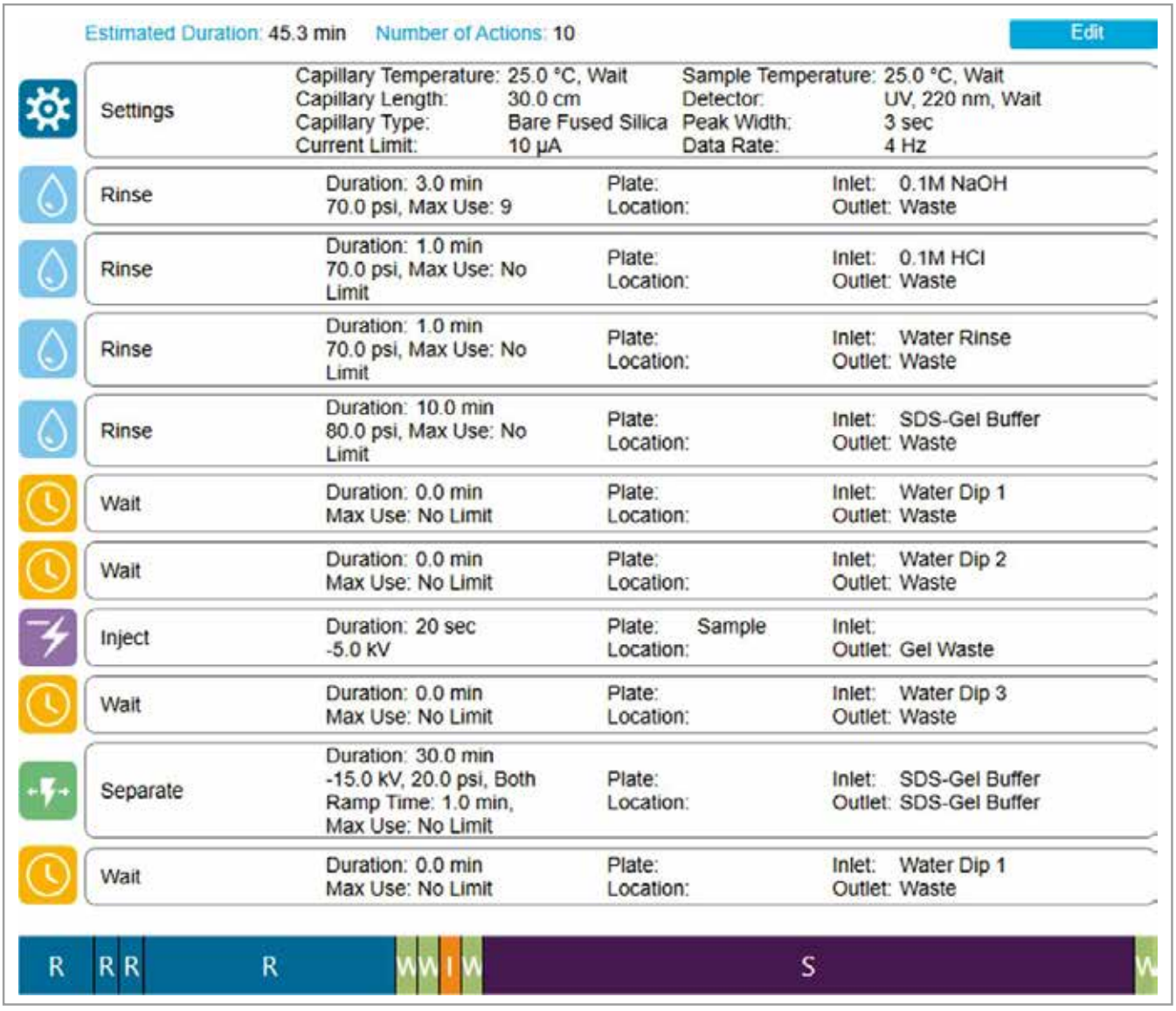


Figure 4. Separation method steps as shown using the BioPhase 8800 system.

the appropriate well of the injection sample inlet plate for analysis on the BioPhase 8800 system. Different incubation buffers and incubation temperatures were evaluated to achieve the optimal sensitivity and minimum sample preparation. 1X, 2X, 4X, 5X, 8X,10X, and 20X dilutions of sample buffer from the SDS-MW kit (100 mM Tris-HCl pH 9.0, 1% SDS) and SDS solutions at different concentrations from 0.01% to 1% were compared in this technical note. The incubation temperature ranging from 40° C to 90° C were also evaluated for the optimal sample preparation conditions.

Sample preparation for AAV CE-SDS-LIF BioPhase

20 µL of AAV sample, diluted as previously described, was mixed with 20 µL of Tris sample buffer, 4 µL of 1M DTT, and 1 µL of 2 mg/mL Chromeo P503 dye[5] and incubated at 60° C for 20 min. After cooling the samples down to room temperature, 154 of DI water was added to the mixture. AAV1, AAV2, and AAV8 were prepared following the sample preparation procedure. 100 µL of the diluted, prepared sample solution was transferred into the well of the injection sample inlet plate for analysis on the BioPhase 8800 system. The leftover 100 µL of the diluted prepared sample solution was transferred to the sample vial for analysis on the PA 800 Plus.

All single capillary electrophoresis analyses were accomplished using a PA 800 Plus Pharmaceutical Analysis system configured with LIF detector and solid-state laser with excitation wavelength that 488 nm and a 600 nm band pass emission filter from Edmund Optics (Barrington, NJ). CE-SDS separations were performed using the EZ-CE cartridge (Part # A55625) with a 20 cm effective length (30 cm total length). A 50 µm ID bare fused silica capillary was filled with the SDS-MW gel-buffer system. Capillary conditioning was: 0.1 M NaOH rinse for 3 minutes at 70 psi,

0.1 M HCl rinse for 1 minute at 70 psi, HPLC grade water rinse for 1 minute at 70 psi and SDS-MW gel buffer rinse for 10 minutes at 80 psi before each run. The applied electric field strength was 500 V/m for all capillary electrophoresis analyses in reversed polarity mode (anode at the detection side). The samples were electrokinetically injected at 5 kV for 20 seconds. The 32 Karat software 10.1 package was used for data acquisition and processing (Part # A79486AC, SCIEX).

The multiplexed separation utilized the BioPhase 8800 system. The gel-buffer system, capillary conditioning, injection, and separation conditions (Figure 6) were the same as those for the single capillary analyses. Separations were accomplished in the BioPhase BFS Capillary Cartridge – 8 x 30 cm. The BioPhase software package was used for data acquisition and processing.

Results and discussion

Sample buffer optimization

Different sample preparations and buffers were evaluated to achieve optimal sensitivity and resolution of the capsid proteins for the AAV 8 serotype on the BioPhase 8800 system using CE-SDS-UV. One of the parameters optimized was the sample buffer. Sample buffer from the SDS-MW kit (100mM Tris-HCL, pH9.0, 1% SDS) was used at 1x-20x dilution range as shown in Figure 5. Data from all7 dilution points were obtained from a 30 min single analysis on the multi-capillary. The analysis shows the best peak intensity was obtained using 1x sample buffer which correlates well with results obtained from previous studies on the PA 800 Plus.[3]

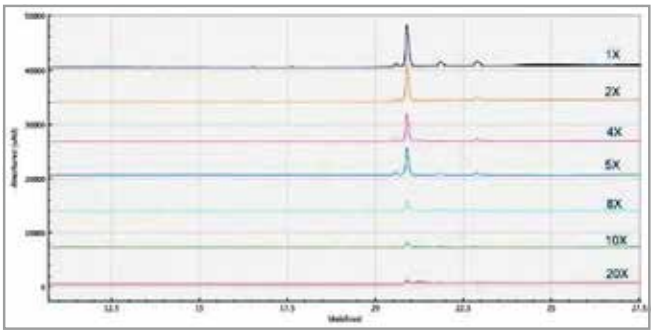


Figure 5. Sample buffer optimization with dilutions from 1x - 20x range using CE-SDS-UV on the BioPhase 8800 system. Best peak intensity of all 3 capsid proteins were obtained using 1x dilution.

Incubation temperature optimization

The optimization of the sample incubation temperature for the AAV8 serotype is shown in Figure 1. The peak intensity of all 3 capsids are optimized at 50° C. With increased incubation temperature greater than 50° C, smaller peaks with increased intensity is observed while the VP3 protein peak intensity decreases. These smaller peaks are likely degradation products of the VP3 proteins. This method is optimized for the AAV 8 sample, the optimal incubation temperature can differ for different serotypes.

% SDS optimization

Figure 6 shows the optimization of the % SDS concentration used for the optimal method. 5 L of 1% - 1.5% SDS provided the optimal peak shape and sensitivity. This provides sufficient amounts for protein binding and minimal residual salt concentration for best efficiency of electrokinetic sample injection.

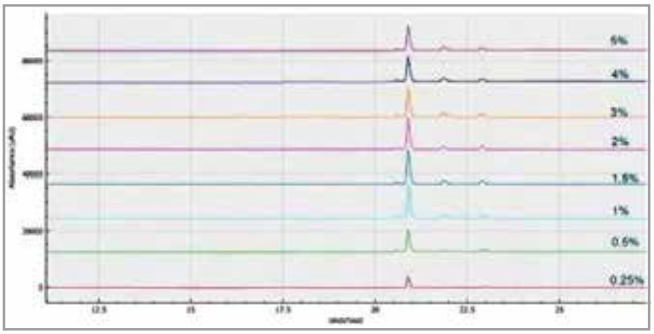


Figure 6. BioPhase 8800 electropherograms from CE-DS-UV sample analysis using various %SDS ranging from 0.25% to 5%. Optimized value obtained was 1 - 1.5% SDS.

Comparison between BioPhase 8800 and PA 800 Plus

To compare the data quality between the multi-capillary and the single capillary platforms, analysis of 3 different AAV serotypes, 1, 2, and 8, respectively was performed on both the PA800 Plus and BioPhase 8800 systems. The sensitivity and migration time of the 3 capsid proteins align well between the 2 systems (Figure 7). Additionally, as expected the migration times obtained for each serotype also correlated well between the 2 systems.

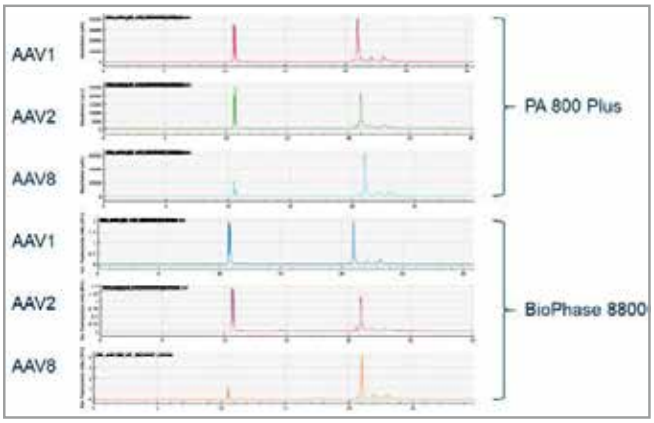


Figure 7. Comparison of CE-SDS-LIF analysis of AAV1, AAV2, and AAV8 on the single capillary PA 800 Plus and the multi-capillary BioPhase 8800 system using an LIF detector. Results obtained on both instruments correlates well. The analysis on the multi-capillary system was completed 3x faster than the single capillary system.

Conclusions

- Multiplexing capability of the BioPhase 8800 allows for 12x faster analysis compared to the single capillary systems for the method optimization of AAV purity analysis by CE-SDS.
- The sample preparation with the Chromeo P503 dye labeling for LIF analysis was simple and did not require any buffer exchange of sample cleanup. This helps in decreasing time for sample preparation and overall workflow completion time.
- Optimized conditions obtained on the multi-capillary system correlates well with the values obtained on the PA 800 Plus system. Thus, providing a seamless and easy method transfer from the single to the multi-capillary platform.

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Enhanced AAV capsid protein analysis using CE-SDS with native fluorescence detection on the BioPhase 8800 system

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As adeno-associated virus (AAV) gene therapies progress toward clinical and commercial viability, robust analytical workflows are essential for ensuring product quality and regulatory compliance.[1,2] Capillary electrophoresis (CE) platforms, such as the BioPhase 8800 system from SCIEX, offer integrated solutions for multi-stage critical quality attribute (CQA) analysis across the AAV lifecycle-from plasmid critical starting material to AAV product.[3] This technical note evaluates the performance of a newly integrated native fluorescence (NF) detection on the BioPhase 8800 system for AAV capsid protein analysis using a CE-SDS workflow. Compared to traditional ultraviolet (UV) detection, native fluorescence (NF) detection provides improved baseline stability and enables label-free detection of viral proteins (VPs), thus simplifying sample preparation and enhancing data processing. While its sensitivity does not exceed that of laser-induced fluorescence (LIF) detection, NF detection offers a balanced alternative with superior repeatability and linearity. Three AAV samples, including serotype 8, serotype 9, and a self-complementary (sc) variant, were analyzed to compare detection modes. Results demonstrate that NF detection enhances workflow efficiency and analytical robustness, supporting its utility in high-throughput AAV protein characterization.

Key features of CE-SDS with NF detection for AAV capsid protein analysis

- **Extension of AAV QC workflow capabilities:** NF extends the existing characterization capabilities for plasmid to AAV capsid protein and genome titer with an additional detection mode, offering flexibility for protein analyses in AAV drug development and QC.
- **Superior performance compared to UV detection:** NF offers ~3× higher sensitivity, tighter %RSDs for capsid protein purity (CPA%), and faster data processing due to a stable, flat baseline, especially beneficial for detecting low-abundance VP peaks and impurities.
- **Label-free workflow vs. LIF detection:** NF eliminates the need for fluorescent dye labeling and thus reduces variability from dye labeling.

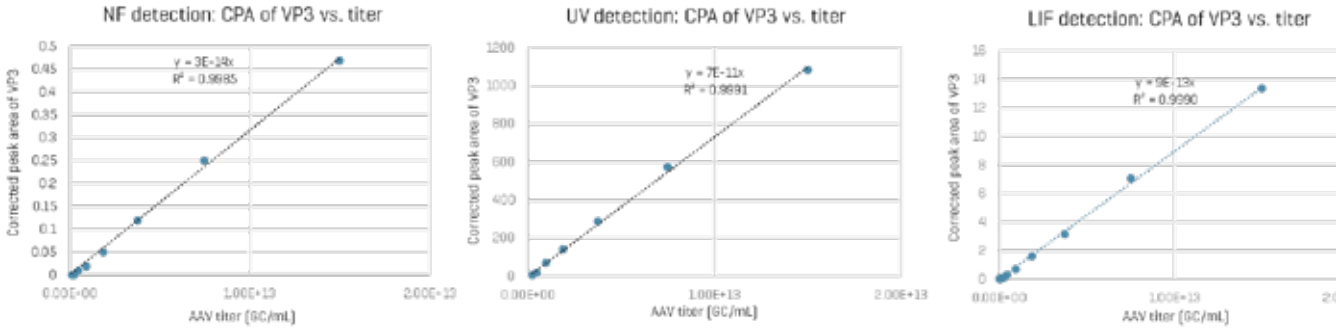


Figure 1: Capsid protein analysis of AAV8-CMV-GFP using CE-SDS on the BioPhase 8800 system with three detection modes. Linearity plots are shown for NF (left), UV (middle), and LIF (right). All three detection modes demonstrate excellent linearity across the tested titer range, with correlation coefficients (R^2) > 0.99. NF detection shows a threefold improvement in limit of detection (LOD) compared to UV, while LIF achieves the highest sensitivity with an LOD of ~1×10^9 GC/mL. NF provides a label-free alternative with improved baseline stability and precision, while LIF remains optimal for ultra-low titer samples.

Introduction

As gene therapy continues to advance, the need for scalable, reproducible, and regulatory-compliant analytical workflows for AAV drug production has become increasingly critical.[1, 2] CE platforms now offer integrated solutions for comprehensive CQA analysis across the AAV lifecycle, from plasmid DNA integrity to capsid protein characterization, within a single system.[3]

The SCIEX BioPhase 8800 system supports a suite of CE-based assays that streamline quality assessments at multiple stages of AAV development, which can reduce technical transfer challenges, accelerate scale-up, and ensure consistency in AAV drug quality. As illustrated in Figure 2, the system enables high-resolution purity analysis of plasmid DNA using the DNA 20 kb Plasmid and Linear kit,[4] RNA integrity evaluation via the RNA 9000 Purity & Integrity kit,[5] and capsid protein purity analysis and VP ratio determination through the CE-SDS Protein Analysis kit.[6] Additionally, a novel approach allows for quantitation of full, partial, and empty capsids by integrating genome titer and capsid titer data.[7]

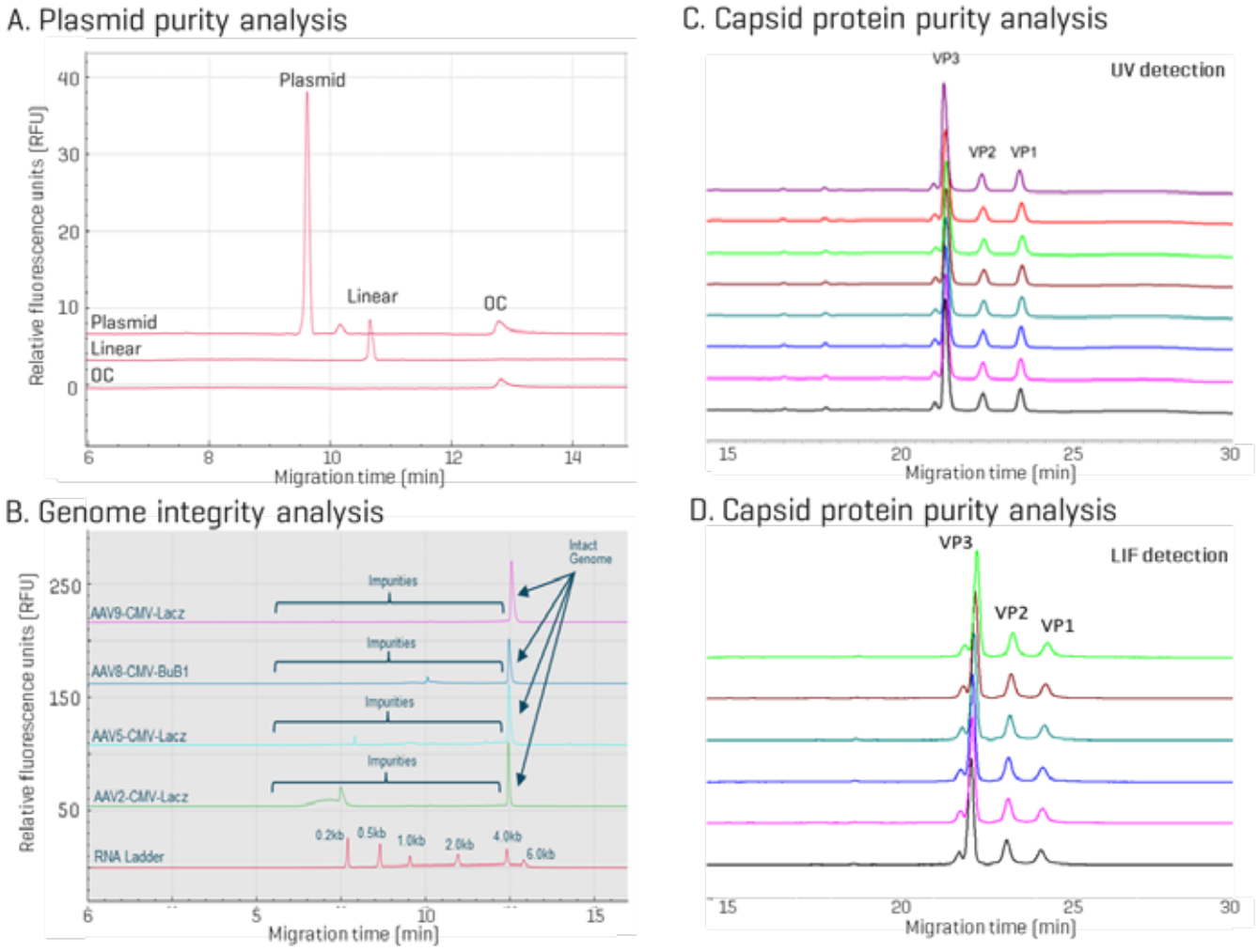


Figure 2. Streamlined CE-based analytical workflows for AAV drug production using the BioPhase 8800 system. Representative electropherograms illustrate the multi-stage quality control enabled by a single platform: (A) Separation of plasmid isoforms using the DNA 20 kb Plasmid and Linear kit; (B) RNA integrity assessment with the RNA 9000 Purity & Integrity kit; (C) and (D) Capsid protein analysis with the CE-SDS Protein Analysis kit using UV and LIF detection, respectively. Additionally, quantitation of full, partial, and empty capsids is derived from genome and capsid titer measurements. Together, these assays demonstrate a unified platform approach for comprehensive AAV CQA analysis.

Traditionally, CE-SDS with UV detection has been widely used for AAV capsid protein analysis. While effective for high-titer samples, UV-based workflows often suffer from baseline fluctuations due to non-analyte absorbance and gel buffer heterogeneity, complicating peak integration and slowing data analysis. Moreover, UV detection lacks the sensitivity required for low-titer or in-process samples. CE-SDS with LIF detection offers improved sensitivity but introduces complexity through dye labeling, which can affect assay accuracy due to variability in dye binding.

This technical note evaluates the performance of a newly integrated NFD on the BioPhase 8800 system. The NFD leverages the intrinsic fluorescence of aromatic amino acids, primarily tryptophan, to enable label-free detection of VPs with enhanced sensitivity and stable baselines compared to UV detection. This eliminates the need for dye labeling and simplifies sample preparation relative to LIF detection.

In this study, three AAV samples (serotype 8, serotype 9, and a self-complementary construct) were analyzed using CE-SDS coupled with UV, LIF, and NF detection on the BioPhase 8800 system with NF. Comparative results demonstrate that NFD provides improved sensitivity, more stable baselines, and simplified data processing relative to UV detection, while avoiding the labeling requirements of LIF. The integration of NFD expands the BioPhase 8800 system's analytical capabilities, offering a more efficient and precise workflow for AAV capsid protein analysis.

Methods

Materials: The BioPhase CE-SDS Protein Analysis kit (P/N: C30085), the BioPhase BFS capillary cartridge - 8 x 30 cm (P/N: 5080121), and the BioPhase sample and reagent plates (4,4,8) (P/N: 5080311) were from SCIEX (Marlborough, MA). (P/N: 5080311) were from SCIEX (Marlborough, MA). The β-mercaptoethanol (β-ME) (P/N: M3148-25ML) was from MilliporeSigma (St. Louis, MO). The Chromeo P503 dye (P/N: 15106) was from ACTIVE MOTIF (Carlsbad, CA). Dithiothreitol (DTT, P/N: V3151) was from Promega (Fitchburg, WI). 10x Phosphate Buffered Saline (PBS) (P/N: AM9624) was from Thermo Fisher Scientific (Waltham, MA).

Samples: The AAV8-CMV-GFP (P/N: SL100833), AAV9-CMV-LacZ (P/N: SL100868) and scAAV9-GFP (P/N: SL100840-sc) were from SignaGen Laboratories (Rockville, MD). And these three samples were used for the capsid purity analysis and viral protein ratio comparison of 3 different detection (UV, LIF, and NF) modes. The packaged AAV8 of pAV-CMV-GFP $1.50x10^{13}$ GC/mL) was from Charles River (Rockville, MD) and was used for comparison of the linearity, LOD, and LOQ determination of the 3 different detection modes (UV, LIF, and NF).

Sample preparation for AAV capsid protein analysis by CE-SDS-UV and CE-SDS-NF on the BioPhase 8800 system with NF: 10 µL of AAV sample solution was mixed with 10 µL of sample buffer from the BioPhase CE-SDS Protein Analysis kit and 3 µL of β-ME and incubated at 70°C for 10 minutes. After the samples were cooled to room temperature, 77 µL of DI water was added to the mixture. The diluted mixture was transferred into the appropriate well of the sample inlet plate. Analysis was then performed on the BioPhase 8800 system with NF using separation conditions described in the application note of the BioPhase CE-SDS Protein Analysis kit. [8]

Sample preparation for AAV capsid protein analysis by CE-SDS-LIF on the BioPhase 8800 system with NF: The AAV sample was mixed with 10 µL of sample buffer from the BioPhase CE-SDS Protein Analysis kit and 2 µL of 1M DTT and incubated at 70°C for 10 minutes. After samples were cooled to room temperature, 1 µL of 1 mg/mL Chromeo P503 dye (dissolved in methanol) was added, and the mixture was incubated at 70°C for another 10 minutes. After the samples were again cooled to room temperature, 77 µL of water was added to the mixture. The diluted mixture was transferred into the appropriate well of the sample inlet plate. Analysis was then performed on the BioPhase 8800 system with NF using separation conditions described previously.[6]

Sample preparation for AAV linearity analysis by CE-SDS-UV and CE-SDS-NF on the BioPhase 8800 system with NF: The packaged AAV8 of pAV-CMV-GFP (1.50×10^{13} GC/mL) was serially diluted 2-fold with 1x PBS. A total of 14 dilutions were made with AAV concentration in a range of 1.50×10^{13} GC/mL to 1.83×10^{9} GC/mL. Each diluted sample was prepared and analyzed following the sample preparation for AAV capsid protein analysis by CE-SDS-UV and CE-SDS-NF on the BioPhase 8800 system with NF.

Sample preparation for AAV linearity analysis by CE-SDS-LIF on the BioPhase 8800 system with NF: The packaged AAV8 of pAV-CMV-GFP (1.50×10^{13} GC/mL) was serially diluted 2-fold with 1x PBS. A total of 15 dilutions were made with AAV

concentration in a range of 1.50 x 10^{13} GC/mL to 9.16 x 10^{8} GC/mL. Each diluted sample was prepared and analyzed following the sample preparation for AAV capsid protein analysis by CE-SDS-LIF on the BioPhase 8800 system with NF.

Instrumentation: The BioPhase 8800 system with UV/LIF/NF (P/N: 5314860) was from SCIEX. The Multi-Therm shaker incubator (P/N: H5000-H) was from Benchmark Scientific (Sayreville, NJ).

Software: BioPhase software package version 1.5 was used for creating run methods and sequences, and for data acquisition and processing.

Results and discussion

Capsid protein purity analysis of AAV8 using CE-SDS with three detection modes: Accurate characterization of AAV capsid proteins is essential for evaluating product quality, consistency, and potency in gene therapy applications.[1] The CE-SDS assay on the BioPhase 8800 system enables high-resolution separation of VPs, including VP1, VP2, VP3, and VP3′, and supports multiple detection modes to accommodate varying analytical needs. In this section, we compare the performance of three detection technologies-NF, UV, and LIF-in analyzing the capsid proteins of three representative AAV samples. Each method offers distinct advantages and limitations in terms of sensitivity, baseline stability, and quantitation accuracy. The following results highlight how these detection modes influence data quality and repeatability in CE-SDS-based capsid protein analysis.

Figure 3 presents the comparative analysis of AAV8-CMV-GFP capsid proteins using CE-SDS on the BioPhase 8800 system with three different detection modes: NF, UV, and LIF.

Panel A displays electropherograms (E-grams) of a single injection analyzed by each detection mode. All three methods successfully resolved the capsid protein components-VP1, VP2, VP3, and VP3′-with comparable peak profiles. However, distinct differences in baseline stability and sensitivity were observed. The UV detection trace shows noticeable baseline fluctuations, particularly in the 15–30 minute range, where the VP peaks and low-abundance impurity peaks migrate. These fluctuations complicate peak integration and obscure minor impurity peaks, especially those migrating between 15 and 20 minutes. In contrast, both NF and LIF detection modes exhibit flat, stable baselines, enabling more straightforward and automated peak integration. While NF improves sensitivity over UV, it does not match the sensitivity of LIF, which clearly detects low-abundance species with signal-to-noise ratios (S/N) greater than 10.

Panel B illustrates repeat injections of the same AAV8-CMV-GFP sample by each detection mode: NF (n=7), UV (n=7), and LIF (n=8). All three detection modes demonstrate excellent repeatability in migration time and peak shape for VP proteins. However, baseline consistency again differentiates the methods. UV traces show variability in baseline drift, while NF and LIF maintain consistent baselines across replicates, supporting more reliable integration and quantitation.

Table 1 summarizes the repeatability metrics for capsid protein purity analysis across detection modes. All methods yielded low relative standard deviations (RSDs) for VP migration times, confirming robust separation performance. NF detection produced comparable capsid protein purity percentages (corrected peak area percentage, CPA%) to UV, but with tighter RSDs, especially for low-abundance peaks, due to improved baseline stability. LIF detection provided the most consistent CPA% values, but its reliance on dye labelling introduces potential bias. Specifically, VP1 and VP2 peaks showed disproportionately high CPA% under LIF due to increased lysine content, which enhances dye binding with P503. This non-stoichiometric labeling can distort intensity-based quantitation, making normalization essential for accurate VP ratio determination.

To further validate these findings, the same CE-SDS experiment was conducted using two additional AAV samples: AAV9-CMV-lacZ and scAAV9-CMV-GFP. Although the electropherograms are not shown in this technical note, the repeatability results are summarized in Tables 2 and 3 below. Consistent with the AAV8-CMV-GFP data presented in Figure 3 and Table 1, all three detection modes demonstrated excellent repeatability in migration time and VP peak profiles. NF detection again showed improved baseline stability and tighter RSDs compared to UV, while LIF maintained the highest sensitivity but required dye labelling. These results reinforce the conclusion that NF detection offers a robust, label-free alternative for capsid protein analysis with performance comparable to LIF and superior to UV in terms of baseline stability and integration precision.

In summary, NF detection offers a label-free alternative with improved baseline stability and quantitation precision over UV, and comparable repeatability to LIF. While LIF remains the most sensitive method, its dependency on dye labelling introduces variability that must be accounted for in quantitative analysis. Future work will focus on normalization strategies to correct for dye-binding bias and improve accuracy in VP ratio determination across detection platforms.

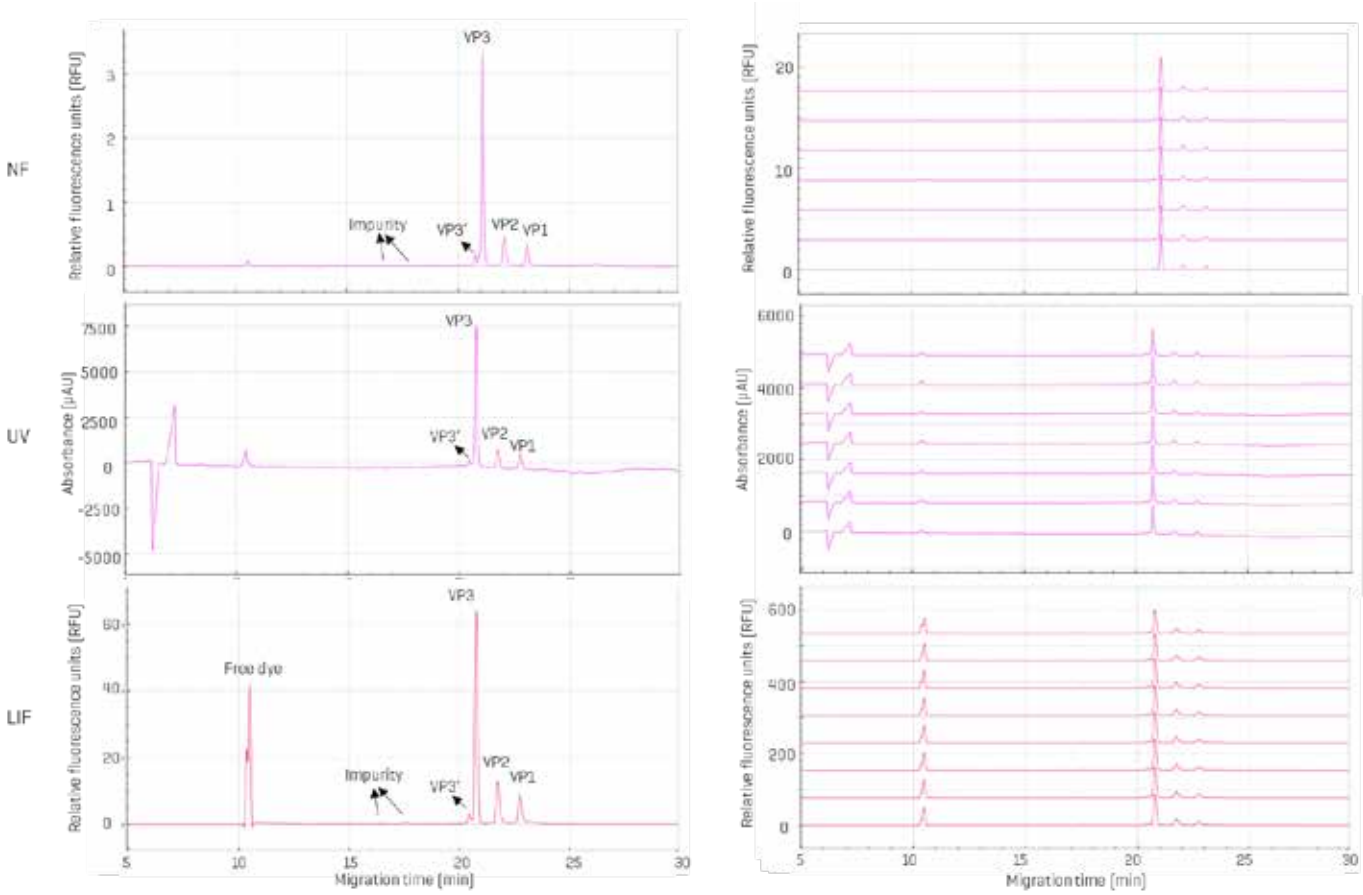


Figure 3. Capsid protein analysis of AAV8-CMV-GFP using CE-SDS on the BioPhase 8800 system with three detection modes. Panel A (left): Electropherograms of a single injection analyzed by NF detection (top), UV detection (middle), and LIF detection (bottom). All three methods resolve VP1, VP2, VP3, and VP3' peaks with comparable profiles. NF and LIF show flat baselines, enabling easier and more accurate peak integration, while UV detection exhibits baseline fluctuations and lower sensitivity, missing low-abundance impurities. Panel B (right): Repeat injections of the same sample analyzed by NF (n=7, top), UV (n=7, middle), and LIF (n=8, bottom). All detection modes demonstrate excellent repeatability in migration time and peak shape. NF and LIF maintain consistent baselines across replicates, supporting robust quantitation and automated data processing.

Table 1. Repeatability comparison of the AAV8-CMV-GFP capsid protein purity analysis using CE-SDS with UV, LIF, and NF detection on the BioPhase 8800 system. This table summarizes the relative standard deviations (RSD%) of migration times and capsid protein purity percentages (CPA%) for VP1, VP2, and VP3 across three detection modes.

AAV8-CMV-GFP	VP3				VP2				VP1			
	MT (min)	RSD% of MT	CPA%	RSD% of CPA%	MT (min)	RSD% of MT	CPA%	RSD% of CPA%	MT (min)	RSD% of MT	CPA%	RSD% of CPA%
LIF (n=8)	20.94	0.61	65.81	0.27	21.92	0.61	17.04	0.82	22.93	0.61	13.44	1.45
NF (n=7)	21.28	0.70	74.68	0.28	22.28	0.70	13.29	1.22	23.32	0.71	8.77	2.94
UV (n=7)	20.85	0.47	74.50	0.78	21.82	0.47	12.98	3.50	22.84	0.48	9.09	5.39

Table 2. Repeatability comparison of the AAV9-CMV-LacZ capsid protein purity analysis using CE-SDS with UV, LIF, and NF detection on the BioPhase 8800 system with NF. This table summarizes the relative standard deviations (RSD%) of migration times and capsid protein purity percentages (CPA%) for VP1, VP2, and VP3 across three detection modes.

AAV9-CMV-lacZ	VP3				VP2				VP1			
	MT (min)	RSD% of MT	CPA%	RSD% of CPA%	MT (min)	RSD% of MT	CPA%	RSD% of CPA%	MT (min)	RSD% of MT	CPA%	RSD% of CPA%
LIF (n=8)	20.52	0.45	70.31	1.18	21.32	0.45	13.99	1.93	22.41	0.45	13.42	1.12
NF (n=7)	20.89	0.82	81.34	0.56	21.70	0.83	9.48	1.26	22.83	0.83	9.18	3.98
UV (n=7)	20.38	0.59	80.84	1.13	21.16	0.60	10.06	5.74	22.25	0.60	9.09	6.57

Table 3. Repeatability comparison of the scAAV9-CMV-GFP capsid protein purity analysis using CE-SDS with UV, LIF, and NF detection on the BioPhase 8800 system. This table summarizes the relative standard deviations (RSD%) of migration times and capsid protein purity percentages (CPA%) for VP1, VP2, and VP3 across three detection modes.

scAAV9-CMV-GFP	VP3				VP2				VP1			
	MT (min)	RSD% of MT	CPA%	RSD% of CPA%	MT (min)	RSD% of MT	CPA%	RSD% of CPA%	MT (min)	RSD% of MT	CPA%	RSD% of CPA%
LIF (n=8)	20.44	0.50	69.02	0.34	21.24	0.51	14.89	0.50	22.33	0.51	12.06	0.98
NF (n=7)	21.20	1.11	80.12	0.48	22.02	1.11	9.56	1.06	23.17	1.10	9.15	2.82
UV (n=7)	20.56	0.87	81.90	1.22	21.35	0.88	9.56	7.10	22.45	0.90	8.55	11.98

Linearity assessment of VP3 quantitation across detection modes: Quantitative accuracy and sensitivity are critical for capsid protein analysis, especially when working with AAV samples across a wide range of titers. To evaluate the linearity of VP3 quantitation, a series of AAV8-CMV-GFP samples with varying genome copy (GC) concentrations were analyzed using CE-SDS on the BioPhase 8800 system with three detection modes: NF, UV, and LIF.

Figure 4 presents the linearity plots of VP3 capsid protein purity percentage (CPA%) versus AAV titer for each detection mode. The left panel shows the NF detection plot, the middle panel displays UV detection, and the right panel illustrates LIF detection. All three methods demonstrated excellent linearity, with $R^2 > 0.99$ across the tested concentration ranges.

Table 4 summarizes the linearity metrics, including the tested titer range, limit of detection (LOD), and limit of quantitation (LOQ), determined using S/N P-P calculated by the BioPhase 8800 software. For this specific AAV8-CMV-GFP sample, NF detection achieved a threefold improvement in LOD compared to UV, detecting down to approximately 1×10^{11} GC/mL. LIF detection exhibited the highest sensitivity, with an LOD of 1×10^{9} GC/mL, making it suitable for ultra-low titer samples.

While LIF offers superior sensitivity, it requires fluorescent dye labeling, which introduces complexity and potential variability in quantitation due to non-stoichiometric dye binding. In contrast, NF detection provides a label-free, streamlined workflow with improved baseline stability and precision over UV, making it a practical alternative for routine capsid protein purity analysis. NF detection also enhances impurity detection and supports consistent peak integration, especially in low-abundance regions.

In summary, all three detection modes are suitable for quantitative analysis of VP3 across a broad titer range. NF detection stands out as a balanced solution, combining improved sensitivity over UV with the simplicity of label-free operation. LIF remains the method of choice for ultra-low titer applications where maximum sensitivity is required.

Table 4. Linearity, sensitivity, and quantitation metrics for VP3 capsid protein analysis of AAV8-CMV-GFP using CE-SDS with UV, LIF, and NF detection on the BioPhase 8800 system.

	Plotted concentration range (GC/mL)	LOQ (GC/mL)	LOD (GC/mL)	R^2
NF	1.17E+11 - 1.50E+13	4.69E+11	1.17E+11	0.9985
UV	4.69E+11 - 1.50E+13	9.38E+11	4.69E+11	0.9991
LIF	9.16E+08 - 1.50E+13	3.66E+09	9.16E+08	0.9990

Table 5. Comparison of VP ratio determination for three AAV samples using CE-SDS with UV, LIF, and NF detection on the BioPhase 8800 system.

	Average of VP1 : VP2 : VP3 ratio		
	AAV8-CMV-GFP	AAV9-CMV-lacZ	scAAV9-CMV-GFP
NF	1.0: 1.5: 7.8	1.0: 1.0: 8.1	1.0: 1.0: 8.0
UV	1.0: 1.4: 7.5	1.0: 1.1: 8.2	1.0: 1.0: 8.6
LIF	1.0: 1.2: 4.5	1.0: 1.0: 4.8	1.0: 1.2: 5.2

VP ratio determination of AAV capsid proteins using CE-SDS with multiple detection modes: The ratio of viral proteins

[VP1:VP2:VP3] in AAV capsids is a critical quality attribute that reflects capsid integrity and assembly efficiency. While the theoretical ratio is approximately 1:1:10, actual measurements can vary depending on the AAV batch, assay type and the detection method used for a given assay type, i. e., CE-SDS analysis. In this study, three AAV samples-AAV8-CMV-GFP, AAV9-CMV-lacZ, and scAAV9-CMV-GFP-were analyzed using CE-SDS on the BioPhase 8800 system with three detection modes.

Table 5 summarizes the VP ratio results obtained from each detection mode. While all methods resolved VP1, VP2, and VP3 peaks, the calculated ratios differed significantly, particularly with LIF detection. This discrepancy is attributed to the inherent differences in detection principles:

UV detection (220 nm) measures absorbance from the peptide backbone, reflecting total amino acid content. It is simple and widely available but has lower sensitivity, making it less preferable for low-concentration samples. VP2 and VP3 may be underrepresented due to their lower amino acid counts.

LIF detection uses dyes such as Chromeo P503 that bind to lysine residues. While highly sensitive and suitable for low-titer samples, dye binding varies across VP proteins due to differences in lysine content. This can lead to overestimation of VP1 and VP2 relative to VP3.

NF detection relies on intrinsic fluorescence from tryptophan residues. It is a label-free detection technique and offers improved baseline stability. Since its sensitivity depends on tryptophan content, sensitivity will vary among VP proteins. VP3 and VP2 may be underrepresented if they contain fewer tryptophan residues.

To address these biases, normalization was applied using the known amino acid composition of VP1, VP2, and VP3 from the AAV8-CMV-GFP sequence. Specifically, the number of tryptophan [# of W], total amino acids [# of AA] and lysine [# of K] was used to adjust the intensity-based VP ratios [shown in Table 6].

After normalization, the VP ratios obtained from LIF detection were comparable to those from UV and NF detection. However, residual differences remained, likely due to factors such as protein folding, accessibility of dye-binding sites, and structural heterogeneity, which affect the dye binding efficiency of the VPs and fluorescence intensity.

These findings highlight the importance of understanding detection-specific biases when interpreting VP ratios. While NF detection offers a practical, label-free alternative with improved precision and baseline stability, LIF remains valuable for ultra-low titer samples, provided that normalization strategies are applied to correct for dye-binding variability.

Table 6. Normalization factors for VP ratio adjustment based on amino acid composition of AAV8-CMV-GFP capsid proteins.

Normalization factors			
	VP1	VP2	VP3
NFD-# of W	15	12	12
UV-# of AA	737	600	534
LIF-# of K	32	22	17

Table 7. VP ratio of AAV8-CMV-GFP capsid proteins after normalization across detection modes.

After Normalization			
	VP1	VP2	VP3
NF	1.0	1.9	9.8
UV	1.0	1.7	10.4
LIF	1.0	1.7	8.5

Conclusions

The integration of NFD into the CE-SDS workflow on the BioPhase 8800 system significantly enhances the analytical capabilities for AAV capsid protein characterization. By leveraging intrinsic fluorescence from tryptophan residues, NFD enables a label-free, high-precision, and time-efficient approach to VP analysis. This advancement complements the streamlined AAV drug production quality control process-from plasmid to capsid-by offering improved sensitivity, reproducibility, and operational simplicity. Below are the key features that highlight the value of NFD in this workflow:

- **Integrated into streamlined AAV QC workflow**: Extends CE-based quality control from plasmid DNA to capsid protein analysis, supporting end-to-end characterization on a single platform.
- **Enhanced sensitivity over UV detection**: Approximately 3× greater sensitivity for AAV samples compared to UV, enabling improved detection of low-abundance impurities and more accurate quantitation at lower titers.
- **Improved precision for CPA% measurements**: Delivers tighter %CVs for capsid protein purity due to consistent baseline and reliable peak integration, especially critical for low-abundance VP peaks.
- **Time-saving during data processing**: Stable, flat baseline simplifies peak integration, reducing manual effort and accelerating analysis-ideal for high-throughput workflows and DoE studies.
- **Label-free workflow**: Eliminates the need for fluorescent dye labeling required in LIF detection, minimizing variability from reagent inconsistencies, manual handling, and labeling efficiency differences between VPs.

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A new approach for high-resolution full and empty AAV capsid analysis utilizing a high-throughput method for comprehensive AAV evaluation on a single CE platform

Featuring the BioPhase 8800 system

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To ensure the safety and efficacy of gene therapies delivered by adeno-associated virus (AAV) vectors, multiple critical quality attributes (CQAs) must be well characterized before regulatory submission.[1] Different analytical platforms are currently being used to characterize multiple CQAs. However, these methods have limitations or cannot be used to analyze all serotypes without significant optimization. In this technical note, we present an approach for high-throughput multiple CQA analysis of AAVs using a single platform: the multi-capillary BioPhase 8800 system. High-accuracy analytical data were collected in a single day for multiple CQAs, including nucleic acid loading and titer, capsid protein profile and ratio, and capsid titer from the same samples.

A new approach was used here to obtain empty, partial, and full capsid ratios by utilizing data for other CQAs (Figure 1). The workflow is serotype-independent and amenable for development and QC. Using a single, multi-capillary platform for multiple AAV CQA analyses saves time and cost and reduces laboratory footprint compared to using different platforms.

Key features of the comprehensive AAV characterization workflow

- High-definition full, partial, and empty analysis is accomplished with a new methodology
- High-throughput, comprehensive analysis of multiple CQAs for the same AAV sample is performed on a single platform within a single day
- High-resolution, serotype-independent capsid profile, ratio, and impurity analyses, and titer determination are achieved with a CE-SDS-LIF workflow
- High-resolution, serotype-independent genome integrity analysis and titer determination are achieved with a CGE-LIF workflow
- Multi-capillary capability enables fast result generation for all methods
- Time and cost saving, and laboratory footprint reduction are attained by using a single tabletop platform for the analysis of multiple CQAs

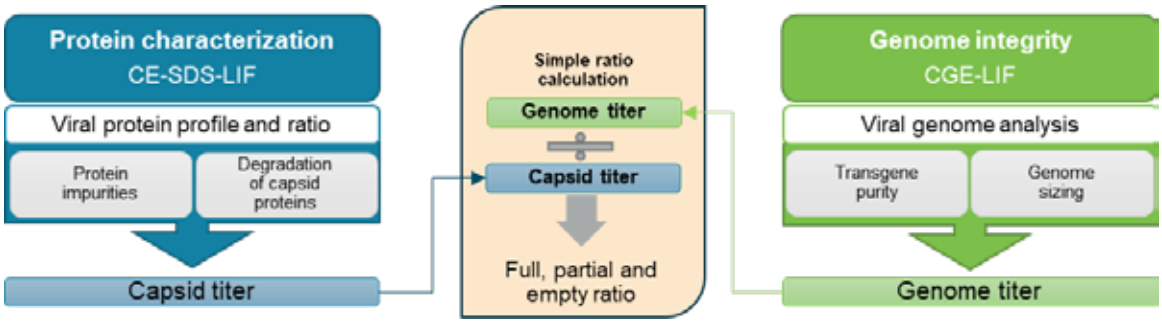


Figure 1. A new approach for high-resolution full and empty AAV capsid analysis as part of a high-throughput, comprehensive analysis suite of AAV CQAs on the BioPhase 8800 system. Each sample is analyzed with 2 workflows: the CE-SDS-LIF for protein characterization including the capsid titer determination; the CGE-LIF for genome integrity analysis including genome titer determination. The percentage of the full capsids is calculated by dividing the genome titer by the capsid titer.

Materials and methods

Materials: The BioPhase CE-SDS Protein Analysis kit (PN C30085), the BioPhase BFS Capillary Cartridge (PN 5080121), the BioPhase Sample and Reagent Plates (PN 5080311), the RNA 9000 Purity & Integrity kit (PN C48231) and Sample Loading Solution (SLS) (PN 608082) were from SCIEX (Framingham, MA). The Chromeo P503 dye (PN 15106) was from ACTIVE MOTIF (Carlsbad, CA). Dithiothreitol (DTT, PN V3151) was from Promega (Fitchburg, WI). QIAquick PCR purification kit (PN 28104) was from Qiagen (Germantown, MD). Single-stranded RNA ladder 0.5-9 kb (PN N0362S) was from New England BioLabs (NEB) (Ipswich, MA). Nuclease-free water (PN AM9932), 10x Phosphate Buffered Saline (PBS) (PN AM9624), and RNA 6000 Ladder (PN AM7152) were from Thermo Fisher Scientific (Waltham, MA).

Samples: The packaged AAV8 of pAV-CMV-GFP (~2.62x10^{13} GC/mL), packaged AAV8 of pAV-CMV-BuB1, and AAV8-CMV-GFP reference standard with full capsids (PN RS-AAV8-FL) were from Charles River (Rockville, MD). The AAV1-CMV-GFP (PN SL100803), AAV2-CMV-GFP (PN SL100812), AAV2-CMV-Lacz (PN SL100854), AAV5-CMV-Lacz (PN SL100857) and AAV9-CMV-Lacz (PN SL100868) were from SignaGen Laboratories (Rockville, MD).

Instrument and software: A BioPhase 8800 system (PN 5083590) equipped with LIF detection was from SCIEX. The excitation wavelength was 488 nm, and the emission wavelength was 520 nm for AAV genome integrity analysis. A 600 nm bandpass emission filter (PN 5085177) from SCIEX was used for CE-LIF analysis of AAV capsid proteins. Data acquisition and analysis were performed using BioPhase software, version 1.0 (SCIEX).

Sample preparation for AAV capsid protein analysis by CE-SDS-LIF on the BioPhase 8800 system: A 20 µL sample of AAV was diluted to 1x10^{11} GC/mL with 1x PBS. The diluted AAV sample was then mixed with 20 µL of sample buffer from the BioPhase CE-SDS Protein Analysis kit and 4 µL of 1M DTT and incubated at 70°C for 10 minutes. After samples were cooled to room temperature, 2 µL of 1 mg/mL Chromeo P503 dye was added, and the mixture was incubated at 70°C for another 10 minutes. After the samples were again cooled to room temperature, 154 µL of water was added to the mixture. AAV1, AAV2, and AAV8 samples were all prepared following the same sample preparation procedure. A 100 µL sample of the diluted, prepared solution was transferred into a sample well on the sample plate. Analysis was then performed on the BioPhase 8800 system using separation conditions described previously.[2]

Sample preparation for AAV capsid titer determination by CE-SDS-LIF on the BioPhase 8800 system: The packaged AAV8 of pAV-CMV-GFP (2.62 x 10^{13} GC/mL) was used as a standard and serially diluted 2-fold with 1x PBS to generate results to build a standard curve. A total of 15 serial dilutions were made with AAV concentration in a range of 2.62 x 10^{13} GC/mL to 1.60 x 10^{9} GC/mL. From each diluted standard solution, 15 µL was removed and added to 15 µL of sample buffer from the BioPhase CE-SDS Protein Analysis kit and 3 µL of 1M DTT and incubated at 70°C for 10 minutes. After the mixture was cooled to room temperature, 1.5 µL of 1 mg/mL Chromeo P503 dye was added, and the mixture was incubated at 70°C for another 10 minutes. After the samples were cooled to room temperature, 115.5 µL of nuclease-free water was added to the mixture to quench the reaction. An AAV8-CMV-GFP reference standard with full capsids from Charles River was used as the test sample, and 15 µL of it was labeled with Chromeo p503 dye as described above. Then, 90 µL of the diluted, prepared sample solution was transferred into a sample well of the sample plate for analysis on the BioPhase 8800 system. After CE separation, results were analyzed using the BioPhase 8800 software. The corrected peak area of the VP3 peak for each dilution point of the standard was plotted against the AAV titer in GC/mL to generate the standard curve. A linear equation was obtained from the standard curve. The capsid titer of the AAV test sample was determined using the corrected peak area of the VP3 peak of the test sample and the linear equation.

Sample preparation for AAV genome integrity analysis by CGE-LIF on the BioPhase 8800 system: The AAV samples were prepared following a simplified workflow for genome release and purification. First, 20 µL of each AAV sample was mixed thoroughly with the binding buffer from the QIAquick PCR purification kit. The manufacturer's instructions were followed except that the column was washed twice, and the AAV genome sample was eluted from the column using 50 µL of 10x diluted elution buffer (Buffer EB from the kit diluted with nuclease-free water). Then, 10 µL of the eluted AAV genome solution was mixed with 40 µL of nuclease-free water and 50 µL of SLS, heated at 70°C for 10 minutes, and immediately cooled on ice for 5 minutes. The final 100 µL mixture was transferred into a sample well of the sample plate. CE separation conditions were the same as described previously.[3]

Sample preparation for AAV genome titer determination by CGE-LIF on the BioPhase 8800 system: Packaged AAV8 of pAV-CMV-GFP (2.62 x 10^{13} GC/mL) was used as a standard and serially diluted 2-fold with 1x PBS to generate results for building a standard curve. A total of 14 serial dilutions were made with AAV concentration in a range of 2.62 x 10^{13} GC/mL to 3.20 x 10^{9} GC/mL. From each diluted standard solution, 110 µL

was used for genome extraction using the QIAquick PCR purification kit, following the manufacturer's instructions, except the column was washed twice, and the AAV genome sample was eluted from the column using 50 µL of 10x diluted elution buffer. Before loading onto the instrument for analysis, 10 µL of the eluted AAV genome solution was prepared as described above. As a test sample, an AAV8-CMV-GFP reference standard with full capsids from Charles River was used for genome extraction using the QIAquick PCR purification kit, following the same process as for each standard dilution. After CE separation, results were analyzed using the BioPhase 8800 software. The corrected peak area of the intact genome peak for each dilution point of the standard was plotted against the AAV titer in GC/mL to generate the standard curve. The genome titer of the AAV test sample was determined using the corrected peak area of the intact genome peak in the test sample and the linear equation.

Calculation of the percentage of AAV full capsids: The percentage of full capsids in the test sample was determined by dividing the genome titer by the capsid titer from the same test sample and multiplying it by 100.

Analytical challenges for AAV analysis

AAVs are safe and effective gene delivery vehicles to drive persistent transgene expression in gene therapy. Over 100 unique clinical trials with recombinant AAV (rAAV) vectors have been approved by US and European regulatory agencies.[1] Rapid growth in this gene therapy space created a need to manufacture rAAVs at a large scale and with high quality. Table 1 shows the reported CQAs and their potential impact on the quality of rAAVs. These CQAs must be characterized and monitored throughout the manufacturing process to ensure product efficacy and safety. The full-to-empty capsid ratio is especially important because full capsids deliver intact transgenes into target cells and directly impact the potency of AAV therapeutics. Nonfunctional empty capsids reduce transduction efficiency and might exacerbate vector-related immunotoxicity. Furthermore, partially filled capsids containing truncated genomes or fragments of host cell nucleic acids or vectors could cause low efficacy, genotoxicity, and immunotoxicity. Therefore, it is essential to use a high-resolution sizing methodology to resolve the intact genome from the partial genome and other impurities. Monitoring the full and empty capsid ratio is critical for process improvements in removing empty and partial capsids for better efficacy and safety of AAV-based gene therapy. Currently, biopharma scientists often use

Table 1. Quality considerations for rAAV.

CQA	Vector Potency	Immunogenicity	Transduction efficiency	Packaging efficiency
Viral protein profile and ratio	●	●	●	●
Genome integrity	●	●	●	
Titer	●	●	●	
Full and empty capsid ratio	●	●	●	
Protein impurities	●	●		
Nucleic acid impurities	●	●		

different platforms to analyze different CQAs. Table 2 compares current technologies to determine multiple AAV CQAs, their limitation, and the cost of each platform. The information in Table 2 indicates that comprehensive AAV analysis of multiple CQAs on different platforms is time-consuming and costly. Additionally, different laboratories are required to house different platforms and specially trained scientists are needed to run different instruments and perform data analysis using additional software. In this technical note, a single multi-capillary electrophoresis system, the BioPhase 8800 system, was utilized to provide a complete set of gene therapy product quality analysis for multiple AAV CQAs (Figure 2). Two main workflows were used. The CE-SDS-LIF workflow was used to analyze viral protein profile and ratio, protein impurities, and capsid titer. The CGE-LIF workflow was used for high-resolution analysis of genome integrity and genome titer. A new approach was employed to determine the percentage of full capsids by a calculation, as shown in Figure 1. Results in this technical note demonstrate that the BioPhase 8800 system provides a fast, high-throughput, comprehensive analysis of multiple AAV CQAs.

Table 2. Comparison of current technologies to determine multiple AAV CQAs. *Abbreviations: ELISA: enzyme-linked immunosorbent assay; SDS-PAGE: Sodium Dodecyl Sulfate-polyacrylamide gel electrophoresis; IEX: ion exchange chromatography; SEC: size exclusion chromatography; MALS: multi-angle light scattering.*

Technology	Limitation	Cost of platform
Real-time PCR (qPCR) *Digital PCR (dPCR)*	*qPCR: Low accuracy and precision* *dPCR: False positive, limited sizing*	*$~20,000 to $~120,000*
ELISA/SDS-PAGE	*Low accuracy and precision*	*$~20,000*
Spectrophotometry	*Impurity interferences; low accuracy*	*$~18,000*
Chromatography (IEX and SEC)	*Needs a large sample amount; cannot distinguish partial from full capsids*	*$~80,000*
SEC-MALS	*Cannot distinguish partial from full capsids* *Assumes 100% mass recovery and needs extinction coefficients*	*$~100,000*
Electron microscopy (EM)	*Low throughput; time-consuming,* *Cannot distinguish partial from full capsids; expensive*	*$~100,000 to $~7 million*
Analytical ultracentrifugation (AUC)	*Low throughput; needs a large sample amount* *Expensive; difficult to implement as a QC release assay*	*$~100,000 to $~400,000*
Charge detection mass spectrometry (CDMS)	*Low throughput; expensive; difficult to validate as a QC assay*	*>$750,000*

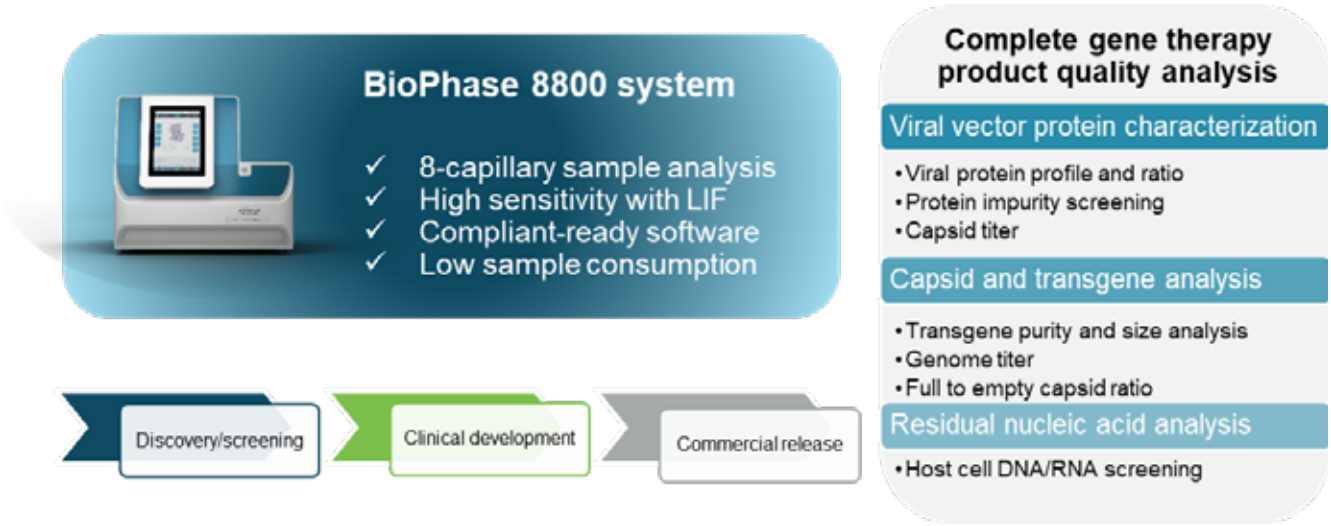


Figure 2. Comprehensive AAV analysis on the BioPhase 8800 system for key AAV CQAs. High-throughput, high-resolution sample analysis is enabled using the pre-assembled cartridge with 8 capillaries. High sensitivity is achieved with laser induced fluorescence detection or LIF. The system comes with compliant-ready software. A small sample volume is sufficient for analysis. This system offers flexible solutions designed for all phases of the drug development process including discovery, development and QC.

High-resolution AAV capsid characterization

The CE-SDS-LIF workflow shown in Figure 3 was used for high-throughput, high-resolution AAV capsid characterization. AAV samples were denatured in the presence of DTT and the sample buffer from the BioPhase CE-SDS protein analysis kit, then labeled with Chromeo p503 dye, and further separated on the BioPhase 8800 system using a BioPhase BFS capillary cartridge. As shown in Figure 4, baseline resolution was achieved in separating VP1, VP2, and VP3 peaks for AAV samples of 3 different serotypes: AAV1, AAV2, and AAV8. These peaks were also well-separated from impurities. Furthermore, baseline resolution was achieved in separating the VP3' (VP3 variant) peak from the VP3 peak for all 3 serotypes. These results indicate that the CE-SDS-LIF workflow works for all 3

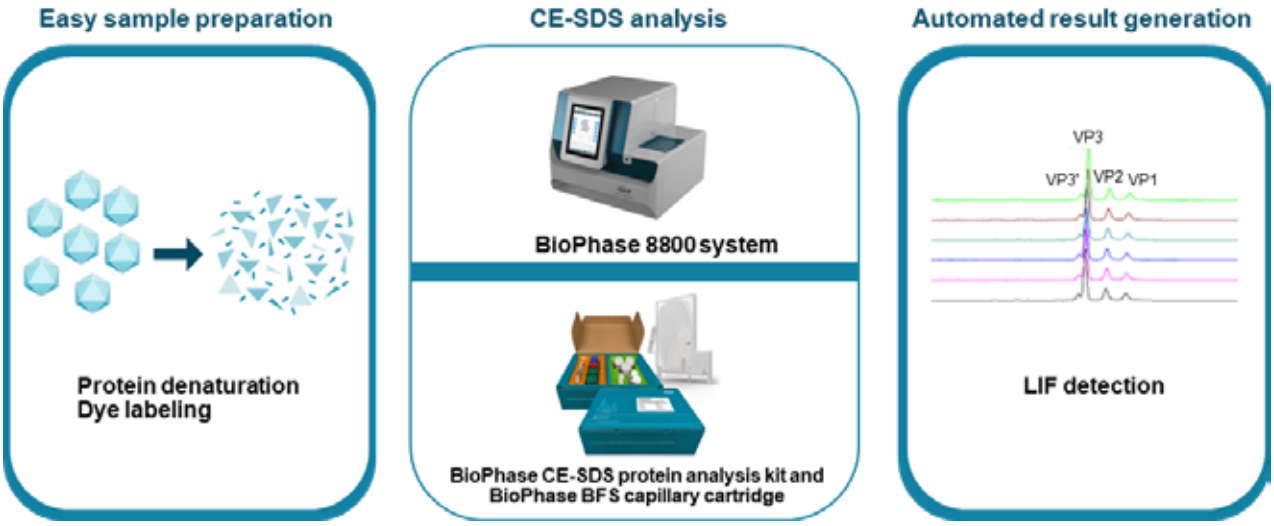


Figure 3. High-throughput, high-resolution CE-SDS-LIF workflow for AAV capsid characterization on the BioPhase 8800 system. Samples were denatured in the presence of SDS, labeled with a fluorescent dye, and separated by CE with LIF detection to achieve high sensitivity. Data were collected and analyzed automatically. Three viral capsid proteins and the VP3 variant peaks were well-separated for easy assessment of their ratio.

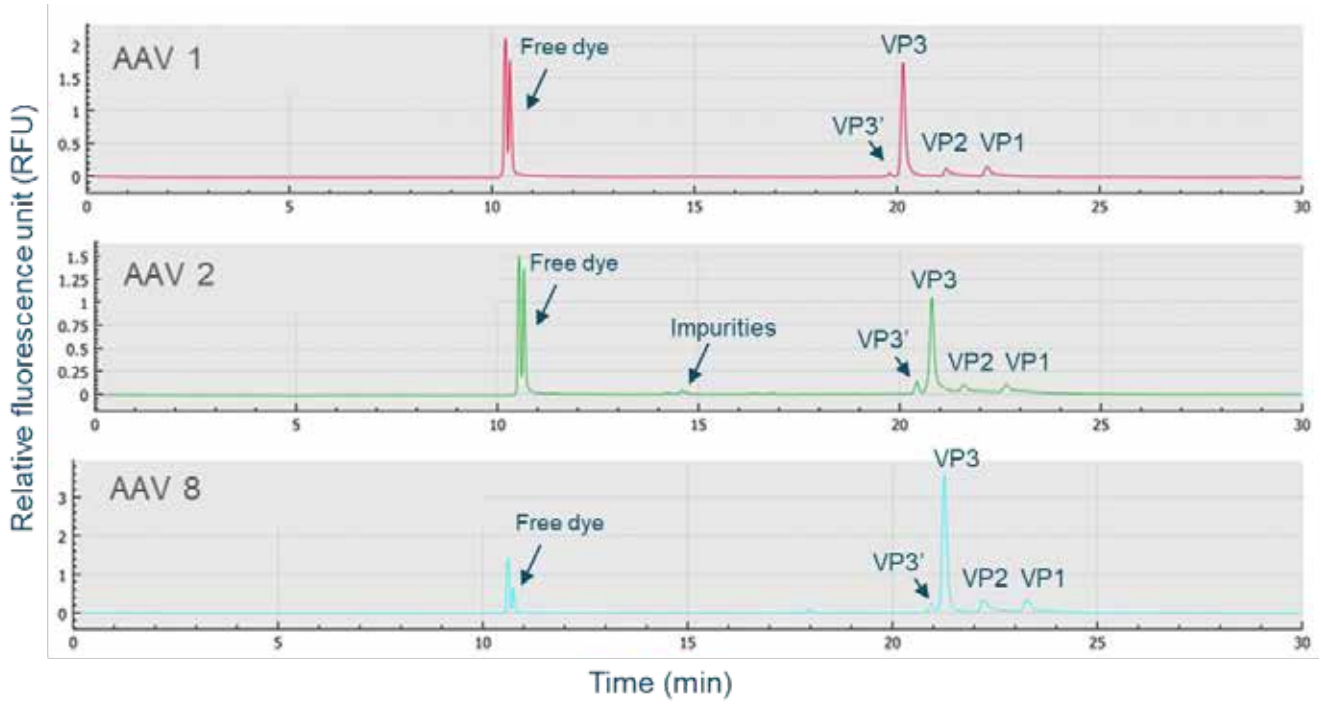


Figure 4. Analysis of capsid proteins from AAV samples of different serotypes using the same CE-SDS-LIF workflow. Samples of 3 different serotypes, AAV1, AAV2, and AAV8 were analyzed on the BioPhase 8800 system. Baseline resolution of VP1, VP2, VP3 and VP3' was achieved for all 3 serotypes. The presence of impurities were detected as well. These results demonstrate that this workflow is serotype-independent.

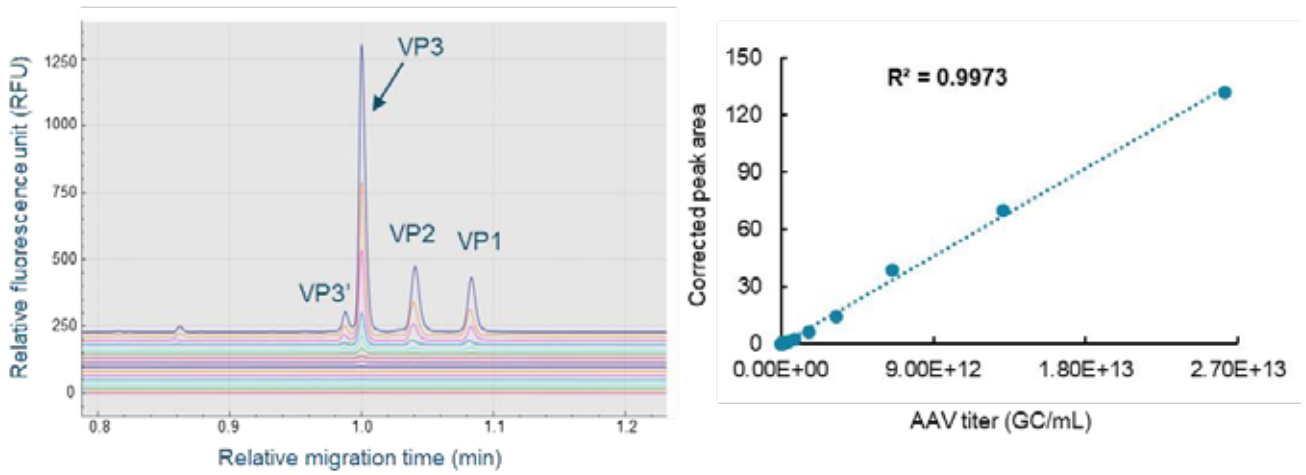


Figure 5. Fast, high-throughput AAV capsid titer determination using the CE-SDS-LIF workflow on the BioPhase 8800 system. The panel on the left is the overlay of electropherograms obtained with samples from different dilution points. The panel on the right is the plot of the corrected peak area of VP3 against the AAV titer in GC/mL with excellent linearity. The LOD was $1.60x10^9$ GC/mL. The LOQ was $6.40x10^9$ GC/mL. The linear detection range was $6.40x10^9$ GC/mL to $2.62x10^{13}$ GC/mL.

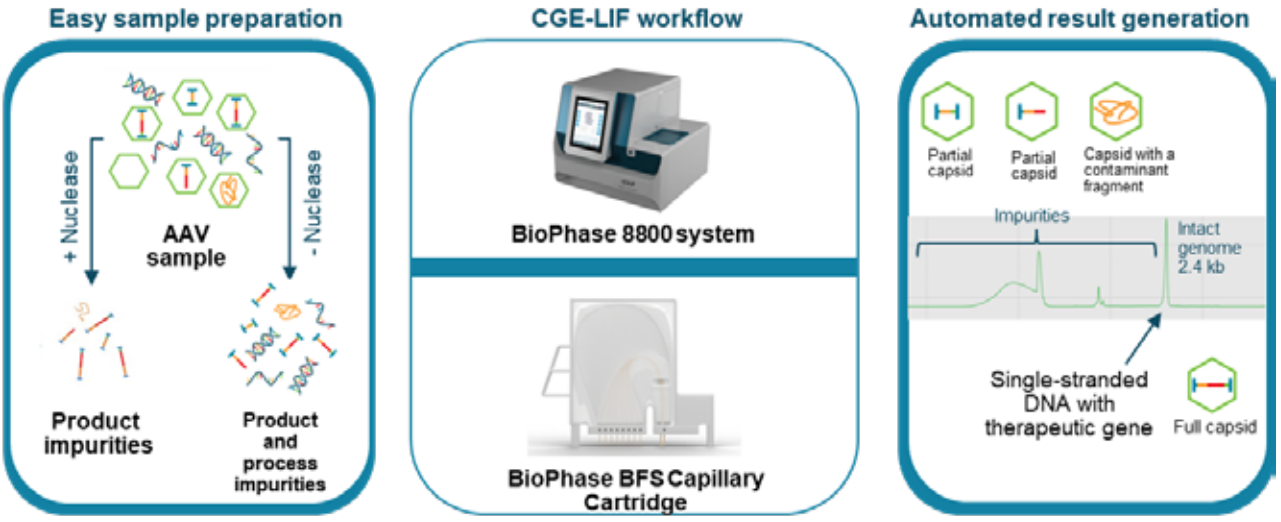


Figure 6. High-throughput, high-resolution CGE-LIF workflow for AAV genome integrity analysis on the BioPhase 8800 system. Two sample preparation options can be used. AAV samples can be treated with nuclease prior to nucleic acid extraction for analysis of product-related impurities. Alternatively, nucleic acids can be released and purified directly from the AAV samples without nuclease treatment for analysis of both product and process related impurities. Extracted nucleic acids were analyzed by CGE-LIF with the use of a fluorescent dye, SYBR™ Green II Gel Stain*, for high sensitivity analysis. The intact genome was well separated from partial genome and other impurities.

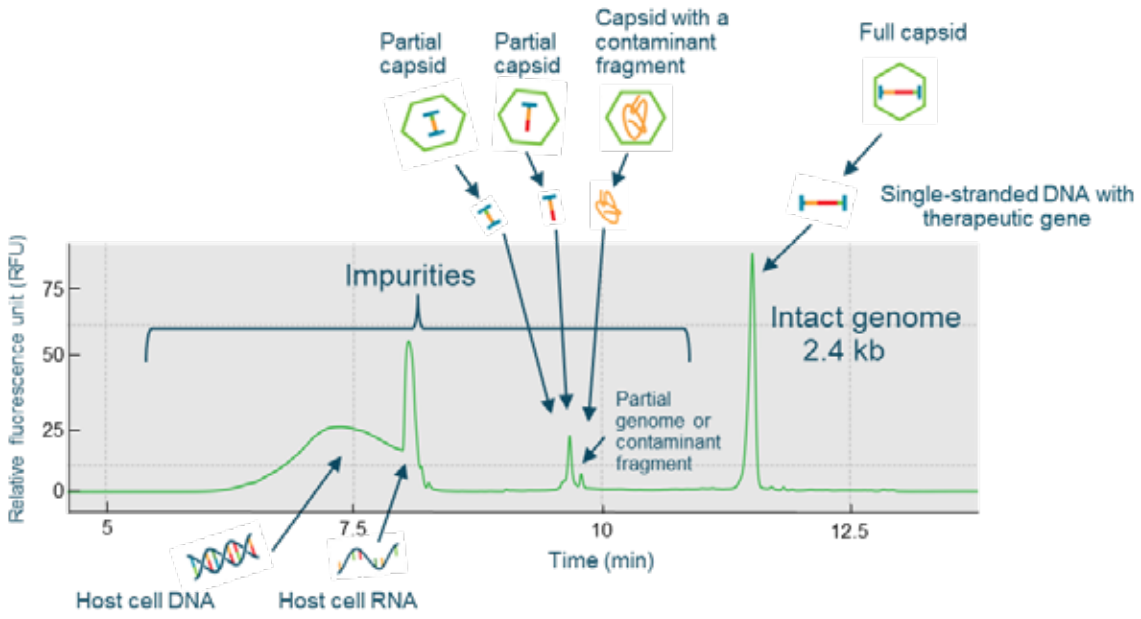


Figure 7. High-resolution genome integrity analysis on the BioPhase 8800 system. The intact genome of 2.4 kb was well separated from potential partial genomes, contaminant fragments, small-sized host cell RNA, and host cell DNA impurities.

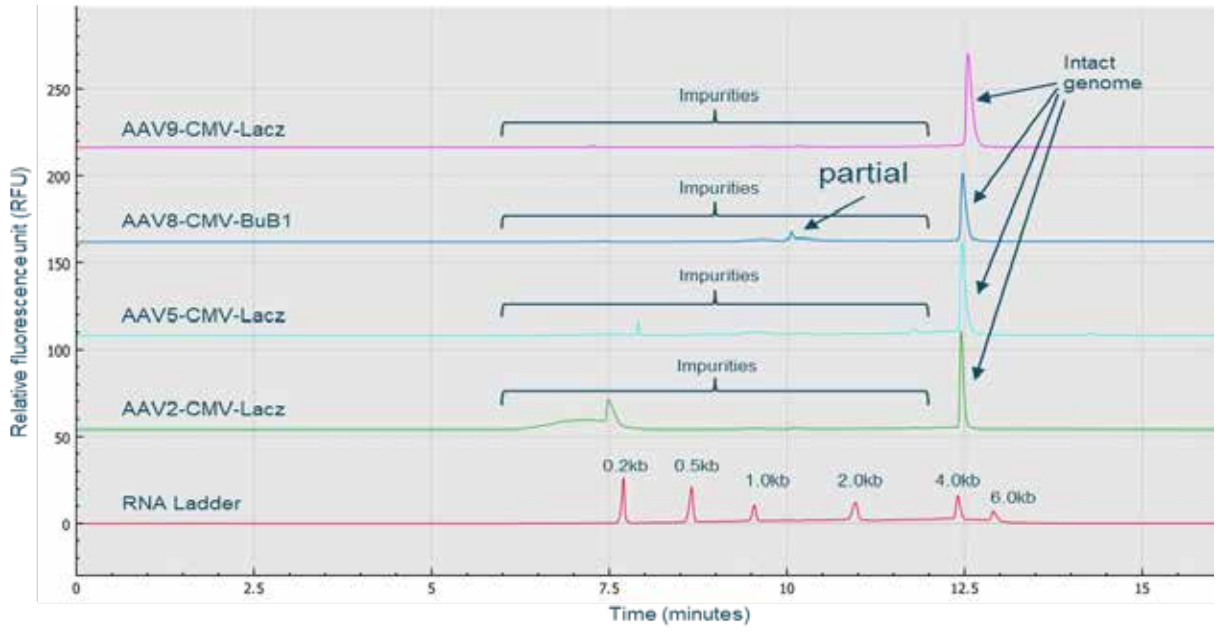


Figure 8. High-throughput, high-resolution genome integrity analysis of multiple AAV serotypes using the CGE-LIF workflow on the BioPhase 8800 system. Nucleic acids extracted from 4 different serotypes, AAV2, AAV5, AAV8, and AAV9 were analyzed. High-resolution separation of the intact genome from impurities was achieved for all 4 serotypes, demonstrating that this workflow can be implemented independent of serotype.

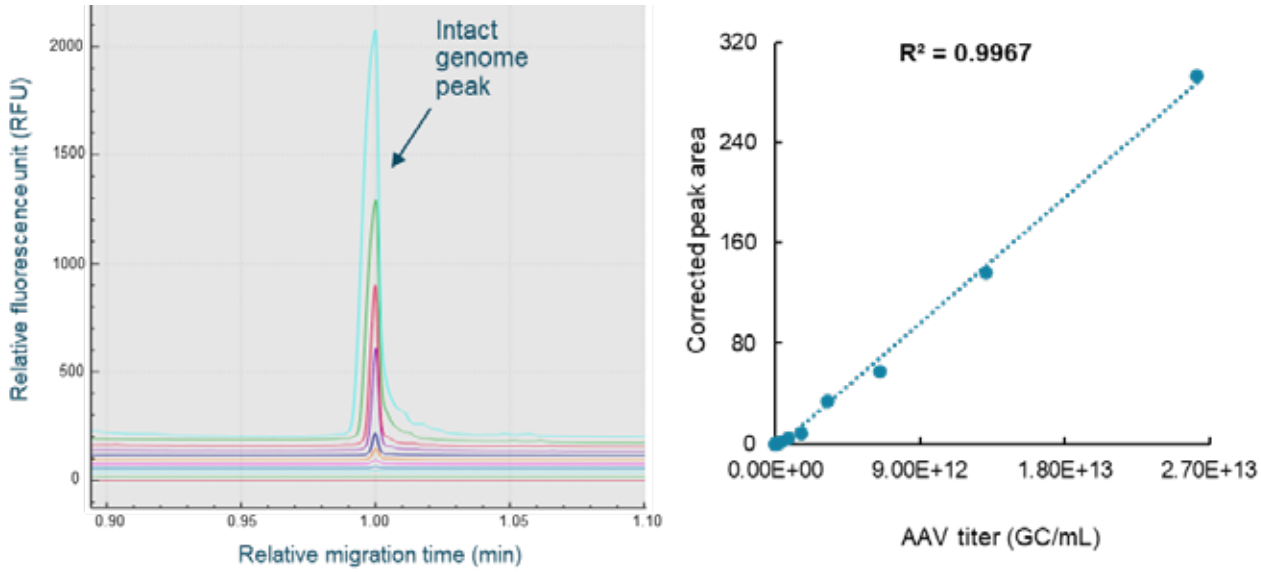


Figure 9. Fast, high-throughput AAV genome titer determination using the CGE-LIF workflow on the BioPhase 8800 system. The panel on the left shows the overlay of electropherograms obtained with samples from different dilution points. The panel on the right shows the linear plot of the corrected peak area of the intact genome peak against the AAV titer in GC/mL. The LOD was $1.28x10^{10}$ GC/mL. The LOQ was $2.56x10^{10}$ GC/mL. The linear detection range was $2.56x10^{10}$ GC/mL to $2.62x10^{13}$ GC/mL.

serotypes and is a serotype-independent workflow. The VP3' and VP3 peaks differ by 8 amino acids due to their difference in translation start site, as reported previously.[4] The baseline resolution between VP3 and VP3' peaks demonstrates the outstanding resolving power of CE on the BioPhase 8800 system.

Fast, high-throughput AAV capsid titer determination

An AAV8 sample with a known titer was used as the standard. It was serially diluted 2-fold for 15 dilutions with 1x PBS buffer. Sample from each dilution point was denatured in the presence of DTT and the sample buffer, labeled with the Chromeo p503 dye, and separated on the BioPhase 8800 system. As shown in the left panel of Figure 5, baseline resolution was obtained for VP1, VP2, VP3, and VP3' peaks. The Optimizer feature on the "Integration" tab in the BioPhase 8800 software was enabled to select a best-fit analysis automatically. After minor adjustments to peak integration parameters, results were exported for simple processing in Microsoft Excel. The corrected peak area for the VP3 peak was plotted against the AAV titer in GC/mL. As shown in the right panel of Figure 5, excellent linear curve fitting was achieved, evidenced by an R^2 value of 0.9973. The limit of detection (LOD) was 1.60 x 10^9 GC/mL. The limit of quantification (LOQ) was $6.40x10^9$ GC/mL with a linear range of about 3 logs. A linear equation was obtained from the linear curve fitting. An AAV8 test sample was processed in the same experiment, using the same workflow as the AAV standard. The corrected peak area of the VP3 peak from this test sample was used to calculate its AAV capsid titer using the equation from this linear curve. The capsid titer for this test sample was 2.65 x 10^{12} GC/mL.

High-resolution AAV genome integrity analysis

The CGE-LIF workflow shown in Figure 6 was used for high-throughput AAV genome integrity analysis. Two sample preparation options can be used. A fast method was used for the analysis of both product- and process-related impurities with direct extraction of the single-stranded DNA genome from the AAV sample using the Qiagen PCR purification kit. An optional benzonase treatment step can be used for the analysis of product-related impurities in the AAV sample. The extracted AAV genome sample was analyzed by CGE-LIF on the BioPhase 8800 system. Figure 7 shows the high-resolution results obtained with an AAV2-CMV-LacZ sample. The 2.4 kb intact genome peak was well-separated from 2 distinct peaks with sizes around 1 kb and small-sized impurities of around 0.2 kb.

The 2 distinct peaks with sizes around 1 kb could be potential partial genome peaks or contaminant fragments from the host cell genome or plasmid vectors. There are 2 groups of small-sized impurities: the one with a sharp peak shape could be host cell RNA impurities, while the broad peak could be small-sized host cell DNA fragments. Since the BioPhase BFS capillary cartridge has 8 capillaries, the same high-resolution genome integrity analysis can simultaneously be performed for up to 8 AAV samples. Figure 8 shows the results obtained with 4 different AAV samples: AAV2-CMV-LacZ, AAV5-CMV-LacZ, AAV9-CMV-LacZ and AAV8-CMV-BuB1, and the RNA ladder. High-resolution of intact genome peaks and partial and small size impurities was achieved for all 4 AAV samples. This result demonstrates that the CGE-LIF workflow on the BioPhase 8800 system is a serotype-independent workflow.

Fast, high-throughput AAV genome titer determination

The same AAV8 sample with known titer used in the aforementioned AAV capsid titer determination was also used as the standard for building the standard curve for AAV genome titer determination. The AAV8 sample was serially diluted 2-fold for 11 dilutions with 1x PBS buffer. The sample from each dilution point was subjected to nucleic acid extraction using the Qiagen PCR purification kit. The extracted nucleic acid samples were heat-treated and analyzed on the BioPhase 8800 system using the multi-capillary BioPhase BFS capillary cartridge. Samples from the 14 dilution points and a test sample were completed in 2 60-minute runs. After CE separation, results were analyzed with the BioPhase 8800 software. Results were exported. Data processing was performed in Microsoft Excel. The corrected peak area of the intact genome peak was plotted against the AAV titer of the standard (Figure 9). Excellent linearity was demonstrated by the R^2 value of 0.9967. The LOD was 1.28 x 10^{10} GC/mL and the LOQ was 2.56 x 10^{10} GC/mL with a linear range of about 3 logs. The AAV genome titer of the AAV8 test sample was 2.21x10^{12} GC/mL.

A new approach for AAV full, partial and empty capsid analysis

Since the capsid titer and the genome titer for the same AAV8 test sample were determined in the previous experiments, the percentage of the full capsids in this test sample was obtained by dividing the genome titer of 2.21 x 10^{12} GC/mL (value A) by the capsid titer of 2.65 x 10^{12} GC/mL (value B) and multiplying by 100, as shown in Figure 10. The percent of full capsids in this AAV8 sample was 83.4%. No additional experiments were needed. As shown in Figure 11, the percentage of full capsids obtained by CE was consistent with the values determined by two orthogonal technologies: transmission electron microscopy (TEM) and AUC.

Benefits of comprehensive AAV analysis of multiple CQAs on a single, multi-capillary CE system

As listed in Table 1, multiple CQAs must be characterized and monitored throughout the AAV manufacturing process to ensure the efficacy and safety of AAV-based gene therapy drugs. Currently, biopharma scientists often use multiple, different platforms for the analysis of all key AAV CQAs. As shown in

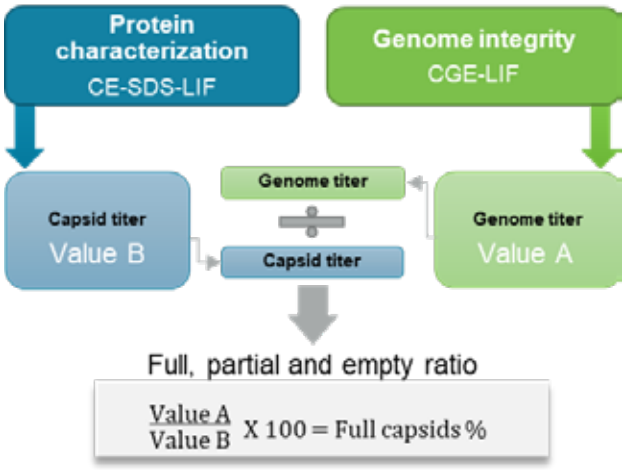


Figure 10. A simple calculation for determining the percentage of full capsids in an AAV sample. The percentage of the full capsids is calculated by dividing the genome titer (value A) by the capsid titer (value B), and multiplying by 100.

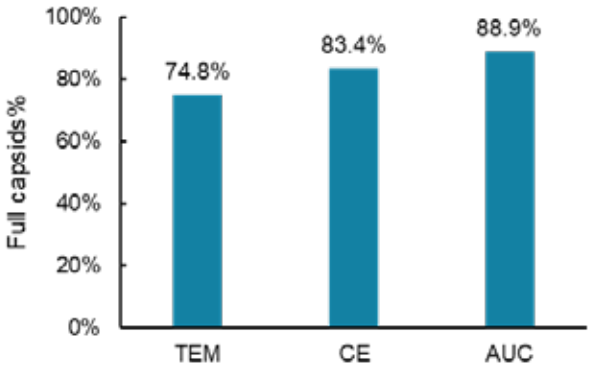


Figure 11. Comparison of the percentage of full capsids determined by three different technologies: TEM, CE, and AUC. The TEM and AUC results were obtained from the vendor of the AAV test samples.

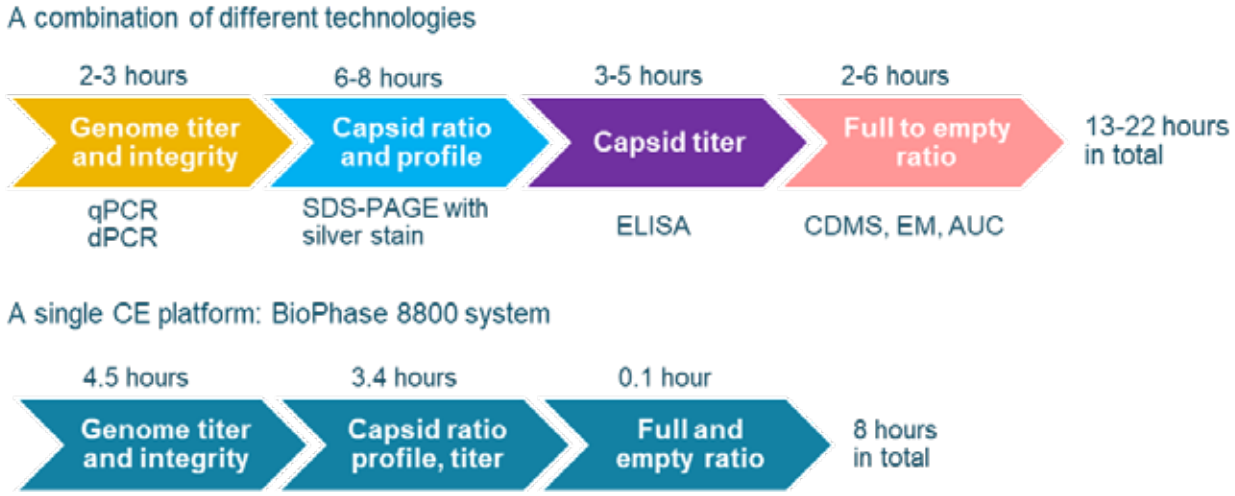


Figure 12. Comparison of the timeline for analysis of all key AAV CQAs per sample between different methods. Time for sample preparation is included. When a combination of 4 different technologies is used, the total amount of time needed for all analyses is 13 to 22 hours.[5-6] However, the same number of CQAs can be analyzed on the BioPhase 8800 system in 8 hours. Therefore, using the BioPhase 8800 system saves significant time compared to an approach using a combination of different platforms.

Figure 12, when other platforms are used, the total time for comprehensive analysis of all key AAV CQAs for an AAV sample would be 13-22 hours including sample preparation time. In addition, multiple laboratories might be needed to house instruments for different platforms. Analysts with different skill sets are also required to perform tests on different instruments. In contrast, a comprehensive analysis of all key CQAs can be done on a single platform, the BioPhase 8800 system, within 8 hours. With its tabletop design, the BioPhase 8800 system would not only save cost in equipment purchasing but also save laboratory space, enabling a streamlined quality test process with high efficiency.

Conclusions

- It is essential to characterize and monitor multiple CQAs to ensure the safety and efficacy of AAV drug products
- High-throughput, serotype-independent, comprehensive analysis of all key CQAs for the same AAV sample was achieved on a single CE platform within a single day
- Fast result generation for all methods was accomplished using the multi-capillary cartridge, saving time and costs
- High-resolution full, partial, and empty capsid analysis as part of a comprehensive set of analytical methods for AAV was achieved on the BioPhase 8800 system
- High-resolution, high-throughput gene therapy platform method development from discovery through quality control (QC) can all be carried out on the BioPhase 8800 system
- Platform methods on the BioPhase 8800 system with fast process times can shorten development timelines, save cost, streamline the QC process and accelerate time to market

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Determination of Full, Partial and Empty Capsid Ratios for Adeno-Associated Virus (AAV) Analysis

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SCIEX, Brea, CA

Introduction

Adeno-associated virus (AAV) is one of the most commonly used delivery vehicles (vectors) in gene therapy development. AAV is a combination of proteins (capsids) forming a closed shell encompassing a single-stranded DNA called a transgene.[1] Manufacturing of AAV vectors produces three types of capsids empty, partial, and full. Considered manufacturing impurities are empty and partial AAVs because they either lack the genomic material or contain only fragments of the genome. The desired product is the full AAV because it includes the full length of the desired genomic material. The presence of these impurities could affect the efficacy and safety of AAV vector products because of their risk of increased immunogenicity of the end product. Additionally, it can inhibit the transduction of full AAVs by competing for vector binding sites on the transfected cells.[2] Therefore, it is essential to determine the amount of these impurities (partial and empty AAVs), as well as the desired drug product (full AAV). Moreover, the ratio of full and empty is a critical quality attribute requirement for any AAV production process and quality control. There are multiple technologies currently being used to assess the full and empty ratio of AAV, such as analytical ultracentrifugation (AUC), transmission electron microscopy (TEM), to name a few. 3,4,5 However, these traditional methodologies have their own set of challenges hence driving the need for an orthogonal technique that is faster and easier to perform.

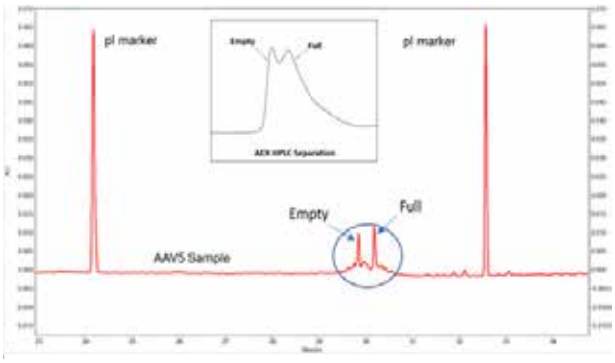


Figure 2. AAV serotype 5 separation. Left pane is cIEF; Right pane is AEX. E is Empty AAV and F is Full AAV.



Figure 1. The PA 800 Plus Pharmaceutical Analysis System

This technical note demonstrates a capillary isoelectric focusing (cIEF)-based method to separate AAV full and empty capsids and assess their ratio. The results shown demonstrate excellent resolution between full and empty AAV as well as potential partial capsid species allowing the assessment of their ratios. The results from this methodology correlate well with orthogonal approaches such as Anion Exchange-High Performance Liquid Chromatography (AEX-HPLC). However, AEX-HPLC provides less resolving power for these species and can struggle to quantify a smaller abundant peak vs. a closely eluting dominant peak.

Key Features

- A high resolution cIEF platform approach, with method optimization workflows for improved separation of AAVs of different serotypes with minimal pI differences(≤0.1 pH unit)
- cIEF profiles and pI values can be determined and used for AAV vector identification
- Provides rapid analysis time with less than 1 hour per sample compared to traditional methods such as AUC and EM which can take days
- This method offers a good correlation of full and empty AAV ratio with orthogonal technologies such as AEX-HPLC.

cIEF Experimental

Instrumentation

All cIEF experiments were performed using a PA 800 Plus Pharmaceutical Analysis System (SCIEX, Brea, CA) equipped with a UV detector and a 280 nm filter (P/N 969136), as shown in Figure 1. Data were collected and analyzed using 32 Karat™ Software. The capillary was N-CHO capillary (SCIEX, P/N 477601) with 20 cm effective length and 30.2 cm total length. The separations were performed at 20° C with the anode at the sample introduction end of the capillary.

Sample Preparation and cIEF Separation Solutions

The cIEF gel (P/N 477497) and cIEF peptide marker kit (PN A58481) were purchased from SCIEX. Broad and narrow range ampholytes were purchased from GE Healthcare. The solutions used in the preparation of the cIEF sample master mix, as well as the separation electrolytes, were prepared according to the cIEF application guide[6].

AAV Serotypes

Proprietary Serotype AAV Samples. A set of two AAV samples of proprietary serotype were used. Sample #1 is the sample with enriched empty capsids, while sample #2 is the sample with enriched full capsids. These two samples were concentrated by 20 times using Amicon Ultra 0.5mL Centrifugal Filters from EMD Millipore. Ten microliters of each AAV sample was mixed with 240 µL of Master Mix solution and transferred to sample vials for cIEF analysis.

AAV Serotype 5 Sample. An AAV5-CMV-GFP with a titer ~1X 1013 GC/mL (Cat# SL100819, Lot# AAV62019) from SignaGen Laboratories was used. The sample was prepared by mixing 3 µL of AAV5 with 24 µL of master mix and transferred to a nanoVial (SCIEX, P/N 5043467) for cIEF analysis.

AAV Serotype 8 (Full and Empty) Sample. Full and Empty AAV 8 samples were purchased from Vigene Biosciences (Lot# 2019.09.12). The titer of empty enriched packaged AAV8 of pAV-CMV-GFP sample was 5.10 X1012 GC/mL, and the titer of full enriched packaged AAV8 of pAV-CMV-GFP sample was 1.10 X1013 GC/mL. Three microliters of each AAV8 sample was mixed with 24 µL of master mix and transferred to a nanoVial (SCIEX, P/N 5043467) for cIEF analysis.

AEX-HPLC Experimental

The AAV5-CMV-GFP (Cat# SL100819, Lot# AAV62019) sample from SignaGen Laboratories was also analyzed using a CIMac SO3-0.1 AAV Analytical Column from BIA separations (PN 110.6157-1.3) on an ACQUITY UPLC H-class PLUS System from Waters Corporation for the AEX-HPLC analysis following the instruction of AAV Analytical Column[7] for AAV full and empty capsid ratio comparison of orthogonal technologies.

Results and Discussion

Existing Methods to Separate AAV Full and Empty Capsids

There are multiple methods to determine the ratio between the full and empty AAV. One such approach is determining the percentage of the full capsids in the total capsids by dividing the number of genome vectors derived from the existing qPCR data by the total capsid number obtained from the ELISA data.[8] However, this method lacks sufficient data accuracy and precision. Another way is to determine the protein and DNA content in the samples by spectrophotometric means using the optical density of AAV samples at 260 nm and 280 nm.[9] This approach is simple, rapid, and easy to operate. However, it requires high purity of the AAV sample to minimize the interference of the impurities with UV absorbance at 260 nm and 280 nm. A third alternative and perhaps the most complex is AUC.[3,] Indeed, AUC can separate full, partial, and empty AAVs. However, analysis is length, expensive, and it demands a large sample amount, data interpretation requires expertise, making this technique unattractive in a GMP environment. TEM is another often used technology in the industry, and it could reliably count the full and empty particles as a population.[4,5] However, it is challenging to distinguish the partial capsids, and it is time-consuming for data interpretation to meet the need for timely results required in quality control.

Perhaps the most popular analytical technique used for the quantitative determination of AAV full/empty is Ion exchange chromatography[10]. However, this method requires a large number of samples and is serotype- dependent. Furthermore, it could not distinguish between partial, full, and empty AAV. Additionally, it lacks the necessary resolving power between full and empty AAV imparing the quantitation of these species, resulting in inaccurate full and empty ratio. Mass spectrometry based approaches such as charge detection mass spectrometry (CDMS) has been recently shown to be able to separate full, partial and empty capsids.11 However, this system is not commercially available.

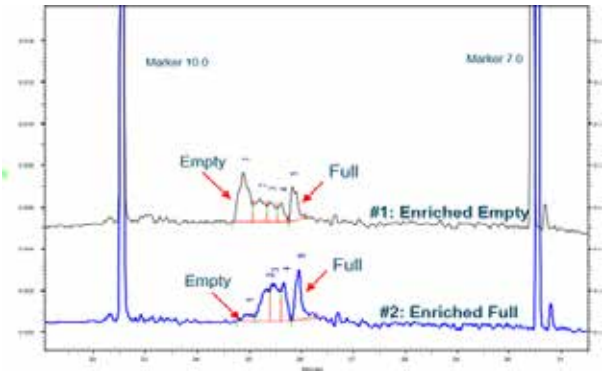


Figure 3. cIEF Results of AAV Samples of a Proprietary Serotype.

cIEF-Based Separation of AAV Full and Empty Capsids

The developed cIEF approach aims to efficiently separate the analytes by their pI (isoelectric point) values. The principle of the method is simple. The full capsids have pI values lower than their empty counterparts due to negative charges from the ssDNA they encapsulate. Therefore, our cIEF approach separates the AAVs and their undesired product-related impurities by taking advantage of their different isoelectric points.

Figure 3 shows the cIEF results of AAV samples of a proprietary serotype. Two samples of the same AAV product with different amounts of full and empty capsids were analyzed. Sample #1 was enriched with empty capsids, while sample #2 was enriched with full capsids. The cIEF profiles of the proprietary AAV samples mobilized between pI marker 7.0 and 10.0. The empty capsid peak is mobilized at a higher pI value while the full capsid peak at lower pI value. Meanwhile, some potential partial capsid peaks are mobilized between the peaks corresponding to the empty and full capsid. Notably, the cIEF profiles were consistent with the profiles obtained by analytical ultracentrifugation (data not shown).

The calculated pI difference between the full and empty AAV serotype 8 is about 0.1 pH units imposing a separation challenge. The broad range ampholytes failed to provide sufficient baseline resolution compromising the quantitation of the full and empty capsids. However, baseline resolution was achieved by using a mixture of broad and narrow range ampholytes. The dark blue circles in Figure 4, highlighted the empty and full capsid peaks of the AAV8 samples.

The single peak in front of the circle was identified as an impurity peak from one of the pI markers, since it was also observed in a blank injection with pI markers. Notably, higher intensity peaks corresponding to the empty capsid was observed in the empty capsid-enriched sample. In comparison, higher intensity of full capsid peaks were found in the full capsid-enriched sample as expected. Finally, we demonstrate in figure 4, that by expertly utilizing the narrow range ampholyte, it is possible to achieve excellent baseline resolution of the AAV full and empty capsids. This high-resolution cIEF method also may also give insights into the charge heterogeneity characteristics of AAVs. Indeed, multiple peaks were observed for the empty as well as full capsids of this set of AAV 8 samples, which could indicate the charge heterogeneity. Further experiments are needed to validate this claim.

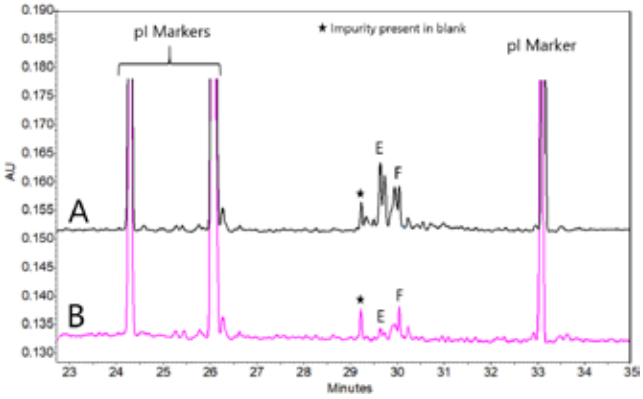


Figure 4. cIEF Results of AAV Serotype 8 Samples. **A** is AAV8 Empty(E) and **B** is AAV8 Full (F).

Distinct pI values for AAV identification

It is worth noting that the pI values of the AAV capsid peaks can be quantitatively determined based on the calibration curve of internal pI markers. The pI value of the AAV8 samples was approximately 7.1(pI value data not shown in Figure 4), while the pI of the AAV samples with proprietary serotype was about 9.0 (Table 1). These results demonstrate that this assay can potentially be used as an AAV vector identification method based on the profile and pI values.

Table 1. Calculated pIs of Separated Peaks Using cIEF Method in Figure 3.

	AAV sample #1		AAV sample #2	
ID	Peak #	pI	Peak #	pI
Empty	1	9.09	1	9.11
	2	8.95	2	8.99
Partial (possible)	3	8.92	3	8.92
	4	8.84	4	8.84
Full	5	8.73	5	8.73

Determination of AAV Full /Empty Capsids Ratio

The ratio of full/partial/empty capsids can be calculated based on the corrected peak areas of the separated capsid peaks in the cIEF electropherograms. The relative content of the full and empty capsids of AAV 8 samples separated in Figure 4, is summarized in Table 2.

Table 2. F/E AAV Determination of AAV8 Samples Separated in Figure 4.

	Empty	Full
AAV8 E SMP	*57%*	*43%*
AAV8 F SMP	*22%*	*78%*

Figure 2 shows the full and empty separation profile of AAV5 sample by both cIEF (left panel) and AEX- HPLC (right panel). As observed, the cIEF method nicely resolves the full from the empty, as well as the partial capsids, compared to the AEX-HPLC method, which showed poor resolution. Additionally partially filed AAVs were clearly observed in the cIEF methods, whereas in the HPLC method it was not. Hence to compare the orthogonality of these two analytical separation approaches, we included the Area % of both partial and full in cIEF and counted as total full AAV and compared with AEX-HPLC. Their absorbance at 280 nm for both technologies was also evaluated for comparison. The results in Table 3, reveals that the ratio of the full and empty capsids determined by cIEF correlates well with that of the AEX-HPLC method.

Table 3. Comparison of AAV5 Full and Empty Capsids Determination Using cIEF and AEX-HPLC Methods.

	Empty	Full *
cIEF	*33%*	*67%*
AEX-HPLC	*31%*	*69%*

**** It is the sum of full and partial peaks for cIEF***

It is worth noting that absorbance at 280 nm of AAV capsids, is prone to over-estimation due to the contribution of extra UV absorbance of the genetic materials in full capsids. Hence a correction factor using the molar extinction coefficients of the full and empty capsids at different wavelengths is needed. For comparative analysis with other orthogonal techniques such as TEM and AUC, using this correction factor for cIEF analysis will improve the accuracy for the quantitative determination of full capsids for the various serotypes.

Conclusions

This technical note demonstrates a cIEF based method for the separation and analysis of AAV full and empty capsids amenable to optimization to different serotypes. The pI profiles can be determined and used for AAV identification. The utilization of the optimal mixture of wide and narrow pH range ampholytes can efficiently improve the separation of AAV samples with small pI differences between full, partial and empty capsids. The sample analysis time for this method is fast, less than 1 hour per sample. The analysis is performed on a well validated and an automated cIEF based platform to obtain reliable and reproducible results across serotypes.

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毛细管等电聚焦方法（ cIEF ）测定腺相关病毒（ AAV ）的等电点

The Detection of Isoelectric Point for Adeno-Associated Virus (AAV) by Capillary Isoelectric Focusing (cIEF) Method

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Keywords： capillary isoelectric focusing(cIEF); adeno-associated virus(AAV); PA 800 Plus

1. 前言

腺相关病毒（Adeno-Associated Virus，AAV）是无包膜的单链线状DNA病毒，由结构蛋白（VP1、VP2、VP3）和遗传DNA构成，属于复制缺陷性的病毒。由于其具有宿主范围广、非致病性、低免疫原性、长期稳定表达外源基因、良好的扩散性能和物理性质稳定等优点，被广泛认为是介导基因治疗的理想载体之一。根据AAV结构特点，整个病毒颗粒的衣壳表面暴露的氨基酸侧链决定了AAV具有特定的等电点（Isoelectric Point，pI）。根据AAV等电点的差异可判别AAV包装的完整性（通常完整包装的AAV等电点低于空心载体等电点）。在成品控制和纯化AAV的工艺中，可利用毛细管等电聚焦（Capillary Isoelectric Focusing，cIEF）检测其等电点。

毛细管等电聚焦是利用中空的毛细管通道进行等电点测定的方式，其优势如下：

1) 中空毛细管可以进行完整病毒颗粒的测定，而不会引起堵塞或污染毛细管的情况；

2) 具有较长的分离距离（30 cm），可得到更优的分离度；

3) 利用3个等电点标准品进行等电点测定，等电点结果更准确。

在毛细管等电聚焦方法中决定样品分离度的主要参数是尿素胶中尿素浓度和两性电解质的选择。在本技术说明通过优化尿素和两性电解质进行了AAV等电点的测定。

2. 试剂耗材

2.1 试剂和耗材

氢氧化钠（Sigma-Aldrich, PN S5881），磷酸（Sigma-Aldrich, PN 345245），醋酸（Sigma-Aldrich, PN A6283），精氨酸（Sigma-Aldrich, PN A5006），亚氨基二乙酸（Sigma-Aldrich, PN 220000），尿素（Amresco, PN 0568），3-10两性电解质（GE Healthcare，PN 17-0456-01），8-10.5两性电解质（GE Healthcare, PN 17-0455-01），cIEF Gel（SCIEX, PN 477497），等电点标准品（SCIEX, PN A58481），二次去离子水（Millipore），N-CHO涂层毛细管（SCIEX, PN 477601），10 kDa超滤管（Millipore, PN UFC501096）。

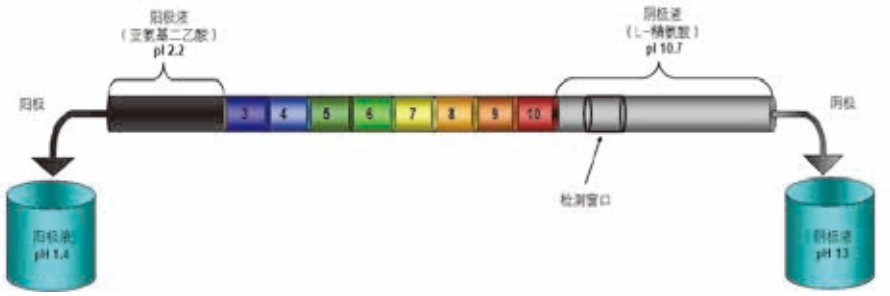


图1. 毛细管等电聚焦的原理示意图。

2.2 试剂的配置

根据SCIEX毛细管等电聚焦分析试剂盒的说明书，进行试剂的配置：200 mmol/L磷酸（阳极液）、300 mmol/L氢氧化钠（阴极液）、350 mmol/L醋酸（化学迁移液）、500 mmol/L精氨酸（阴极稳定液）、200 mmol/L亚氨基二乙酸（阳极稳定液）、4.3 mmol/L尿素（毛细管冲洗液）、尿素胶（尿素浓度根据优化条件进行配置）。

2.3 样品及前处理方法

腺相关病毒（adeno-associated virus, AAV）由国内某生产企业提供。样品的浓度为0.02 mg/mL，利用10 kDa超滤管进行样品浓缩至1.4 mg/mL。

根据SCIEX毛细管等电聚焦分析试剂盒的说明书，选择10.0、9.5和7.0的等电点标准品，配置可容纳多个样品的预混液，提高实验的重现性。如表1，再将10 µL样品添加至一份预混液中，充分涡旋混合。尿素胶中尿素浓度和两性电解质根据优化条件进行配置。

表1. 毛细管等电聚焦实验中样品预混液的配置。

试剂	多个样品（n）所需体积（µL）
尿素胶	200×（n+1）
两性电解质	12×（n+1）
阴极稳定液	20×（n+1）
阳极稳定液	2×（n+1）
等电点标准品（10.0）	2×（n+1）
等电点标准品（9.5）	2×（n+1）
等电点标准品（7.0）	2×（n+1）

3. 实验

3.1 实验参数

仪器：PA 800 Plus；毛细管：50 µm I.D. N-CHO涂层毛细管，20/30.2 cm（有效/总长度）；毛细管温度：20 ℃；样品储存温度：10 ℃；进样条件：25 psi，99 s；聚焦条件：25 kV，15 min；迁移条件：30 kV，30 min；检测器及波长：紫外检测器（UV），280 nm。

3.2 尿素浓度优化

固定样品预混液中的其它试剂，进行了尿素胶中尿素含量的优化，分别配置0、1、2、3、4 mol/L尿素胶，进行样品预混液的配置。

3.3 两性电解质优化

根据样品等电点的大致范围，选择3-10和8-10.5的两性电解质进行优化，3-10和8-10.5两性电解质的混合比例为0:1；1:3；1:1；3:1；1:0。

4. 结果

4.1. 尿素浓度的优化

通常溶液pH等于蛋白的等电点时，蛋白稳定性较差，容易发生析出沉淀现象。在样品预混液中添加尿素，可促进蛋白在毛细管等电聚焦实验中的溶解，使样品稳定迁移至毛细管窗口并进行检测。此次实验优化中，尝试了0-4 mol/L的尿素浓度，结果如图2；可见在3 mol/L尿素浓度下，可将AAV分为较明显的两个组份，且响应值良好，因此在毛细管等电聚焦实验中，选择3 mol/L尿素进行AAV的分析。

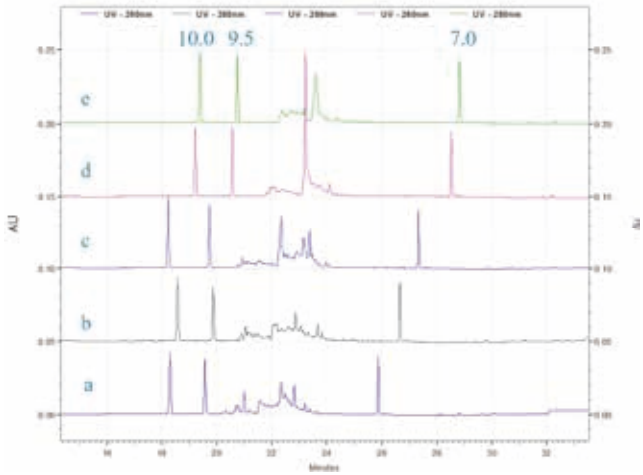


图2. 不同尿素浓度的实验结果（a: 0 mol/L; b:1 mol/L; c: 2 mol/L; d: 3 mol/L; e: 4 mol/L）。

4.2 两性电解质的优化

两性电解质的范围和浓度会影响蛋白的分离，将3-10和8-10.5范围的两性电解质以不同比例混合，进行分离度的优化。此次实验中，尝试了3-10和8-10.5共5个比例的混合，分离结果如图3；随着8-10.5两性电解质的添加，AAV的分离度有改善，但8-10.5两性电解质含量过高会影响AAV的迁移。结果中可发现3-10和8-10.5两性电解质比例在3:1条件下对AAV的分离最优。

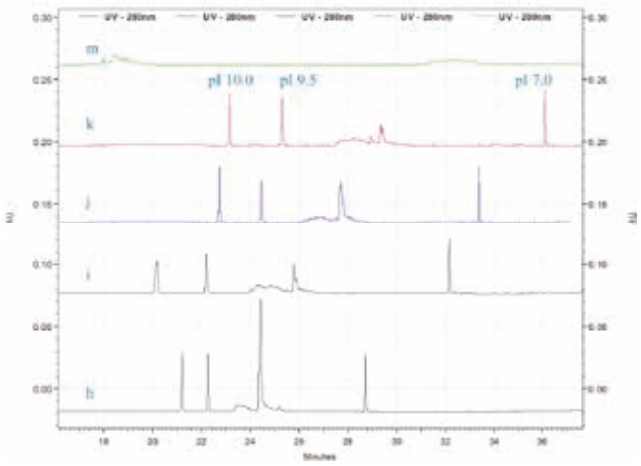


图3. 不同两性电解质的实验结果（3-10 :8-10.5为h: 1:0; i : 3:1; j: 1:1; k: 1:3; m: 0:1）。

4.3 在优化条件下对AAV的等电点测定

在最优条件下（3 mol/L尿素，3-10与8-10.5两性电解质为3:1），对AAV样品进行了等电点测定，如图4。由于AAV包装的不完整性，样品可能存在完整包装、未包装遗传物质及包装部分遗传物质的病毒颗粒，这些病毒颗粒的等电点可能由于遗传物质是否完整存在不同的等电点。在此毛细管等电聚焦条件下，AAV可分为5个峰，其中5号峰含量最高，如表2。

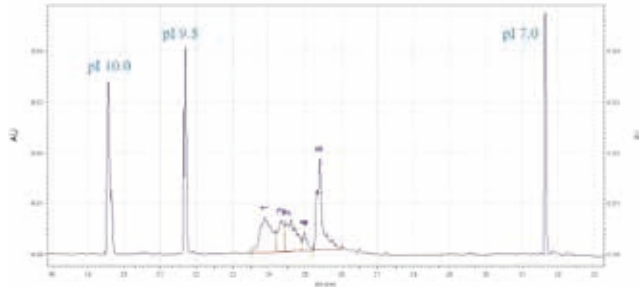


图4. 最优实验条件下对AAV等电点的测定。

4.4 方法重复性的考察

在最优条件下考察了方法的多针重复性（n=5），如图5。以含量最高的峰为主峰（Main Peak, MP），迁移时间和峰面积百分比的RSD值分别为0.20%和1.34%，如表5。

表2. 最优条件下AAV的等电点测定。

峰	等电点	峰面积%
1	8.89	27.86
2	8.78	12.45
3	8.74	20.97
4	8.62	5.08
5	8.50	33.65

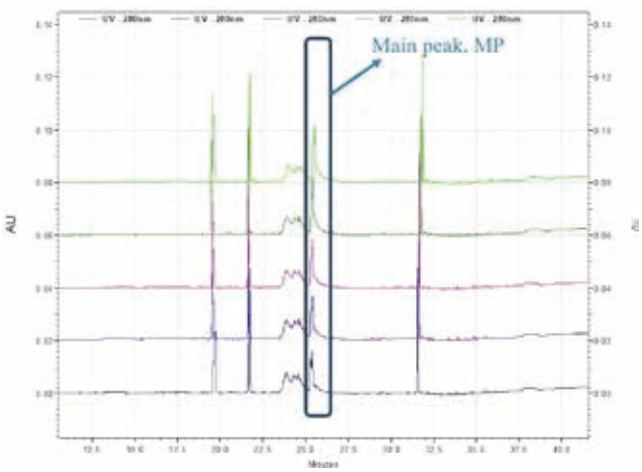


图5. 最优方法下的重复性考察。

表5. 重复性考察中等电点范围及主峰等电点和主峰面积%的RSD。

n=5	等电点范围	主峰等电点	主峰面积%
n1	8.54-8.95	8.54	32.59%
n2	8.51-8.91	8.51	32.95%
n3	8.50-8.89	8.50	33.64%
n4	8.50-8.89	8.50	33.56%
n5	8.50-8.90	8.50	32.96%
RSD	0.20%	1.34%	

5. 总结

毛细管等电聚焦方法可对完整病毒颗粒进行等电点测定，本技术说明通过优化尿素浓度和两性电解质，确定了在尿素浓度为3 mol/L、两性电解质为(3-10):(8-10.5) =3:1的条件下，可实现对AAV的等电点分析。所分析AAV样品的等电点范围为8.50-8.89，在方法重复性考察中迁移时间和峰面积百分比的RSD分别为0.20%和1.34%。方法稳定可靠，可用于AAV的等电点测定。

Genome Sizing Methodology for Transgene Integrity Analysis of Adeno-Associated Viruses (AAV)

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Introduction

Adeno-associated virus (AAV) is a small (25-nm) virus that is composed of a non-enveloped icosahedral protein shell called capsid and a single-stranded DNA genome of about 4.7 kb. With its excellent safety profile and high efficiency in transducing a broad range of target tissues, AAV vector has become an attractive choice for gene therapy.[1] Two recombinant AAV (rAAV) based drugs have been approved by the FDA: Zolgensma by AveXis for spinal muscular atrophy and Luxturna by Spark Therapeutics for inherited blindness. Many more are in clinical trial. One of the most commonly used methods for the production of rAAV vectors is the triple-transfection method which involves co-transfection of permissive cells such as HEK293 cells with three plasmids: one containing the transgene of interest flanked by the AAV inverted terminal repeats (ITRs), a packaging plasmid containing rep and cap genes, and a third plasmid encoding adenoviral helper genes. Gradient ultra-centrifugation is often used for purification of packaged rAAV vectors (Full capsids) from cellular debris contaminants, host cell DNA and RNA as well as Empty AAV capsids. However, remnants of contaminants can still be present in the viral vector product after purification. In addition, errors made in encapsidation of the intact genome containing the transgene during rAAV production can lead to heterogeneous

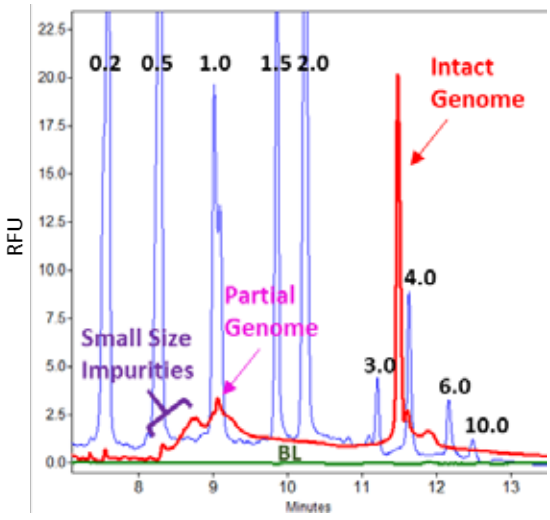


Figure 2: Genome Size Analysis of an AAV8 Sample. Blue trace: RNA size standards with sizes in kilobases marked in black numbers; Red trace: AAV8 sample; Green trace: blank control.



Figure 1. The PA 800 Plus Pharmaceutical Analysis System (A) and the EZ-CE Cartridge (B).

populations with both intact genome and partial or smaller than unit-length genomes. The quality of Full capsids has a direct impact on the efficacy of the treatment including outcome of both preclinical and clinical studies. Therefore, it is crucial to accurately assess the quality and the correct length/size of the genome encapsidated in the vector. Verification of genome size has been traditionally done by denaturing agarose-gel electrophoresis and southern blot.[2] Both are time consuming and with limited resolution on size determination. In this technical note, we describe the development of novel CE-LIF based methods for accurate determination of size purity of AAV genome. Our results indicate that these methods are capable of detecting intact and partial AAV genome as well as small size impurities.

Key Features

- Excellent separation resolution between intact and partial AAV genomes from small size impurities
- Sample separation is complete within 15 minutes, compared to hours to days with denaturing agarose gel and multiple hours with southern blot
- Quick assessment of AAV genome size purity can be achieved in 30 minutes through a simplified, screening workflow, compared to hours with denaturing agarose gel or southern blot
- A more comprehensive workflow that provides a more definitive answer
- Both the screening and comprehensive workflows are suitable for multiple AAV serotypes

- Excellent separation repeatability with CVs of less than 0.5% for migration time

Methods

Materials: The LIF Performance Test Mix (PN: 726022), Nano vials (PN 5043467) and pre-assembled EZ-CE Capillary Cartridge (PN A55625, Figure 1B) were from SCIEX, Framingham, MA. Urea (PN 29700), Nuclease-free water (PN AM9932), SYBR Green II RNA gel stain, 10,000x concentrate in DMSO (PN S7564), 10x DNase I buffer (PN AM8170G) were obtained from Thermo Fisher Scientific, Waltham, MA. Polyvinyl-pyrrolidone (PVP, PN 437190), benzonase (PN E1014-5KU), 0.5 M EDTA, pH 8.0 (PN E7889-100ML), Transcript RNA markers 0.2-10 kb (PN R7020) and 10x Tris Borate EDTA (TBE) buffer (PN 574795), Molecular Biology Grade, Amicon Ultra-0.5 centrifugal filter unit with MW cut off of 100 kDa (PN UFC510024) were from Millipore Sigma, St. Louis, MO. The 5 µm syringe filter (PN 4650) was from PALL Corporation, Port Washington, NY. Rainin LTS filter tips were from Mettler Toledo, Oakland, CA. QIAquick PCR purification Kit (PN 28104) and Proteinase K (PN 19131) were from Qiagen, Germantown, MD. Packaged AAV8 and AAV formulation buffer (1X PBS with 0.001% Pluronic F68) were from Vigene Biosciences, Rockville, MD. AAV5 and AAV2 were from Signagen, Rockville, MD.

Instrument and Software: A PA 800 Plus Pharmaceutical Analysis System (Figure 1A) equipped with LIF detector and solid-state laser with excitation wavelength at 488 nm and a 520 nm band pass emission filter were from SCIEX, Framingham, MA. Data acquisition and analysis were performed using 32 Karat™ Software 10.3.

Preparation of the Separation Buffer: To make 100 mL of the separation buffer, 1 g of PVP was added to 60 mL nuclease-free water in a 250 mL glass beaker and allowed to sit at room temperature for 10 minutes, the solution was swirled gently to help PVP to be completely dissolved. Then, 24 g of urea was added. The solution was mixed with a clean stirring bar for about 20 minutes without heating. After urea was completely dissolved, 10 mL of the 10x TBE buffer was added. After one more minute of stirring, nuclease-free water was added until the total volume was 100 mL. The separation buffer contained 1% PVP, 1x TBE, pH 8.3 and 4 M urea. This buffer should be good for one month if stored at 2ºC to 8ºC in 10 mL aliquots. Before sample run, the required aliquot of buffer was warmed up to room temperature and filtered through a 5 µm filter. SYBR Green II dye was added at a 1 to 25,000 dilution. About 7.5 mL of this dye-containing buffer was used for each set of 8 injections.

LIF Calibration: To ensure consistent response of LIF detector throughout this study, the LIF detector was calibrated using LIF Calibration Wizard and Performance Test Mix (PN 726022) following the instructions in the PA 800 Plus System Maintenance Guide (PN A51964).

Sample Storage: AAV samples and the Sigma Transcript RNA Markers were aliquoted at 5 to 20 µL upon first thawing and stored at -80ºC freezer to avoid multiple freeze-thaw cycles.

Sample Preparation for AAV Genome: Filter tips were used for all steps to minimize unintended nucleic acid degradation. Nucleic acid impurities in AAV samples were digested in a 20 µL reaction that contained 10 µL sample, 7 µL of AAV formulation buffer, 2 µL of 10x DNase I buffer and 1 µL of benzonase that was diluted 10 fold in 1x DNase I buffer (10mM Tris-HCl, pH 7.5 at 25°C, 2.5 mM $MgCl_2$, 0.1 mM $CaCl_2$). The digestion was carried out at 37°C for 30 minutes and terminated by addition of 2 µL of 50 mM EDTA and 278 µL of AAV formulation buffer. The benzonase and degraded nucleic acid were removed by using an Amicon centrifugal filter unit with 100 kDa cutoff. The filter unit was prewetted by centrifuging 400 µL of AAV formulation buffer through it at 14,000 x g for 2 minutes in a microfuge followed by decanting the filtrate and any residual buffer inside the filter. After benzonase treated AAV sample was loaded, the filter was centrifuged at 14,000 x g for 2 minutes. The concentrated sample was collected by inverting the filter unit in a clean collection tube and centrifuged at 1,000 x g for 2 minutes. AAV formulation buffer was added to the concentrated sample to increase the total volume to 95 µL. AAV capsids were disassembled by adding 5 µL of 20 mg/mL Proteinase K and incubated at 37°C for 2 hours. The AAV genome released from AAV capsids was purified using QIAquick PCR purification Kit following manufacturer's instructions except the columns were washed twice. The AAV genome sample was eluted from the column using 30 µL of nuclease-free water. Ten microliters of the eluted AAV genome sample was heated at 70ºC for 2 minutes, followed by 5 minutes on ice before transferring to a Nano vial and analyzed on a PA 800 Plus System. Any deviation from this procedure is described in results section.

Sample Preparation for RNA Markers: The Sigma Transcript RNA markers were diluted in nuclease-free water to 1 ng/µL, heat-treated and loaded onto the instrument as described above for the AAV genome sample.

Preparation of Buffer Trays and Sample Trays: Vial positions for buffer trays are indicated in Figure 3. All solutions were pipetted with filter tips. Each "NF Water" vial was filled with 1.5 mL nuclease-free (NF) water. Waste vial was filled with 1 mL NF water. "Sep Buffer" and "TBE Buffer" vials were filled with 1.5 mL of separation buffer containing SYBR Green II dye or 1x TBE.

Instrument Setup: Instrument set up is shown in Figures 4-8.

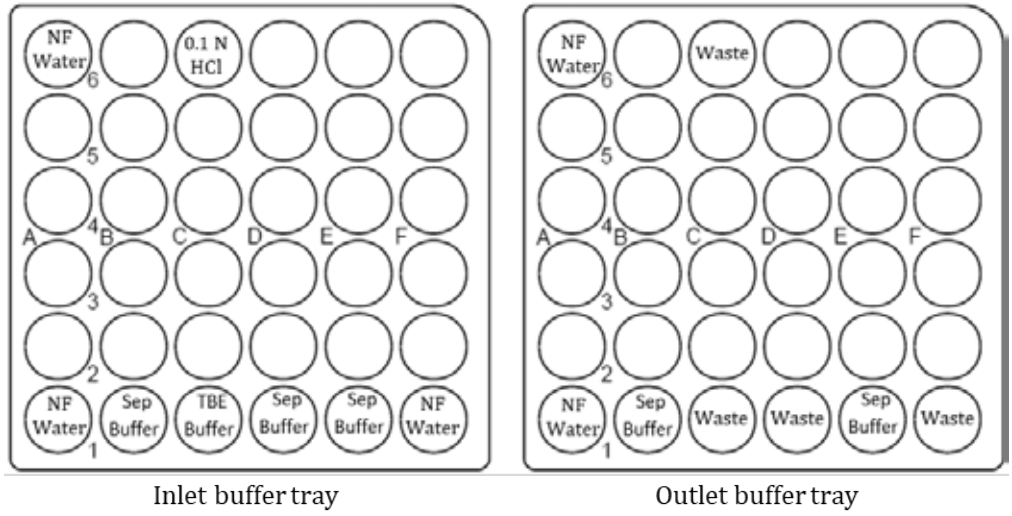


Figure 3. Buffer Tray Setup. Schematic setup for Inlet (left) and Outlet (right) buffer trays for conditioning, shut down and up to 8 sample injections.

The Initial Conditions and LIF Detector Initial Conditions were the

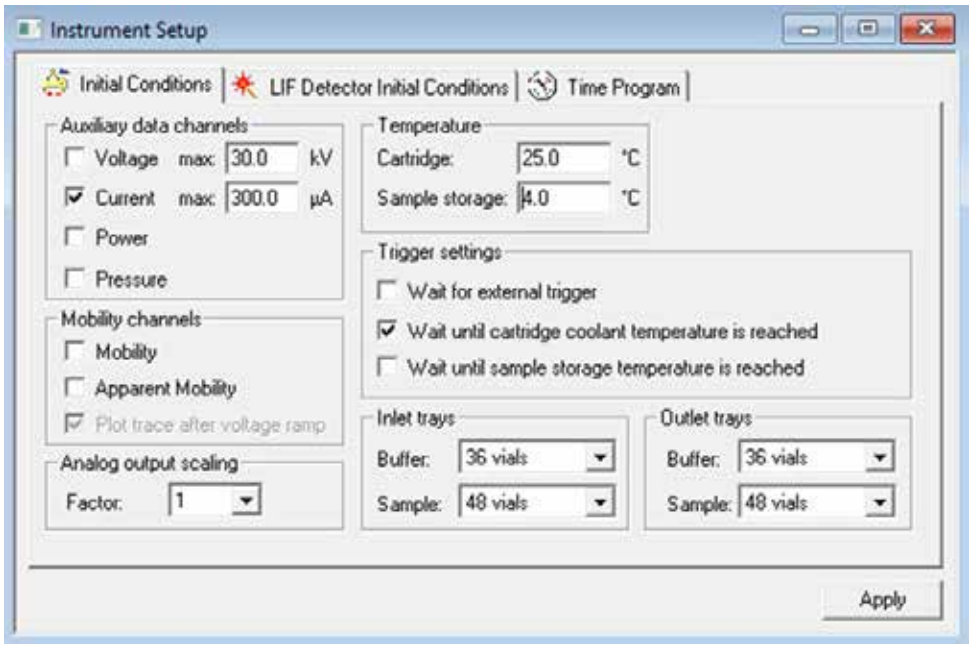


Figure 4. Settings for “Initial Conditions” Tab.

same for the Conditioning, Separation and Shut Down methods.

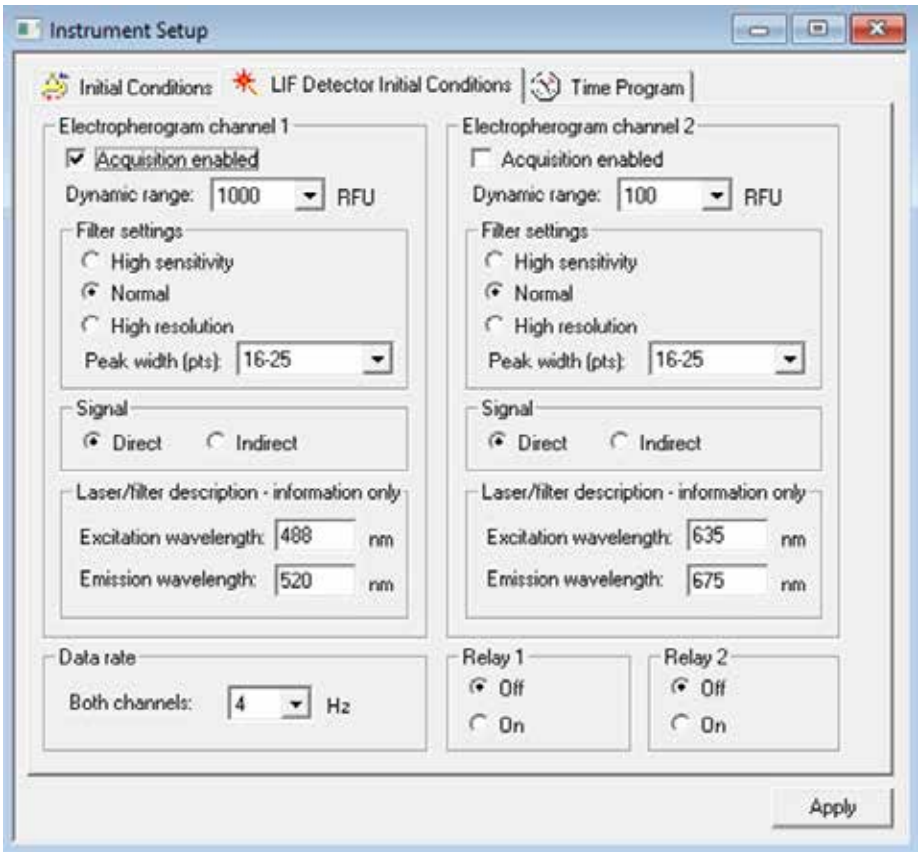
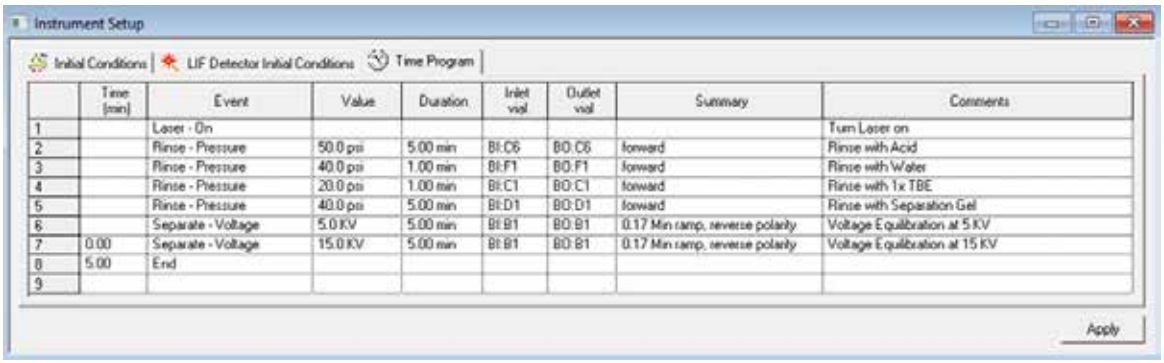


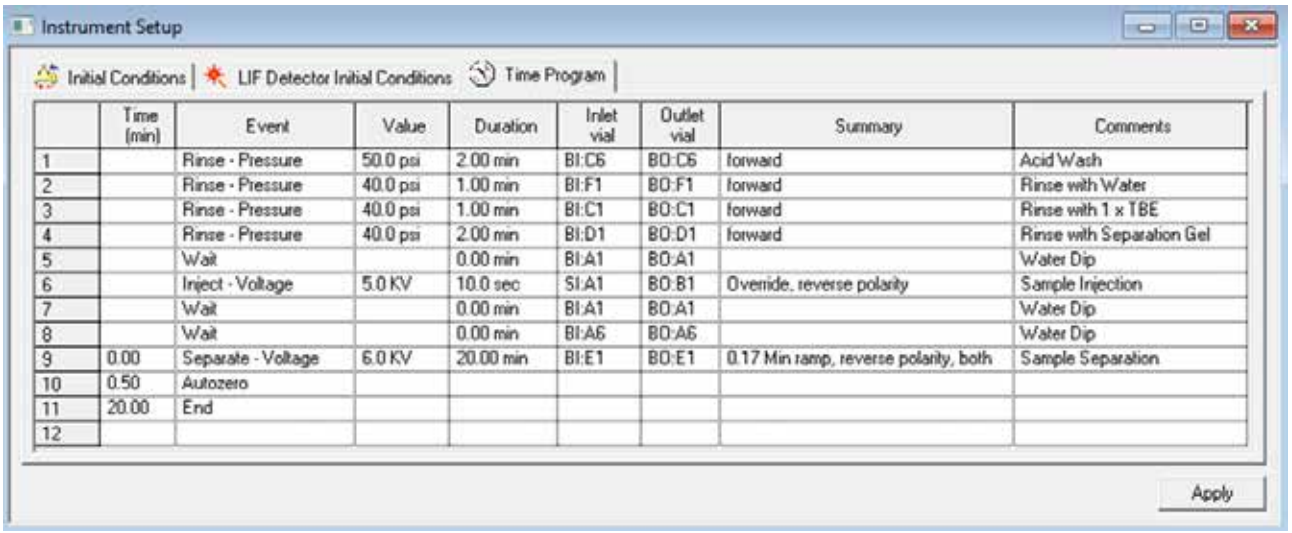
Figure 5. Settings for “LIF Detector Initial Conditions” Tab.



	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1		Laser - On						Turn Laser on
2		Rinse - Pressure	50.0 psi	5.00 min	BI:C6	BO:C6	forward	Rinse with Acid
3		Rinse - Pressure	40.0 psi	1.00 min	BI:F1	BO:F1	forward	Rinse with Water
4		Rinse - Pressure	20.0 psi	1.00 min	BI:C1	BO:C1	forward	Rinse with 1x TBE
5		Rinse - Pressure	40.0 psi	5.00 min	BI:D1	BO:D1	forward	Rinse with Separation Gel
6		Separate - Voltage	5.0 KV	5.00 min	BI:B1	BO:B1	0.17 Min ramp, reverse polarity	Voltage Equilibration at 5 KV
7	0.00	Separate - Voltage	15.0 KV	5.00 min	BI:B1	BO:B1	0.17 Min ramp, reverse polarity	Voltage Equilibration at 15 KV
8	5.00	End						
9								

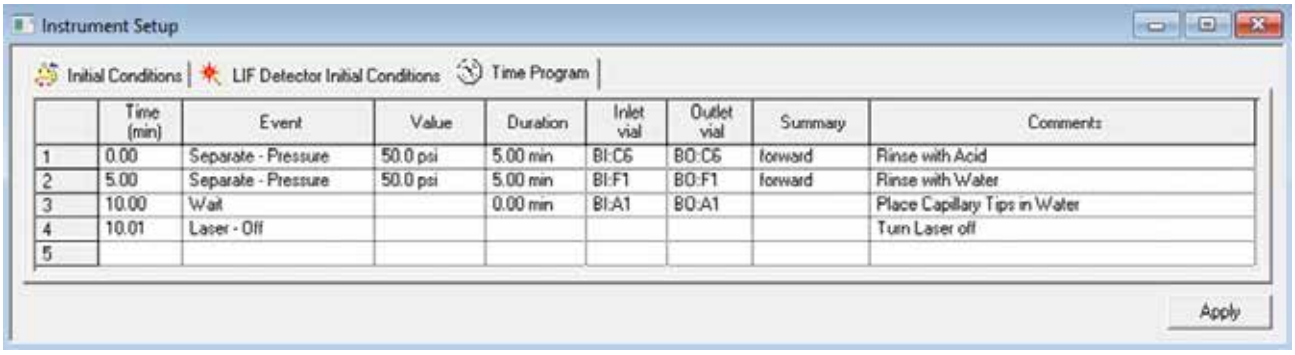
Figure 6. Time Program Settings for the Conditioning Method.

small size impurities between 0.5 to 1 kb were also detected. In



	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1		Rinse - Pressure	50.0 psi	2.00 min	BI:C6	BO:C6	forward	Acid Wash
2		Rinse - Pressure	40.0 psi	1.00 min	BI:F1	BO:F1	forward	Rinse with Water
3		Rinse - Pressure	40.0 psi	1.00 min	BI:C1	BO:C1	forward	Rinse with 1 x TBE
4		Rinse - Pressure	40.0 psi	2.00 min	BI:D1	BO:D1	forward	Rinse with Separation Gel
5		Wait		0.00 min	BI:A1	BO:A1		Water Dip
6		Inject - Voltage	5.0 KV	10.0 sec	SI:A1	BO:B1	Override, reverse polarity	Sample Injection
7		Wait		0.00 min	BI:A1	BO:A1		Water Dip
8		Wait		0.00 min	BI:A6	BO:A6		Water Dip
9	0.00	Separate - Voltage	6.0 KV	20.00 min	BI:E1	BO:E1	0.17 Min ramp, reverse polarity, both	Sample Separation
10	0.50	Autozero						
11	20.00	End						
12								

Figure 7. Time Program Settings for the Separation Method.



	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1	0.00	Separate - Pressure	50.0 psi	5.00 min	BI:C6	BO:C6	forward	Rinse with Acid
2	5.00	Separate - Pressure	50.0 psi	5.00 min	BI:F1	BO:F1	forward	Rinse with Water
3	10.00	Wait		0.00 min	BI:A1	BO:A1		Place Capillary Tips in Water
4	10.01	Laser - Off						Turn Laser off
5								

Figure 8. Time Program Settings for the Shut Down Method.

Results and Discussions

Analysis of AAV Genome of Different Sizes: Packaged AAV8 of pAV-CMV-GFP (Vigene) and AAV5 of pAAV-CMV-GFP (Signagen) were treated with proteinase K to release the AAV genome.[3] Nucleic acids purified with QIAquick from these samples were analyzed by CE-LIF on a PA 800 Plus Pharmaceutical Analysis System with a sample injection condition of 5 kv for 3 seconds. In Figure 9, the red trace was obtained with the RNA size standard. The blue trace was obtained with the AAV8 genome sample at 5 µL with a titer of 1.10 x 10^{13} GC/mL. The intact genome (2.8 kb) as well as the addition, a small peak slightly larger than 2.8 kb was present.

This may be the same intact genome but with secondary structure. There is also a shallow peak larger than 10 kb. Further experiments need to be conducted to determine if this was due to a multimeric AAV genome. The green trace was obtained with the AA5 genome sample at 5 µL with a titer of 1 x 10^{13} GC/mL. The intact genome (2.35 kb) as well as the small size impurities between 0.5 to 1 kb were detected. The size of short impurities in AAV5 samples was smaller than those in AAV8 samples. This might be related to the differences in production processes for making these two samples. For example, different amounts of nucleases can be used in treating the cell lysate. In addition, there can be differences in how the AAV Full capsids were

collected at the end of the ultra-centrifugation procedure. It is important to note that the RNA size standards migrate slower

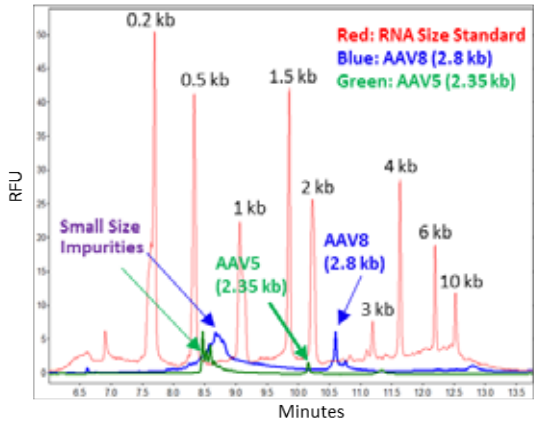


Figure 9: Analysis of AAV Samples of Different Genome Sizes.

than the single stranded AAV genome of the same sizes. This is due to differences in base composition in these nucleic acid fragments as well as the differences related to ribose in RNA versus deoxyribose in single stranded DNA.

Benefit of Benzonase Treatment: Small size impurities were present in AAV samples as shown in Figure 9. These impurities could be degraded nucleic acids from the host cell and from the plasmid vector that were not well separated from the Full AAV capsids. Benzonase is an unselective endonuclease that attacks and degrades all forms of DNA and RNA (single stranded, double stranded, linear and circular) down to oligonucleotides that are 2–5 bases in length. An Empty AAV8 sample produced as a by-product during the production of a packaged AAV8 containing an intact genome size of 4.5 kb was obtained from Vigene. This Empty AAV8 sample at 10 µL with a titer of 1.06 x 10^{13} GC/mL was treated with benzonase and filtered through an Amicon Ultra-0.5 centrifugal filter unit with MW cut off of 100 kDa to remove benzonase as well as degraded nucleic acid species. The filtrate was also collected. As a control, another Empty sample at 10 µL that was not treated with benzonase was also applied to the Amicon filter. The filtrate was also collected. Since Empty AAV samples often contain a low amount of Full AAV capsids, the Empty samples were treated with Proteinase K after they were collected from the Amicon filter device. Nucleic acids from the two Empty samples (with or without benzonase treatment) and the two filtrate samples were purified and analyzed on a PA 800 Plus System. Results in Figure 10 demonstrated that the amount of small size impurities was significantly reduced in the benzonase treated sample than in sample not treated with the enzyme. In addition, a significant amount of small size impurities was present in the filtrate from the Empty AAV sample not treated with benzonase. In contrast, much lower amount of these impurities was present in filtrate from the benzonase treated Empty AAV sample indicating that the benzonase was effective in degrading the small size impurities outside of the capsids. As expected, a small amount of intact AAV genome shown by the red arrow was detected in the two empty AAV samples regardless of benzonase treatment. However, it was not detected in the two filtrate samples.

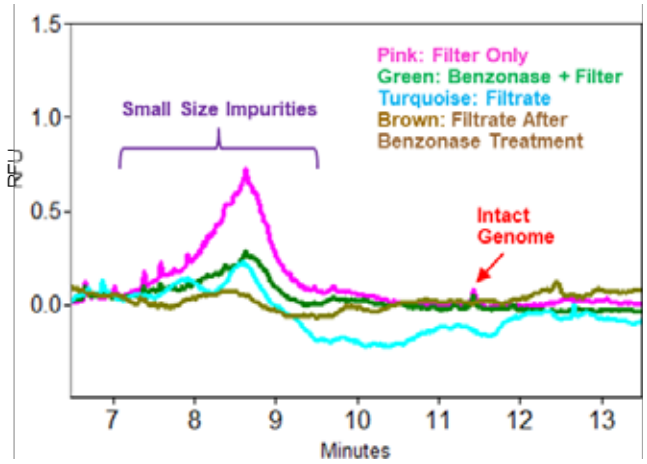


Figure 10: Benzonase Treatment. Use of benzonase treatment led to decrease in small size impurities in an empty AAV8 sample. Sample injection: 5 kv, 3 seconds.

Separation of Partial AAV Genome from the Intact AAV Genome: As shown in Figure 9, the intact AAV genome and small size impurities were detected in two AAV samples with relatively smaller intact genome sizes: AAV8 (2.8 kb) and AAV5 (2.35 kb). No partial genome was detected. An AAV8 sample with a larger intact genome size (4.5 kb) was tested next. In this experiment, both Full and Empty AAV8-4.5 kb samples at 10 µL with a titer of 1.06 x 10^{13} GC/mL were treated with benzonase to degrade nucleic acid impurities outside of the capsids. After benzonase digestion, samples were filtered with Amicon filter, treated with protein K before nucleic acid extraction and separation on a PA 800 Plus System. Samples were injected at 5 kv for 3 seconds. As a control, another pair of Full and Empty AAV8-4.5 kb samples were processed in the same way as described above but without benzonase treatment. Results obtained with these four samples were shown in Figure 11. The peak for the intact genome was present in all 4 samples. However, the peak height was much higher in the two Full samples than in the two Empty samples. The corrected peak areas for the intact genome peak were 10944 and 9656 in Full samples treated with or without benzonase, and 571 and 573 in Empty samples treated with or without benzonase. Therefore, this analysis method has the potential to be used as a method for titer determination upon further development. In addition to the intact genome peak, the two Full samples also had a peak

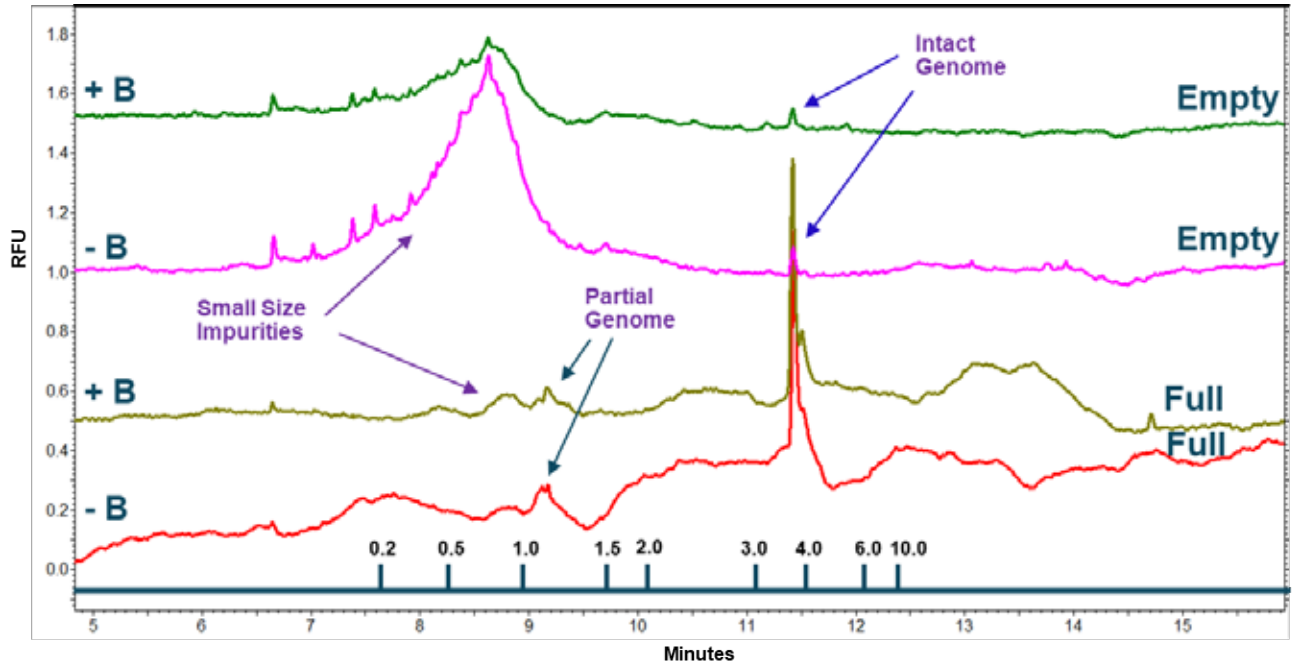


Figure 11: Analysis of Full and Empty AAV8-4.5 kb Samples on a PA 800 Plus System. Samples were treated with (+B) or without (-B) benzonase, passed through Amicon filter followed by nucleic acids extraction and separation on a PA 800 Plus System. The titer for the Full and empty sample was 1.06 x 10^{13} GC/mL. The vertical blue bars represent the migration time positions of RNA size standards with their sizes indicated in kilobases.

that is slightly larger than 1 kb. This could be the partial genome peak (1.3 kb) without the transgene. Sequencing or restriction fragment analysis will be needed to confirm this hypothesis. The small size impurity peak is minimal in both Full samples indicating that these Full samples were of high purity. As expected, the small size impurity peak in Empty sample without benzonase treatment (pink trace) was significantly higher than in the one treated with the enzyme (green trace). In Figure 2, the Full AAV 8-4.5 kb samples treated with benzonase and Proteinase K was injected at 5 kv for 10 seconds. The intact genome, partial genome and low amount of small size impurities were detected (red trace) The signal to noise ratio for the partial genome peak was 8.10. The green trace was obtained with nuclease-free water that went through the entire process of benzonase and proteinase K treatment as well as the nucleic acid purification using QIAquick PCR purification kit. Good baseline was obtained with this blank control.

Confirmation of Presence of Partial AAV Genome in Sample Not Treated with Benzonase and Filtering: The partial genome peak observed in Figure 11 was relatively small. In order to confirm this partial is a true peak, another experiment was performed with the Full AAV8-4.5 kb sample at 10 µL with a titer of 1.06 x 10^{13} GC/mL but without benzonase treatment and filtering. After Proteinase K treatment, the purified nucleic acids were injected on a PA 800 Plus System for 3 seconds, 10 seconds and 20 seconds, respectively at 5 kv. Results are shown in Figure 12. Both the intact genome and the partial genome peaks are present in all injections with increasing signal intensity corresponding to increasing injection time. The small size impurity peaks were also observed, which migrated between 8.5 to 9 minutes, faster than the partial genome peak which was detected around 9.2 min.

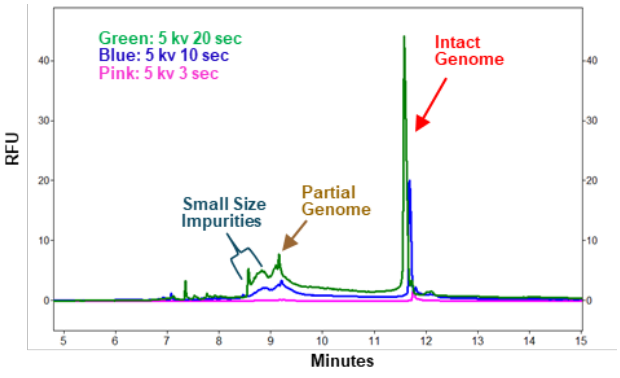


Figure 12: Analysis of Full AAV8-4.5 kb Sample with Different Electrokinetic Injection Conditions (3 to 20 Seconds). Sample was not treated with benzonase and was not filtered.

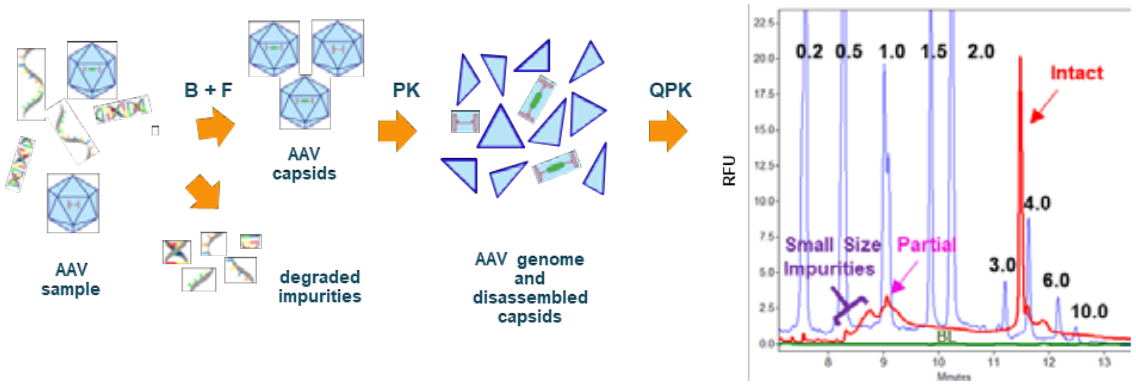


Figure 13. Standard Workflow for AAV Transgene Integrity Analysis by CE-LIF. AAV samples were treated with benzonase (B) to degrade small size impurities outside of capsids. Degraded nucleic acids were removed by filtering (F). Purified AAV capsids were treated with proteinase K (PK) to release AAV genome which was purified with QIAquick PCR purification kit (QPK) and analyzed on a PA 800 Plus System by CE-LIF using a separation gel buffer containing 1% PVP, 1x TBE, 4 M Urea and SYBR Green II.[4] Blue trace: RNA size standards; Red trace: AAV genome sample extracted from packaged AAV8-4.5 kb. Green trace: blank control.

Standard Workflow for AAV Genome Integrity by CE-LIF: Based on the results shown in Figures 9 to 12, the standard workflow for AAV genome integrity analysis was established and is shown in Figure 13. Briefly, the AAV samples were first treated with benzonase. Degraded nucleic acids and lower MW impurities outside of the capsids were removed by filtering through Amicon filter with 100 kDa MW cutoff. The purified AAV capsids were treated with Proteinase K to release the viral genome. The AAV genome samples were purified using QIAquick PCR purification kit and separated on a PA 800 Plus System by CE-LIF.

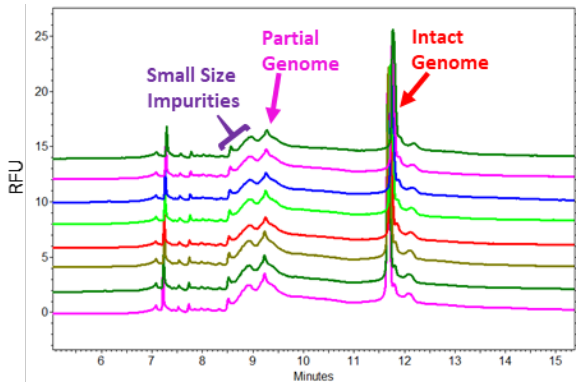


Figure 14: Repeatability Test for Sample Separation. The sample prepared from AAV8-4.5 kb (10 µL with a titer of 1.06 x 10^{13} GC/mL) in standard workflow was injected at 5 kv for 10 seconds for 8 times.

Repeatability Test for Sample Separation: A sample prepared from AAV8-4.5 kb (10 µL with a titer of 1.06 x 10^{13} GC/mL) in standard workflow was injected at 5 kv for 10 seconds for 8 times. As demonstrated in Figure 14, peak patterns in 8 runs were very consistent. The CV for migration time for the partial and intact genome peaks was 0.17% and 0.30% respectively.

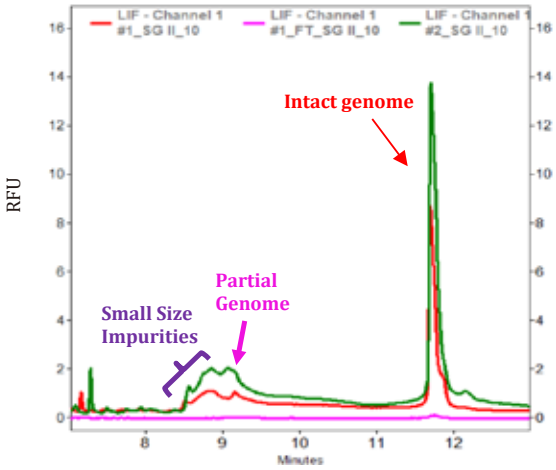


Figure 15: AAV Genome Size Purity Analysis Using a Simplified Workflow. The Green trace was obtained with AAV sample treated with Proteinase K. The red trace was obtained with sample applied to the Qiagen column without prior proteinase K treatment. Pink trace was obtained by digesting the filtrate from the Qiagen column with Proteinase K and then applied to another Qiagen column for purification of nucleic acids.

Quick AAV Size Purity Test Through a Simplified Workflow: Since the standard workflow takes about 3 hours to complete, a simplified workflow was tested. In this short workflow, the AAV8-4.5 kb sample (5 µL with a titer of 1.06 x 10^{13} GC/mL) was not treated with benzonase or proteinase K. Instead, it was mixed thoroughly with the binding buffer from the QIAquick PCR purification kit and applied to the Qiagen column. Filtrate was collected from this sample, then, digested with proteinase K. As a control, another 5 µL sample was treated with Proteinase K before Qiagen column. Nucleic acids were purified and separated on a PA 800 Plus System by CE-LIF. Figure 15 shows that although the signal level obtained from this simplified workflow was lower than samples prepared with Proteinase K, the same peak pattern including the intact genome containing the trasngene, the partial genome and small size impurities were well conserved. It takes only about 30 minutes to test a sample from sample preparation to getting results from a PA 800 Plus System using the simplified workflow. As expected, a very low amount of intact genome was obtained from the filtrate, indicating that a very low number of capsids were not opened in the presence of high concentration of Guanidine HCl present in the Qiagen binding buffer. Similar results were also obtained with an AAV2 sample from Signagen (data not shown). This simplified workflow may be useful for quick in-process quality check during AAV manufacturing process.

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Conclusions

- Capability to detect intact and partial genome helps users to get a quick assessment of the size quality of the AAV genome
- Simplified screening workflow takes 30 minutes, good for fast in-process quality check during AAV manufacturing process
- Standard comprehensive workflow provides a method for more in-depth analysis in 3 hours
- Both benzonase treatment and filtering help remove the small size impurities, simplifying data interpretation
- Methods can be applied to various AAV serotypes
- Preassembled EZ-CE cartridge saves time for users
- Urea is less toxic than denaturant used in denaturing agarose gel such as formaldehyde, sodium hydroxide and glyoxal
- CE-LIF methods generate less waste than denaturing agarose gel and southern blot

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Characterization of lentiviral vector quality attributes using capillary electrophoresis (CE) platform technology

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Introduction

As lentiviruses are increasingly being used as gene and cell therapy vectors, the inherent complexity of the lentiviral particle and the limited analytical methods to support the engineering, manufacturing and quality assessment of cell therapy products create challenges for clinical development. In this technical note, 2 simplified multi-capillary electrophoresis-based workflows are presented to address the key characterization and in-process attribute evaluation of lentivirus vectors (LVVs). The proteomic analysis workflow provides both protein profiling results and p24-based titer (total particle) determination. The genomic analysis workflow provides information about the genome integrity as well as the residual nucleic acids in LVV samples.

Cell and gene therapy products have the potential to offer cures for many diseases and are an area of significant focus for biotechnology and biopharma. More than 89% of cell and gene therapies in development use viral vectors for gene delivery.[1] The LVV is now one of the most commonly used viral vector platforms in pre-clinical development. It offers many advantages over other gene delivery technologies, including stable integration of genetic material into the host genome and the ability to deliver genetic sequences as large as 9 kb.[2]

While these benefits make the LVV an attractive technology for cell and gene therapy applications, better analytical methods to extensively characterize and monitor multiple LVV quality attributes, including physical titer, protein profiling, genome integrity and residual nucleic acids assessment, are important to ensure product efficacy and safety.

This technical note outlines a platform-based approach that uses capillary electrophoresis (CE) to mitigate the technical difficulties in characterizing LVVs for the assessment of multiple quality attributes (Figure 1). Figure 1A illustrates the proteomic analysis of the LVVs, which can be used to monitor protein profiles and determine the p24 titer of the LVV products using a SDS-CGE-LIF workflow. Figure 1B demonstrates the genomic analysis of LVVs to determine the genome integrity of the LVV sample and the residual nucleic acids using a CGE-LIF workflow.

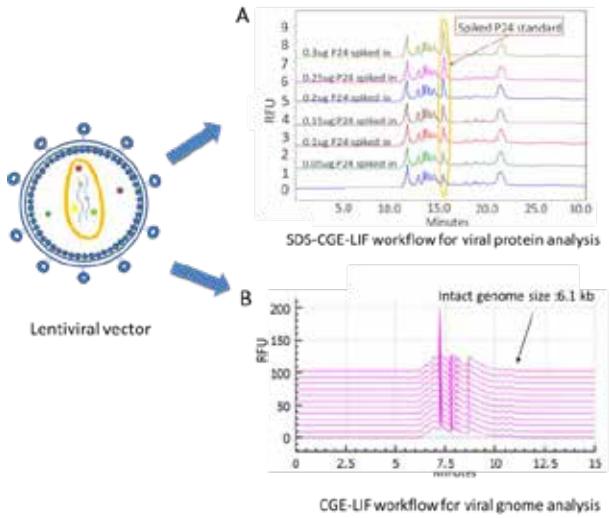


Figure 1. Two workflows for the comprehensive characterization of **LVVs** on a CE-based platform.

Key features

- Robust methods to characterize and monitor quality attributes of LVVs at both protein and genome levels using a CE-based platform method
- Single protein analysis using a CGE-SDS-LIF workflow provides the p24 titer and the protein profile of LVV samples
- Genomic analysis using a CGE-LIF workflow provides information on the genome integrity as well as the residual nucleic acids in LVV samples
- The multiplexing ability of the BioPhase 8800 system expedites the comprehensive characterization of LVV products to enable faster decision making during process development and streamline quality monitoring during the downstream purification process

Materials and methods

Chemicals for protein analysis: The Chromeo P503 dye (PN: 15106) was from Active Motif (Carlsbad, CA). The CE-SDS Protein Analysis Kit (PN: C30085) with the SDS-MW separation gel buffer, 10 kD internal standard, basic wash, acidic wash and sample buffer was from SCIEX (Framingham, MA).

Chemicals for genome analysis: Nuclease-free water (PN: AM9932), SYBR™ Green II RNA gel stain, 10,000x concentrate in DMSO (PN: S7564), RNase-free DNase I (PN: AM2222), 10x DNase I buffer (PN: AM8170G) and 10x phosphate buffered saline (PBS, PN: AM9624) were obtained from Thermo Fisher Scientific (Waltham, MA). Polyvinyl-pyrrolidone (PVP, PN: 437190), benzonase (PN: E1014-5KU), 0.5M EDTA, pH 8.0 (PN: E7889-100ML), transcript RNA markers 0.2-10 kb (PN: R7020) and 10x tris borate EDTA (TBE) buffer (PN: 574795) and molecular biology grade water were from Millipore Sigma (St. Louis, MO). The 5 µm syringe filter (PN: 4650) was from PALL Corporation (Port Washington, NY). The Rainin LTS filter tips were from Mettler Toledo (Oakland, CA). The QIAamp Viral RNA Mini Kit (PN: 52904) was from Qiagen (Germantown, MD). LVVs with titer of approximately 1 x 10^9 transduction units per mL (TU/mL) were from SignaGen Laboratories (Rockville, MD). The single-stranded RNA ladder 0.5-9 kb (PN: N0362S) was from New England BioLabs (Ipswich, MA). The sample loading solution (SLS, PN: 608082) and ethanol (200 proof) were from AAPER Alcohol and Chemical Co. (Shelbyville, KY).

Samples: In-process LVV samples were from an internal project at St. Jude Children's Research Hospital (Memphis, TN). Pre-made lentivirus materials (LV-CAG-GFP, PN: SL100270; LV-ChAT-GFP, PN: SL100303; LV-CAG-Cre-mCherry, PN: SL100284; LV-EF1a-GFT-T2A-fLuc-Puro, PN: SL100329) were from SignaGen Laboratories (Rockville, MD).

Sample preparation for protein analysis: Samples were prepared as previously described in a published technical note.[3] For the preparation of the Chromeo P503 working solution, 1 mg of Chromeo P503 dye (lyophilized powder) was reconstituted in 1 mL of methanol. After reconstitution, the dye label was stored at 2-8°C for up to 6 months, according to the manufacturer's instructions. For sample preparation, 5 µL of lentivirus solution was mixed with 5 µL of sample buffer provided in the CE-SDS Protein Analysis Kit and incubated at 70°C for 10 minutes. The sample solution was incubated for another 10 min at 70°C after mixing with 0.5 µL of 1 mg/mL Chromeo P503 dye working solution. After cooling to room temperature, 39.5 µL of DI water was added to the reaction mixture and the diluted sample was transferred to the sample vial for protein-level analysis using SDS-CGE-LIF.

Sample preparation for genome analysis: Preparation of the separation gel buffer and RNA ladder was performed as previously described in a published technical note.[4] For the preparation of the LVV RNA genome, RNA was extracted using the QIAamp Viral RNA Mini Kit. First, 25 µL of each LV sample was diluted with 45 µL of 1x PBS and mixed thoroughly with 280 µL of the lysis buffer containing 10 ng/mL carrier RNA. After a brief spin, 280 µL of 100% ethanol was added, the sample was thoroughly mixed and the resulting solution was loaded onto the spin column. The column was washed with buffers from the kit. The LVV RNA genome sample was eluted with 40 µL of nuclease-free water. Before loading the sample onto the instrument for analysis, 20 µL of the eluted LV genome sample was mixed with 30 µL of SLS, heated at 70°C for 2 minutes and then immediately cooled on ice for at least 5 minutes. The benzonase and DNase I treatment procedures were implemented to remove nucleic acid impurities outside of LV. Samples were digested in a 30 µL reaction that contained 25 µL of LV sample in nuclease-free water, 1 µL of 1x PBS, 3 µL of 10x DNase I buffer and 1 µL of benzonase that was diluted 10-fold with 1x DNase I buffer (10mM Tris-HCl, pH 7.5 at 25°C, 2.5mM $MgCl_2$, 0.1mM $CaCl_2$) or 1 µL of DNase I. The digestion was carried out at 37°C for 30 minutes and terminated by adding 3 µL of 50mM EDTA and heating at 65°C for 10 minutes. The resulting sample was subjected to RNA extraction using the QIAamp Viral RNA Mini Kit, according to the manufacturer's instructions.

Capillary electrophoresis methods: The methods used for CE separations were described in previous technical notes.[3,4]

Instruments and software: The BioPhase 8800 system equipped with a laser-induced fluorescence (LIF) detector was from SCIEX (Framingham, MA). The excitation wavelength of the LIF detector was 488 nm. The emission wavelength was set to 520 nm for genome integrity analysis and 600 nm for protein analysis. The BioPhase BFS capillary cartridge (8 x 30 cm, PN: 5080121) and Sample and Reagent Plates (PN: 5080311) were from SCIEX (Framingham, MA). The Multi-Therm shaker incubator (PN: H5000-H) was from Benchmark Scientific (Sayreville, NJ). Data acquisition and analysis were performed using BioPhase software, version 1.0 (SCIEX; Framingham, MA).

Results and Discussion

Analysis of lentiviral proteome

Workflow of lentiviral protein analysis

The proteome of the lentivirus is composed of 5 structural and several non-structural proteins (Figure 1). Each protein plays a

vital role in the life cycle of the lentivirus.[5] Glycoprotein complexes in the envelope enable the virus to attach to and fuse with target cells to initiate the infectious cycle. The p24 protein in the capsid is usually used to determine total particle titers.[6]

Here, a separation-based method for lentiviral protein analysis was developed with 2 purposes. First, the accurate total particle titer of the lentivirus sample was determined by quantifying the separated p24 capsid protein without interference from other proteins. Second, the well-separated protein profile was utilized for purity assessment of lentiviral proteins and for process optimization or process monitoring in LVV manufacturing. [3]

The protein analysis workflow uses a 2-step sample preparation protocol that includes the disassembly of the lentivirus particles and covalent labeling with a fluorophore. The resulting p503 dye-tagged lentivirus proteome was loaded onto the BioPhase 8800 system for quantitative SDS-CGE-LIF separation and analysis. This workflow and the resulting electropherogram are shown in Figure 2. The evaluation results of the workflow in a previous study[3] demonstrated that it provided sensitive titer determination based on precise quantification of the separated p24 protein peak with 4 orders of magnitude for detection, a linearity of r^2 = 0.9953 and LOQ of 0.4 ng/mL. It also provided a protein purity assessment of the lentiviral vector samples with excellent reproducibility.

Protein analysis of in-process samples

To further evaluate how this workflow can be applied in LVV production, 5 in-process LVV samples from an internal project were analyzed using the protein analysis CGE-SDS-LIF workflow. The 5 in-process elute dilute samples were from the same cell harvest but processed under different conditions in the downstream purification process of the lentivirus production with titer ranging from 10^7 -10^8 TU/mL. These in-process samples, together with a LV-CAG-GFP reference material with a titer of about 10^9 TU/mL, were prepared and loaded onto the BioPhase 8800 system, which allowed up to 8 samples to be analyzed in parallel. Analysis of the 5 in-process samples and the reference material was completed within 1 hour (Figure 3). The higher peak area of protein peaks in the LV-CAG-GFP relative to the 5 in-process samples was due to the relatively higher titer of LV-CAG-GFP sample. The difference in the protein profiles between the commercial LV-CAG-GFP sample and the internal in-process samples might be due to their variations in production processes.

The identification of the p24 protein and VSV-G proteinwere confirmed by spiking p24 standard in the LVV samples when analyzing using the protein analysis CGE-SDS-LIF workflow and LC-MS analysis of the peak fractions (data not shown in this technical note). The formulation protein in the elute dilute sample #3 (Figure 3A) was a matrix protein added to the formulation buffer to stabilize the LVV. The quantification of the p24 peak in each in-process sample can be used to monitor the downstream purification steps (Figure 3B). The relative quantification of p24 and VSV-G can be used to monitor the presence of free p24 in in-process LVV samples. Figure 4A shows the zoomed-in electropherogram of the 5 in-process samples. The areas of the p24 peak and the VSV-G peak of each sample were plotted to reveal a strong correlation between p24 and VSV-G (Figure 4B). These results indicate that there was no free p24 present in these in-process samples.

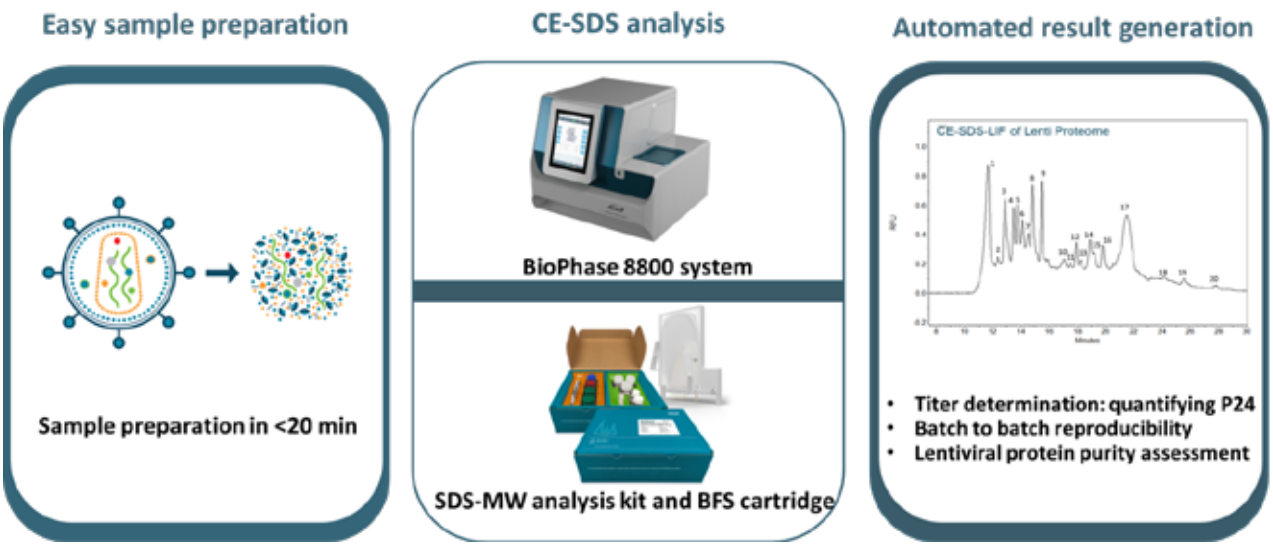


Figure 2. Workflow for LVV protein profiling and p24 titer analysis.

p 3

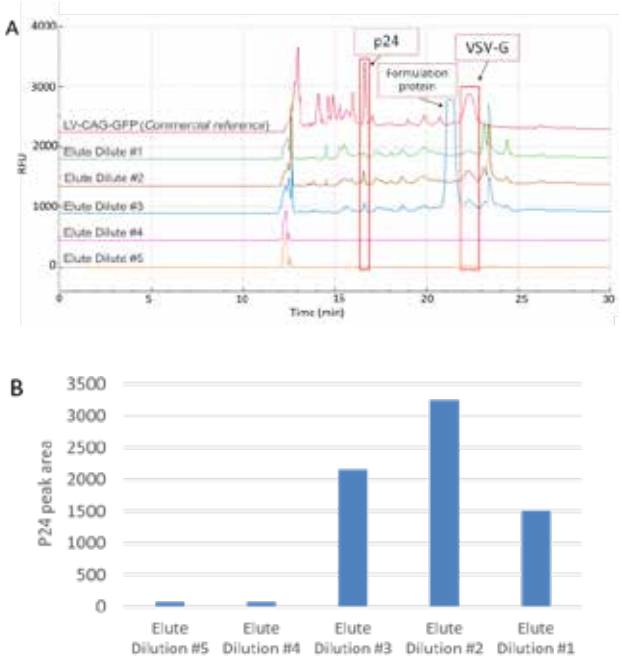


Figure 3. Protein analysis of 5 in-process LVV samples. A) Comparison of protein profiles of in-process samples and a commercial reference material. B) Bar graph of the p24 peak area of the 5 in-process LVV samples.

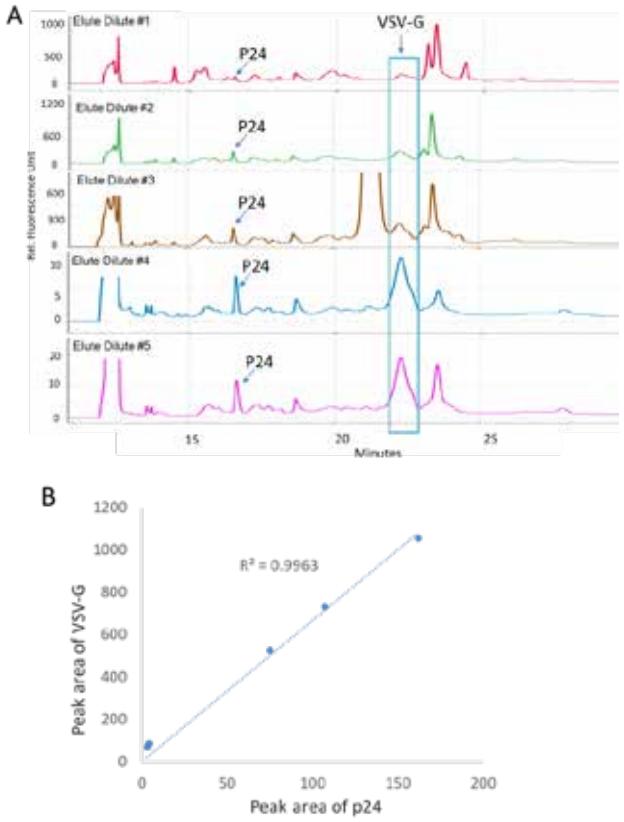


Figure 4. Correlation of p24 peak and VSV-G peak of 5 in-process LVV samples. A) Zoomed in electropherogram of the 5 in-process samples. B) Plot of peak area of p24 peak and VSV-G peak.

Analysis of lentiviral genome

Workflow for genome integrity analysis of LVV

Genomic analysis is another important quality attribute of LVV samples during manufacturing. While many methods have been established to determine viral titer, none currently analyze the integrity of the RNA genome within the lentiviral vectors.[5] Reverse transcription-quantitative PCR (RT-qPCR) can detect the presence of short fragments from the RNA genome. However, this method cannot detect deletion mutants or impurities and measurements that follow both RT and PCR amplification tend to have high variability.[7] Here, we demonstrate parallel amplification-free genome integrity analysis of LVVs by CE-LIF on the BioPhase 8800 system to accelerate analysis time and potentially speed up LVV-related product development processes.

Nucleic acids were first extracted from LVV samples with the Qiagen viral RNA extraction kit, then analyzed on BioPhase 8800 system (Figure 5). Results are shown in the electropherogram in the panel on the right. In this example, the intact genome at 6.1 kb was well-separated from the partial genome and other impurities with excellent repeatability.

Figure 6 shows results obtained from analyzing 4 commercially available, research-grade LVVs with different genome sizes. Nucleic acid extraction with the Qiagen viral RNA extraction kit was performed on 25 µL samples of each LVV with a titer of 1 x 10^9 TU/mL in the presence of the carrier RNA. The arrows indicate peaks that correspond to intact genomes. RNA ladders are shown in the 2 traces at the bottom. This genome integrity method can be used to analyze a wide range of genome sizes.

Genome integrity analysis of in-process samples

Figure 7 shows the results obtained for genome integrity analysis of the 5 in-process LVV samples together with a commercial LV-GFP reference material. Samples were injected using pressure injection and separated at a capillary temperature of 30°C. The intact genome that separated from the small molecule nucleic acids can be used to monitor the genome integrity and the

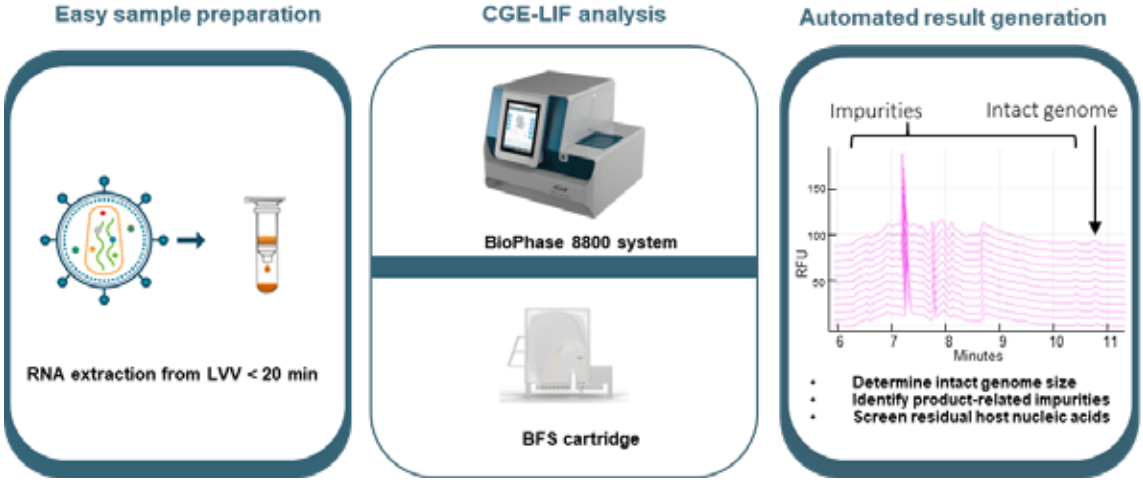


Figure 5. Workflow for LVV genome integrity analysis.

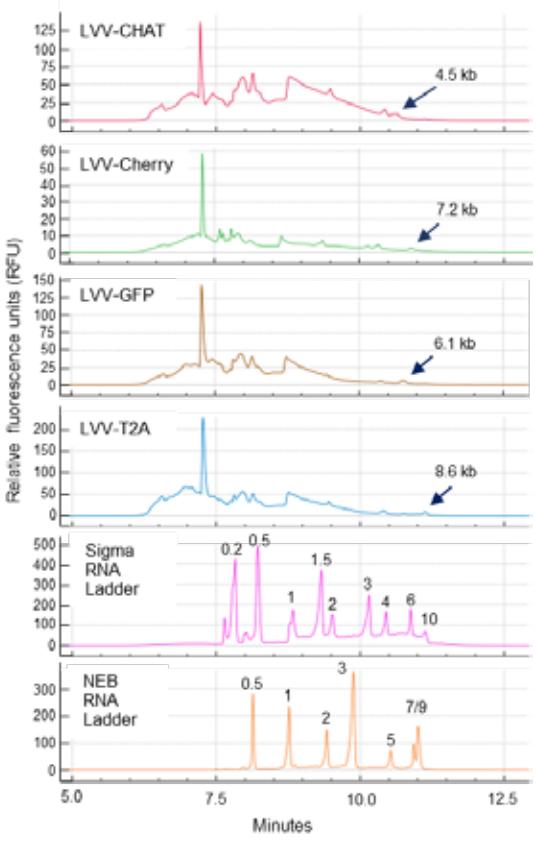


Figure 6. Analysis of LVVs with various theoretical genome sizes. Nucleic acid extraction with the Qiagen viral RNA extraction kit was performed in the presence of the carrier RNA on 25 µL samples of LVVs with a titer of 1 x 10^9 TU/mL.

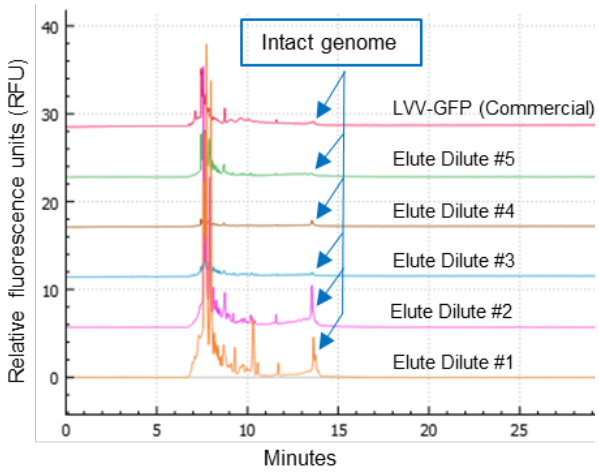


Figure 7. Genome integrity analysis of in-process samples.

presence of residual nucleic acids in the samples during process development.

Residual nucleic acids screening workflow

Figure 8 shows results obtained from an in-depth genome integrity analysis of LV-GFP obtained from a commercial vendor. The top panel shows control results that were obtained without treating the LVV sample before nucleic acid extraction. The middle panel shows results obtained when samples were treated with benzonase before extraction. The sample in the lower panel was treated with DNase I before extraction. The areas in purple circles indicate the impurities that were present outside of the LVVs, as

these peaks were present in the control condition but not in the benzonase- or DNase I- treated samples.

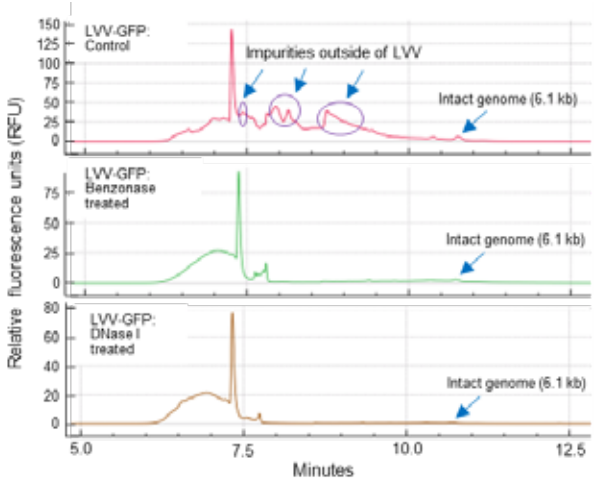


Figure 8. Residual nucleic acids screening workflow.

Conclusions

- CE technology demonstrates unique capabilities for quality attribute monitoring of LVVs at both protein and genome levels
- Precise quantification of key proteins and informative proteome profiles were utilized for characterization and monitoring of the in-process LVV products using the SDS-CGE-LIF workflow for protein analysisGenome integrity and residual nucleic acids were monitored for the eluting dilute of LVVs in different purification steps using the CGE-LIF genome analysis workflow
- Both the protein- and genome-level analyses were performed on a high-throughput BioPhase 8800 system to provide a comprehensive, ultrafast characterization of the quality attributes of the LVVs

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CGE-LIF方法对慢病毒中宿主细胞残留DNA片段大小的分布分析

Size analysis of residual host cell DNA in Lentivirus by CGE-LIF

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1. 引言

哺乳动物细胞系如今已经成为表达制备生物制品最广泛的宿主。在已经上市和正在进行临床试验的生物制品中，70%来源于哺乳动物细胞系，如幼仓鼠肾细胞系、中国仓鼠卵巢细胞(CHO)、小鼠骨髓瘤 NS0 细胞系以及人胚肾细胞系(HEK-293)。宿主细胞残留DNA（HCD）一种可能存在于最终的产品中的工艺相关杂质，其残留问题受到了生物制药用户的广泛关注，存在制瘤性和感染性。现有研究表明，可能引发致病的功能基因至少在200 bp以上，因此残留DNA片段越大，风险等级越高。美国食品药品监督管理局（FDA）关于人类基因治疗新产品生产指导文件中明确指出HCD的片段要小于200 bp；国家药品监督管理局（NMPA）生物制品药学部同样在《基因治疗产品药学研究与评价技术指导原则》的征求意见稿中指出，HCD的片段要小于200 bp。因此基因治疗终产品中需要合适的方法检测HCD的片段大小分布情况。

本文以慢病毒样品为例，使用SCIEX PA 800 Plus药物分析系统，结合激光诱导荧光检测器（LIF）和dsDNA 1000 试剂盒（货号477410）（图1），对宿主细胞残留DNA进行片段分布检测。



图1. PA 800 Plus药物分析系统与dsDNA 1000 试剂盒

2. 试剂及方法

2.1 仪器和试剂

PA 800 Plus制药分析系统，匹配LIF检测器激光诱导荧光检测器（SCIEX公司，激发波长488 nm，发射波长520 nm）。DNA 涂层毛细管（100 μm内径）、dsDNA 1000固体凝胶、SYBR Gold 荧光染料购自Thermo Fisher（Carlsbad, CA, USA）；10× Tris Borate-EDTA（TBE）缓冲液、200 bp、500 bp、1000 bp的marker缓冲液购自Sigma Aldrich（St Louis, MO，USA）。

凝胶缓冲液配置：将20 mL去离子水加入固体凝胶中，搅拌直到固体凝胶完全溶解，使用1×TBE 缓冲液对上述溶液进行10倍稀释，得到分离凝胶缓冲液；取10 mL凝胶缓冲液与1 μL SYBR Gold染料混合均匀，供CGE-LIF分析使用。

2.2 样品及前处理

慢病毒样品由国内某基因治疗公司提供，利用湖州申科HCD磁珠法提取试剂盒进行的纯化。样品处理流程如图2所示：

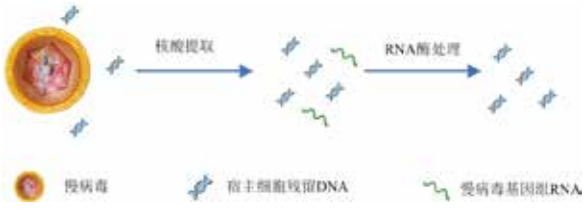


图2. 慢病毒HCD分布分析样品前处理流程

样品残留 DNA 提取

采用样品残留 DNA 提取试剂盒，具体操作按试剂盒说明书进行。取 100 µL供试品，加入蛋白酶 K 溶液，混匀后 55 ℃水浴 1 h，使膜蛋白降解、与 DNA 结合的蛋白质降解、DNA 充分游离；加入 200 µL 异丙醇和 10 µL 磁珠，使游离DNA 与磁珠充分结合，分离磁珠；加入各 700 µL 洗涤液 A 和 B，使磁珠和洗涤液混匀，完成 2 次磁珠洗涤；将离心管开盖干燥 10 min，除去残留乙醇；加入100 µL 预热洗脱液，混匀后 70 ℃水浴 10 min，分离磁珠；上清即为 DNA 纯化液，经紫外可见分光光度计测定浓度。

2.3 CGE-LIF方法设置

毛细管：DNA涂层毛细管，100 µm内径，30/40.2 cm（有效/总长度）；进样条件：0.2 psi，10 s；分离电压：-7.8 kV，20 min；毛细管温度：20 ℃；样品室温度：10 ℃。毛细管的预处理：使用去离子水在20 psi压力下冲洗5 min，凝胶缓冲液在20 psi压力下冲洗5 min；-7.8 kV加电平衡10 min。针与针间冲洗：使用凝胶缓冲液在20 psi压力下冲洗2 min。

3. 结果与分析

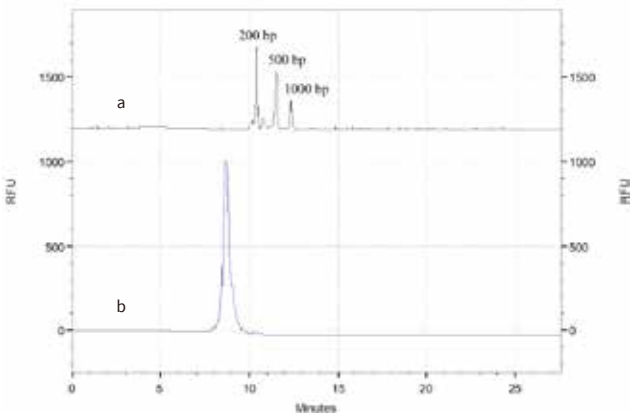


图3. CGE-LIF 法对慢病毒的HCD片段大小分析。a, marker（200,500,1000 bp）；b, 慢病毒DNA提取物；

利用CGE-LIF方法，通过与marker的对比，可准确判定慢病毒样品HCD的片段大小是否＜200 bp，从而确定样品是否符合格。如图3为慢病毒样品中HCD分布分析的谱图，通过与marker比对，可以看到样品中残留的核酸片段都在200 bp以下；由于慢病毒基因组RNA已被酶切破坏，因此在＞1000 bp的范围内未检测到任何DNA及RNA片段。通过CGE-LIF的方法，可以快速准确的判定慢病毒样品中HCD的分布情况，进而可以帮助用户更好的对工艺的优化和种产品的质控。

4. 结论

该毛细管凝胶电泳（CGE-LIF）方法具有如下特点：

- **高效分离**：可按照片段大小对不同DNA进行分离，结合DNA marker，可评估DNA的片段大小分布。
- **灵敏度**：可检测10 pg/mL的片段浓度1，若在前处理的过程中对DNA进行浓缩处理，可获得更高的检测灵敏度， 满足各种残留DNA检测的要求。
- **操作方便**：具有商品化的样品前处理试剂盒和分离试剂盒。
- **符合法规**：仪器具有操作（OQ）认证，操作软件能够进行权限设置和审计追踪。

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CGE-LIF方法对重组腺病毒疫苗中宿主细胞残留DNA片段大小的分布分析

Size analysis of residual host cell DNA in recombinant adenovirus vaccine by CGE-LIF

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SCIEX, China

1. 引言

哺乳动物细胞系已经成为表达制备生物制品最广泛的宿主。在已经上市和正在进行临床试验的生物制品中，70%的宿主细胞来源于哺乳动物细胞系，如中国仓鼠卵巢细胞(CHO) 、人胚肾细胞系(HEK-293) 。通过细胞培养生产生物制品时，需要在下游工艺中去除所有的源于宿主细胞残留的杂质，如胞质蛋白、基因及潜在病毒等。其中，来源于宿主细胞的外源性 DNA 具有传递肿瘤或病毒相关基因的可能性，因此宿主细胞残留 DNA（ HCD ）作为一种可能存在于终产品中的工艺相关杂质，受到了生物制药用户的广泛关注。HCD 已作为生物制品安全性评价的一个重要指标。

研究表明，当HCD片段大于200 bp以上时，引发致病的可能性高。因此，不仅要对HCD总量进行定量分析，还需要对其大小分布进行考察。美国食品药品监督管理局（ FDA ）关于人类基因治疗新产品生产指导文件中明确指出HCD的片段要小于200 bp；国家药品监督管理局（ NMPA ）生物制品药学部同样在《基因治疗产品药学研究与评价技术指导原则》的征求意见稿中指出，HCD的片段要小于200 bp。基于核酸片段大小分析的毛细管凝胶方法（ CGE ），可以帮助用户清晰的判定样品中HCD的分布情况，帮助用户更好的判断样品是否满足法规要求。

近来，HCD分布的分析也受到疫苗生产企业的广泛关注，本文以重组腺病毒疫苗样品为例，使用SCIEX PA 800 Plus药物分析系统，结合激光诱导荧光检测器（ LIF ）和dsDNA 1000 试剂盒（ 货号477410 ）（ 图1 ），对其HCD进行片段分布检测。



图1. PA 800 Plus药物分析系统与dsDNA 1000 试剂盒

2. 试剂及方法

2.1 仪器和试剂

PA 800 Plus药物分析系统，匹配LIF检测器（ SCIEX公司，激发波长488 nm，发射波长520 nm ）。DNA 涂层毛细管（ 100 μm内径 ）、dsDNA 1000固体凝胶购自SCIEX公司；SYBR Gold 荧光染料购自Thermo Fisher（ Carlsbad, CA, USA ）；10× Tris Borate-EDTA（ TBE ），200 bp、500 bp、1000 bp的marker缓冲液购自Sigma Aldrich（ St Louis, MO, USA ）。

凝胶缓冲液配置：将20 mL去离子水加入固体凝胶中，搅拌直到固体凝胶完全溶解，使用1× TBE 缓冲液对上述溶液进行10倍稀释，得到分离凝胶缓冲液；取10 mL凝胶缓冲液与1 uL SYBR Gold染料混合均匀，供CGE-LIF分析使用。

2.2 样品及前处理

重组腺病毒疫苗样品由国内某疫苗生产企业提供，基因组为双链DNA，大小约为30 kbp，利用PreSEQ™ HCD磁珠法提取试剂盒（PN：4400799）进行的纯化。样品前处理流程及空白对照设计如图2所示，为了排除宿主细胞本身基因组的影响，重组腺病毒样品首先用DNA酶进行处理，去除样品中存在的HCD；之后用提取试剂盒抽提核酸，其中主要为病毒本身的基因组，记作空白对照。

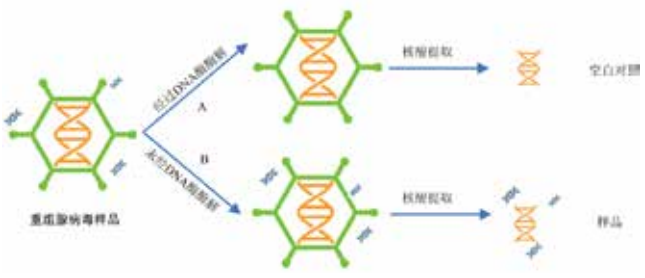


图2. 重组腺病毒疫苗HCD分布分析样品前处理流程。A）空白对照；B）样品；

样品残留 DNA 提取

采用样品残留 DNA 提取试剂盒，具体操作按试剂盒说明书进行。取 100 µL供试品，加入10 µL 5 M NaCl，再加入70 µL蛋白酶 K 溶液，混匀后 56 ℃水浴 30 min，使膜蛋白降解、与 DNA 结合的蛋白质降解、DNA 充分游离；冷却至室温后，加入360 µL裂解液，再加入30 µL混匀后的磁珠和400 µL的结合溶液，涡旋混匀，使游离DNA与磁珠充分结合，15000 × g高速离心15 s分离磁珠，丢弃上清液；加入各 300 µL 洗涤液，使磁珠和洗涤液混匀，15000 × g高速离心15 s分离磁珠，完成 2 次磁珠洗涤；将离心管开盖干燥 5 min；加入50µL洗脱液，混匀后 70 ℃水浴 7min，分离磁珠；上清即为 DNA 纯化液，经紫外可见分光光度计测定浓度。

2.3 CGE-LIF方法设置

毛细管：DNA涂层毛细管，100 µm内径，30/40.2 cm（有效/总长度）；进样条件：0.2 psi，10 s；分离电压：-7.8 kV，20 min；毛细管温度：20 ℃；样品室温度：10 ℃。毛细管的预处理：使用超纯水在20 psi压力下冲洗5 min，凝胶缓冲液在20 psi压力下冲洗5 min；-7.8 kV加电平衡10 min。针与针间冲洗：使用凝胶缓冲液在20 psi压力下冲洗2 min。

3. 结果与分析

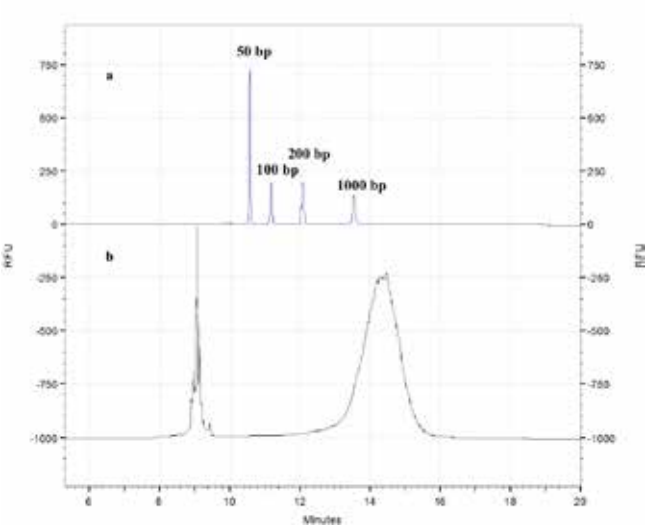


图3. CGE-LIF 法对重组腺病毒的HCD片段大小分析。a, marker（50 bp,100 bp, 200 bp, 1000 bp）；b, 重组腺病毒疫苗样品（未经DNA酶处理）；

为了证明CGE-LIF方法对重组腺病毒疫苗样品中HCD分布分析的可行性，采用未经DNA酶处理的重组腺病毒疫苗样品进行核酸的提取，其中包含重组腺病毒基因组和HCD。如图3所示，通过与 DNA marker对比，HCD片段小于200 bp，符合产品的质量要求；由于是未经DNA酶处理，推断大于1000 bp的核酸为重组腺病毒基因组。

在考察生产工艺中是否会有残留的DNA片段，也可设计未接种病毒的细胞培养液按照常规工艺流程进行培养，其中不含有重组腺病毒，属于一种工艺监控的空白对照。对该空白对照进行CGE-LIF分析，可从生产工艺角度反馈产品中是否可能会有 > 200 bp的核酸残留。

通过CGE-LIF的方法，可以快速准确判定重组腺病毒疫苗样品中HCD的分布情况，进而可以帮助用户更好进行工艺优化和产品质控。

4. 结论

该毛细管凝胶电泳方法具有如下特点：

- **高效分离**：按照片段大小对不同DNA进行分离，可结合DNA marker评估DNA的片段大小分布。
- **灵敏度**：可检测10 pg/mL的片段浓度[1]，若在前处理的过程中对DNA进行浓缩处理，可获得更高的检测灵敏度， 满足各种残留DNA检测的要求。
- **操作方便**：具有商品化的样品前处理试剂盒和分离试剂盒。
- **符合法规**：仪器具有操作（OQ）认证，操作软件能够进行权限设置和审计追踪。

参考文献

1. TechNote：CE-LIF方法分析宿主细胞残留DNA的片段分布（RUO-MKT-02-9259-ZH-A）

毛细管电泳-激光诱导荧光（CE-LIF）法分析基因治疗产品的DNA限制酶酶切片段

Analyzing the Restriction Enzyme Fragments of Gene Therapy Product by Capillary Electrophoresis with Laser-Induced Fluorescence (CE-LIF) Detection

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Keywords： Capillary Electrophoresis- Laser-Induced Fluorescence (CE-LIF); Restriction Enzyme Fragments; Gene Therapy Product

1 前言

基因治疗是现代生物技术与临床医学等多学科交叉融合而形成的针对人类重大疾病，如，恶性肿瘤、心血管疾病、遗传性疾病和自身免疫性疾病等进行治疗研究的新手段。随着近些年基因治疗相关研究和技术的快速发展，将有越来越多的基因治疗产品进入临床研究和批准上市，寻求合适的分析技术方法，对基因治疗产品进行质量控制，将是该技术领域亟待解决的问题之一。

对基因治疗产品的质控要点中包含了对其的鉴别实验，而鉴别实验往往须分别从核酸和蛋白水平进行。其中，在核酸水平上，需要选用适当的限制酶，使切得的DNA片段能比较充分的反应基因治疗产品核酸的情况，并能在电泳时获得良好的分析[1]。而毛细管电泳（Capillary Electrophoresis, CE）是近些年发展快速的一类新型的分离技术，以其自动化程度高、分辨率高、重复性好、灵敏度高、试剂消耗少和环境污染小等特点在生物技术的研发和质控方面扮演了十分重要的角色。

本研究以一种单纯疱疹病毒（Herpes Simplex Virus, HSV）为载体的基因治疗产品的核酸为研究对象，利用限制性内切酶（Nde I）将核酸特异性酶切为不同长度的DNA片段，采用激光诱导荧光（Laser-Induced Fluorescence, LIF）检测器，建立了CE-LIF的分析方法，对该基因治疗产品载体进行了限制性酶切图谱的鉴别分析，考察了CE-LIF方法的稳定性，并比较了CE方法与琼脂糖凝胶法在结果上的异同。

2 材料与方法

2.1 仪器

PA 800 Plus药物分析系统（SCIEX, USA），32 Karat™软件10.1，激光诱导荧光检测器（激发波长，488 nm，检测波长，520 nm），MA3涡旋混合器（IKA, Germany）。

2.2 试剂与耗材

dsDNA 1000 kit（SCIEX, PN 477410），包含：DNA 涂层毛细、dsDNA 1000 Gel Buffer、dsDNA Test Mix和Orange G Reference Marker。其它试剂：SYBR Gold nucleic acid (Invitrogen™, PN S11494), DL15000 DNA Marker（Solarbio, PN M1700）, Tris Borate EDTA（10 × TBE, PN Sigma T4323）。

2.3 样品

样品来自中检院重组室，为一种HSV载体基因治疗产品中核酸的Nde I限制性酶切片段。

2.4 CE条件

DNA涂层毛细管，50 μm内径；30/40 cm有效/总长度；进样：0.5 psi，3 s；分离电压：-7 kV；分离时间：40 min；样品室温度：4 ℃；毛细管温度：25 ℃；激发波长：488 nm，检测波长：520 nm。

背景电解质（Background Electrolyte，BGE）：取20 mL 二次去离子水（Double Deionized Water, DDI Water）加入ds DNA 1000 Gel buffer中，使固体Gel buffer充分溶解，并充分混合均匀。然后每10 mL Gel buffer中加入1 μL SYBR Gold的染料，并再次涡旋混合均匀，超声除去气泡，此溶液作为分离所用的BGE。

3 结果与讨论

3.1 CE-LIF对DNA限制酶酶切片段分析方法的建立

为得到最优实验条件，本研究对BGE中胶的浓度、进样电压和时间、分离电压、分离温度及毛细管长度对各片段峰分离度和分析时间的影响分别进行了比较。在考察BGE中胶的浓度时，取1×TBE对2.4中的BGE稀释5倍和10倍，随着BGE中胶浓度的降低，样品整体分离度下降。随着进样电压和时间的提高，分离时进入毛细管的样品量增加，容易产生样品的过载，造成分离度下降。分离电压的提高（从-7 kV到-15 kV），虽然会使得分析速度变快，但高电压带来较多的焦耳热，会使得分离度进一步下降。毛细管的温度（从15 ℃到30 ℃）会影响BGE的黏度，毛细管温度升高，BGE黏度下降，样品的分离度会随之下降。本研究考察了有效长度为30 cm和40 cm的毛细管，结果表明对分离度影响不大，最终选择30 cm作为有效长度可进一步缩短分析时间。

最终，本研究确定的最优分离条件为：采用未稀释的BGE，以0.5 psi压力进样3 s，分离电压为-7 kV，毛细管有效长度为30 cm，毛细管温度为25 ℃。其典型电泳谱图为图1 A。

与琼脂糖凝胶结果相比较，CE能够对酶切片段获得良好的分离，并且检测结果更加客观和准确。图1B为琼脂糖凝胶法对该基因治疗产品内部核酸的限制性内切酶片段的分析结果，结果显示，共分离得到7个片段。通过对比可知，CE-LIF法共得到了8个片段峰，比琼脂糖凝胶法多检测得到了1个片段。并且，4、5和6号片段，在CE上能够获得良好的分离，而在琼脂糖凝胶法上，分离度相对较差，判断起来人为主观性较强。在高于15 kbp的范围内，CE法能够检测得到2个片段（7和8），琼脂糖凝胶法上只能观察到1个片段（7）。

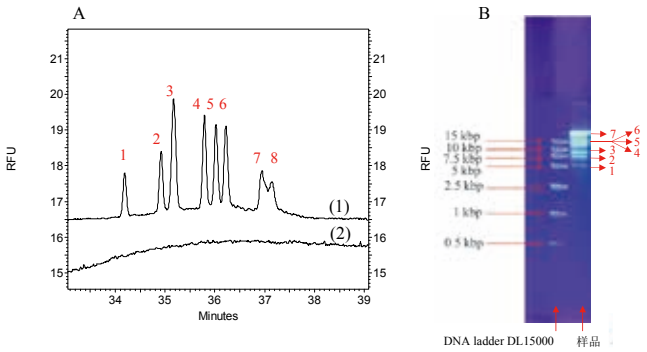


图1. A、(1) CE-LIF法对样品的限制性内切酶切片段的分析，(2) 空白（以DDI Water为样品）；B、琼脂糖凝胶法对样品的限制性内切酶切片段的分析。电泳条件：DNA涂层毛细管，50 μm内径；30/40 cm有效/总长度；进样：0.5 psi，3 s；分离电压：-7 kV；分离时间：40 min；样品室温度：4 ℃；毛细管温度：25 ℃；激发波长：488 nm，检测波长：520 nm。

3.2 DNA限制酶酶切片段长度的计算

采用3.1中的最优CE条件，对DNA ladder DL15000进行分析，得到1 kbp, 2.5 kbp, 5 kbp, 7.5 kbp, 10 kbp和15 kbp, 6个片段长度。通过软件进行线性方程的拟合，其线性回归系数为0.99。最终以此线性方程计算样品中各个片段的长度。其结果参见表1。结果显示，CE法和琼脂糖凝胶法获得的片段长度结果相接近。CE法可通过软件直接对Ladder进行线性拟合，利用样品中各个片段的迁移时间来计算片段长度，方法简单准确。

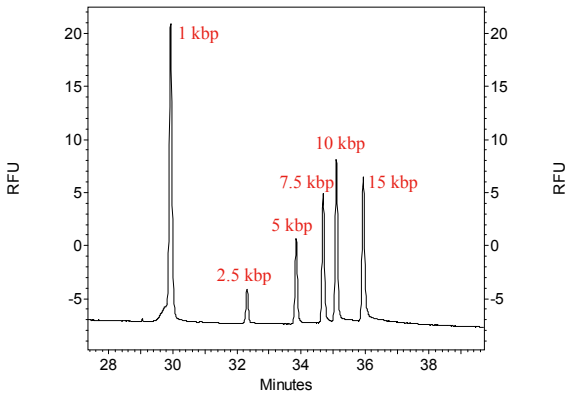


图2. DNA ladder DL15000典型电泳谱图。电泳条件与图1相同。

表1. CE-LIF法与琼脂糖凝胶法对DNA片段长度的测定结果比较。

峰号 Peak No.	CE-LIF（kbp）	琼脂糖凝胶法（kbp）
1	4.87	4.64
2	7.20	6.72
3	8.25	8.06
4	11.47	11.45
5	12.91	12.85
6	14.29	14.43
7	20.33	20.43
8	22.19	NA

表2. 精密度实验结果（N=6）。

峰号 Peak No.	迁移时间 Migration time		校正峰面积百分比 Corrected peak area%		片段长度 ragment size	
	均值 (Mean)/min	RSD%	均值 (Mean)	RSD%	均值 (Mean)/kbp	RSD%
1	33.82	0.31	7.51	2.46	4.87	5.32
2	34.54	0.31	10.66	4.64	7.20	5.77
3	34.80	0.30	22.66	3.10	8.25	5.70
4	35.41	0.30	14.64	1.72	11.47	5.52
5	35.64	0.30	13.68	3.31	12.91	5.60
6	35.84	0.31	13.66	2.78	14.29	5.53
7	36.40	0.30	10.03	5.65	20.33	5.13
8	36.76	0.32	7.11	12.38	22.19	5.18

3.3 精密度实验

本研究为了考察该方法的精密度，取同一份样品使用最优条件连续进样6针，考察迁移时间、校正峰面积百分比和片段长度的精密度。如表2所示，各峰迁移时间的RSD均小于0.32%，1~8峰校正峰面积百分比的RSD均小于5.65%，各峰的片段大小计算结果RSD均小于5.77%，证明该方法精密度良好。

4 结论

本研究建立了CE-LIF法对HSV载体基因治疗产品中核酸的限制性内切酶酶切片段分析的方法。并对比了CE-LIF法和琼脂糖凝胶法在分离度和片段长度计算结果两个方面的差异。结果显示，CE-LIF能够获得更好的分离度以及检测出更多的酶切片段，且酶切片段大小计算结果准确。本研究对CE-LIF法的精密度进行了考察。结果表明，对各个酶切片段的迁移时间、校正峰面积百分比表现出了良好的重复性。

CE-LIF法具有比琼脂糖凝胶法分离度高、迁移时间和校正峰面积百分比重现性好和自动化程度高等优点。在基因治疗产品的质量控制中，通过对实际样品和对照品的酶切片段数量和大小的比较，来实现核酸水平上，对基因治疗产品的鉴别。

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高通量BioPhase™ 8800系统：用于不同片段长度寡核苷酸纯度分析

High throughput BioPhase™ 8800 system: used for purity analysis of oligonucleotides with different fragment lengths

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Key words: BioPhase™ 8800, oligonucleotides , CGE, Chemical Synthesis

1.引言

寡核苷酸在生命科学领域有广泛应用，如作为核酸载体用于蛋白表达以及药物递送、用于构建纳米结构用于生物传感、作为探针用于基因检测等。目前合成技术得到的寡核苷酸中会存在杂质即错误序列，其中难以除去的杂质是 (n–1)缺失序列 (与完整长度为 n 的寡核苷酸相比仅相差一个核苷酸)，从而导致产物纯化比较困难。基因治疗产品药学研究与评价技术指导原则中提到核酸类载体一般应分析错误序列、不完整序列、降解片段等的残留水平，因此需要对其纯度进行分析。目前合成DNA片段可以通过高通量芯片合成仪一次可以得到多条不同片段长度的单链DNA，因此需要高通量的分析仪器应用于寡核苷酸的纯度分析。BioPhase™ 8800多通道毛细管电泳系统搭配毛细管凝胶法基于核酸片段大小的差异进行分离，具有分辨率高、可实现单核苷酸差异的分离以及定量准确等优势；同时BioPhase™ 8800多通道毛细管电泳系统采用了八通道分离毛细管，大大缩短了方法开发和优化的时间，能够有效加快DNA样品分析检测进程。

本文以不同片段长度的寡核苷酸为例，在BioPhase™ 8800多通道系统上采用毛细管凝胶电泳方法搭配SCIEX成熟稳定的凝胶电泳分析试剂盒对样品进行纯度分析。本文通过BioPhase™ 8800多通道系统可以在一次实验中实现不同浓度的样品进行条件优化，得到最佳上样浓度条件。同时也展示了在一次实验中得到8个不同片段的纯度结果，满足多个样品同时检测的需求。

2. 试剂及方法

2.1 试剂及样品

SDS-MW试剂盒中使用的试剂：SDS Gel buffer (SCIEX, A30341)、pH 9.0样品缓冲液 、0.1 M盐酸溶液 、0.1 M氢氧化钠溶液、50 µm内径的熔融石英毛细管(SCIEX, 338451)，总长为30.2 cm。

样品:不同片段长度的寡核苷酸由某核酸片段生产企业提供，浓度均在5 µM，S1-S9片段给长度分别为22 nt, 23 nt, 24 nt, 33 nt, 38 nt, 39 nt, 48 nt, 72 nt, 145 nt，其中S5-S9样品具有染料修饰。

2.2 样品及前处理

制备不同浓度样本溶液：将样品置于1.5 mL离心管中，加入超纯水将核酸样品稀释至0.1 µM，准确移取100 µL稀释的样品溶液加入到装有样品管的CE专用试剂瓶中，作为待测溶液。

2.3 仪器及方法设置

CE-SDS方法实验条件：采用BioPhase™ 8800多通道毛细管电泳分析系统，UV检测器，采集频率：4 Hz，采集波长260 nm；毛细管卡盒：50 µm × 30 cm BioPhase 8800 熔融石英毛细管卡盒 (PN 5080121)；毛细管温度：25 ℃；样品室温度：15 ℃；进样条件：-5 kV，20 s；分离条件：-10 kV，40 min。

毛细管针间冲洗和胶填充：0.1 mol/L氢氧化钠溶液在70 psi压力下冲洗3 min，然后用0.1 mol/L盐酸溶液在70 psi压力下冲洗1 min，再用去离子水在70 psi压力下冲洗1 min，最后用SDS凝胶分离缓冲液在70 psi压力下冲洗10 min。

3. 结果与分析

3.1 实验方法开发

毛细管的温控对样品分离结果很重要，一般情况下温度升高出峰变快，峰型更尖锐，温度降低迁移时间变慢，相应的分离度有所改善；本文以片段差异较小的S1和S3混合样品的分离进行考察，其它实验条件固定，仅改变毛细管的温度；实验结果表明，毛细管温度为25 ℃时，混合样品中的两个片段峰分离度更好，可以实现基线分离，因此，选择25 ℃作为混合样品的最终毛细管温度；其毛细管凝胶电泳分离结果如下图1所示。

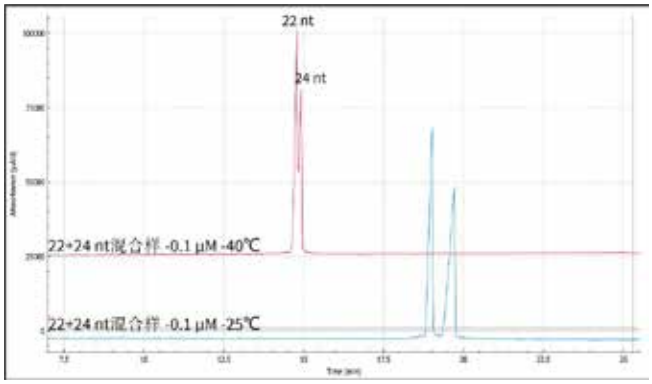


图1. S1和S3混合样品温度条件优化的结果

3.2 不同片段长度寡核苷酸纯度分析结果展示

确认最终实验条件后，将片段长度分别为22-145 nt的9个单链DNA样品分别放置在BioPhase™ 8800系统的不同通道中进行分析，一次实验即可得到不同片段长度样品的纯度结果，具体分离结果如下图2所示；S5、S6、S7、S8、S9样品均存在染料修饰，S5、S7和S9的荧光修饰表现出峰型宽钝情况，不同的染料修饰峰型宽钝程度不一样，可能是染料修饰对样品的构象有一定影响导致；而S6和S8样品的染料修饰则对样品迁移时间有一定的影响，导致样品出峰时间不完全按照片段长度大小。其中S1、S2、S3片段长度仅相差一个核苷酸，从分离谱图可以看出这三个样品可以基线分离。S1-S9样品纯度分别为92.43%、89.77%、93.42%、97.78%%、93.84%、91.91%、99.04%、95.27%、91.07%，结果表明毛细管凝胶电泳方法适用于不同片段寡核苷酸的分离。

3.3 BioPhase™ 8800多通道分析系统毛细管凝胶电泳方法重复性考察

在BioPhase™ 8800系统的A-H通道中放置是同样体积的S7样品，同时对8个通道的样品同时连续进样6次，考察单次进样中8通道间的一致性，以及单通道内连续进样6针的重复性；实验结果表明单次进样8通道间表现出良好的一致性，电泳图见图3a，统计结果见表1，主峰的迁移时间的RSD值< 1.0%，并且校正峰面积百分比的RSD值也< 1.0%；单通道6针进样重复性好，电泳图见图3b，统计结果见表2，主峰的迁移时间的RSD值< 1.0%，并且校正峰面积百分比的RSD值也< 1.0%。实验结果证明：BioPhase™ 8800系统不同通道之间以及单通道连续6针进样重复性良好。

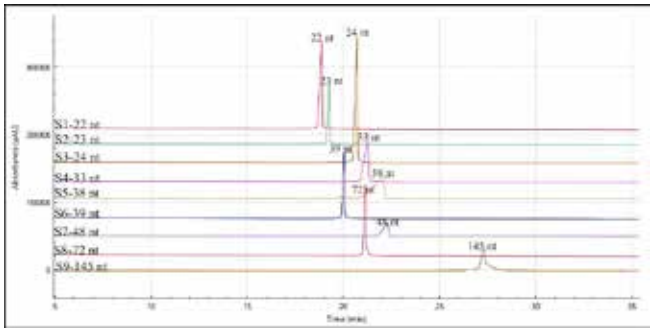


图2. 不同片段寡核苷酸样品分离结果

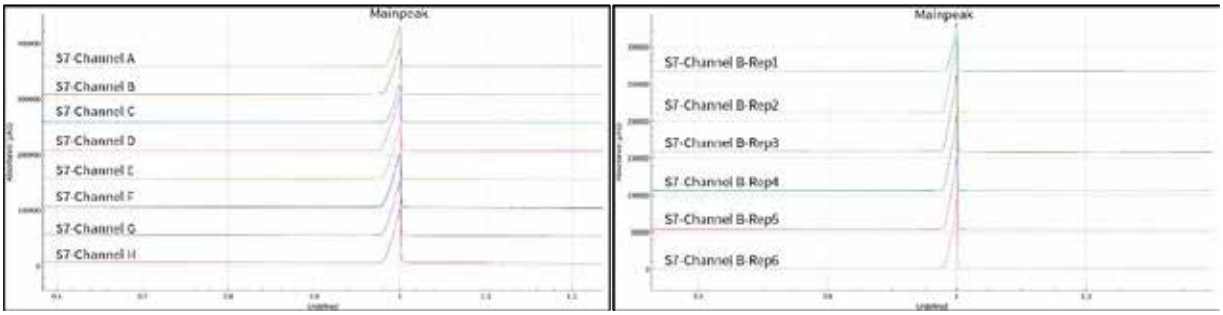


图3. S7样品在不同通道间以及同一通道内分离结果

表1. S7样品在A-H不同通道重复性结果

编号	迁移时间 (min)	校准峰面积百分比 %
Channel A	17.01	98.83
Channel B	16.98	98.78
Channel C	16.97	99.26
Channel D	17.05	98.94
Channel E	17.07	98.88
Channel F	17.02	98.95
Channel G	17.16	99.27
Channel H	17.05	99.31
RSD	0.36	0.22

表2. S7样品在B通道6针重复性结果

编号	迁移时间 (min)	校准峰面积百分比 %
Rep-1	16.98	98.81
Rep-2	16.94	98.82
Rep-3	16.88	98.58
Rep-4	16.88	98.82
Rep-5	16.89	98.97
Rep-6	16.89	98.67
RSD	0.24	0.14

4. 结论

毛细管凝胶电泳法是依据核酸片段大小的差异进行分离，广泛应用于核酸的纯度分析。本文在BioPhase™ 8800多通道系统中采用毛细管凝胶电泳法搭配SCIEX 成熟稳定的SDS-MW分子量分析试剂盒，可有效应用于寡核苷酸纯度分析，主要表现出如下优点：

1. BioPhase™ 8800多通道系统可以一次实验同时运行8个样品，大大节约了样本分析检测时间，显著提高检测通量和分析效率；
2. 毛细管凝胶电泳法适用性广，可以分离不同片段长度的寡核苷酸，分离度高。
3. 实验结果重复性好，同一样品8个通道间以及单个通道内一致性高，迁移时间及校正峰面积百分比的RSD值均小于1% ，表明毛细管凝胶电泳方法重复性好。

毛细管凝胶电泳法对SgRNA的纯度分析

Purity analysis of SgRNA by capillary gel electrophoresis

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Keywords: CGE, SgRNA, Purity

1. 引言

向导RNA（SgRNA, small guide RNA）在RNA编辑过程中可以引导尿苷残基插入或缺失到动质体中，可以与pre-RNA配对，属于小型非编码RNA。SgRNA在CRISPR-Cas9基因编辑系统中具有准确识别靶基因序列的作用，其效果可影响编辑的效率、是否发生脱靶等。SgRNA的长度通常为60-80 nt，长的可达100 nt 左右；常用的HPLC方法对片段较长的SgRNA分离度不够，并且需要用到有机溶剂，对环境有影响；毛细管凝胶电泳法可以对片段长度为99 nt 的SgRNA样品进行有效分离并对其纯度进行分析。

本文以100 nt，99 nt，97 nt，95 nt和90 nt的RNA标准品为例，考察了这些片段的分离情况，并对99 nt的SgRNA样品进行了纯度分析。SCIEX单链DNA分析试剂盒（ssDNA 100-R）基于核酸片段大小进行分离，可以将100 nt与99 nt片段长度的RNA标准品分离，并且100 nt 与97 nt、95 nt、90 nt RNA标准品的分离度大于1。因此对于长至100 nt左右的SgRNA样品，可以实现其N+1和N-1的杂质分离及准确定量。

2. 试剂及样品

2.1 试剂

ssDNA 100-R试剂盒（SCIEX，477480），包含DNA涂层毛细管（477477）、ssDNA 100-R Gel凝胶粉末（77621）、Tris-Borate Buffer 、7 M Urea 、pd(A) 40-60 Test Mix（477626）；

其它试剂：去离子水(Millipore)。

2.2 样品及前处理方法

样品: 100 nt、99 nt、97 nt、95 nt、90 nt片段长度的RNA标准品和 99 nt的 SgRNA均由某药企提供；样品初始浓度均为0.05 nmol/μL;

制备Tris-Borate-Urea Buffer溶液：将135 mL的DDI水添加到含有Tris-Borate干粉的试剂瓶中，使用磁子在800 rpm下搅拌溶液20至30 min，确保硼酸完全溶解。缓慢将干燥的7 M尿素添加到Tri-硼酸盐缓冲液中，同时继续搅拌溶液；在室温下搅拌溶液2 h，直至尿素完全溶解且缓冲液澄清；

制备ssDNA 100-R Gel Buffer溶液：向冻干的凝胶粉末中加入5.0 mL经0.45 μm滤膜过滤后的Tris-Borate-Urea缓冲液；用干净且干燥的磁子在800 rpm下搅拌溶液4-6 h，直到凝胶完全溶解；

制备样本溶液：将样品置于1.5 mL离心管中，加入无酶水稀释至终浓度为1ng/μL ；准确移取20 μL稀释后的样品溶液加入到NanoVial样品管中，用于后续检测；

制备混合样本溶液：将样品置于1.5 mL离心管中，加入无酶水将混合物稀释至样品终浓度为0.5 ng/μL、标准品浓度为0.25 ng/μL ；准确移取20 μL样品溶液加入到NanoVial样品管中，用于后续检测；

3. 仪器及方法

采用SCIEX PA 800 Plus药物分析系统，匹配紫外检测器，采集频率：4 Hz，检测波长为：254 nm；窗口狭缝：100 ×200 μm ；DNA涂层毛细管，100 μm内径，20/ 30.2 cm(有效长度/总长度)；进样条件：-5 kV， 4 s；分离电压：-9.0 kV，45 min；毛细管温度：50 ℃；样品室温度：15 ℃。

毛细管平衡方法：分别用去离子水、TBE 尿素 Buffer在20 psi气压下各冲洗毛细管2 min、然后在50 psi气压下填充ssDNA 100-R Gel 10 min；分别用3 kv、9 kv进行预分离，反向电极，时间分别为5 min，10 min；

凝胶填充方法：在50 psi气压下填充ssDNA 100-R Gel 10 min；用9 kv电压进行预分离，反向电极，时间10 min；

每隔5次分离，应使用凝胶填充法更换一次毛细管中的 ssDNA 100-R Gel。

4. 结果与分析

4.1 100 nt 与99 nt、97 nt、95 nt、90 nt的RNA标准品的分离结果

考察100 nt 与99 nt、97 nt、95 nt、90 nt的RNA标准品的分离，分离结果如下图1。

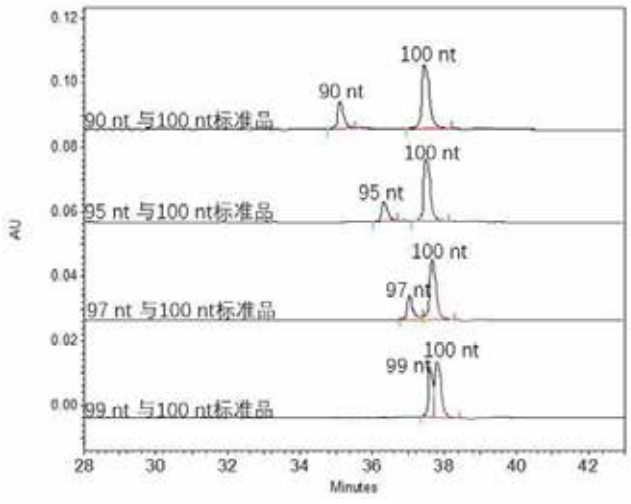


图1. 100 nt与99nt、97nt、95nt、90nt的RNA标准品的分离谱图。

毛细管凝胶电泳法依据核酸片段大小进行分析，100 nt与99 nt、97 nt、95 nt、90 nt的RNA标准品的分离度见表1；100 nt与99 nt片段长度的RNA标准品能够分离，分离度0.6，而100 nt 与97 nt、95 nt、90 nt RNA标准品的分离度均大于1。

表1. 100 nt与99 nt、97 nt、95 nt、90 nt的RNA标准品分离度结果

片段长度	分离度
100 nt与99 nt	0.6
100 nt与97 nt	1.9
100 nt与95 nt	3.2
100 nt与90 nt	6.0

4.2 99 nt 片段长度SgRNA样品的纯度分析

片段长度为99 nt 的SgRNA样品纯度分析结果如下图2。毛细管凝胶电泳法依据核酸片段大小进行分析，通过与100 nt片段长度标准品谱图出峰位置对比，确认99nt样品谱图中峰2是100 nt片段，因此99 nt片段长度 SgRNA样品的纯度为92.54%。

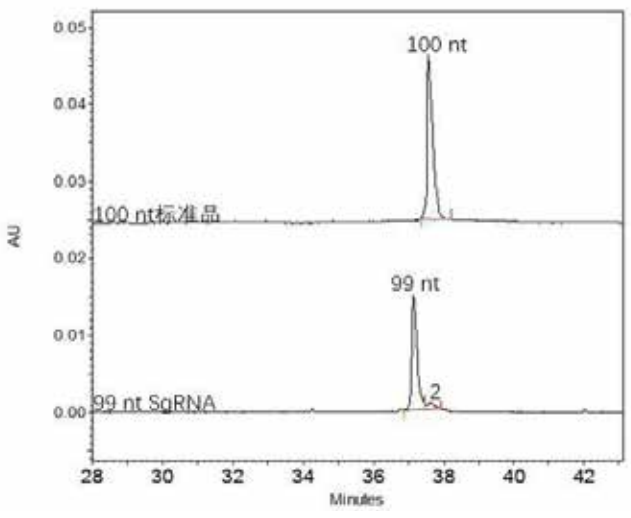


图2. 毛细管凝胶电泳法对99 nt 片段长度SgRNA纯度分析的电泳图

5. 结论

本文采用SCIEX 成熟稳定的ssDNA 100-R单链寡核苷酸分析试剂盒，可以实现100 nt与99 nt、97 nt、95 nt、90 nt的RNA标准品的分离，其中100 nt 与97 nt、95 nt、90 nt RNA标准品的分离度均大于1；因此，对于100 nt左右的SgRNA样品，可以实现其N+1和N-1的杂质分离及准确定量。该方法可有效应用于SgRNA样品与其杂质的分离及纯度分析。

毛细管凝胶电泳法对单链寡核苷酸的纯度分析

Purity analysis of oligonucleotides by capillary gel electrophoresis

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Keywords: CGE, Oligonucleotides, Primer

1. 引言

寡核苷酸是一类由人工合成的若干个短链核苷酸的总称，它可作为DNA合成的引物（Primer）、基因探针（Probe）等，在现代分子生物学研究中具有广泛的用途。此外，随着生物技术的不断发展，寡核苷酸也可作为一种新型的治疗药物，通过作用于信使RNA（mRNA）来调控疾病基因转录翻译，可以达到传统药物无法替代的效果，尤其是对遗传疾病、肿瘤、代谢类疾病及罕见病等非常有优势，被认为是继抗体药物及细胞免疫疗法之后的下一代新型药物。目前在研的寡核苷酸药物种类很多，包括反义寡核苷酸（antisense oligonucleotide，ASO）、小干扰核糖核酸（small interfering RNA，siRNA）、微小核糖核酸（microRNA，miRNA）、核酸适配体（aptamer）等。其中ASO是一种单链寡核苷酸，是常用的基因调控工具之一，已被开发为基因治疗药物。目前寡核苷酸药物几乎都是采用化学方法合成，用高效液相色谱分离纯化，该方法获得的寡核苷酸容易被杂质和截短的错误序列（又叫失败序列）所污染，其中难以除去的杂质是大量（n–1）缺失序列（与完整长度为n的寡核苷酸相比仅相差一个核苷酸），从而导致产物纯化十分困难。因此，迫切需要开发一种可实现单核苷酸差异分离的方法，用于单链寡核苷酸以及引物的纯度分析。

本文以两条片段长度相差1 nt的寡核苷酸引物（33 nt和34 nt）为例，基于SCIEX成熟稳定的单链DNA分析试剂盒（ssDNA 100-R），利用毛细管凝胶电泳方法对其进行了分离。该方法基于核酸片段大小进行分离，分辨率高，可实现单核苷酸差异的分离；定量准确，可用于单链寡核苷酸的纯度测定。

2. 试剂及方法

2.1 试剂及样品

试剂：本实验采用ssDNA 100-R试剂盒 (SCIEX, 477480)，其中包含DNA涂层毛细管（SCIEX, 477477），总长为65 cm，内径100 µm；ssDNA 100-R Gel凝胶粉末 (SCIEX, 77621)、Tris-Borate Buffer (SCIEX, 338481)、7 M Urea (SCIEX，338481)、pd(A) 40-60 Test Mix (SCIEX, 477626)、磁子 (Sigma-Aldrich)、0.45 µm滤膜 (Sigma-Aldrich)、去离子水 (Millipore)。

样品: 两条合成的寡核苷酸引物混合物，浓度分别为388 µM，片段长度分别为33 nt和34 nt。

2.2 样品及前处理

制备Tris-Borate-Urea Buffer溶液：先用甲醇清洗磁子，并用去离子水将磁子冲洗干净；将135 mL的去离子水添加到含有Tris-Borate干粉的试剂瓶中，使用磁子在800 rpm下搅拌溶液20至30 min，确保硼酸完全溶解。缓慢将干燥的7 M尿素添加到Tri-硼酸盐缓冲液中，同时继续搅拌溶液；在室温下搅拌溶液2 h，直至尿素完全溶解且缓冲液澄清；将Tris-Borate-Urea缓冲液储存在2-8 ℃条件下，保质期为30天；

制备ssDNA 100-R Gel Buffer溶液：向冻干的凝胶粉末中加入5.0 mL经0.45 µm滤膜过滤后的Tris-Borate-Urea缓冲液；用干净且干燥的磁子在800 rpm下搅拌溶液4-6 h，直到凝胶完全溶解；将凝胶缓冲液储存在2-8 ℃条件下，保质期为30天。

制备寡核苷酸样本溶液：将样品置于1.5 mL离心管中，加入无酶水稀释至终浓度为77.6 µM；准确移取20 µL稀释后的样品溶液加入到PA 800 Plus 纳升样品管中，用于后续检测；

2.3 仪器及方法设置

采用SCIEX PA 800 Plus药物分析系统，匹配紫外检测器，采集频率：4 Hz，检测波长为：254 nm；窗口狭缝：100 ×200 μm ；DNA涂层毛细管，100 μm内径，20/ 30.2 cm (有效长度/总长度)；进样条件：-3 kV, 4 s；分离电压：-9.0 kV, 40 min；毛细管温度：30 ℃；样品室温度：15 ℃。毛细管活化方法、凝胶填充方法、分离方法、关机方法设置如下图；



图1. 毛细管活化方法时间程序表

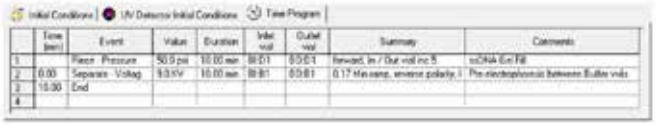


图2. 凝胶填充方法时间程序表



图3. 分离方法时间程序表



图4. 关机方法时间程序表

缓冲液的入口盘及出口盘顺序摆放如下图5所示：

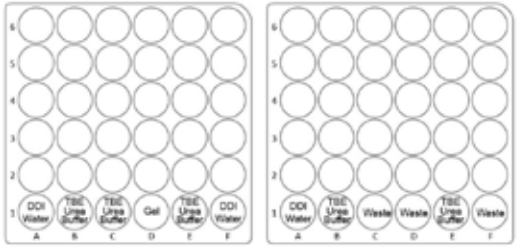


图5. 左侧的缓冲剂入口托盘 (BI) 和右侧的缓冲剂出口托盘 (BO)

每个试剂的体积如下：

- 去离子 水：1.5 mL 去离子 水。
- TBE 尿素缓冲剂：1.5 mL Tris-硼酸盐-尿素缓冲剂
- 凝胶：
  - 对于五次或更少的运行：200 μL ssDNA 100-R Gel。
  - 对于六次或更多次连续运行：1.5 mL ssDNA 100-R Gel。

  注释： 每隔五次运行，应使用凝胶填充法更换一次毛细管中的 ssDNA 100-R Gel。
- 废液：1 mL 去离子 水

3. 结果与分析

3.1 毛细管凝胶电泳法分离结果

样品为两条等浓度的寡核苷酸引物，长度分别为33 nt和34 nt；其毛细管凝胶电泳分离结果如下图；

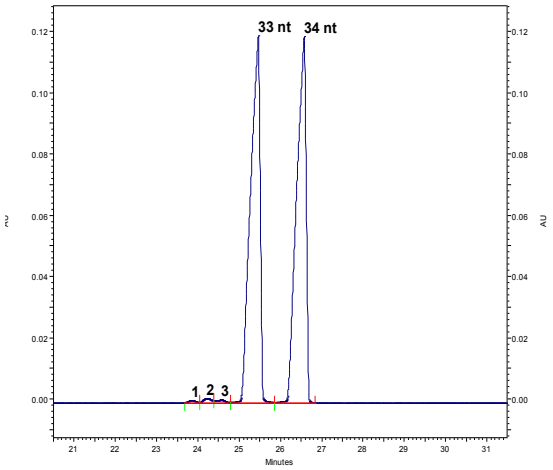


图6. 两条寡核苷酸引物的毛细管凝胶电泳分离谱图

表1. 两条寡核苷酸引物的毛细管凝胶电泳分离结果

PK#	峰名	迁移时间(min)	校正峰面积 %	分离度
1	杂质1	23.86	0.38	0.00
2	杂质2	24.21	0.59	0.89
3	杂质3	24.57	0.50	0.65
4	33 nt	25.46	50.76	1.50
5	34 nt	26.57	47.77	2.28

毛细管凝胶电泳法依据核酸片段大小进行分析，其中峰4和5分别对应33 nt和34 nt的两条等浓度的寡核苷酸引物，并且峰响应值一致；峰1-3是该样品中的杂质峰。如表1所示，毛细管凝胶电泳法可实现对单核苷酸差异的寡核苷酸引物实现基线分离，分离度为2.28。

3.2 重复性考察

为了进一步评估毛细管凝胶电泳方法的稳定性，对该样品进行3次重复进样，结果如表2。33 nt和34 nt两条引物迁移时间的RSD值分别为1.17 %和1.14 %；33 nt和34 nt两条引物校准峰面积%的RSD分别为1.14%和 0.26%，结果表明该方法重复性良好。

表2. 33 nt和34 nt寡核苷酸毛细管凝胶电泳分析的重复性

	33 nt寡核苷酸		34 nt寡核苷酸	
	迁移时间 (min)	校准峰面积%	迁移时间 (min)	校准峰面积%
Rep-1	29.41	51.02	30.66	48.02
Rep-2	29.82	51.01	31.08	47.78
Rep-3	30.10	51.01	31.36	47.98
RSD	1.17%	0.01%	1.14%	0.26%

4. 结论

毛细管凝胶电泳法是依据核酸片段大小的差异进行分离，广泛应用于核酸的纯度分析。本文采用SCIEX 成熟稳定的ssDNA 100-R单链寡核苷酸分析试剂盒，可有效应用于引物及寡核苷酸药物的质量控制。毛细管凝胶电泳法主要表现出如下优点：

1) 分离度高，可实现单碱基差异的寡核苷酸分析：33 nt和34 nt的寡核苷酸可实现基线分离，分离度可达2.28。
2) 分析速度快，30 min内可以完成分离。
3) 操作简单，无需标记，可以直接使用UV检测器。

Development of purity analysis methods for synthetic prime editing guide RNAs (pegRNAs) with high-level secondary structures for prime editing research applications

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Prime editing (PE) is a novel variation on CRISPR systems that utilizes a Cas9 nickase fused to a reverse transcriptase and a prime editing guide RNA (pegRNA) to achieve high editing efficiency and precision.[1] However, the length requirement of pegRNAs at 120–250 nucleotides (nt) and the potential for secondary structures due to complementary or partially complementary sequences present analytical quality control (QC) challenges for purity analysis of chemically synthesized pegRNA molecules. Here, a method based on capillary gel electrophoresis (CGE) was developed to mitigate the technical challenges by thoroughly disrupting the hydrogen bonds in pegRNA molecules and maintaining their denatured state during analysis. This method can offer high resolution and provide purity quantification of these pegRNAs.

Introduction

PE is a gene-editing strategy that can perform targeted, small insertions, deletions, and substitutions without introducing double-stranded breaks in the genome.[1,2] The pegRNAs utilize a novel Cas9 reverse transcriptase fusion protein, guide the protein to the targeted location in the genome and provide a site-specific DNA repair template for reverse transcriptase priming.[3] This technology has the advantages of high precision, efficiency and flexibility over conventional genome-editing approaches, and it shows the potential to correct genetic variants associated with human diseases.[3]

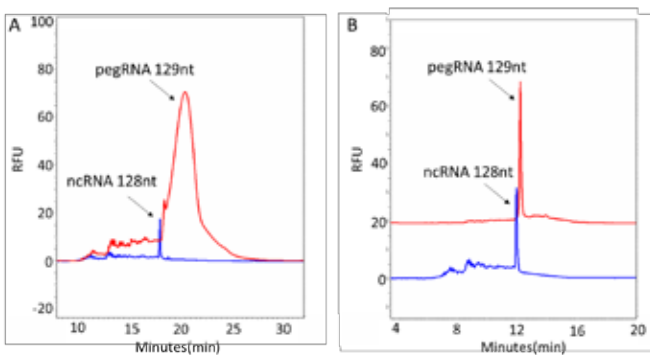


Figure 1. Method optimization for pegRNA purity analysis on a PA 800 Plus system. Analysis of pegRNA at 129 nt and non-complementary RNA (ncRNA) at 128 nt is shown before (panel A) and after (panel B) application of optimized denaturing strategies.

In some PE applications, synthetic RNA oligonucleotides are favored because they allow for the addition of stabilizing chemical modifications, and they are readily amenable to high-throughput screening, which is essential as pegRNA design rules are still being understood. However, the length requirement of 120–250 nucleotides (nt) and the complexity of pegRNAs present quality control (QC) challenges in analytical method development. The high-level secondary structures of pegRNAs can lead to shifts of migration times for pegRNAs that do not resolve in a size-dependent manner. Using common denaturants alone in the analytical analysis will not entirely disrupt the hydrogen bonds of these molecules or maintain the denatured state, resulting in inaccurate determination of the purity of the chemically synthesized pegRNAs. Therefore, due to diverse research and therapeutic interest in PE, there is a need to develop purity analysis methods that can overcome the formation of energetically favorable secondary structures of these chemically synthesized RNA molecules.

This technical note outlines a robust method that uses capillary gel electrophoresis (CGE) to mitigate the technical difficulties involved in the purity analysis of pegRNAs with high-level secondary structures (Figure 13). When synthetic pegRNAs are analyzed on a capillary electrophoresis (CE) system under standard conditions, these secondary structures produce peaks with higher structure isoforms that significantly interfere with the purity analysis (panel A in Figure 1). However, with optimized denaturing strategies, fast and reliable purity determination of pegRNA has been achieved (panel B in Figure 1).

Key features of pegRNA purity analysis

- Innovative purity analysis workflow for chemically synthesized pegRNAs with high-level secondary structures
- Purity determination of pegRNA from the high-resolution separation of the full-length product from its impurities
- A high level of repeatability with relative standard deviation% (RSD%) of <3% was achieved as a result of accurate and stable capillary temperature control on the PA 800 Plus system
- Fast quality assessment of chemically synthesized pegRNAs within 30 minutes per sample

Materials and methods

Materials: The Sample Loading Solution (SLS, PN 608082) and the ssDNA 100-R kit (PN 477480) containing DNA capillaries, ssDNA 100-R gel (lyophilized), Tris-Borate buffer, 7M Urea, and pd(A) 40-60 test mix were from SCIEX (Framingham, MA). Nuclease-free water (PN AM9932) and SYBR Green II RNA gel stain, 10,000X concentrate in DMSO (PN S7586) were from Thermo Fisher Scientific (Waltham, MA). Recombinant Human RNA Helicase A protein (PN ab114300) was from Abcam (Cambridge, UK). The single guide RNAs (sgRNAs) and pegRNA oligonucleotides were chemically synthesized and provided by Integrated DNA Technologies (Coralville, IA).

Instruments and software: A PA 800 Plus system equipped with a laser-induced fluorescence (LIF) detector and an ultraviolet (UV) detector was from SCIEX. The excitation wavelength was at 488 nm, and the emission wavelength was at 520 nm when using the LIF detector. The wavelength was 254 nm when using a UV detector. The nanoVials (PN 5043467) from SCIEX were used for sample loading. Data acquisition and analysis were performed using 32 Karat software version 10.

Methods

Tris-Borate-Urea buffer reconstitution: A total of 135 mL of nuclease-free water was added to the bottle containing the dry Tris-Borate buffer. The buffer solution was stirred for about 20–30 minutes using a magnetic stir bar until the solid was completely dissolved. The dry 7M Urea was then slowly added to the Tris-Borate buffer. The solution was continuously stirred for up to 2 hours at room temperature until the 7M Urea was completely dissolved. The reconstituted Tris-Borate-Urea buffer should be good for 30 days if stored at 2°C to 8°C after preparation. The entire bottle of buffer should be brought to ambient temperature before use. The required volume to be used for the day was removed and filtered through a 0.2 µm disposable syringe filter into a clean container.

ssDNA 100-R gel buffer reconstitution: A certain amount (varied in the method optimization) of 0.2 µm filtered Tris-Borate-Urea buffer was added to the lyophilized gel to reconstitute the gel buffer. After the gel buffer vial was securely capped, the gel mixture was gently stirred with a stirring bar for 4 to 6 hours or overnight until the gel was completely dissolved. The gel buffer was then filtered through a 0.45 µm disposable syringe filter. The prepared gel buffer can be used for up to 30 days after preparation if stored at 2°C to 8°C.

Cartridge assembly: A DNA capillary (PN 477477) from SCIEX was installed according to the instructions in the ssDNA 100-R kit application guide.[4] The total capillary length was 30.2 cm, with 20 cm as the length from the detection window. A 100 x 200 µm aperture was used for better resolution when using a UV detector. Since the inner wall of the DNA capillary is coated, the cartridge assembly should be carried out promptly. Excessive exposure to air may damage the inner coating and cause clogging. The capillary ends must be immersed in liquid (water or buffer) as soon as the cartridge assembly is complete to prevent the coating from drying out.

Preparation of buffer trays and sample trays: Vial positions for buffer trays are indicated in Figure 2. Each water vial was filled with 1.5 mL double deionized (DDI) water. The waste vial was filled with 0.8–1.0 mL DDI water. The gel vial was filled with 1.3 mL ssDNA 100-R gel buffer. The gel vial was capped and sonicated to remove the air bubbles before being loaded onto the PA 800 Plus system for analysis. Specifically, the gel vial was sonicated five times for 30 seconds each time, allowing the air bubbles to rise to the surface after each 30-second interval. The buffer vials were filled with 1.5 mL Tris-Borate-Urea buffer.

Instrument setup: The Initial Conditions and UV Detector Initial Conditions were set up as indicated in Figure 3 and Figure 4, respectively. The same setup was used for all three methods: the new capillary-conditioning method, the gel-filling method and the separation method.

Gel filling and separation: The Time Program settings for the new capillary-conditioning method are shown in Figure 5. Figure 6 and Figure 7 show the Time Program settings for the gel-filling and separation methods, respectively.

Sample preparation: The RNA oligonucleotides were diluted with nuclease-free water to about 0.5–1 µM and then loaded into the sample insert tube (a minimum sample volume of 40 µL) or the nanoVial (a minimum sample volume of 5 µL). Sample vials were loaded onto the sample inlet tray for analysis.

To analyze oligonucleotides with ultra-low concentration, the SYBR Green II RNA gel stain and the LIF detector were used to achieve high sensitivity. First, the Tris-Borate-Urea buffer was mixed with the SYBR Green II RNA gel stain at a ratio of 25,000 to 1. The settings for the LIF Detector Initial Conditions are shown in Figure 8.

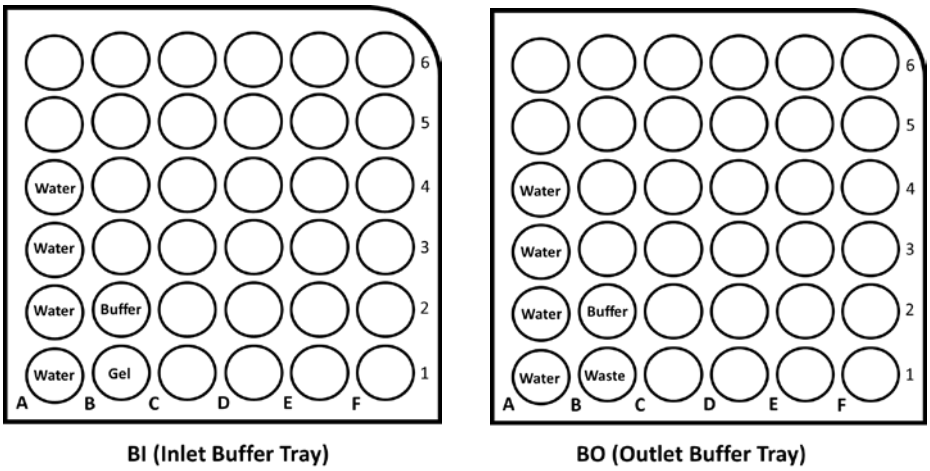


Figure 2. Schematic setup for inlet (left) and outlet (right) buffer trays.

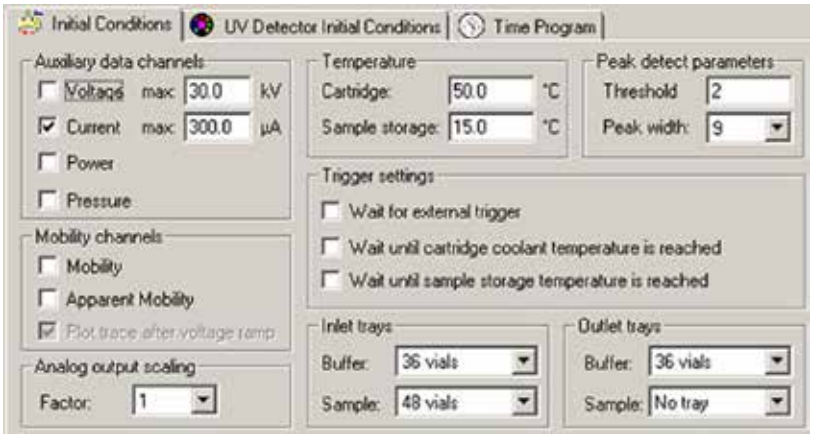


Figure 3. Settings for the Initial Conditions tab.

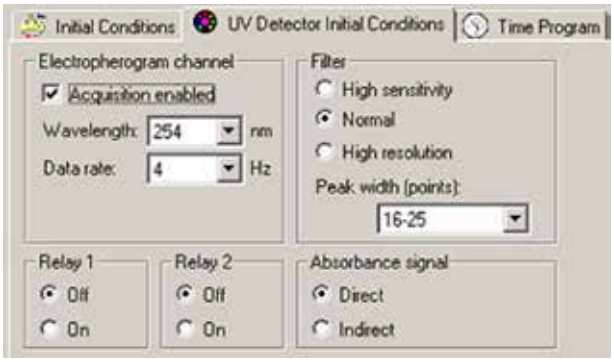


Figure 4. Settings for the UV Detector Initial Conditions tab.

Initial Conditions | UV Detector Initial Conditions | Time Program

	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary
1		Rinse - Pressure	20.0 psi	20.00 min	BI:B1	BO:B1	forward
2							

Figure 5. Time Program settings for the new capillary-conditioning method.

Initial Conditions | UV Detector Initial Conditions | Time Program

	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary
1		Rinse - Pressure	70.0 psi	5.00 min	BI:B1	BO:B1	forward
2		Wait		0.00 min	BI:A2	BO:A2	
3		Wait		0.00 min	BI:A3	BO:A3	
4		Separate - Voltage	3.0 KV	2.00 min	BI:B2	BO:B2	0.17 Min ramp, reverse polarity
5		Separate - Voltage	5.0 KV	2.00 min	BI:B2	BO:B2	0.17 Min ramp, reverse polarity
6	0.00	Separate - Voltage	9.3 KV	10.00 min	BI:B2	BO:B2	0.17 Min ramp, reverse polarity
7	1.00	Autozero					
8	10.00	End					
9							

Figure 6. Time Program settings for the gel-filling method.

Initial Conditions | UV Detector Initial Conditions | Time Program

	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary
1		Wait		0.00 min	BI:A1	BO:A1	
2		Inject - Voltage	1.5 KV	4.0 sec	SI:A1	BO:B2	Override, reverse polarity
3		Wait		0.00 min	BI:A1	BO:A1	
4	0.00	Separate - Voltage	9.3 KV	20.00 min	BI:B2	BO:B2	0.17 Min ramp, reverse polarity
5	1.00	Autozero					
6	20.00	End					
7							

Figure 7. Time Program settings for the separation method.

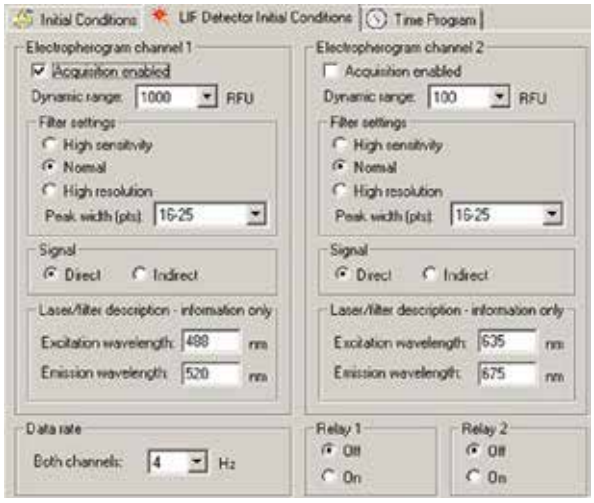


Figure 8. Settings for the LIF Detector Initial Conditions tab.

Results and discussion

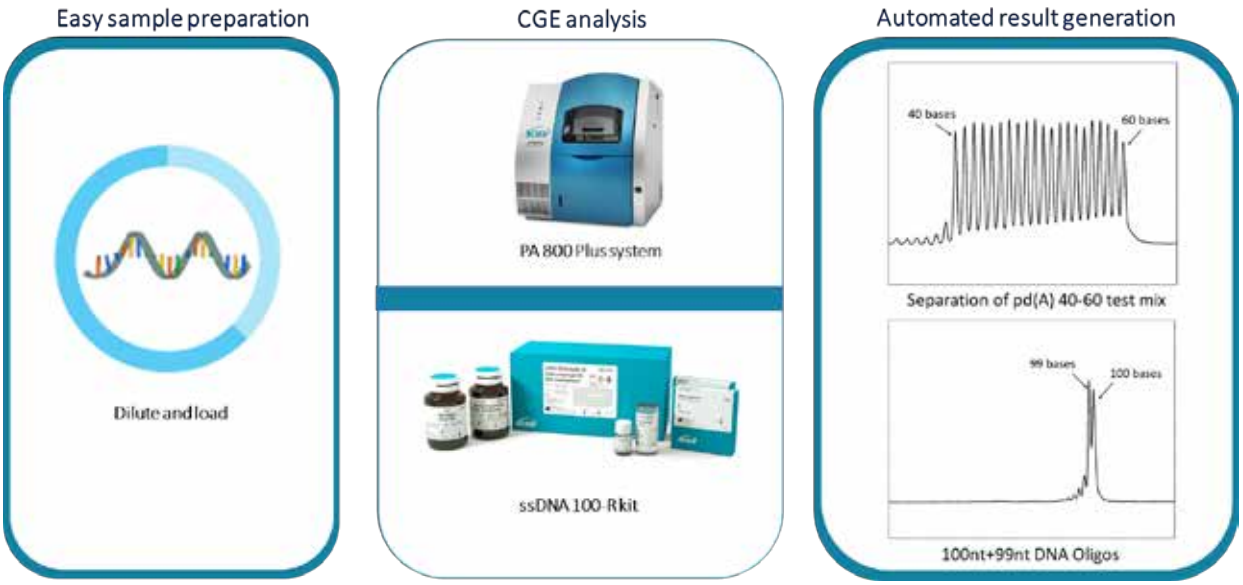


Figure 9. Oligonucleotide purity analysis workflow.

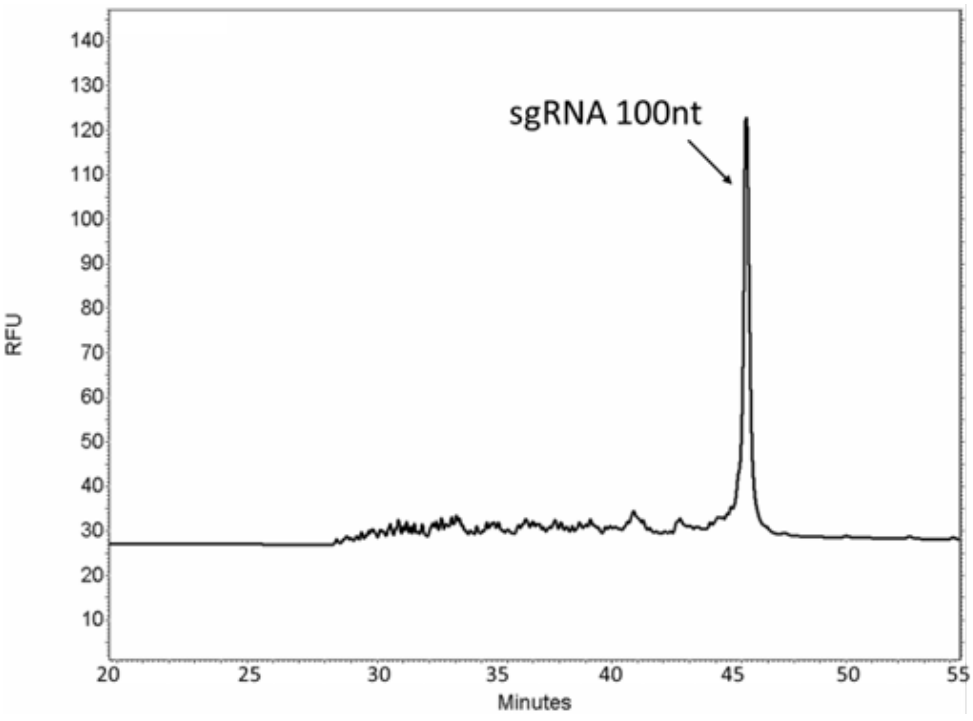


Figure 10. Purity analysis of 100 nt sgRNA using the ssDNA 100-R kit and following the application guide.

Workflow of oligonucleotide purity analysis with single-base resolution

The workflow of oligonucleotide purity analysis shown in Figure 9 utilizes the ssDNA 100-R kit and the PA 800 Plus system with simple dilute-and-load sample preparation. The ssDNA 100-R kit is suitable for both small-sized RNA and DNA purity analysis. It features a replaceable gel buffer and a coated capillary for maximum reproducibility, and it provides rapid separation and analysis of oligonucleotides between 10 and 100 bases with single-base resolution. The results shown on the right side of Figure 9 demonstrate the separation of a poly d(A) 40-60-mer oligonucleotides test mix with single-base resolution (upper panel) and the separation of the 1:1 mixture of a 99 nt and 100 nt DNA oligonucleotide (lower panel).

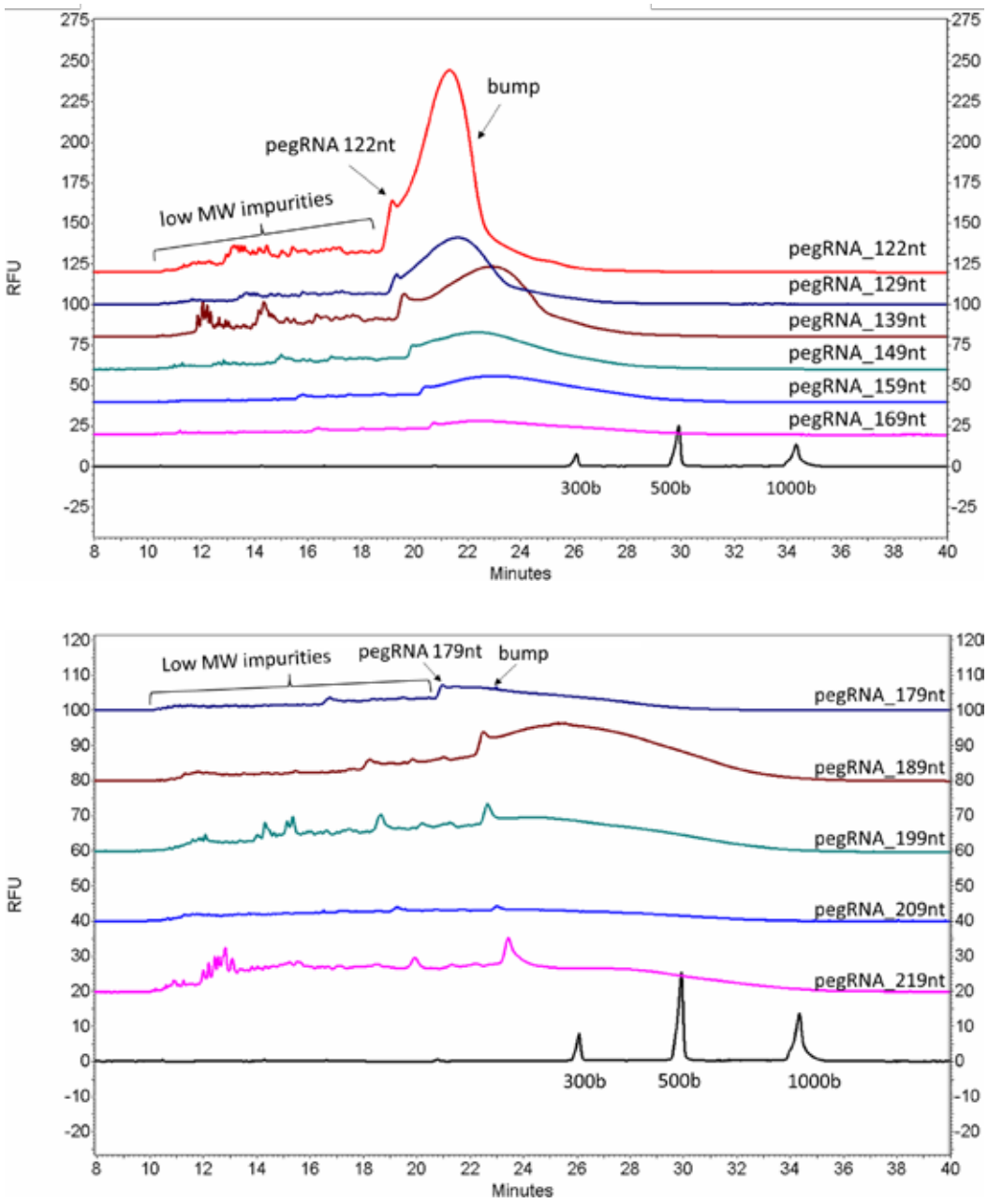


Figure 11. Analysis of pegRNAs using the ssDNA 100-R kit with optimized gel matrix for long oligonucleotides, but without optimization of the denaturation of the pegRNAs. The upper panel is from size 122 nt to 169 nt. The lower panel is from size 179 nt to 219 nt.

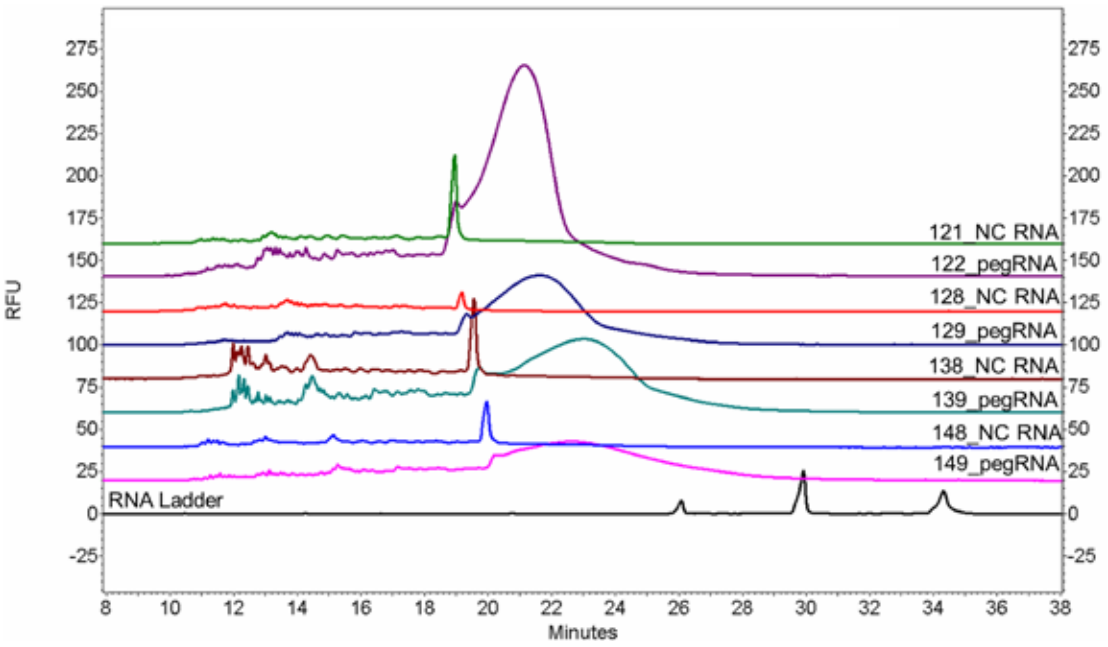


Figure 12. Comparison of the results of pegRNA and NC RNA analysis using the ssDNA 100-R kit with optimized gel matrix for long oligonucleotides.

The purity analysis of a 100-nt single guide RNA (sgRNA) is shown in Figure 10. The purity of the synthesized product can be accurately determined by separating the full-length product (FLP) from its lower molecular weight in-process impurities.

Method development for purity analysis of pegRNAs

The gel matrix in the ssDNA 100-R kit, which is suitable for oligonucleotide analysis up to 100 nt, needs to be optimized for pegRNA analysis due to the larger length requirement (120–250 nt) for pegRNAs. Therefore, using 10 mL of 0.2 µm filtered Tris-Borate-Urea buffer for reconstitution of the lyophilized gel has been selected to provide the optimal resolution and separation time for the RNA ladder in the 100 nt to 250 nt range (data not shown in this technical note).

pegRNA oligonucleotides with lengths of 122 nt, 129 nt, 139 nt, 149 nt, 159 nt, 169 nt, 179 nt, 189 nt, 199 nt, 209 nt and 219 nt, together with an RNA ladder (50–1,000 bases), were analyzed with an optimized gel matrix and the ssDNA 100-R kit (Figure 11). All the pegRNA samples show a sharp peak followed by a big bump.

To investigate the root cause of the abnormal peak shape of pegRNAs, four non-complementary RNAs (NC RNAs) at various lengths (121 nt, 128 nt, 138 nt and 148 nt) were designed with the minimal possibility of forming secondary structures and analyzed together with pegRNAs of similar lengths (122 nt, 129 nt, 139 nt and 149 nt). As shown in Figure 12, all NC RNAs showed a singular peak without the big bump, indicating that the abnormal peak shape of pegRNAs was probably due to their secondary structures.

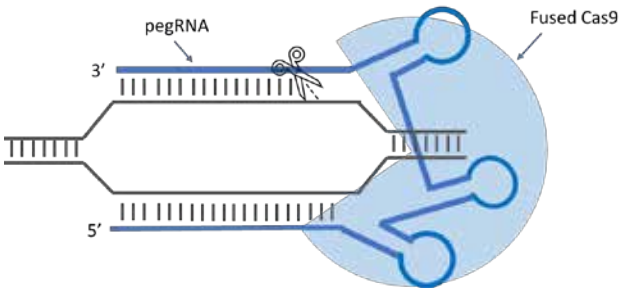


Figure 13. Schematic of pegRNA used for PE. PE utilizes a fusion protein of Cas9 nickase and a reverse transcriptase (light blue), and a long guide RNA, called a pegRNA (dark blue).

Different denaturing methods had thus been applied to the pegRNA oligonucleotides during sample preparation, including treating pegRNA with heat, formamide and helicase. However, similar results were obtained, as shown in Figure 11, without significant improvement in the peak shape. The secondary structure of pegRNA presents a big challenge for purity analysis.

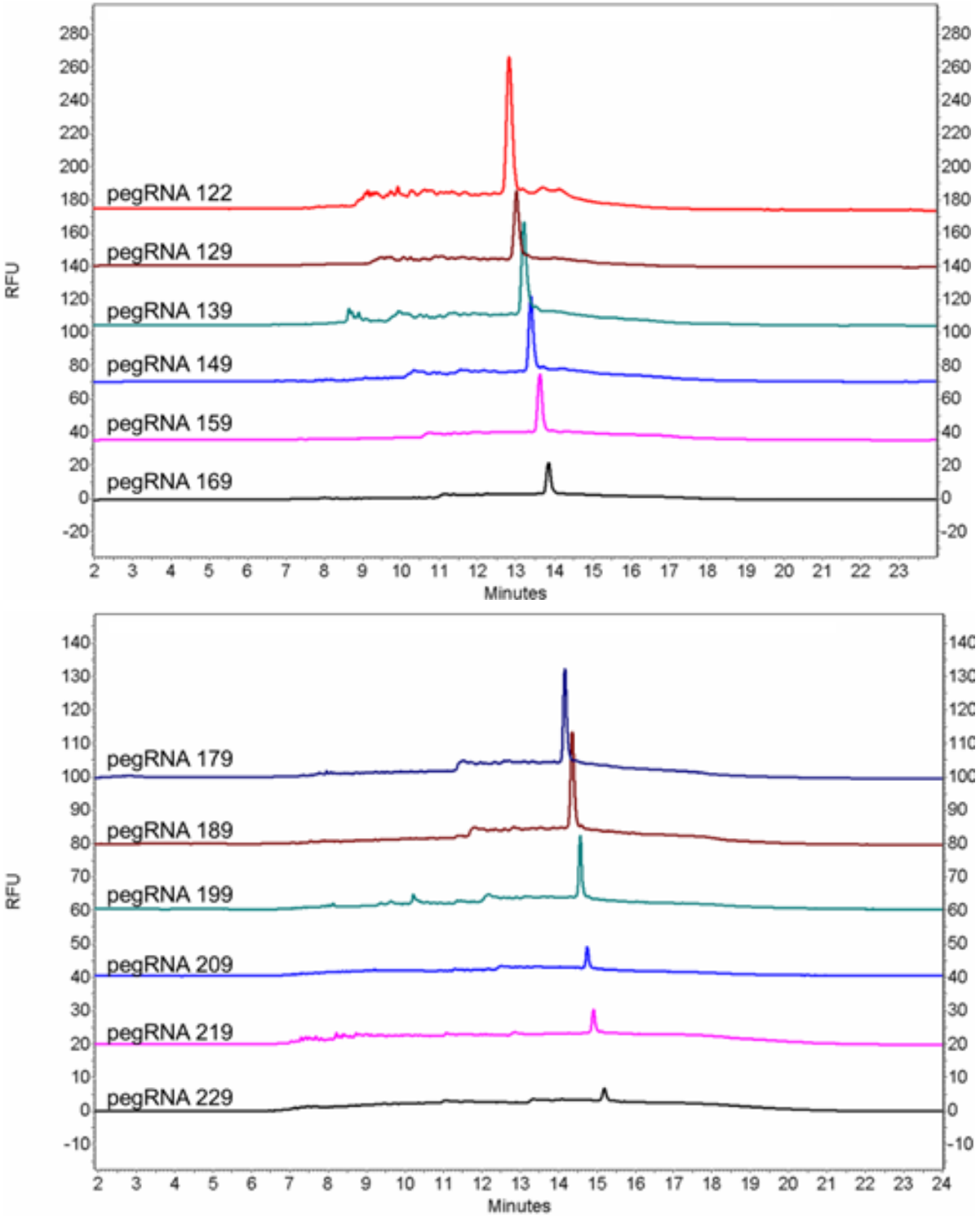


Figure 14. pegRNA analysis using the optimized denaturing strategies and the optimized gel matrix for long oligonucleotides. The upper panel is from size 122 nt to 169 nt. The lower panel is from size 179 nt to 219 nt.

Therefore, the primary focus of this technical note was to develop a robust denaturing technique for pegRNA with high levels of secondary structure (Figure 13). After systematically exploring possible strategies for denaturing pegRNA, the optimized denatured status of pegRNA oligonucleotides. Accurate and repeatable purity analysis of pegRNAs was achieved after establishing the optimized sample treatment and capillary electrophoresis conditions.

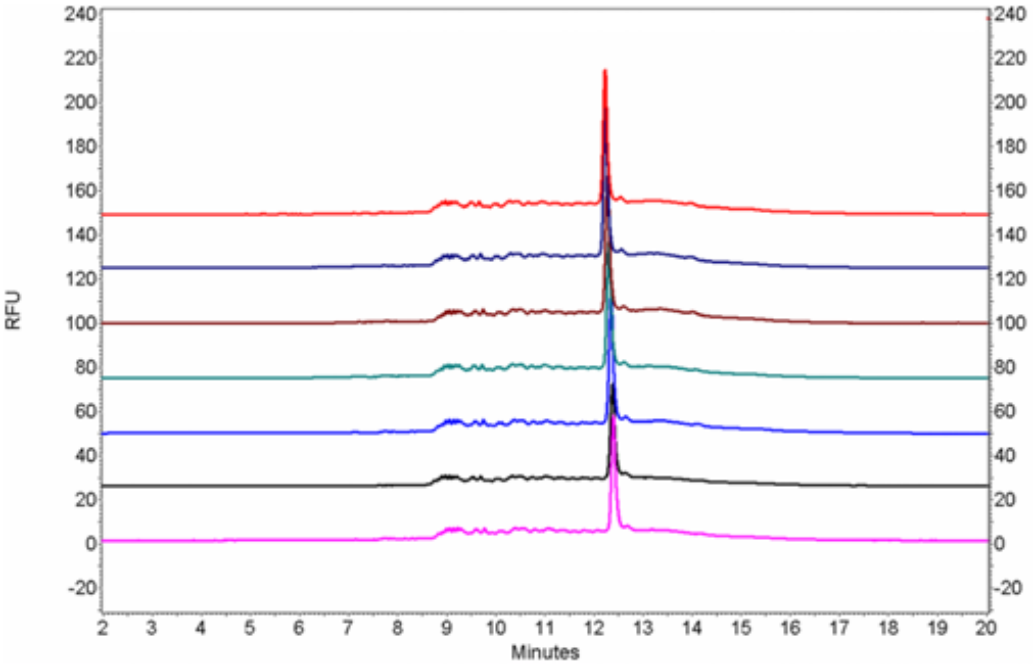


Figure 15. Repeatability of pegRNA analysis using the optimized denaturing strategies and the optimized gel matrix for long oligonucleotides.

methods have been achieved by maintaining the denatured status of pegRNA during the entire separation process in addition to the one-step denaturing of pegRNA oligonucleotides. The pegRNA samples were prepared by mixing with formamide and heating at 65°C for 10 minutes. Then, the capillary temperature was increased to 50°C to maintain the pegRNA sample under a denatured state during the separation. As shown in Figure 14, a single, sharp peak has been obtained for all the tested pegRNA samples with the increased separation temperature.

A repeatability study of a representative pegRNA with a size of 129 nt was performed by seven consecutive injections. As shown in Figure 15, peak profile, migration time and signal level were very consistent between the seven injections. The RSD% of migration time (MT) and the corrected peak area% (CPA%) of FLP were 1.61% and 2.45%, respectively. The liquid cooling feature of the capillary temperature on the PA 800 Plus system enables stable and accurate temperature control during the separation, therefore providing a robust platform for maintaining the

Conclusions

An easy and fast workflow for the purity analysis of complex pegRNA molecules has been described in this technical note. High levels of sequence complementarity in pegRNAs result in remarkable overlapping higher-structure isoforms, presenting a considerable obstacle for purity analysis. After trialing various denaturation methods, the ability to resolve pegRNA sequences into a single, sharp peak has been achieved and %FLP can be determined. A stable and high temperature enabled by the capillary temperature control system on the PA 800 Plus system facilitates the maintenance of the denatured state of pegRNAs during the entire separation process, leading to optimal accuracy and repeatability with RSD% of <3% for the purity determination of pegRNA. It provides a convenient and robust method for quality assessment of chemically synthesized pegRNA within 30 minutes per sample.

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