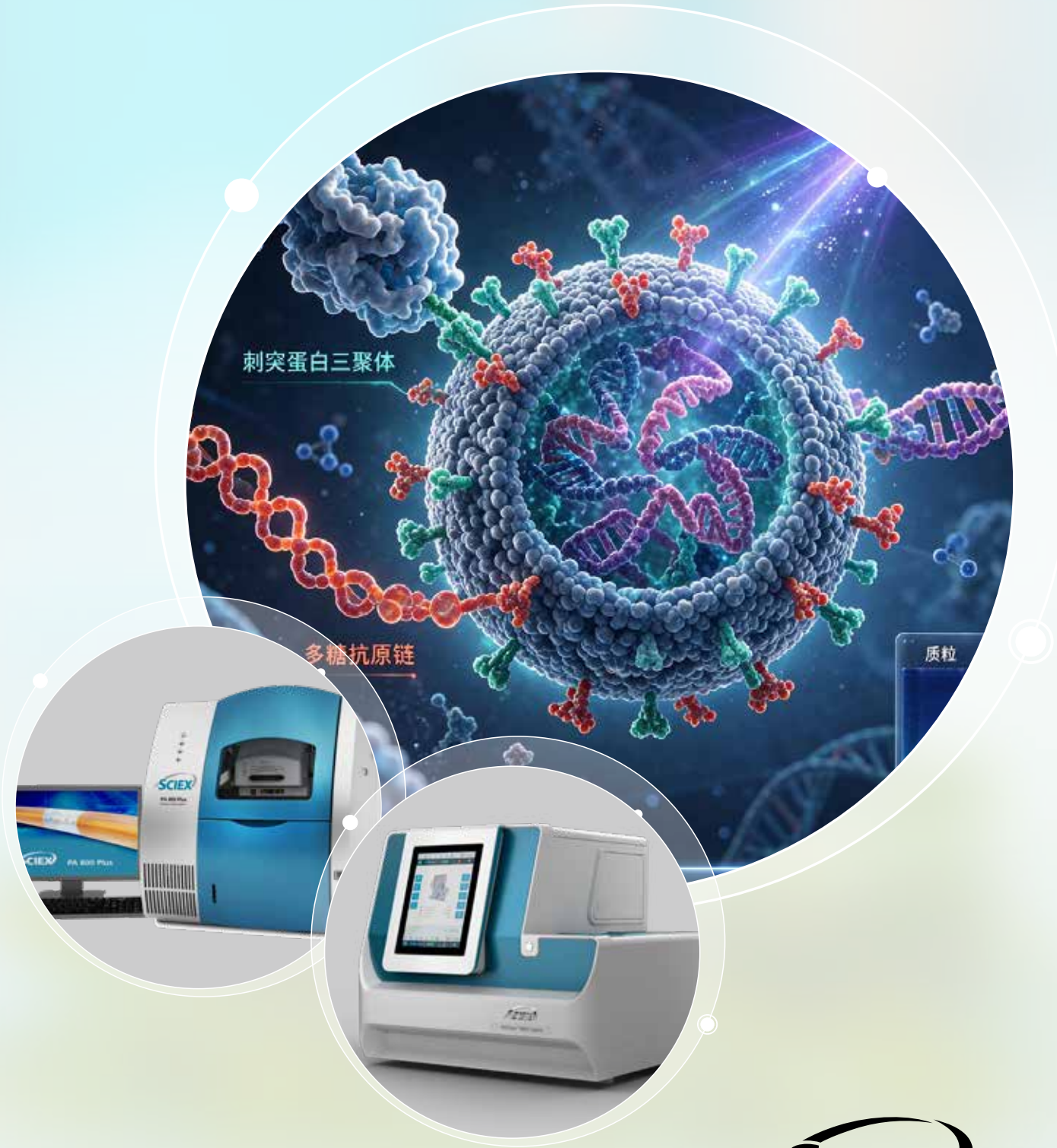
毛细管电泳 (CE)

—— 疫苗产品应用文集



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。

疫苗领域

疫苗产品类型多样，涵盖重组蛋白疫苗、多糖及结合疫苗、病毒样颗粒疫苗以及 mRNA 疫苗等，其质量控制需重点关注抗原纯度、结构完整性、分子大小分布、糖基或多糖组成、核酸完整性及相关工艺杂质等关键质量属性。毛细管电泳 (CE) 技术可针对蛋白抗原、多糖组分和核酸类成分提供高分辨率、高灵敏度的分离分析能力，适用于疫苗研发、工艺优化、批间一致性评价和质量放行等环节，是疫苗产品质量研究与控制中的重要分析平台。

目录

1. 毛细管凝胶电泳用于 mRNA 疫苗原液及制剂的完整性分析 5
2. mRNA-LNP nucleic acid assessment from distinct formulations by multi-capillary gel electrophoresis 8
3. Comprehensive characterization of mRNA-LNP samples using an optimized sample preparation method and CGE-LIF workflow 17
4. 高灵敏度、高稳定性的 CGE 方法对 mRNA PolyA 尾巴分布的分析—— 使用 SDS-MW 试剂盒的方法 24
5. Analysis of mRNA poly(A) tails with single-nucleotide resolution by capillary gel electrophoresis with UV detection (CGE-UV) 27
6. 使用 BioPhase™ 8800 系统分析 mRNA- LNP 等电点及稳定性 34
7. High-resolution characterization of both sgRNA and Cas9 mRNA in a single analysis for CRISPR/Cas9 applications . 36
8. Addressing an unmet need: Developing a new method for purity analysis of large-sized RNA drugs 44
9. BioPhase™ 8800 系统进行不同长度环状 RNA 疫苗的高通量纯度分析 51
10. CGE-LIF 方法对较长（4000 nt 以上）环状 RNA 的纯度分析 54
11. Accurate and robust mRNA-LNP encapsulation efficiency analysis by capillary gel electrophoresis with laser-induced fluorescence detection (CGE-LIF). 56
12. 毛细管凝胶电泳方法用于 mRNA 疫苗中宿主残留 DNA(HCD) 的分布分析 64
13. 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 67

14. CGE-LIF 方法分析不同工艺阶段狂犬疫苗中宿主残留 DNA 的片段大小分布 70

15. CGE-LIF 方法对重组蛋白类新冠疫苗中宿主细胞残留 DNA 片段大小的分布分析 73

16. 毛细管电泳法对宫颈癌疫苗 HPV 纯度和等电点的分析 76

17. CE-SDS 方法对百日咳疫苗相关毒素的纯度分析 81

18. 毛细管胶束电动色谱法对多糖蛋白结合疫苗中游离载体蛋白的检测 84

毛细管凝胶电泳用于mRNA疫苗原液及制剂的完整性分析

Analysis of purity and integrity of mRNA vaccine by capillary gel electrophoresis

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Keywords: Capillary Gel Electrophoresis, LIF, mRNA vaccine, Purity, Integrity

1. 引言

新冠疫情的爆发使市场对于mRNA疫苗的关注度提升到了空前的高度，相比于传统疫苗mRNA疫苗在安全性、有效性、开发速度和规模化生产能力等方面具有显著优势。然而，mRNA本身不稳定，极易被无处不在的核酸酶降解，使得mRNA疫苗在生产、工艺、长期储存等过程存在挑战。因此，一种简单高效的mRNA疫苗纯度分析和分子大小表征方法对其质量、疗效、安全性和优化制造工艺至关重要。

毛细管凝胶电泳法（CGE-LIF）是有效分离核酸的方法，具有分辨率高、分离分析时间短、定量准确、自动化程度高等优势，适用于从几十至上万长度核酸的分离，可有效用于mRNA疫苗纯度及完整性的分析检测。

本文利用SCIEX PA 800 Plus药物分析系统，采用CGE-LIF方法对mRNA疫苗进行纯度和完整性进行表征。该方法分离度高、重复性好，适用于不同阶段mRNA疫苗原液及LNP包裹的mRNA制剂的纯度和完整性表征。

2. 试剂及方法

2.1 试剂和样品

试剂：聚乙烯吡咯烷酮 (PVP, Sigma PN 437190)、 10 × TBE buffer (Sigma, PN 574795)、尿素 (Sigma, PN U5378)、SYBRTM Green Ⅱ RNA Gel Stain (10000 × concentrate in DMSO, Thermo, PN S7564)、SLS (SCIEX, PN608082)、去离子水 (Millipore)。

样品：RNA 9000 Ladder（500nt、1000nt、1500nt、2000nt、2500nt、3000nt、4000nt、5000nt、6000nt、9000nt) (Thermo Fisher，PN AM7152)、样品1 (mRNA疫苗原液，浓度为1000 ng/μL，片段长度为4000 nt)、样品2 (LNP包裹的mRNA疫苗制剂：主要包括向导RNA(gRNA, 100nt以内)和目标核酸(4000nt左右)，核酸总浓度为 500 ng/μL)。

2.2 试剂及样品溶液制备

背景缓冲液制备：分别称取0.2 g PVP和 4.8 g尿素，加入50 mL的离心管中，用1 × TBE溶液 (10 × TBE溶液用去离子水稀释10倍)充分溶解后定容至20 mL，加入2 μL 染料 (即1:20000稀释染料)，混匀即得含1%PVP和4M尿素的背景缓冲液。

样品制备：a) RNA 9000 Ladder用SLS溶液先稀释至10.0 ng/μL，再根据实验需要用SLS稀释至不同浓度。样品1用SLS溶剂稀释至1.0 ng/μL。样品2先用2%的TritonX 1:1混匀，静置10 min，破乳，破乳后的样品再用SLS溶剂稀释至5.0 ng/μL。

2.3 仪器及方法

采用SCIEX PA 800 Plus药物分析系统，匹配LIF检测器，采集频率：4 Hz，激发波长488 nm，发射波长520 nm；毛细管：熔融石英毛细管，50 μm内径，20/30.2 cm (有效/总长度)；毛细管温度：25 ℃；样品室温度：8 ℃；进样条件：电压进样，-5 kV，3 s；分离条件：-5 kV，20 min。

毛细管预冲洗：序列运行前毛细管先用0.1 N HCl进行50 psi冲洗10 min，再用去离子水进行50 psi冲洗10 min，最后用背景缓冲液进行70 psi冲洗10 min。每次运行开始时，毛细管先使用去离子水进行50 psi冲洗2 min，再用背景缓冲液进行70 psi冲洗10 min。

3. 结果与讨论

3.1 RNA 9000 Ladder重复性

RNA 9000 Ladder分离结果如图1，结果显示，在15 min内完成RNA 9000 Ladder所有10个marker (500 nt-9000 nt)的分离，且各峰的分离度好，所有峰之间的分离度均超过1，说明该方法适用RNA样品长度范围广。

连续3针运行，重复性结果如图1和表1，各峰迁移时间重复性RSD值在0.05%-0.18%，校准峰面积百分比RSD值在0.71%-2.55%。说明该方法重复性好，可用于RNA样品纯度分析。

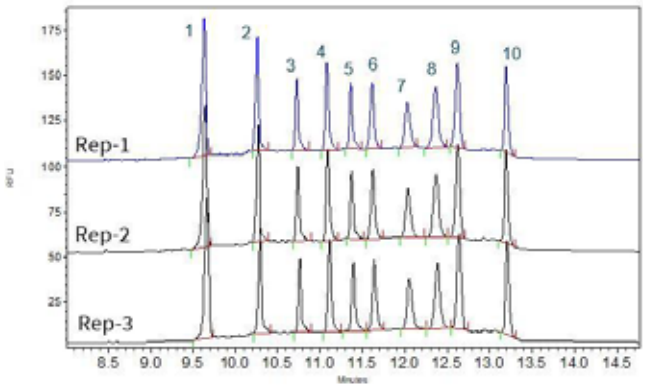


图1. RNA ladder的重复性分离结果

表1. RNA 9000 Ladder各峰的重复性结果 (n=3)

Pk #	1	2	3	4	5	6	7	8	9	10
Length(nt)	500	1000	1500	2000	2500	3000	4000	5000	6000	9000
RSD%(MT)	0.18	0.17	0.16	0.15	0.13	0.12	0.11	0.1	0.08	0.05
RSD%(CAP)	1.8	0.71	1.65	1.09	2.55	1.25	2.26	1.12	1.73	0.98

注：MT指迁移时间；CAP指校准峰面积百分比

3.2 RNA 9000 Ladder检测灵敏度结果

将RNA 9000 Ladder溶液稀释至一系列不同浓度 (2.0 ng/μL、1.0 ng/μL、100.0 pg/μL、10.0 pg/μL、1.0 pg/μL)，当溶液浓度为1.0 pg/μL时，所有10个marker依然能够明显检出 (如图2所示)，说明该方法检测灵敏度高。

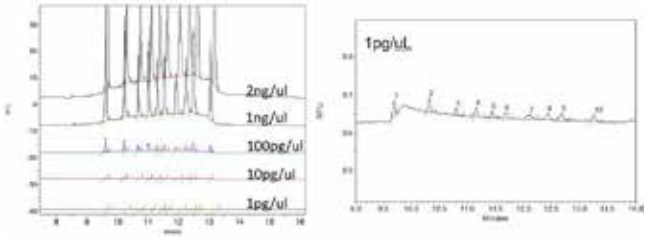


图2. 不同浓度下RNA 9000 Ladder电泳图谱

3.3 mRNA疫苗结果样品检测结果

利用该CGE-LIF方法对mRNA疫苗样品进行分离检测，结果如图3，a为mRNA原液纯度分析电泳图；b为LNP包裹的mRNA制剂纯度分析电泳图，其中9min左右的峰为gRNA（指导mRNA在真核细胞内的编辑和删除），目标片段出峰位置在14 min左右；c为RNA 9000 Ladder混标的电泳图。

从电泳图谱上可看出，mRNA原液和制剂中主峰的出峰位置一致，说明该方法稳定性好，可用于纯化过程纯度监控以及稳定性放行对过程。从图3b可看出，100 nt以内的gRNA和4000 nt左右的mRNA能够清晰的分离和检测，说明该CGE-LIF方法适合分析范围从几十到上万长度的RNA分离检测。

mRNA原液样品(a)纯度检测结果为66.39%，主峰片段长度计算结果为4574 nt；LNP包裹的mRNA制剂样品(b)纯度检测结果为36.22%，主峰片段长度计算结果为4436 nt，符合实际分子量值范围。

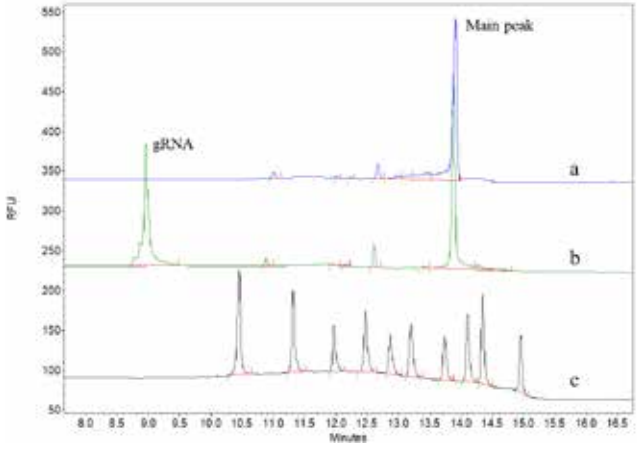


图3. mRNA疫苗原液及制剂样品CGE-LIF分离结果电泳图。
(a: mRNA原液；b: LNP-mRNA制剂；c: RNA 9000 Ladder)

4. 结论

本文采用CGE-LIF方法进行mRNA疫苗纯度及完整性分析，通过RNA 9000 Ladder混标进行重复性和检测灵敏度的考察，各峰迁移时间重复性RSD值在0.05%-0.18%，校准峰面积百分比RSD值在0.71%-2.55%，展示了CGE-LIF方法用于RNA样品纯度及完整性分析良好的稳定性和较高的检测灵敏度。同时，mRNA原液和制剂主峰迁移时间重复性高度一致，说明CGE-LIF方法迁移时间重复性高，可用于纯化过程mRNA疫苗纯度监控以及稳定性考察、产品放行等不同阶段质量控制。该方法下，LNP包裹的mRNA制剂能够清晰地分离和检测到片段在100 nt以内的gRNA和长度在4000 nt左右的目标RNA片段，展示了CGE-LIF方法的较宽的分析范围，适用于多种不同mRNA疫苗产品的质量评估。

mRNA-LNP nucleic acid assessment from distinct formulations by multi-capillary gel electrophoresis

Implications for vaccines and RNA medicine characterization using the BioPhase 8800 system and the RNA 9000 Purity & Integrity Analysis kit

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The use of lipid nanoparticles (LNPs) to deliver mRNA products against SARS-CoV-2 during the COVID-19 pandemic demonstrated the power and efficacy of this drug-delivery device for RNA-based vaccines.[1] In addition to vaccine development, the application of mRNA-LNPs has expanded widely into gene editing and the treatment of aggressive malignancies. As a result, the demand has increased for high-throughput and robust analytical technologies used to ensure that mRNA-LNPs are properly synthesized and loaded with the intended nucleic acid product. In this technical note, we show a high-throughput, robust analysis of nucleic acid integrity and size in mRNA-LNP samples using the multi-capillary BioPhase 8800 system and the RNA 9000 Purity & Integrity Analysis kit.

A simplified nucleic acid extraction method and sample analysis workflow (Figure 1) using capillary gel electrophoresis and laser-induced fluorescence (CGE-LIF) were used to confirm that samples in the mRNA-LNP collection contained different nucleic acid loads. The in vitro transcribed RNA product used to manufacture these mRNA-LNPs was analyzed, and a linearity curve covering approximately 2.5 orders of magnitude was generated. The BioPhase Analysis tool was used to calculate the lower limit of detection (LLOD), the lower limit of quantification (LLOQ) and the upper limit of detection (ULOD), and to guide the identification of low-level impurities or nucleic acid fragmentations associated with manufacturing mRNA-LNPs.[2]

Key features of mRNA-LNP analysis by CGE-LIF

- The BioPhase 8800 system and the RNA 9000 Purity & Integrity Analysis kit provide robust characterization for in vitro transcribed mRNA utilized for the mRNA-LNP synthesis
- Higher and lower molecular weight products seen in nucleic acid species extracted from mRNA-LNPs can be characterized with high resolution on the BioPhase 8800 system
- The BioPhase Analysis tool provides automated purity and accurate size analysis for the optimal characterization of nucleic acids loaded into mRNA-LNPs

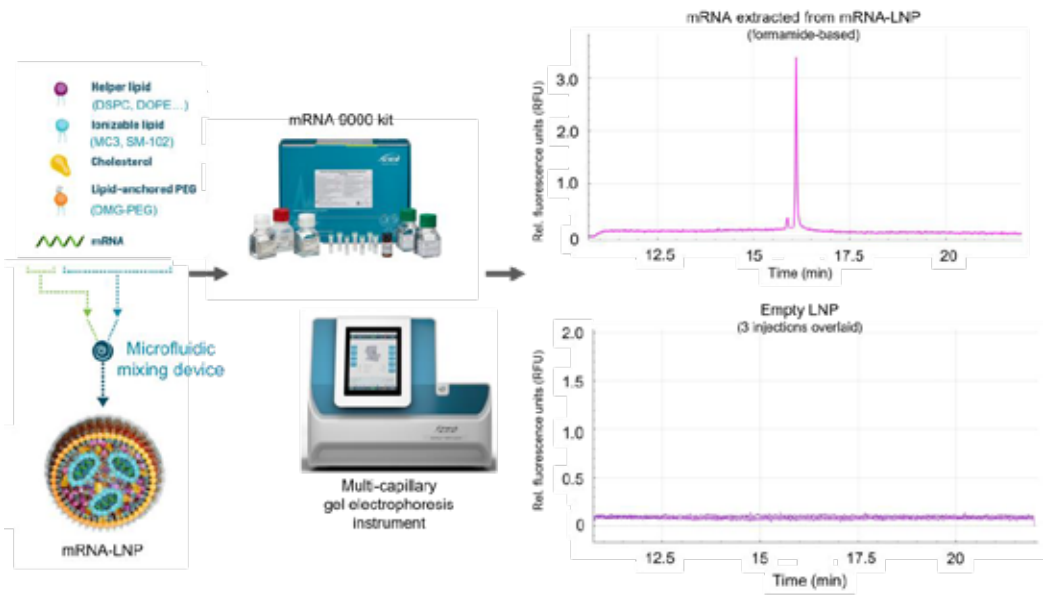


Figure 1. Simplified workflow to assess nucleic acid integrity of mRNA-LNPs with the multi-capillary BioPhase 8800 system and the RNA 9000 Purity & Integrity Analysis kit. The top panel shows a representative profile of RNA extracted from an MC3-based mRNA-LNP product. The bottom panel shows the overlay of 3 empty MC3-LNP products.

p 1

Methods

Materials: The RNA 9000 Purity & Integrity Analysis kit (Figure 1, P/N C48231) containing the Nucleic Acid Extended Range Gel, SYBR Green II RNA Gel Stain*, acid wash (regenerating solution), CE-grade water and the ssRNA ladder (50-9000 bases), in addition to the pre-assembled BioPhase BFS capillary cartridge (8 capillaries, 30 cm total length, P/N 5080121), disposable BioPhase sample and reagent plates (P/N 5080311) and the sample loading solution (SLS, P/N 608082), were from SCIEX (Framingham, MA).

Sample preparation: mRNA controls and SINTEF proprietary mRNA-LNP samples carrying 5 different nucleic acid payloads (1.929 kb) based on the ionizable lipid MC3 (DLin-MC3-DMA) and SM-102 formulations were stored at -80°C until analysis. For CGE-LIF analysis under denaturing conditions, 10 µL of the respective mRNA-LNP sample was mixed with 20 µL of 0.3% Triton X-100 in CE-grade water, 50 µL of formamide solution (SLS) and 10 µL of CE-grade water. Samples were incubated at room temperature for 20 minutes and then heated for 10 minutes at 70°C using a thermal cycler. Next, samples were chilled on ice for at least 5 minutes and transferred to the multi-capillary sample plate.

Non-denaturing mRNA-LNP sample preparation conditions involved mixing 10 µL of the respective mRNA-LNP sample with 20 µL of 0.3% Triton X-100 in CE-grade water. Samples were incubated at room temperature for 20 minutes and then heated for 5 minutes at 70°C using a thermal cycler. Samples were chilled on ice for at least 5 minutes and 60 µL of CE-grade water was then added before transferring them to the multi-capillary sample plate. Figure 2 illustrates sample preparation conditions, analysis, and capabilities of the BioPhase 8800 system.

mRNA linearity analysis: The free mRNA product (1.929 kb, 1mg/mL) was diluted to 0.1 mg/mL. This solution was then serially diluted by half to yield a 15-point dilution series that terminated at approximately 6.10 x 10^{-6} mg/mL. Samples of each concentration were heated in 50 µL aliquots for 5 minutes at 70°C using a thermal cycler. Samples were immediately chilled on ice for at least 10 minutes before transferring them onto the multi-capillary sample plate for CGE-LIF analysis.

RNA ladder sample preparation: The RNA ladder sample was prepared as described in the user manual for the RNA 9000 Purity & Integrity Analysis kit.[3] Briefly, 2 µL of the ladder was mixed with 48 µL of formamide solution (SLS) and heated at 70°C for 5 minutes using a thermal cycler. Samples

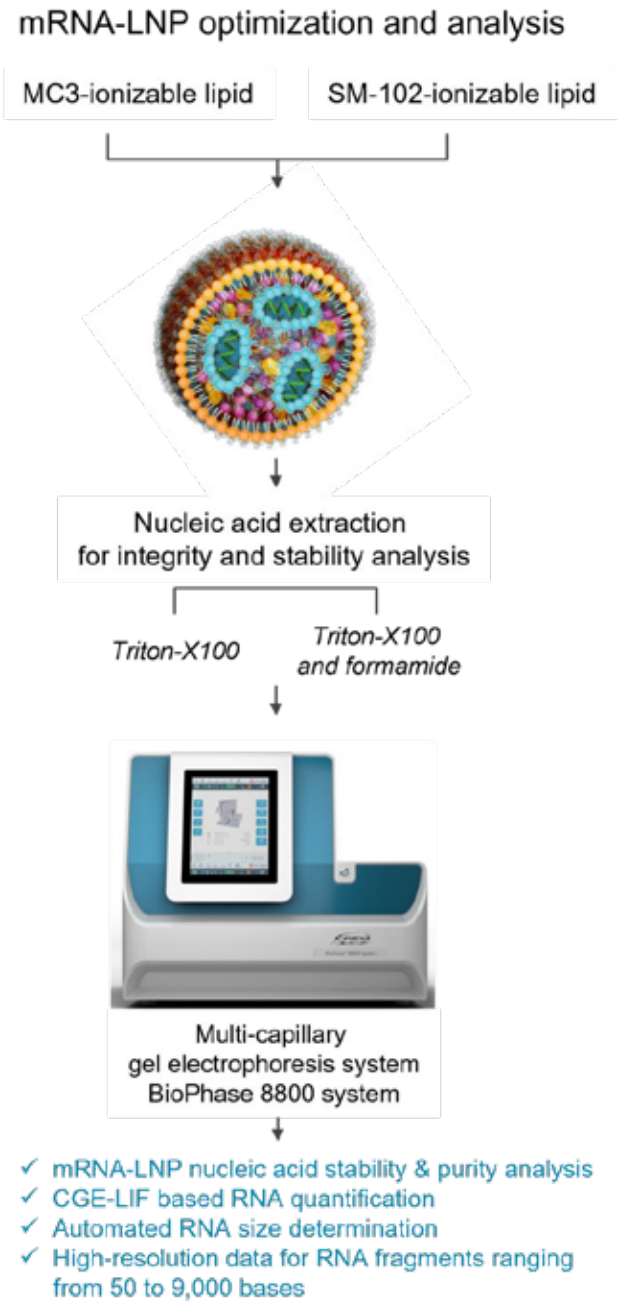


Figure 2. mRNA-LNP nucleic acid analysis workflow. MC3 and SM-102 LNP formulations were treated under denaturing and non-denaturing conditions. The RNA 9000 Purity & Integrity Analysis kit and the BioPhase 8800 system accurately determined the size of the main nucleic acid product loaded into 6 different mRNA-LNP formulations.

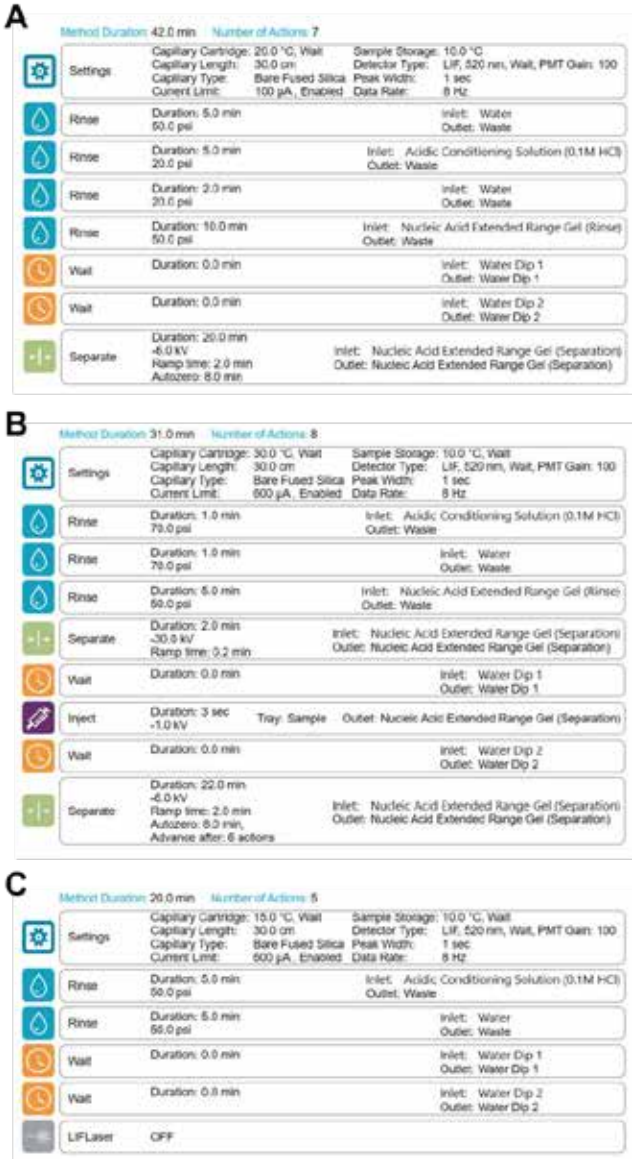


Figure 3. Methods implemented on the BioPhase 8800 system. (A) Parameters are shown for the conditioning method, (B) the optimized separation method using electrokinetic injection and (C) the shutdown method.

were chilled on ice for at least 5 minutes and then 60 µL of CE-grade water was added before transferring them to the multi-capillary sample plate.

Instrument and software: A BioPhase 8800 system (P/N 5083590) equipped with LIF detection was from SCIEX (Framingham, MA). The excitation and emission wavelengths used were 488 nm and 520 nm, respectively. Data acquisition and analysis were performed using BioPhase software, version 1.2.20 (SCIEX, Framingham, MA).

Data analysis: Signal intensity, corrected peak area (CPA) and corrected peak area% (CPA%) were determined for the main product, nucleic acid impurities and higher molecular weight (HMW) species using the BioPhase software. Values were tabulated in a spreadsheet to calculate percent, average and standard deviation and to create graphical representations of the data.

Results and discussion

Sample preparation for mRNA-LNP nucleic acid analysis

A critical step for product-specific attribute assessment of mRNA-LNP products is the extraction of encapsulated nucleic acid from the LNPs. Factors that are commonly considered to optimize mRNA-LNP standard operating procedures (SOPs) for nucleic acid integrity, stability, and quantification assays include extraction reagents, the timing of extraction procedures and nucleic acid labeling products that increase the sensitivity of measured samples. All reagents and extraction procedures should be assessed to develop reliable SOPs to assess final product qualification criteria or analytical development, for example by following ICHQ2 guidelines.[2]

The results presented in this technical note highlight the applicability of the fully-automated, multi-capillary BioPhase 8800 system for the assessment of mRNA-LNPs and for the complex optimization of mRNA-LNP product qualifications. The detection settings for the extracted nucleic acid from mRNA-LNPs were based on electrokinetic injection and LIF using the SYBR Green II RNA Gel Stain (Figure 3). This approach allowed effective detection and identification of low-level impurities and other higher-order nucleic acid structures contained in the LNPs.

Figure 4 illustrates representative electropherograms obtained from an MC3-based LNP carrying an intermediate payload of nucleic acid (MC3-LNP process 3). This mRNA-LNP sample contained 50% MC3, 38.5% cholesterol, 10% DSPC and 1.5% DMG-PEG. The top panel of Figure 4 indicates the nucleic acid profiling achieved by treating the MC3- based mRNA-LNP with a Triton X-100 solution. In contrast, the bottom panel of Figure 4 shows the result from the treatment of the same mRNA-LNP sample with Triton X-100 solution enhanced with ~55% formamide (SLS). The non-denaturing sample preparation (top panel) yielded 3 major products in the LNP. Product A corresponded to 4.75% of the detected nucleic acid in the sample and might indicate the presence of uncapped mRNA

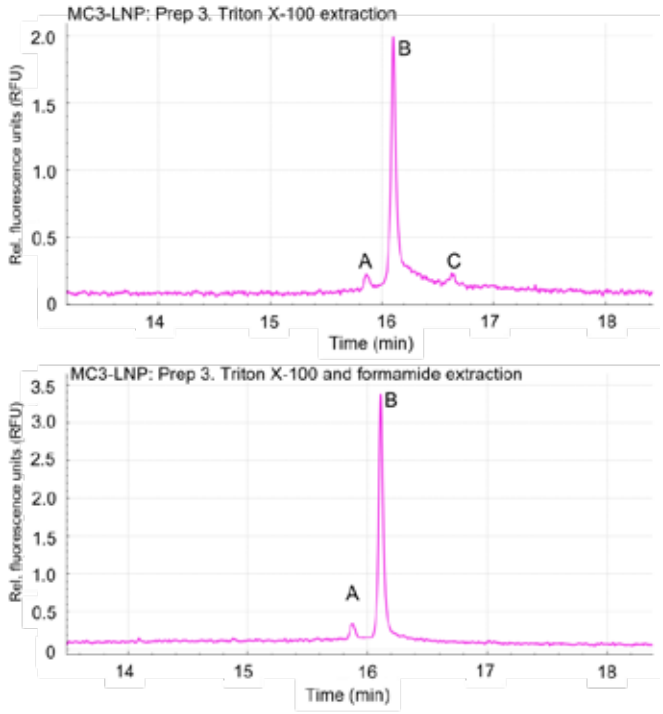


Figure 4. mRNA-LNP denaturing conditions disrupt higher-order nucleic acid structures. Under standard conditions (top panel), the MC3-LNP sample with an intermediate mRNA payload (prep 3) showed a higher molecular order structure or HMW product, C. The presence of formamide in the denaturing conditions (bottom panel) resulted in a disruption or disassociation of the HMW product, as only the minor product (A) and main product (B) were detected under denaturing conditions.

product. Product B corresponded to 74.06% of the detected nucleic acid and is the main mRNA product. Finally, product C corresponded to 21.19% of the detected nucleic acid and is a HMW product. In contrast, the denaturing sample preparation (bottom panel) yielded only products A and B that corresponded to 7.14% and 92.86% of the detected nucleic acid, respectively. No HMW products were detected using denaturing conditions.

These results are consistent with previous nucleic acid characterizations of mRNA-LNPs. Previous studies have shown that under non-denaturing conditions, RNA can form tertiary structures that can be reversed with formamide or heat treatment.[4] Additional studies have indicated that RNA can form lipid complexes that are detectable by mass spectrometry.[5] This indicates that RNA and lipid interactions can occur spontaneously after mRNA-LNP preparation and accumulate during storage. Therefore, the sample preparation conditions for mRNA-LNPs are important to consider to accurately assess the nucleic acid content of LNP particles.

Confirmation of non-denaturing sample preparation conditions and mRNA-LNP product identification by size

To further demonstrate that denaturing sample preparation is important for the analysis of nucleic acid extracted from mRNA-LNP molecules, 2 different mRNA-LNP formulations were assessed using the BioPhase 8800 system and LIF. Both prep 3 and prep 5 of the mRNA-LNP contained 50% MC3, 38.5% cholesterol, 10% DSPC and 1.5% DMG-PEG, however, the 2 preps contained different total amounts of mRNA. Figure 5 illustrates the nucleic acid profiling in the presence of Triton X-100 compared to a Triton X-100 and formamide-based extraction procedure. Prep 3 and prep 5 of the mRNA-LNP were analyzed in parallel with the RNA ladder. This approach allowed the size to be determined for the main product, impurities and HMW products.

The expected size for the main RNA product was 1.929 kb. The electropherograms for prep 3 and prep 5 of the mRNA-LNP indicated that the main nucleic acid products eluted before the 2 kb marker. These results suggest that the integrity of the mRNA was maintained during mRNA-LNP synthesis. These results further confirmed that the HMW complexes were disrupted in the presence of Triton X-100 and formamide.

To further characterize the integrity of mRNA payloads into LNPs, the BioPhase 8800 system was used to calculate the size of the putative free mRNA product and the main mRNA product extracted from mRNA-LNPs by using the Point-to-Point fit type analytical feature. Preps 1-5 of the MC3- and SM-102-based LNPs all contained 50% MC3, 38.5% cholesterol, 10% DSPC and 1.5% DMG-PEG but these preparations contained different total amounts of mRNA. The calculated sizes for the main product extracted from preps 1-5 of the MC3 mRNA-LNP and of the SM-102 formulation were highly accurate, with %error values <2% (Table 1).

Together, these results indicate that the mRNA-LNP sample preparation conditions are important for assessing the purity and integrity of nucleic acids encapsulated by LNPs. Using this workflow, multi-capillary CGE-LIF can be applied to identify RNA main products, assess content purity and achieve high-throughput sample processing to establish product-specific attributes for mRNA-LNPs.

Loaded and empty mRNA-LNP sample analysis by CGE-LIF

Empty LNP samples were analyzed in parallel with loaded mRNA-LNP samples to test whether LNP components, such as lipids, impurities, degradants, and nucleic acid extraction reagents, affected the CGE-LIF analysis of encapsulated nucleic acids. CGE-LIF was used to assess matrix effects caused by the lipid composition of the LNP (50% MC3, 38.5% cholesterol, 10% DSPC and 1.5% DMG-PEG) on the output signal.

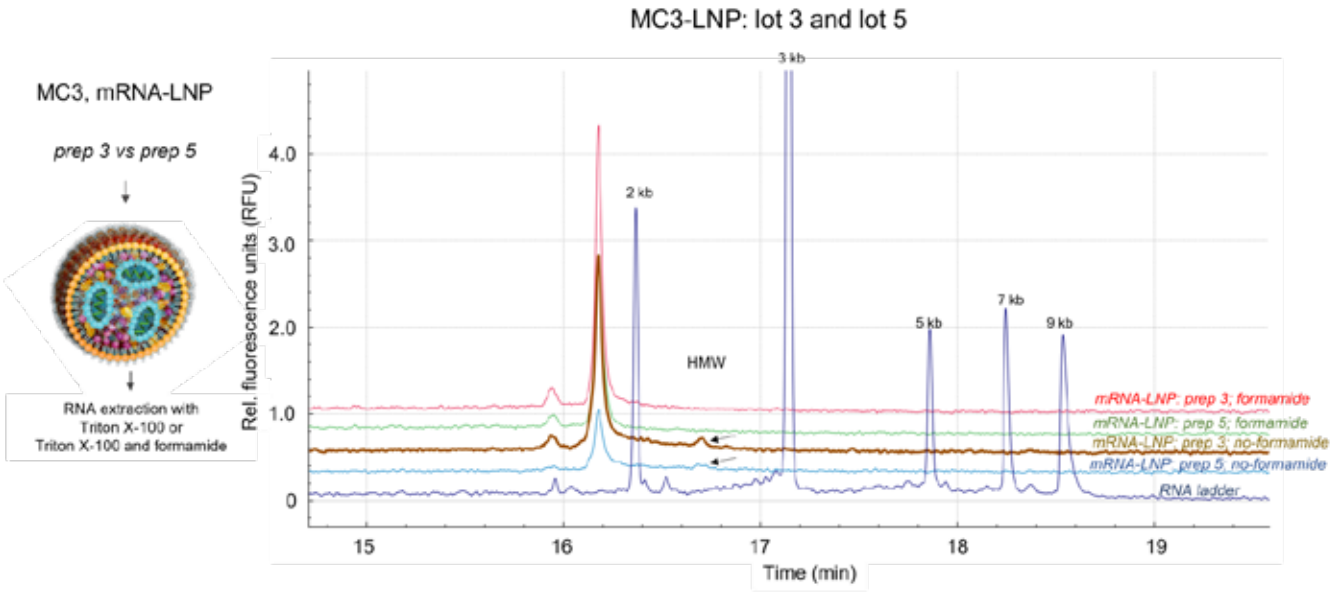


Figure 5. mRNA-LNP higher order structures disrupted under denaturing conditions. Nucleic acid products extracted from 2 different MC3-based formulations processes, mRNA-LNP prep 3 and prep 5, showed no HMW products in the presence of formamide. Arrows point to the presence of RNA HMW products in the absence of formamide treatment. The red and brown traces show prep 3 with and without formamide treatment, respectively. The green and blue traces show prep 5 with and without formamide treatment, respectively. The dark blue trace shows the RNA molecular ladder from the 9000 Purity & Integrity Analysis kit for size determination.

Figure 1 illustrates the abbreviated workflow used to assess the loaded mRNA-LNP sample and the empty LNPs using the BioPhase 8800 system and the validated RNA 9000 Purity & Integrity Analysis kit. The loaded mRNA-LNP and empty LNP samples were treated with denaturing conditions using a Triton X-100 solution and a formamide solution (SLS) prior to CGE-LIF analysis.

The top electropherogram (Figure 1) represents the nucleic acid detected from mRNA-LNP prep 3 (pink trace). This characterization showed the presence of a minor peak and the main RNA product. In contrast, the electropherogram on the bottom illustrates the CGE-LIF signal obtained by analyzing empty LNPs (purple traces, n=3). No effect on the background signal (RFU) was observed, suggesting that the mRNA-LNP matrix effects are negligible. Therefore, CGE-LIF with the BioPhase 8800 system could differentiate nucleic acids from other components that make up the mRNA-LNP in these samples without requiring complex nucleic acid purification.

Nucleic acid characterization from differently loaded mRNA-LNPs

Five differently loaded MC3-based LNPs and a loaded SM-102 mRNA-LNP formulation, containing 50% MC3 or SM-102, 38.5% cholesterol, 10% DSPC and 1.5% DMG-PEG and differing in the initial amount of mRNA for encapsulation, were analyzed by CGE-LIF for nucleic acid integrity and relative content. The results demonstrated that the BioPhase 8800 system could differentiate these mRNA-LNP samples based on the nucleic acid extracted using the Triton X-100 and formamide solution.

Figure 6 illustrates the relative nucleic acid content for these samples based on the average CPA of the main RNA product only. Prep 2 of the mRNA-LNP had the least nucleic acid encapsulated by the LNPs, highlighted by an average CPA value of 0.13 with a %CV of 6%. In contrast, prep 5 of the mRNA-LNP

Table 1. mRNA product and impurity identification by size. The BioPhase software nucleic acid analysis tool used the RNA ladder to accurately calculate the size of the main expected product (1.929 kb) in differently loaded mRNA-LNP formulations.

Product	RNA, minor product size (nt)	RNA, main product size (nt)	%Error from expected size
MC3-LNP1	*1810*	*1902*	*1.42%*
MC3-LNP2	*1828*	*1918*	*0.58%*
MC3-LNP3	*1835*	*1928*	*0.04%*
MC3-LNP4	*1838*	*1931*	*0.09%*
MC3-LNP5	*1858*	*1951*	*1.13%*
SM-102-LNP1	*1856*	*1950*	*1.09%*

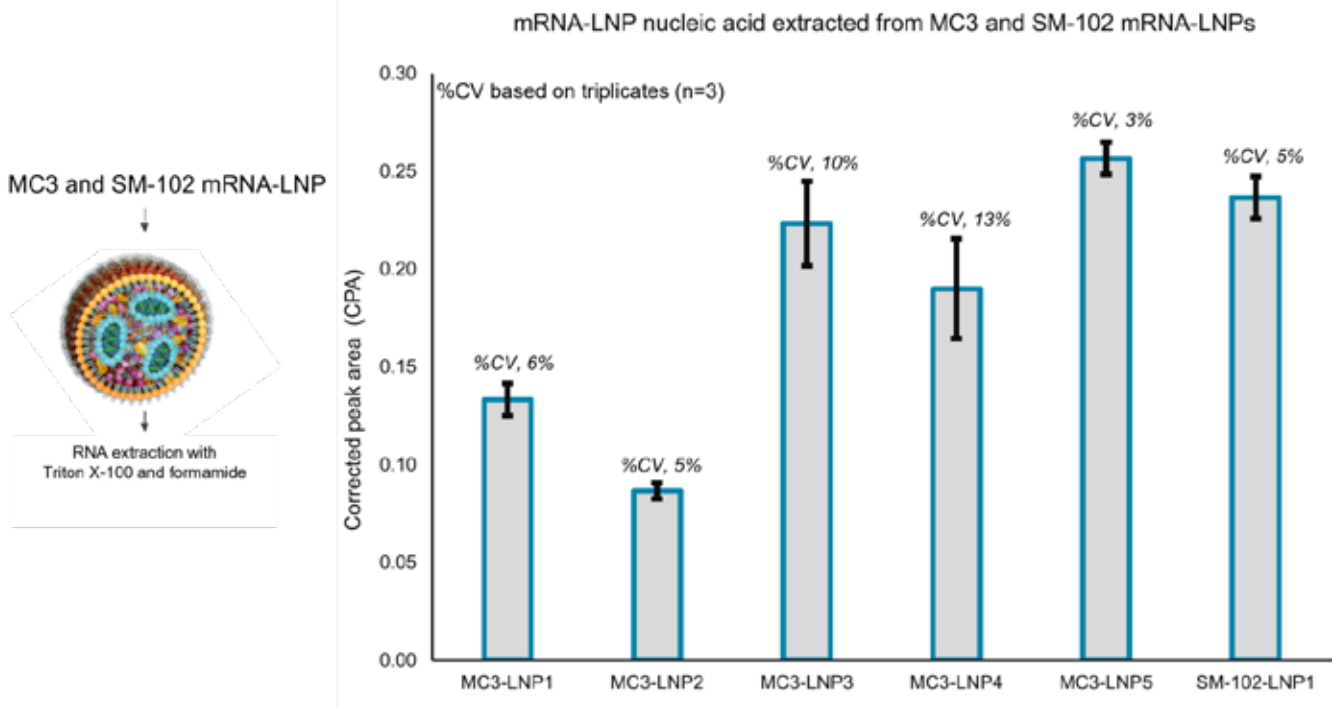


Figure 6. Nucleic acid characterization from distinct mRNA-LNPs. Six different mRNA-LNP formulations, which included 5 MC3 and 1 SM-102 mRNA-LNPs, were evaluated for nucleic acid content based on CPA values obtained for the main product using Triton X-100 and formamide extraction conditions. The error bars represent the variation of 3 injections.

had the highest nucleic acid content with an average CPA of 0.26. Prep 3 of the mRNA-LNP had an intermediate nucleic acid content with an average CPA value of 0.22. The SM-102 mRNA-LNP sample had nucleic acid content that was in between that of preps 3 and 5 of the MC3-LNP, with a CPA value of 0.24 and %CV of 5%. These results demonstrate the ability of the BioPhase 8800 system to distinguish between mRNA-LNP formulations that are representative of R&D activities. This type of application positions this analytical platform for robust assessment of manufacturing processes of mRNA-LNP products.

Table 2 reports the CPA for each MC3-based mRNA-LNP formulation. In contrast to the results shown in Figure 6, this analysis included the nucleic acid content represented by both the main and minor peaks. In general, the minor peak contributed <10% to the nucleic acid levels. This observation suggests that impurities represented by minor peaks that exceed 10% of nucleic acid content should be monitored during the assessment of new production lots or manufacturing processes. The %CV calculations for the main and minor peaks ranged from 2.95% to 14.48%, based on 3 sequential injections. These values indicate that RNA-lipid matrix interactions might occur during the analysis or that RNA-destabilization can occur from distinct formulation conditions. Together, these results provided a deeper characterization of the RNA main products and impurities that were packaged in this mRNA-LNP collection.

Relative nucleic acid characterization from MC3 and SM-102 mRNA-LNP formulations

Recently, tissue-specific nanoparticles have been used to deliver RNA payloads for various therapeutic applications.[1] As a result, the demand for nucleic acid characterization with proven performance for evaluating mRNA-LNP formulations with varying lipid compositions has greatly increased. This technical note demonstrates the ability to assess the nucleic acid integrity and content of mRNA-LNP formulations containing different ionizable lipids. The 2 formulations consisted of 50% MC3 or SM-102, 38.5% cholesterol, 10% DSPC and 1.5% DMG-PEG. Each formulation was evaluated with the same amount of mRNA (1.929 kb) used for encapsulation and the nucleic acid content was analyzed by CGE-LIF with the BioPhase 8800 system.

Figure 7, panel A shows the nucleic acid profiling from an MC3-based mRNA-LNP formulation under denaturing conditions (blue trace) and the corresponding empty LNP (red trace). Panel B shows the nucleic acid profiling of an SM-102-based mRNA-LNP formulation under denaturing conditions (purple trace) and the

Table 2. Nucleic acid content assessment with the BioPhase 8800 system. CPA analysis revealed distinct mRNA content among 5 mRNA-LNP formulations based on MC3 lipids.

Formulation sample	Main and minor peaks (CPA)	%CV (n=3)
MC3-LNP1	*0.14*	*5.70%*
MC3-LNP2	*0.10*	*4.22%*
MC3-LNP3	*0.24*	*8.88%*
MC3-LNP4	*0.20*	*14.48%*
MC3-LNP5	*0.28*	*2.95%*

corresponding empty LNP (dark blue trace). Panel C shows that the minor and major RNA products have similar contents, regardless of which ionizable lipid was used in the mRNA-LNP formulations.

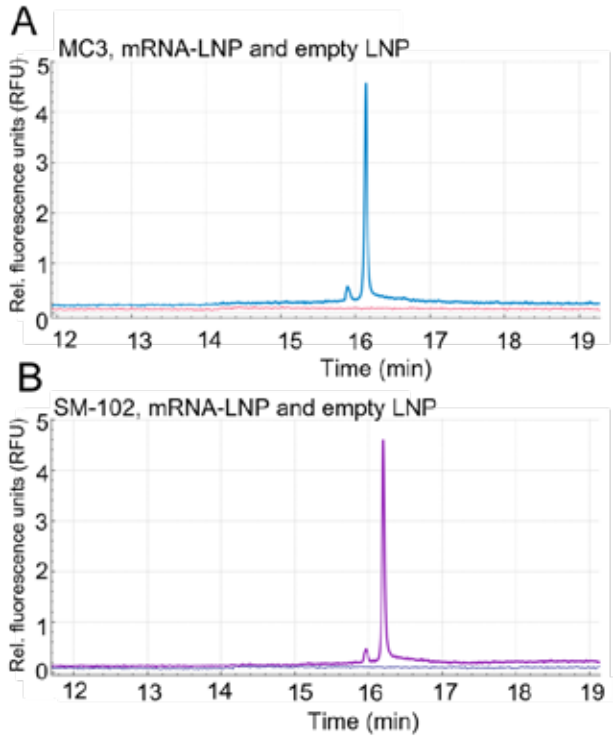
In summary, the use of the BioPhase 8800 system and the ready-to-use RNA 9000 Purity & Integrity Analysis kit demonstrated consistency in the mRNA extracted from mRNA-LNPs made of different ionizable lipids. These analyses can therefore be beneficial for the mRNA-LNP field by expanding on the applications of RNA-based products for tissue-specific nanomedicines.

mRNA-LNP nucleic acid linearity, LLOQ, LLOD and ULOD

The free mRNA product (1.929 kb) used to load the different mRNA-LNP formulations was used to generate a linearity curve in a water-formamide matrix. The lipid compositions and mixing ratios of mRNA-LNP formulations are diverse and therefore it can be challenging to replicate specific matrix compositions. The water-formamide matrix used in this study provided a readily reproducible matrix for the detection of nucleic acid products used for mRNA-LNP synthesis.

An electrokinetic injection was used on the BioPhase 8800 system to assess linearity (Figure 8). The samples were serially diluted and heated at 70°C for 10 minutes using a water-formamide matrix. The linearity of the samples ranged from 0.1 mg/mL to 6.10x10^{-6} mg/mL. These nucleic acid products were also detected with LIF using the SYBR Green II RNA Gel Stain. Figure 8 illustrates representative electropherograms from the serially diluted samples of free mRNA in the water-formamide matrix. As demonstrated by the electropherograms, the free mRNA did not include HMW products.

In summary, this study produced a 9-point linearity curve that spanned approximately 2.5 orders of magnitude (R^2 = 0.9999). The linear plotting and regression modeling were based on the average corrected peak area produced from 3 injections. The open circles indicated the specific corrected peak from each individual injection. The ULOQ was 1.56 x 10^{-3} mg/mL, the LLOQ was 1.22 x 10^{-5} mg/mL and the LLOD was 6.10 x 10^{-6} mg/mL (Figure 9).



C

mRNA-LNP sample	RNA, minor product		RNA, main product	
	CPA	CPA%	CPA	CPA%
MC3-LNP3	0.02	7.31%	*0.25*	*92.79%*
SM-102-LNP1	0.02	*7.17%*	*0.24*	*92.93%*

Figure 7. Nucleic acid content profiling from mRNA-LNP formulations with distinct ionizable lipids. (A) MC3 and (B) SM-102 mRNA-LNP formulations with a similar amount of mRNA starting material were evaluated for nucleic acid content by CGE-LIF. (C) Table illustrating the CPA and CPA% values for the minor and main products observed in these 2 formulations.

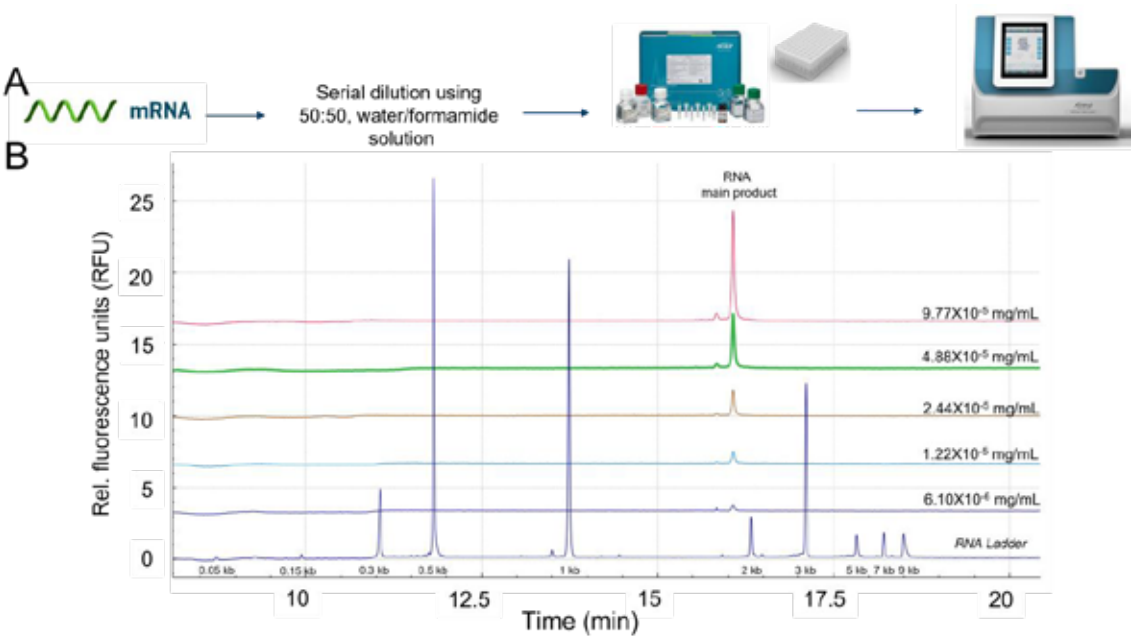


Figure 8. Representative overlayed electropherograms of serially diluted unencapsulated mRNA for linearity curve and nucleic acid analysis by CGE-LIF. The free mRNA or unencapsulated nucleic acid product was serially diluted by a factor of 2 and samples were analyzed simultaneously using the multi-capillary electrophoresis system. The RNA 9000 Purity & Integrity Analysis kit was used to analyze the main product and impurities of the in vitro transcribed mRNA. Panel A shows the sample preparation workflow for mRNA analysis using the BioPhase 8800 system. Panel B shows representative electropherograms from the serially diluted samples of free mRNA in the water/formamide matrix. These electropherograms highlight the presence of the main product that elutes before to the 2 kb maker (blue trace, RNA ladder).

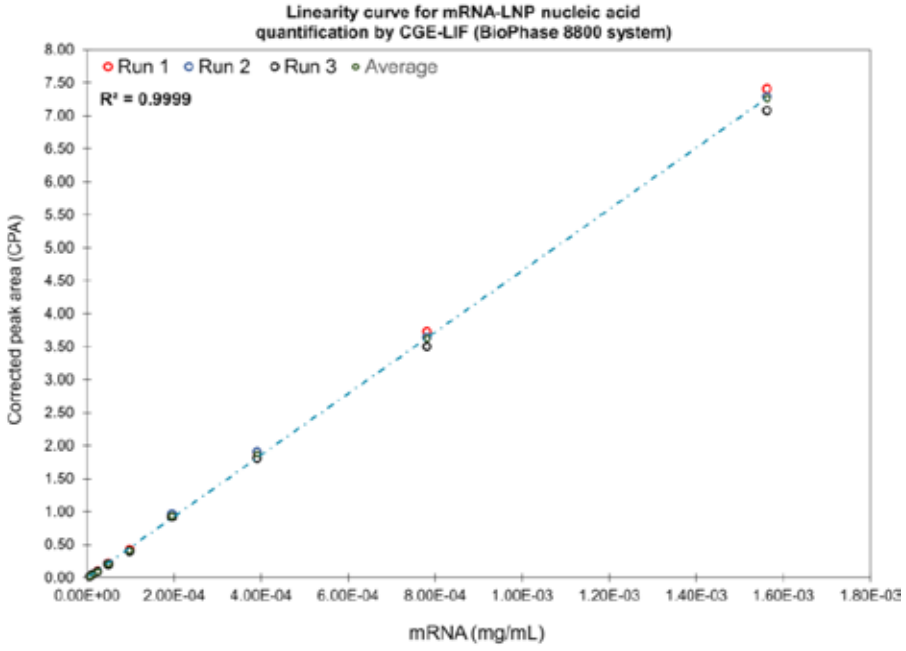


Figure 9. Linearity of mRNA for nucleic acid analysis. The CPA of the main RNA product was used to construct a linearity curve across 9 mRNA concentrations. The regression analysis was performed on the average of 3 injections. The R^2 value for the linear regression analysis was 0.9999. The LLOD was 6.10 x 10^{-6} mg/mL, the LLOQ was 1.22 x 10^{-5} mg/mL and the ULOD was 1.56 x 10^{-3} mg/mL.

Conclusions

- A simplified workflow for RNA extraction from mRNA-LNPs using Triton X-100 and formamide showed compatibility with the BioPhase 8800 system and the RNA 9000 Purity & Integrity Analysis kit
- HMW products commonly seen in nucleic acid species extracted from mRNA-LNPs can be dissociated by formamide treatment
- The BioPhase 8800 system equipped with LIF provided robust reproducibility for nucleic acid extracted from mRNA-LNPs, showing strong evidence that the mRNA-LNPs samples were loaded differently with RNA products
- A linearity curve using the unencapsulated mRNA product in a water/formamide matrix covered approximately 2.5 orders of magnitude, with an LLOD of 6.10 x 10^{-6} mg/mL, an LLOQ of 1.22 x 10^{-5} mg/mL and a ULOD of 1.56 x 10^{-3} mg/mL

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Comprehensive characterization of mRNA-LNP samples using an optimized sample preparation method and CGE-LIF workflow

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This technical note presents the characterization of mRNA-LNP samples using an optimized sample preparation method developed in a previous study.[1] The method eliminates the heat incubation during sample preparation of both mRNA and mRNA-LNP formulations to prevent heat-induced degradation, thereby significantly enhancing the accuracy of mRNA integrity analysis. Following sample preparation, the samples are analyzed using capillary gel electrophoresis with laser-induced fluorescence detection (CGE-LIF), a robust and sensitive technique well-suited for evaluating mRNA integrity, purity, stability, and encapsulation efficiency (EE%) in LNPs.

In this study, the optimized method was further validated and applied to characterize several critical quality attributes (CQAs) of mRNA-LNP samples during their production. First, the repeatability of mRNA integrity analysis was assessed for both mRNA drug substance (DS) and mRNA-LNP drug product (DP), demonstrating excellent consistency and reliability. Second, the method's quantitation capability was confirmed by establishing linearity across a range of mRNA concentrations, with R^2 values $\geq$ 0.994 across six independent runs. Third, a forced degradation study under elevated temperatures was performed to assess mRNA stability, revealing clear temperature-dependent degradation patterns. Finally, the method was applied to determine EE%, achieving an average of 93.8% with high reproducibility.

Overall, this optimized sample preparation and CGE-LIF analysis method provides a reliable, accurate, and comprehensive approach for characterizing mRNA-LNP products, supporting the development, quality control, and regulatory evaluation of high-quality mRNA-based therapeutics and vaccines.

Key features

- **Heat-free, optimized sample preparation:** Eliminates heat-induced degradation during processing, ensuring accurate and reliable mRNA integrity analysis.
- **High precision and reproducibility:** Delivers excellent repeatability (RSD < 0.5% for migration time; RSD < 2.5% for purity) and robust quantification (R^2 $\geqslant$ 0.994) across multiple runs.
- **Comprehensive characterization:** Enables effective assessment of mRNA stability, EE% (average 93.8%), and integrity using a sensitive CGE-LIF platform—supporting quality control of mRNA-LNP therapeutics.

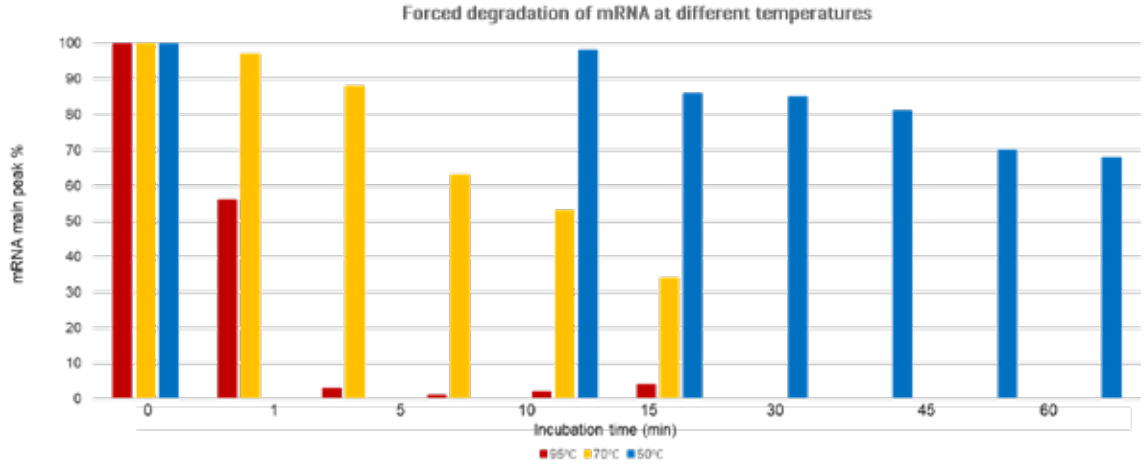


Figure 1. Degradation kinetics of mRNA in mRNA-LNP samples under different thermal stress conditions. The percentage of intact mRNA was normalized to the 0-minute time point and plotted over time at 95°C (red), 70°C (yellow), and 50°C (blue). The results illustrate that higher temperatures accelerate mRNA degradation.

Introduction

mRNA-based therapeutics and vaccines have emerged as a transformative class of biopharmaceuticals, offering rapid development timelines, scalable manufacturing, and flexible design capabilities.[2] LNPs are widely used to encapsulate and deliver mRNA in vivo, protecting it from enzymatic degradation and enabling efficient cellular uptake.[3] As mRNA-LNP formulations progress through development into clinical and commercial manufacturing, there is a growing need for robust, high-resolution analytical methods to characterize CQAs such as mRNA integrity, purity, stability, and EE%.[4, 5]

However, the inherently fragile nature of mRNA poses significant analytical challenges, particularly during sample preparation, where conventional heat-based methods can lead to degradation and inaccurate assessments.[6] To address this issue, we previously developed an optimized sample preparation protocol that avoids heat incubation and instead utilizes Triton X-100 and formamide with shaking at room temperature. This gentle approach preserves RNA integrity while enabling efficient release of mRNA from LNPs.

In this technical note, we apply and further validate the optimized method for the comprehensive characterization of mRNA-LNP samples using CGE-LIF. CGE-LIF is a highly sensitive and reproducible platform ideally suited for analyzing mRNA size, purity, and degradation profiles. The method is evaluated across key analytical dimensions, including repeatability, quantitation capability, stability under thermal stress, and EE%. Together, these assessments support the robustness, reliability, and applicability of the optimized workflow for the development and quality control of mRNA-based therapeutics and vaccines.

Materials

Materials: The RNA 9000 Purity & Integrity kit (P/N: C48231) was from SCIEX (Framingham, MA) and contained Nucleic Acid Extended Range Gel, SYBR™ Green II RNA Gel Stain*, Acid Wash/Regenerating Solution, CE Grade Water and ssRNA Ladder. The BioPhase BFS capillary cartridge - 8 x 30 cm (P/N: 5080121), BioPhase sample and reagent plates (4,4,8) (P/N: 5080311) and sample loading solution (SLS, or formamide, P/N: 608082) were from SCIEX. The 0.2 µm syringe filter (P/N: 4612) was from PALL (Port Washington, NY). Rainin LTS filter tips were from Mettler Toledo (Oakland, CA). Nuclease-free water (NFW, P/N: AM9932) and Surfact-Amps X-100 (10% (v/v) (Triton X-100, P/N: 28314) were obtained from Thermo Fisher Scientific (Waltham, MA). The firefly luciferase (FLuc) mRNA (P/N: L-7602, 1929 nucleotides) was from TriLink BioTechnologies (San Diego, CA). In this technical note, it was used as a model to represent the DS of an mRNA-based vaccine. Moderna-like lipid nanoparticles (LNPs) encapsulating CleanCap® FLuc mRNA, also supplied by TriLink, were formulated by SINTEF (Trondheim, Norway) and used as the model DP for mRNA-LNP vaccine analysis in this technical note.

Sample preparation

Sample preparation for calibration curve: FLuc mRNA was diluted with CE-grade water, and formamide (50% final concentration) was added to each sample to achieve an 8-point calibration curve of mRNA at a concentration from 0.5 to 13.5 µg/mL.

Optimized sample preparation for mRNA DS sample: 10 µL of the mRNA was mixed with 40 µL of CE-grade water. Finally, 50 µL of formamide (50% final concentration) was added, and the sample was incubated at room temperature for 10 min.

Optimized sample preparation for the mRNA-LNP sample: 10 µL of the mRNA-LNP sample was mixed with 15 µL of Triton X-100 to achieve a final concentration of 1.2%. The mixture was shaken at 800 rpm at room temperature (RT) for 10 min. Subsequently, 25 µL of CE-grade water was added and the sample mixed thoroughly. Finally, 50 µL of formamide (50% final concentration) was added, and the sample was incubated at room temperature for 10 min.

Instrument methods

A BioPhase 8800 system (P/N 5083590) equipped with LIF detection was from SCIEX. The excitation and emission wavelengths used were 488 nm and 520 nm, respectively. Data acquisition and analysis were performed using BioPhase software, version 1.2.20.

The conditioning method, separation methods (pressure injection mode or electrokinetic injection mode), and the

shutdown method used in this technical note were described in the application guide of the RNA 9000 Purity & Integrity kit.[10]

Results and discussion

Optimized mRNA and mRNA-LNP sample preparation method: As discussed in the previous technical note,[1] the fragile nature of mRNA presents significant challenges during the preparation of samples for mRNA-based therapeutics and vaccines. Heat incubation, commonly used during the preparation of mRNA drug substances and mRNA-LNP drug products, can lead to mRNA degradation, compromising the accuracy of analytical results. To address this, an optimized sample preparation protocol was developed that avoids heat incubation to preserve mRNA integrity.

For mRNA sample analysis, 50% formamide was added to denature the mRNA prior to loading onto the BioPhase 8800 system. For mRNA-LNP samples, the optimized protocol involved treatment with 1.2% Triton X-100 to disrupt the lipid nanoparticles, followed by the addition of formamide and shaking at 800 rpm at room temperature for 10 minutes. This approach maximized mRNA release while minimizing degradation. In addition, pressure injection was identified as the preferred method for sample introduction, particularly for samples with low concentration or high salt content.

The performance of this optimized sample preparation method was further evaluated for the characterization of both mRNA and mRNA-LNP samples. The refined protocol significantly improved the reliability and reproducibility of mRNA integrity analysis, thereby supporting the development of high-quality mRNA-LNP drug products essential for advancing mRNA-based therapeutics and vaccines.

In this technical note, Fluc mRNA was used as a model to demonstrate the optimized method for assessing mRNA integrity, stability (of both mRNA and mRNA-LNPs), and LNP EE% during mRNA-LNP manufacturing.

Excellent repeatability of mRNA integrity analysis: To ensure consistent and reliable mRNA integrity analysis, the method's repeatability was evaluated for both mRNA DS and mRNA-LNP DP samples. The results demonstrated excellent repeatability, as indicated by the low %RSD values for both the migration time (MT) of the mRNA main peak and sample purity.

Figure 2 presents the overlaid electropherograms of mRNA DS analyzed across eight capillaries using the multi-capillary BioPhase 8800 system. The calibrated migration time was applied to align the main peak across all eight capillaries. Table 1 summarizes the repeatability data, showing %RSD values of 0.3% for migration time and 0.7% for purity of the mRNA main peak, both indicating high analytical precision.

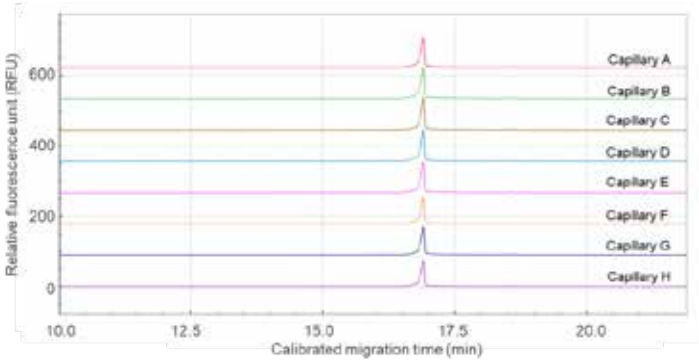


Figure 2: Overlaid electropherograms of mRNA drug substance analyzed across eight capillaries on the BioPhase 8800 system. Calibrated migration time was used to align the main peak, demonstrating high consistency across capillaries.

Table 1: Repeatability results for the analysis of the mRNA DS across eight capillaries.

Sample prep. #	Migration time (min)	CPA%
A	16.9	87.2
B	16.9	86.8
C	16.9	87.7
D	16.9	86.6
E	17.0	87.7
F	16.9	87.8
G	16.8	87.0
H	16.9	86.1
Average	**16.9**	**87.1**
%RSD	**0.3%**	**0.7%**

Similarly, Figure 3 shows overlaid electropherograms of the mRNA-LNP drug product analyzed on four different capillaries, each with three replicates. Again, the calibrated migration time was used to align the main peaks across the traces. Table 2 presents the repeatability data for the mRNA-LNP samples, with %RSD values of 0.5% for migration time and 2.3% for purity, confirming the method's robustness and consistency.

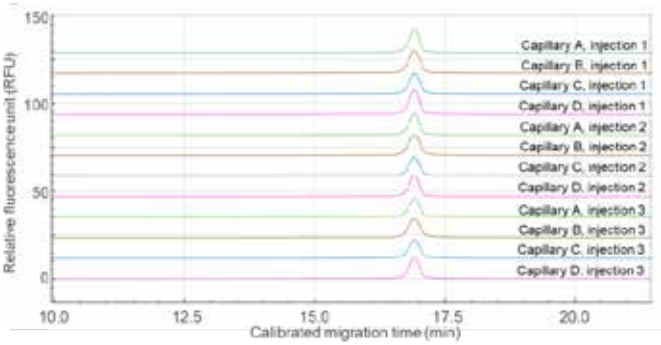


Figure 3: Overlaid electropherograms of mRNA-LNP drug product analyzed on four capillaries with three replicates each. Calibrated migration time alignment confirms consistent mRNA peak detection and minimal variability.

Overall, the optimized sample preparation and analysis protocol demonstrates high repeatability and reproducibility for mRNA integrity assessment. This robust method provides a reliable analytical tool for the quality control of both mRNA drug substances and mRNA-LNP drug products.

Table 2: Repeatability results for analysis of the mRNA-LNP DP across four capillaries with three replicates.

Sample preparation #	Migration time (min)	CPA%
Capillary A-injection #1	17.1	86.0
Capillary B-injection #1	17.0	83.8
Capillary C-injection #1	17.0	80.5
Capillary D-injection #1	17.0	81.3
Capillary A-injection #2	17.2	83.6
Capillary B-injection #2	17.1	80.2
Capillary C-injection #2	17.1	80.2
Capillary D-injection #2	17.1	82.6
Capillary A-injection #3	17.3	81.0
Capillary B-injection #3	17.2	84.8
Capillary C-injection #3	17.2	81.9
Capillary D-injection #3	17.1	83.2
Average	**17.1**	**82.4**
%RSD	**0.5%**	**2.3%**

Quantitation capability: To evaluate the quantitation capability of the optimized method, we assessed its linearity across a range of mRNA concentrations over six different days. The response was plotted against concentration, and the resulting calibration curves—shown in Figure 4—demonstrated excellent linearity, with coefficients of determination (R^2) consistently no less than 0.994. The limit of detection (LOD) was determined to be 0.25 µg/µL for the Fluc mRNA standard.

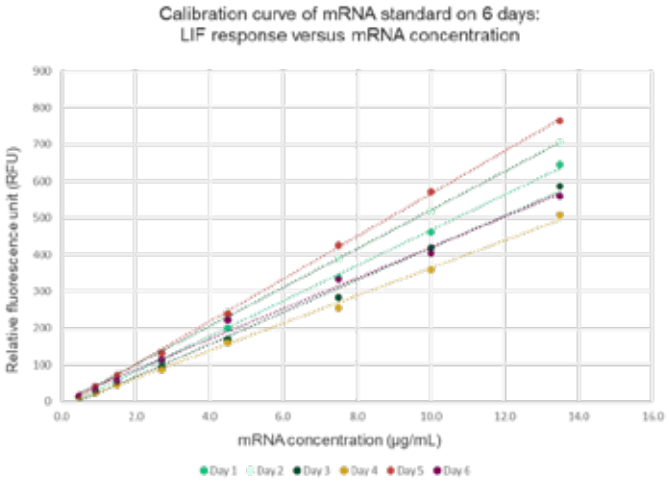


Figure 4: Calibration curves generated from Fluc mRNA standards analyzed over six different days to evaluate the linearity of the optimized method. Each curve demonstrates a strong linear relationship between signal response and mRNA concentration, with R^2 values ≥ 0.994, confirming excellent quantitation capability and method reproducibility.

Some variation in calibration curve intensities was observed across the six runs, with approximately 15% variability in signal response between days. This variation can be attributed to several factors. First, the mRNA standards were freshly prepared for each run, potentially introducing slight differences in concentration or handling. Second, minor inconsistencies in the amount of dye added to the separation gel during each preparation may have affected signal intensity. Additionally, detector variability between runs could also contribute to the observed differences. Given these factors, a calibration curve should be freshly prepared alongside the samples for each analytical run to ensure accurate and consistent quantitation.

Overall, this optimized method provides a reliable and robust analytical tool for the quantitation of mRNA in mRNA-LNP samples, supporting the development and quality control of high-quality mRNA-based therapeutics and vaccines.

Forced degradation study: To investigate the stability of mRNA-LNP samples under various stress conditions, a forced degradation study was conducted using the optimized method. The samples were subjected to different temperatures (95°C, 70°C, and 50°C) for varying incubation times.

Figure 1 shows the percentage of intact mRNA detected at different incubation times and temperatures, normalized to the 0-minute time point. The plots illustrate the degradation

kinetics of mRNA in mRNA-LNP samples under each temperature condition. As expected, higher temperatures accelerated mRNA degradation. At 95°C, degradation was rapid, with a substantial reduction in intact mRNA (red bars in Figure 1). At 70°C, degradation occurred more gradually, showing a progressive decline in the intact mRNA signal (yellow bars). At 50°C, degradation proceeded at the slowest rate, with only a modest decrease in intact mRNA over time (blue bars).

In addition to quantifying the loss of intact mRNA, the extent of degradation was evaluated by measuring the smear signal, representing fragmented RNA. As an example, Figure 5 shows the electropherograms of mRNA-LNP samples incubated at 70°C for 1 minute (Figure 5A) and 30 minutes (Figure 5B). Over time, the intensity of the main peak decreased while the smear signal increased, indicating progressive degradation.

Figure 6 presents a time-course analysis of mRNA degradation at 70°C for both mRNA drug substance (DS) and mRNA drug product (DP) samples. The data demonstrate a consistent decrease in intact mRNA and a corresponding increase in smear signal with longer incubation times, further demonstrating the method's ability to detect and quantify both intact and degraded species.

These results highlight the critical impact of temperature on mRNA stability and underscore the importance of stringent temperature control during the manufacturing, storage, and handling of mRNA and mRNA-LNP products. The optimized method proved effective in monitoring both intact and degraded mRNA, confirming its robustness and reliability for stability studies.

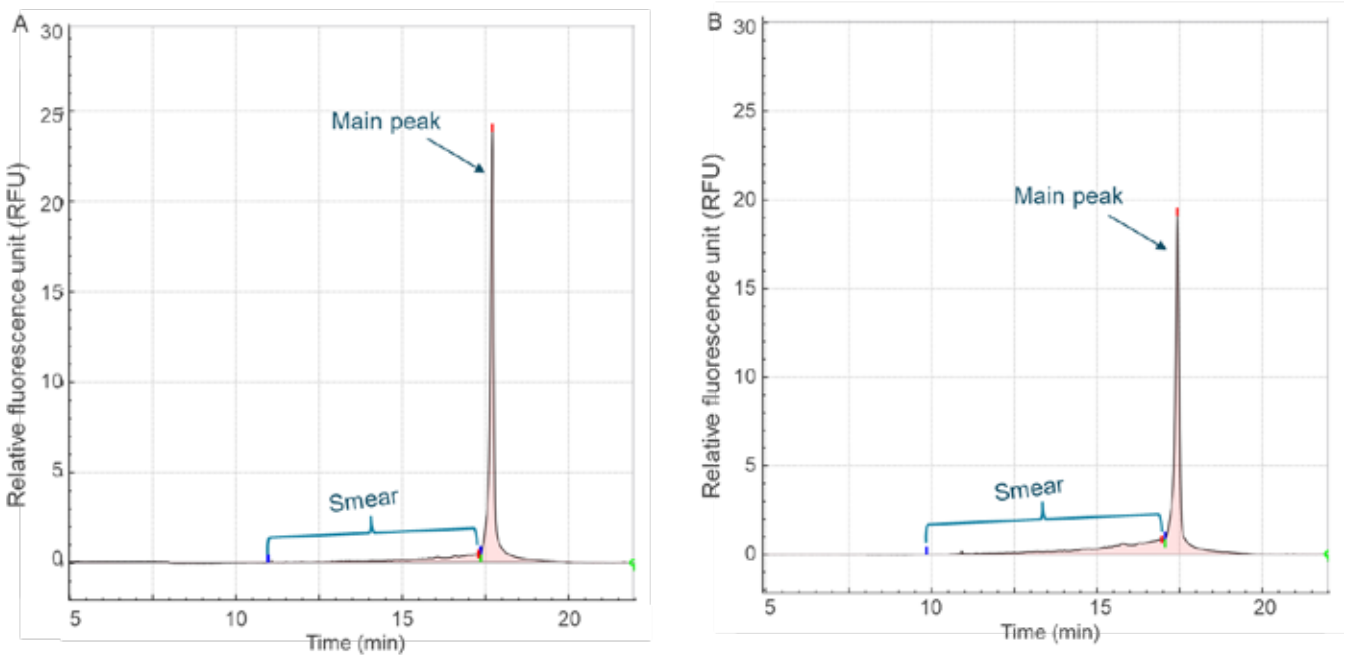


Figure 5: Electropherograms of mRNA-LNP samples incubated at 70°C for 1 minute (A) and 30 minutes (B). The intensity of the intact mRNA peak decreased, while the smear signal—representing degraded mRNA—increased over time, indicating progressive thermal degradation.

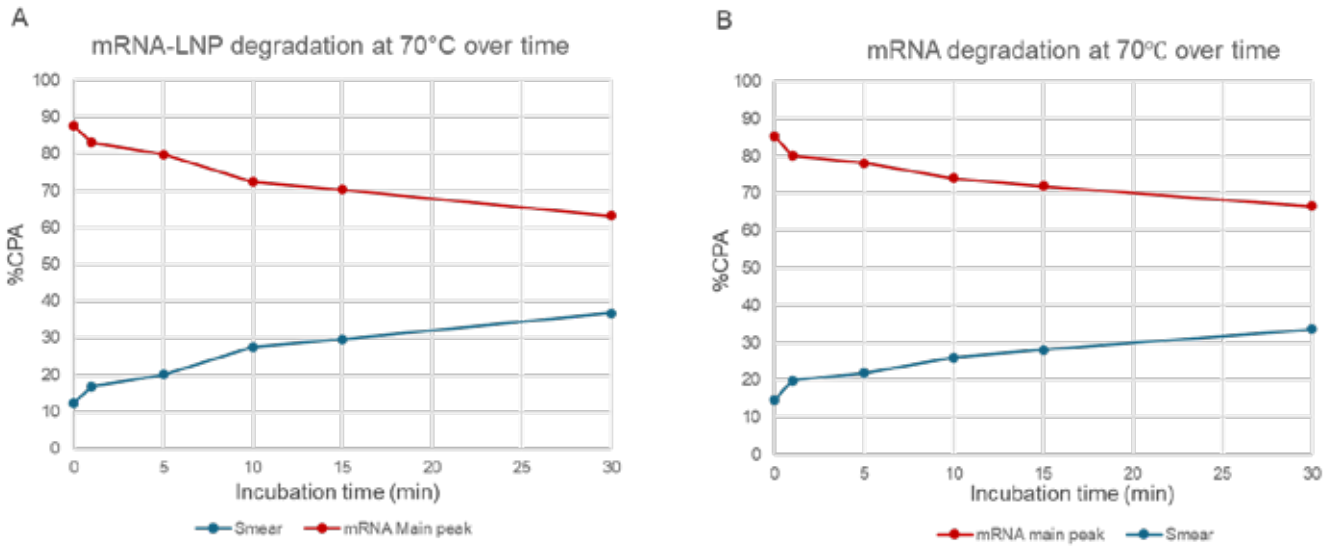


Figure 6: Time-course degradation profiles of mRNA drug DS and mRNA DP samples incubated at 70°C. The percentage of intact mRNA decreased while the degradation smear increased over time, demonstrating the method's capability to quantify both intact and degraded RNA species.

EE% determination: To determine the EE% of the mRNA-LNP drug product, samples and calibration curves were prepared in a 96-well plate format. Two calibration curves were generated: one for the measurement of total mRNA (using Triton X-100 to disrupt the LNPs during sample preparation) and the other for the quantification of free, unencapsulated mRNA. All samples were prepared in triplicate for both free RNA and total RNA analyses.

EE% was calculated based on the difference between the measured total RNA and free RNA concentrations. The results, summarized in Table 3, show an average EE% of 93.8%, with a relative standard deviation (RSD) of 0.9% across three replicates. This high EE% indicates that the vast majority of mRNA was successfully encapsulated within the lipid nanoparticles, minimizing the amount of unencapsulated (free) RNA. The inclusion of Triton X-100 in the sample preparation step effectively disrupted the lipid nanoparticles, enabling accurate quantitation of total mRNA.

These results confirm that the optimized method is reliable and precise for determining EE%, a critical parameter, for evaluating the quality and performance of mRNA-LNP formulations. High EE% ensures effective protection and delivery of mRNA, supporting the development of stable and efficacious mRNA-based therapeutics and vaccines.

Table 3: EE% of mRNA-LNP DP determined from triplicate measurements of total RNA and free RNA. The average EE was 93.8% with a relative standard deviation (RSD) of 0.9%, demonstrating high encapsulation and excellent reproducibility of the optimized method.

Sample preparation	Total RNA, µg/mL	Free RNA, µg/mL	EE	Average	RSD
#1	55.98	3.71	93.4%		
#2	50.80	3.45	93.2%	93.8%	0.9%
#3	50.08	2.66	94.7%		

Conclusions

In summary, this technical note demonstrates the effectiveness of the optimized release method for characterizing mRNA-LNP samples using capillary gel electrophoresis (CGE). The key findings are:

- Optimized, heat-free sample preparation: Eliminates heat incubation during sample prep to prevent mRNA degradation, significantly improving the accuracy and integrity of mRNA analysis.
- Excellent repeatability and quantitation capability: Ensures consistent and reliable mRNA integrity results for both mRNA drug substances (DS) and mRNA-LNP drug products (DP), with low %RSD in peak migration time and purity across multiple capillaries.
- Outstanding quantitation capability: Demonstrates excellent linearity ($R^2 \geqslant 0.994$) across a wide range of mRNA concentrations over six independent runs, enabling accurate and reproducible quantitation.
- Robust forced degradation assessment: Evaluates mRNA and mRNA-LNP stability under thermal stress (95°C, 70°C, 50°C), highlighting temperature-dependent degradation patterns and the method's sensitivity in detecting both intact and degraded species.
- Accurate EE% determination: Measures the total and free RNA to determine EE%, achieving an average EE of 93.8% with high precision and reproducibility.
- Comprehensive characterization: The optimized method supports the development of high-quality mRNA-LNP products, essential for advancing mRNA-based therapeutics and vaccines.

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高灵敏度、高稳定性的CGE方法对mRNA PolyA尾巴分布的分析——使用SDS-MW试剂盒的方法

A method with high separation and good stability for mRNA PolyA tail distribution analysis

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Keywords: CGE, mRNA, PolyA尾巴分布

1. 引言

mRNA疫苗是在第一代减毒/灭活疫苗和第二代亚单位疫苗基础上发展起来的第三代疫苗。mRNA疫苗保持了能胞内表达蛋白的优点，且没有整合到宿主DNA 的风险。在真核生物中，poly(A)尾巴几乎存在于每个mRNA上。mRNA的PolyA尾巴有助于维持mRNA的稳定性、运输和转译效率，影响mRNA的半衰期，通常PolyA尾巴的长度在几十至几百个核苷酸之间。 PolyA尾巴的添加效率和分布是mRNA疫苗的质量控制参数之一，美国药典在“mRNA疫苗和治疗药物质量分析指导”中提到mRNA原料药以及mRNA药物均需要对PolyA尾巴进行表征。Gao 等[1]采用毛细管凝胶电泳法搭配ssDNA试剂盒对mRNA PolyA尾巴分布进行了分析，可以实现单碱基分离效果。

本文开发了一种使用SDS-MW 试剂盒进行PolyA尾巴分析的方法，在同样保证单碱基分离的基础上，可提供更快的分析速度、更高的稳定性和灵敏度。通过对pd(A) 40-60 Test Mix标准品进行测试确认该方法可以实现单碱基分离效果，然后分别测试两个不同片段长度的mRNA PolyA样品，同样可以实现单碱基分离效果，验证了该方法具有优异的分离性能；同时利用30-70 nt片段PolyA样品考察方法重复性，结果表明方法稳定性良好，适合用于mRNA PolyA尾巴的分布分析。

2. 试剂及方法

2.1 试剂及样品

SDS-MW试剂盒包含的试剂：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、10kD蛋白内标 (SCIEX，A26487)、10-225 kDa MW分子量标准品(SCIEX，A22196) 、50 μm内径熔融石英毛细管(SCIEX，338451)；去离子水(Millipore)；

PolyA尾巴部分的酶切与纯化试剂：RNase-free-ddH2O(Vazyme，PN P071）、RNase T1酶(Thermo，EN0541)、LiCl(Sigma-Aldrich ，L116328) 、Tris(Sigma-Aldrich，RDD008)、HCl(Sigma-Aldrich，258148)、无酶水(Thermo，4387936)、甲醇(Sigma-Aldrich，322415)、磁力架(Thermo，12321D)、EDTA(Sigma-Aldrich ，4008-M)、mRNA Capture Beads 2.0 (Vazyme，PN N403)。

样品: 1个已经酶切纯化后的mRNA PolyA尾巴样品由南京诺唯赞企业提供，片段长度分别为30-70 nt，浓度约46 ng/μL；

2.2 溶液制备

2.2.1 试剂及样品溶液制备

试剂制备：按照试剂说明书进行配制；

pd(A) 40-60 Test Mix标准品溶液制备：向 pd(A) 40-60 Test Mix瓶中添加 500 μL 去离子水，然后混合均匀，可以按照15 μL一管进行

等份体积分装，储存于-20 ℃；取一管分装后的Test Mix置于1.5 mL离心管中，加入去离子水稀释7.5倍；准确移取100 µL稀释后的溶液加入到装有样品管的CE专用试剂瓶中，用于上机检测；

样本溶液制备：样品不需要额外稀释，准确移取20 µL样品溶液加入到装有样品管的CE专用试剂瓶中，用于上机检测；

2.3 仪器及方法设置

仪器：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 ；熔融石英毛细管，50 µm内径，20/ 30.2 cm (有效长度/总长度)；进样条件：-5 kV， 20 s；分离电压：-10 kV，40 min；毛细管温度：25 ℃；样品室温度：15 ℃。

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 pd(A) 40-60 Test Mix标准品分离结果

pd(A) 40-60 Test Mix标准品是一个包含40-60个A碱基的混合物，在同样进样条件下使用SDS-MW试剂盒方法样品出峰更快，灵敏度更高，并且也能实现与ssDNA试剂盒方法相同的单碱基分离效果，因此该方法可以用作mRNA PolyA尾巴分布分析，如下图1。

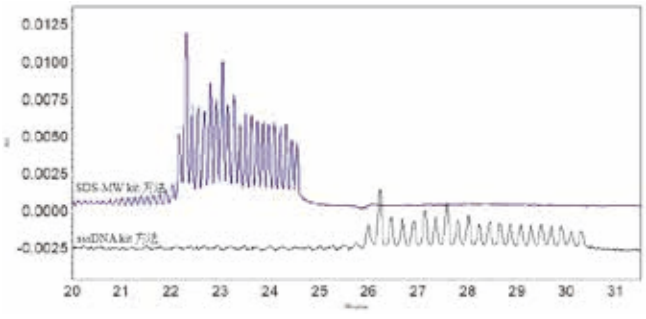


图1. pd(A) 40-60 Test Mix的分离结果

3.2 Poly A尾巴分布分析

使用SDS-MW分析试剂盒方法对30-70 nt PolyA样品进行分离，分离结果如图2，从结果谱图上看30-70 nt PolyA样品中有两部分，每一部分片段均能实现单碱基分离，两个片段校正峰面积比例分别为36.61%和63.39%，表明该方法对实际样品的分离也能达到单碱基分离效果。

3.3 方法稳定性考察

为了进一步评估方法的稳定性，对同一管30-70 nt PolyA样品进行3次连续进样，3次结果高度一致，具体分离结果如表1。样品中30 nt和70 nt两个片段的3针迁移时间RSD值分别为0.09 %和0.10 %；30 nt和70 nt两个片段的3针校准峰面积百分比的RSD分别为1.18 %和 0.69 %，结果表明该方法重复性好。

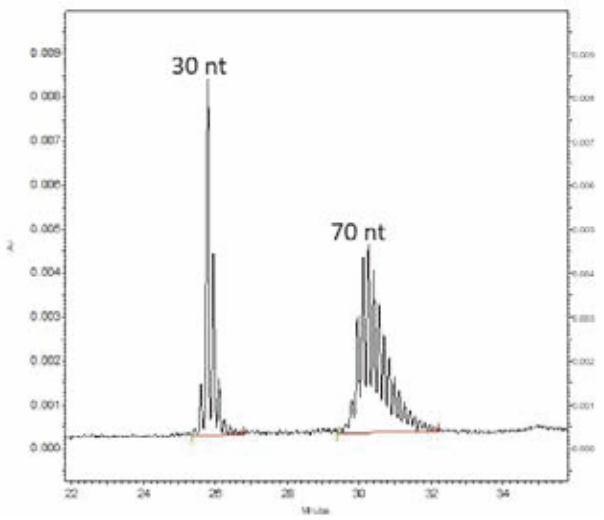


图2. 30-70 nt PolyA样品的分离结果

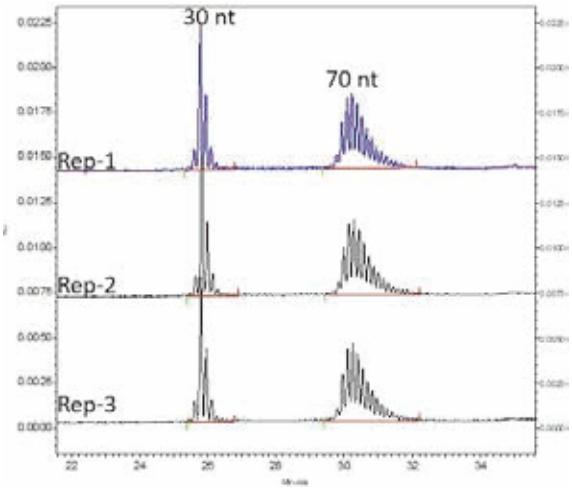


图3. 30-70 nt PolyA样品的3针重复性结果

表1. 30-70 nt PolyA样品3针重复性结果

名称	30 nt片段		70 nt片段	
	迁移时间 (min)	校准峰面积百分比%	迁移时间 (min)	校准峰面积百分比%
Rep-1	25.80	36.54	30.26	63.46
Rep-2	25.83	37.30	30.30	62.70
Rep-3	25.78	37.30	30.24	62.70
RSD %	0.09	1.18	0.10	0.69

4. 结论

毛细管凝胶电泳方法分离原理主要依据核酸片段大小的差异进行分离，本文采用SCIEX 成熟稳定的SDS-MW分子量分析试剂盒，可有效应用于mRNA PolyA尾巴分布分析。该方法主要表现出如下优点：

1) 分离度高，可实现单碱基差异分离：pd(A) 40-60 Test Mix、30-70 nt PolyA样品均可实现单碱基分离效果。

2) 稳定性好：30-70 nt PolyA样品中两个片段的3针迁移时间RSD值分别为0.09 %和0.10%，校准峰面积百分比的RSD分别为1.18 %和 0.69%。

5. 参考文献

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Analysis of mRNA poly(A) tails with single-nucleotide resolution by capillary gel electrophoresis with UV detection (CGE-UV)

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The poly(A) tail is an essential part of messenger RNA (mRNA). For mRNA-based therapeutics and vaccines, the length of the poly(A) tail is a critical quality attribute (CQA) since it directly affects the stability and translation efficiency of the mRNA. In this technical note, we describe a workflow for mRNA poly(A) tail analysis by CGE-UV using the ssDNA 100-R kit on the PA 800 Plus Pharmaceutical Analysis system. Single-nucleotide resolution was obtained over an extended size range of 9 to 156 nucleotides (nt), enabling accurate poly(A) tail length determination and peak quantitation with excellent assay repeatability. The simple sample preparation and ready-to-use ssDNA 100-R kit on a 21 CFR Part 11 compliance-ready platform make this a potential workflow for development of a QC release test.

Key features of mRNA poly(A) tail analysis by CGE-UV on the PA 800 Plus system

- **Exceptional resolving power:** Achieve single-nucleotide resolution critical for accurate poly(A) length determination and peak quantitation of each poly(A) tail species
- **Extended size coverage:** Sustain single-nucleotide resolution in the size range of 9 to 156 nt
- **Excellent assay repeatability:** Obtain consistent poly(A) tail profiles in multiple injections
- **Streamlined workflow:** Achieve direct measurement of poly(A) tail length and distribution with streamlined sample preparation, using the ready-to-use ssDNA 100-R kit on a 21 CFR Part 11 compliance-ready platform. This method has the potential to be developed for QC testing

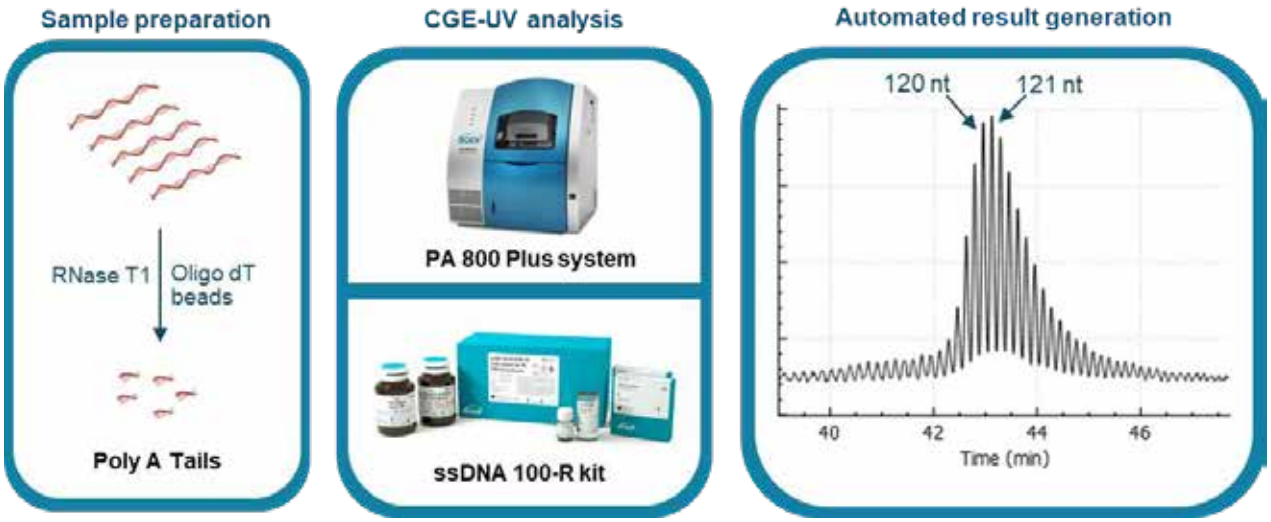


Figure 1. Workflow for mRNA poly(A) tail analysis by CGE-UV. The mRNA sample is digested with RNase T1 first. The poly(A) tails released are then purified with oligo dT conjugated magnetic beads and separated on the PA 800 Plus using the ssDNA 100-R kit. The electropherogram in the right panel demonstrates single-nucleotide resolution in a typical poly(A) tail profile obtained with an mRNA sample.

Introduction

Since the approval of mRNA vaccines against SARS-CoV-2virus, in vitro transcribed (IVT) mRNA has gained popularity in vaccine development, protein replacement therapies, and cancer immunotherapies. The poly(A) tail length is a CQA for mRNA-based therapeutics. One challenge in studying poly(A) tails is that they are difficult to sequence and accurately measure. Various analytical methods have been developed to characterize this CQA. Ion-pair reversed-phase liquid chromatography (IPRP-LC) allows quantification of the percentage of mRNA containing a poly(A) tail but lacks the ability to determine the specific length of the poly(A) tail. Next-generation sequencing (NGS) can achieve single-nucleotide resolution in separating poly(A) tail length. However, its implementation for release testing of manufactured mRNA poses complexity and requires powerful bioinformatics software tools. In this technical note, single-nucleotide resolution was achieved across an extended size range from 9 to 156 nt using a CGE-UV workflow, allowing for precise determination of poly(A) tail length and accurate quantitation of peaks with outstanding assay repeatability.

Methods

Materials: The ssDNA 100-R kit (P/N 477480) containing DNA Capillary, ssDNA 100-R Gel (lyophilized), Tris-Borate buffer, 7M Urea, and pd(A) 40-60 Test Mix; Universal Vials (P/N A62251), Universal Vial Caps (P/N A62250), PCR Microvials (P/N 144709), CE Grade Water (P/N C48034), and NanoVial (P/N 5043467) were from SCIEX (Framingham, MA). Nuclease-free water (NFW, P/N AM9932), RNase T1 (1000 U/µL, P/N EN0541), Dynabeads Oligo $(dT)_{25}$ (P/N 61002), and Zeba Spin Desalting columns (7K MWCO, 75 µL, P/N 89877) were from Thermo Fisher Scientific (Waltham, MA). The 10x RNase H Reaction Buffer (Component #B0297SVIAL) of the RNase H kit (P/N M0297L) was from New England Biolabs (Ipswich, MA). The firefly luciferase (FLuc) mRNA (P/N: L-7602) was from TriLink BioTechnologies (San Diego, CA). The FLuc mRNA contains a 5' Cap and a 3' poly A tail, mimicking an mRNA drug substance. The 120 nucleotide (nt) polyA size marker was custom-synthesized by Integrated DNA Technologies (Coralville, IA).[1] The Agencourt SPRIPlate 96S Super Magnet (P/N A32782) was from Beckman Coulter Life Sciences (Indianapolis, IN). The 0.2 µm (P/N: 4612) and the 0.45 µm (P/N 4654) syringe filters were from PALL (Port Washington, NY). The LABQUAKE rotator (model 400100) was from Barnstead International (Dubuque, IA).

Instruments and software: A PA 800 Plus Pharmaceutical Analysis System (P/N A66528) equipped with an ultraviolet (UV) detector was from SCIEX. The UV wavelength used was 254 nm. Data acquisition and analysis were performed using 32 Karat software version 10.

Tris-Borate-Urea (TBU) buffer preparation: This step must be done one or a few days before running samples. To rehydrate the buffer, 135 ml of 0.2 µm filtered deionized water was added to the bottle containing the dry Tris-Borate from the ssDNA 100-R kit. The buffer solution was mixed with a clean magnetic stirring bar for about 20–30 minutes until boric acid was completely dissolved. The dry 7M Urea was then slowly added to the Tris-Borate buffer while stirring the solution. The urea was completely dissolved after about 2 hours and the solution became clear. 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: Five milliliters of freshly filtered TBU buffer was added to the bottle with lyophilized gel. The gel bottle was then placed onto a rotator in a cold room (2°C to 8°C) for 72 hours with gentle rotation or stirred at room temperature for 5 to 6 hours. The prepared gel buffer can be used up to 30 days after preparation if stored at 2°C to 8°C. The gel buffer was brought to ambient temperature and filtered through a 0.45 µm disposable syringe filter before use.

Cartridge assembly: A DNA capillary (PN 477477) from ssDNA 100-R kit was installed according to the instructions in the ssDNA 100-R kit application guide.[2] The total capillary length was 30.2 cm, with 20 cm as the length to 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) when 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 2A. Each CE Water vial was

filled with 1.5 mL CE Grade Water. The waste vial was filled with 1.0 mL CE Grade Water. The 100-R Gel vial was filled with 1.5 mL ssDNA 100-R Gel buffer, and the TBU Buffer vials were filled with 1.5 mL TBU buffer.

Instrument setup: The Initial conditions and UV detector initial conditions were set up as indicated in the ssDNA 100-R kit application guide.[2] The same setup was used for all methods: the new capillary-conditioning method, the gel-filling method, the separation method and the shutdown method. The time program settings for the capillary-conditioning method and for the gel-filling method are provided in the ssDNA 100-R kit application guide.[2] Time program settings for the separation method are shown in Figure 2B.

Sample preparation: First, the FLuc mRNA was digested with RNase T1 to release the polyA tails. The RNase T1 reaction with a total volume of 100 µL was assembled by mixing 63 µL of 1 mg/mL (100 pmoles) FLuc mRNA that was preheated at 70°C for 5 minutes and chilled on ice for 5 minutes, 10 µL of the 10 x RNase H reaction buffer, 24.5 µL of NFW, and 2.5 µL of the RNase T1. [1] The mixture was incubated at 37°C for 1 hour in a thermal cycler. Secondly, the released polyA tails were purified using the Dynabeads Oligo $(dT)_{25}$. For 100 pmoles of mRNA samples, 1 mg (or 200 µL) of the Dynabeads Oligo $(dT)_{25}$ was washed and resuspended in 100 µL of the binding buffer following the manufacturer's instructions. The RNase T1 digested Fluc mRNA sample (100 µL) was added to 100 µL of the binding buffer and heated at 65°C for 2 minutes in a thermal cycler to unfold possible secondary structures. Immediately after the heat treatment, the sample was placed on ice. In a 500 µL microcentrifuge tube, the 200 µl of mRNA and binding buffer mixture were then mixed with 100 µL of Dynabeads Oligo $(dT)_{25}$ prepared as described above and mixed thoroughly by vortex. The released polyA tails in the RNase T1 treated FLuc mRNA sample were annealed to the oligo $(dT)_{25}$ on the Dynabeads by rotating continuously on a rotator for 30 to 60 minutes at room temperature. The tube was then placed on the magnet for 1-2 minutes. The supernatant was carefully removed while the tube was still on the magnet. Next, the tube was removed from the magnet, and beads were resuspended in 200 µl of washing buffer B. The beads were washed 3 times with the washing buffer B. To elute the polyA tails, 50 µL of ice-cold NFW was added to the beads. The mixture was transferred to a PCR tube, heated at 80°C for 2 minutes in a thermal cycler, and then immediately placed on the magnet. After 1-2 minutes on the magnet, the eluted polyA tails were transferred to a new RNase-free tube. Finally, the sample was desalted with the Zeba column following the instructions from the manufacturer. About 45 µl of purified polyA tail was collected at the end, and 10 ul of this sample was transferred to a Nanovial for CE analysis.

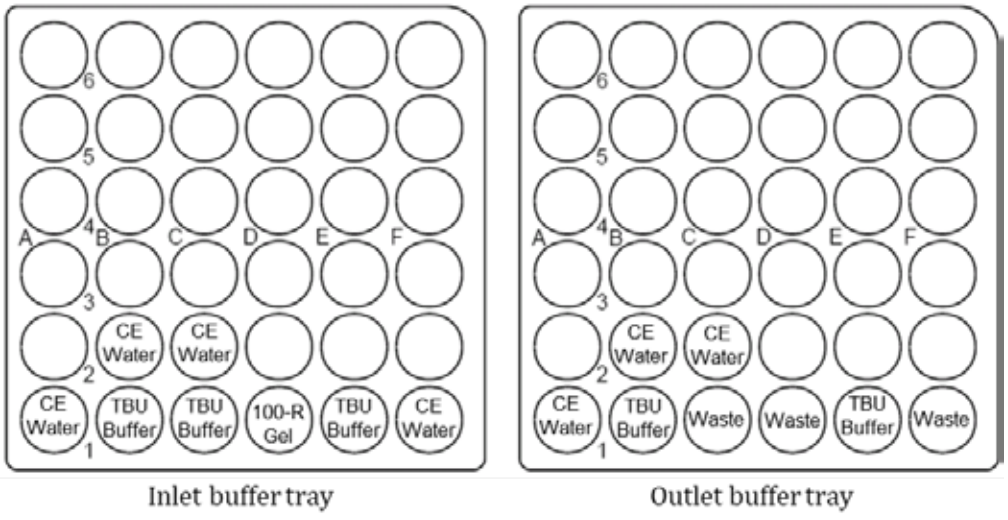


Figure 2. Schematic setup for inlet (left) and outlet (right) buffer trays.

Initial Conditions | UV Detector Initial Conditions | Time Program

	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1		Wait		0.00 min	BI:A1	BO:A1		Water dip to clean the capillary tip
2		Inject - Voltage	5.0 KV	10.0 sec	SI:A1	BO:B1	Override, reverse polarity	Sample injection
3		Wait		0.00 min	BI:B2	BO:B2		Water dip to prevent sample carry-over
4		Wait		0.00 min	BI:C2	BO:C2		Water dip to prevent sample carry-over
5	0.00	Separate - Voltage	11.0 KV	55.00 min	BI:E1	BO:E1	0.17 Min ramp, reverse polarity, both	Separation in ssDNA 100-R gel
6	1.00	Autozero						
7	55.00	End						
8								

Figure 3. Time Program settings for the separation method.

Poly(A) tail analysis workflow by CGE-UV with single nucleotide resolution

The workflow of Poly(A) tail analysis by CGE-UV is illustrated in Figure 1. The mRNA sample is digested with RNase T1 first. The poly(A) tails released are then purified with oligo dT conjugated magnetic beads and separated on the PA 800 Plus system using the ssDNA 100-R kit that contains a replaceable gel buffer and a coated capillary for enhanced reproducibility. The electropherogram in the right panel demonstrates single-nucleotide resolution in a typical poly(A) tail profile obtained with an mRNA sample.

Poly(A) tail length determination

To determine the poly(A) tail length, the poly(A) tails released from the TriLink FLuc mRNA sample were mixed with a 120 nt size marker before CE separation on the PA 800 Plus. As a control, the poly(A) tail samples were also analyzed alone. Results obtained with the poly(A) tail sample and the spiked-in 120 nt size marker are shown in the upper panel of Figure 4, while the results obtained with the poly(A) tail sample alone are shown in the lower panel. Single-nucleotide resolution was achieved in both separation results and maintained throughout the entire poly(A) tail profile. The arrow in the upper panel indicates the 120 nt full-length size marker comigrating with one of the poly(A) tail peaks in the sample, indicating the length of this poly(A) tail peak is 120 nt. This 120 nt peak and the 121 nt peak are the most abundant poly(A) tail species in this sample, consistent with the theoretical 120 nt poly(A) tail length design by TriLink. This sample's poly(A) tail profile included a length distribution from 97 nt to 156 nt. This heterogeneity in poly(A) length may be caused by transcription slippage of the RNA polymerase used to synthesize the FLuc mRNA, as indicated previously.[3]

Single-nucleotide resolution maintained in the size range of 9 nt to 156 nt

Figure 5 shows results obtained with a 120 nt size marker (in the pink trace) and with poly(A) tails from the TriLink FLuc mRNA (in the blue trace). The 120 nt full-length product for this size marker and n-1 species down to the size of 9 nt were well separated with single nucleotide resolution. The single-nucleotide resolution was also achieved in analyzing the poly(A) tails from the TriLink FLuc mRNA with a length distribution of 97 to 156 nt. These results demonstrate a sustained single-nucleotide resolution in the size range of 9 nt to 156 nt. For mRNA-based therapeutics, it was shown that a poly (A) tail length of approximately 100 nt is optimal to minimize degradation.[4] However, it has been reported that short poly(A) tails are associated with highly expressed, well-translated genes.[5] Some very stable natural transcripts, such as beta-actin, were shown to have a short poly(A) tail of less than 30 nucleotides. Therefore, the poly(A) tail analysis workflow by CGE-UV is suitable for analyzing mRNA-based therapeutics and natural transcripts.

Excellent assay repeatability

To assess the assay repeatability, poly(A) tails from the TriLink FLuc mRNA sample were injected 4 times, and the results are shown in Figure 6. Each panel displays the electropherogram from 1 injection. The poly(A) tail profiles in all 4 injections were consistent, with 120 nt and 121 nt peaks as the most abundant species, and single nucleotide resolution was maintained throughout the profile for each

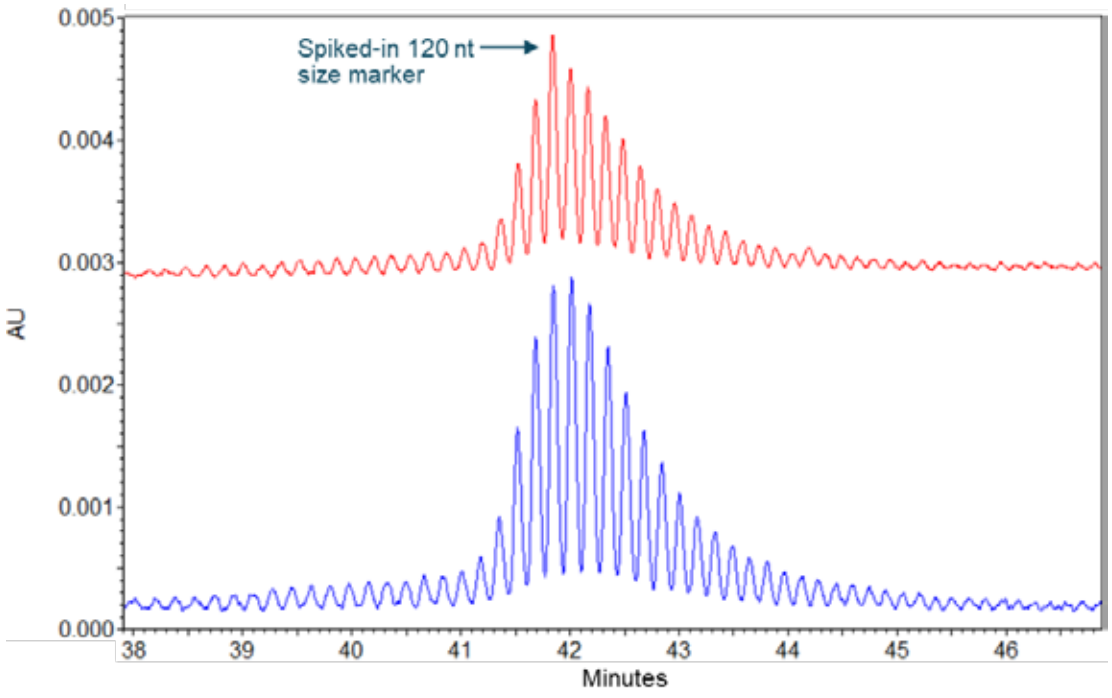


Figure 4: Analysis of polyA tails from TriLink FLuc mRNA. Upper panel: Electropherogram of poly(A) tails from TriLink FLuc mRNA with spiked-in 120 nt size marker. Lower panel: Electropherogram of poly(A) tails from TriLink FLuc mRNA without the spiked-in size marker.

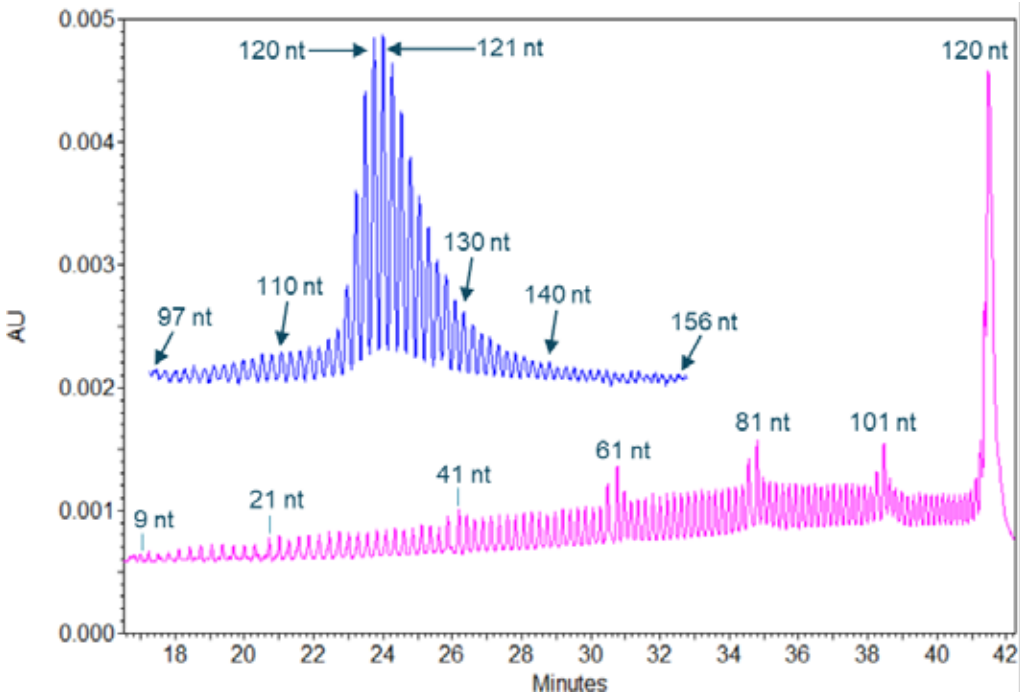


Figure 5: Single-nucleotide resolution over a size range from 9 nt to 156 nt. The Blue trace: poly(A) tails from TriLink FLuc mRNA. Pink trace: 120 nt poly(A) size marker and its n-1 species.

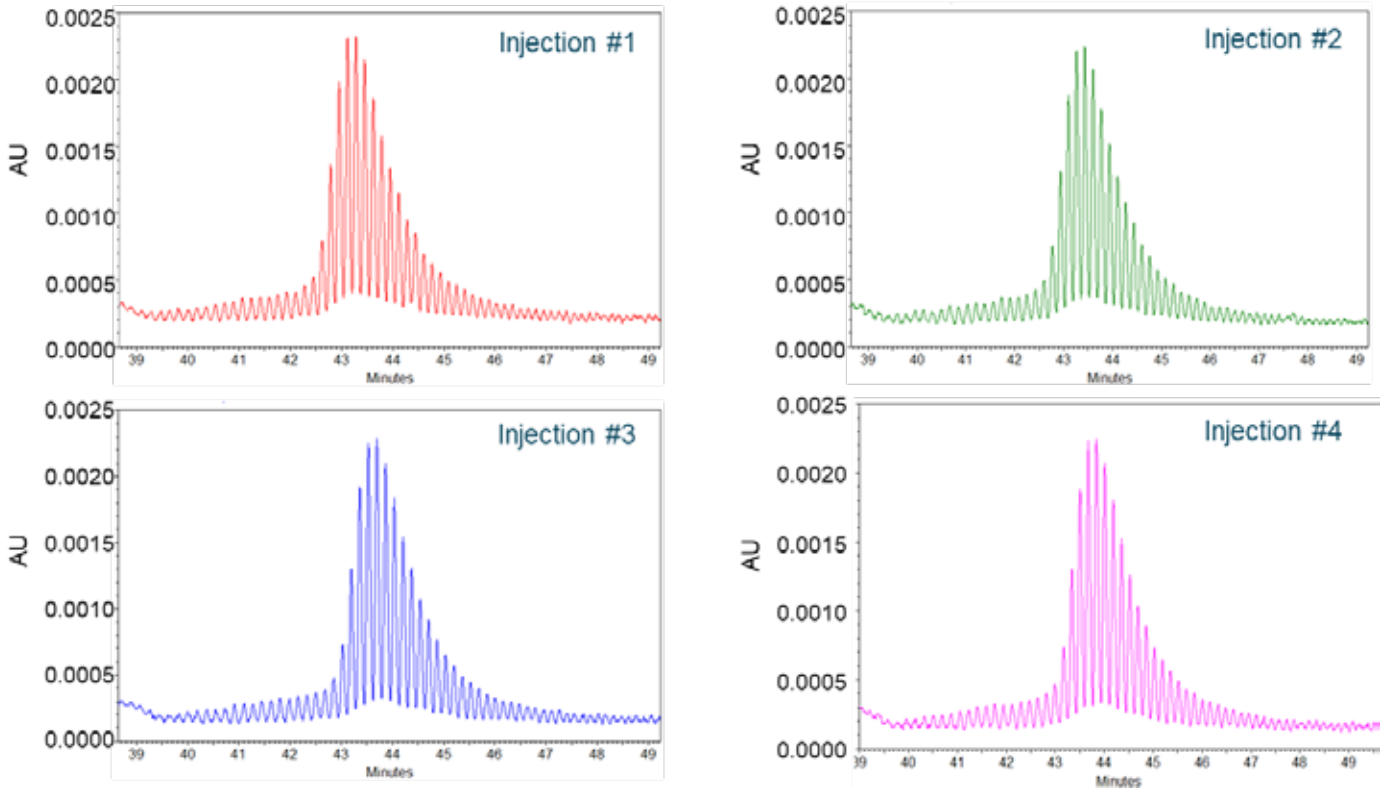


Figure 6: Injection repeatability. The same sample was injected 4 times. Each panel shows the electropherogram from 1 injection.

injection. The CVs for the migration times and the corrected peak area percentage (CPA%) of the 120 and 121 nt species across 4 injections were better than 0.60% and 2.50%, respectively, demonstrating excellent assay repeatability.

Peak quantitation of poly(A) tail species

Maintaining single-nucleotide resolution throughout the entire poly(A) tail profile enabled the execution of peak integration. The CPA% of each poly(A) tail species of the TriLink FLuc mRNA sample was determined using the 32 karat software, and results were summarized in Figure 7. The poly(A) tail distribution of the TriLink FLuc mRNA covers 60 fragments in a size range of 97 nt to 156 nt. Each fragment peak had a signal-to-noise ratio of 5 or higher. The most abundant 2 species (dark green) are the 120 nt and the 121 nt fragments, each representing about 9%. These 2 species, together with 17 additional species with lengths ranging from 116 nt to 132 nt, cover about 80% of all poly(A) tails. This quantitative information adds value to the characterization and quality monitoring of mRNA-based therapeutics.

Size (nt)	CPA%	Size (nt)	CPA%	Size (nt)	CPA%
97	0.15	117	2.65	137	0.70
98	0.23	118	4.99	138	0.61
99	0.25	119	7.60	139	0.49
100	0.28	120	9.09	140	0.47
101	0.35	121	9.42	141	0.40
102	0.32	122	8.63	142	0.36
103	0.38	123	7.45	143	0.31
104	0.45	124	6.10	144	0.27
105	0.48	125	5.06	145	0.23
106	0.50	126	4.13	146	0.29
107	0.57	127	3.31	147	0.19
108	0.66	128	3.04	148	0.20
109	0.68	129	2.19	149	0.15
110	0.76	130	1.82	150	0.17
111	0.75	131	1.55	151	0.23
112	0.84	132	1.26	152	0.17
113	0.96	133	1.10	153	0.16
114	1.03	134	0.99	154	0.15
115	1.15	135	0.82	155	0.11
116	1.48	136	0.66	156	0.10

Figure 7. CPA% of poly(A) tails of the TriLink FLuc mRNA.

Conclusions

- Single-nucleotide resolution was achieved with the CGE-UV workflow, enabling accurate and direct measurement of poly(A) tail length
- The single-nucleotide resolution was sustained across the entire poly(A) tail profile in the size range of 9 to 156 nt, facilitating peak integration of each individual poly(A) tail species
- Excellent assay repeatability was demonstrated by multiple injections of the same poly(A) tail sample with CVs for the migration times and the CPA% of the 120 and 121 nt species across 4 injections better than 0.60% and 2.50%, respectively
- Potential of CGE-UV workflow for development of QC assay for poly(A) tail analysis with simple and fast sample preparation and ready-to-use ssDNA 100-R kit on a 21 CFR Part 11 compliance-ready platform

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使用BioPhase™ 8800系统分析mRNA- LNP等电点及稳定性

Isoelectric points analysis and stability analysis of mRNA-LNP by BioPhase™ 8800 system

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Keywords: BioPhase™ 8800; cIEF; mRNA-LNP; empty LNP; stability

1. 前言

近几年mRNA疫苗的快速发展，并逐渐被探索用于基因治疗、肿瘤治疗等众多领域。mRNA分子很不稳定，容易被外源及内源核酸酶降解，需要脂质纳米颗粒（LNP）进行包裹和递送。LNP是一种高效的药物递送系统，通常由四部分结构脂组成： 阳离子脂质、中性脂质、 两性脂质和PEG-脂质，与mRNA通过自组装工艺形成复杂的球形或圆柱形结构。已有Technical Note [1]对cIEF方法表征mRNA-LNP的等电点的工作做了详细描述。在前面Technical Note基础上，本文同时分析了mRNA-LNP和空载LNP的等电点，发现两者的等电点存在差异。而且通过mRNA-LNP等电点分布的变化可以反映其稳定性，表现在mRNA-LNP样品是否会有酸性变异体或者碱性变异体增多。

本文使用BioPhase™ 8800系统，通过cIEF方法表征mRNA-LNP和空载LNP的等电点，同时对mRNA-LNP的稳定性进行评估。主要内容包括：

1. cIEF方法同时表征mRNA- LNP和空载LNP的等电点，两者的等电点存在差异： mRNA-LNP的等电点比空载LNP等电点偏大，而且前者的紫外吸收强度高；
2. cIEF评价mRNA- LNP的产品稳定性，随着放置时间的增加，酸性变异体增多，适用于生产工艺质量评估及产品稳定性研究；
3. BioPhase™ 8800多通道毛细管系统，可一次分析8个样品，极大地提升了检测效率。

2. 仪器及方法

BioPhase™ 8800系统，配备紫外检测器（UV，波长280 nm），BioPhase软件1.2.20（SCIEX）。用于BioPhase™ 8800系统系统的中性涂层毛细管卡盒 8 × 30 cm（SCIEX PN 5080119）；BioPhase™ 8800系统启动盘套装（SCIEX PN 5080311），包含4块96孔样品盘、4块缓冲液盘、8块出口盘。

cIEF卡盒使用前，先使用 Neutral Capillary Conditioning Solution，70 psi冲洗5 min。然后运行分离方法，进行样品分离分析。

BioPhase™ 8800系统系统分离方法时间程序见图1。



图1. BioPhase™ 8800系统系统分离方法时间程序

3. 试剂及样品

试剂： BioPhase Capillary Isoelectric Focusing (cIEF) kit（SCIEX, PN C30101）， cIEF Peptide Marker Kit（SCIEX，A58481），两性电解质（Pharmalyte 3-10, Cytiva）。 4 M 尿素-cIEF胶配置方法请参照

《BioPhase™ 8800 cIEF等电聚焦分析试剂盒说明书》。

样品：含有相同浓度的阳离子脂质体的mRNA-LNP 样品和空载LNP样品由某疫苗企业提供。

前处理：制备足够分析8个样品用的样品预混液，每份样品预混液的配比如下：100 µL 4 M 尿素-cIEF胶、6 µL两性电解质（Pharmalyte 3-10）、10 µL 阴极稳定剂、1 µL阳极稳定剂和各1µL的pI 标准品（pI 10.0, 9.5, 5.5）。之后取5 µL mRNA-LNP样品与120 µL 样品预混液混合，涡旋混匀，若样品盐浓度高于50 mM，可脱盐处理。用移液器将样品与预混液的混合溶液转移至96孔板（8×12），当分析样品不足8个时，需要在同一列的样品孔中添加100 µL的预混液。

4. 结果与讨论

4.1 mRNA-LNP 样品与空载LNP样品等电点的差异分析

采用cIEF方法分析mRNA-LNP 样品和空载LNP样品的等电点，结果如图2。通过两个样品等电点比较，可以看出包裹mRNA的LNP等电点相对于空载LNP偏大，mRNA-LNP 主峰等电点为7.07，空载LNP主峰等电点为6.91。推测包裹mRNA后，可能导致LNP的表面电荷发生变化，增大了等电点。同时，包裹mRNA后的LNP信号强度显著增加，推测是mRNA的紫外吸光度影响了LNP在280nm的吸收强度。此外，cIEF方法有助于评估mRNA-LNP空载率。

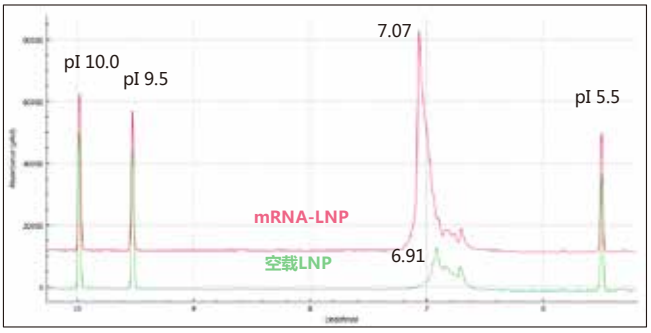


图2. mRNA-LNP 样品与空载LNP样品等电点的结果

4.2 mRNA-LNP 制剂的稳定性评估

mRNA-LNP 样品储存于4℃环境，分别在第1天，第15天和第30天进行cIEF方法的分离分析，来评估mRNA-LNP制剂的稳定性。使用BioPhase分析软件处理mRNA-LNP 样品的3个数据，如图3，可以确认到随着时间的增加，样品的酸性变异体增多，这些酸性变异体可能是由于mRNA暴露在LNP表面产生的。表明随着时间的增加，样品的异质性增加，均一性改变，稳定性变差。因此，cIEF方法可以有效评估mRNA-LNP的稳定性。

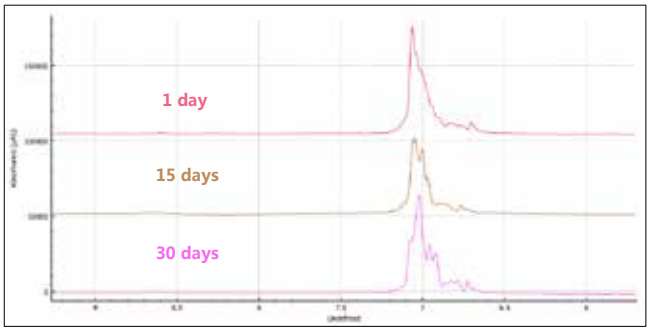


图3. mRNA-LNP 制剂的稳定性评估

5. 结论

cIEF可以用于分析mRNA- LNP和空载LNP的等电点，两者的等电点存在差异：包裹mRNA的LNP的等电点比空载LNP等电点偏大，而且其紫外吸收强度高； cIEF可用于评价mRNA- LNP的产品稳定性，随着放置时间的增加，酸性变异体增多； BioPhase™ 8800多通道毛细管系统，可一次分析8个样品，单个样品分析时间仅需5 min左右，能够极大的缩短mRNA-LNP工艺筛选、批量放行检测及稳定性考察等阶段的样品检测时间。

6. 参考文献

[1] SCIEX Tech Note：CIEF方法分析脂质纳米颗粒包裹的mRNA疫苗（LNP- mRNA）的等电点（RUO-MKT-02-14720-ZH-A）

High-resolution characterization of both sgRNA and Cas9 mRNA in a single analysis for CRISPR/Cas9 applications

BioPhase 8800 system and the RNA 9000 Purity & Integrity Kit

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SCIEX, USA

Introduction

RNA vaccine and CRISPR (clustered regularly interspaced short palindromic repeats) reagents manufacturers are often challenged to accurately characterize and quantify differently sized RNA molecules, impurities, and degraded RNA species present in vaccines or personalized medicinal products [1,2]. To help overcome these challenges, in this technical note, we present an analytical kit-based solution for the characterization of both RNA integrity in the final product and RNA fragmentation. Using the multi-capillary electrophoresis platform, we demonstrate an efficient and timesaving workflow to assess various critical quality attributes (CQAs) for potential mRNA vaccines and CRISPR reagents (Figure 1). Excellent reproducibility was obtained for the purity content of the main product of CRISPR/Cas9 gene editing system with a %CV of <2%. These results demonstrate the ability of the RNA 9000 Purity & Integrity Kit to separate single-stranded RNA products in the range of 50 to 9,000 bases.



The validated RNA Purity & Integrity Kit includes ssRNA ladder in the range of 50 to 9,000 bases, SYBR™ Green II RNA Gel Stain*, separation gel, acid wash reagent, CE-grade water, and LIF performance test mix.

Key features

- Ability to acquire high-resolution data for RNA fragments ranging from 50 to 9,000 bases using the RNA 9000 Purity & Integrity Kit
- Excellent %CV, < 2%, for the corrected peak area% of the main product of the Cas9 mRNA component of the CRISPR/Cas9 gene editing system
- Ready-to-use, validated kit and methods enable efficient RNA fragment characterization using the high-throughput BioPhase 8800 system

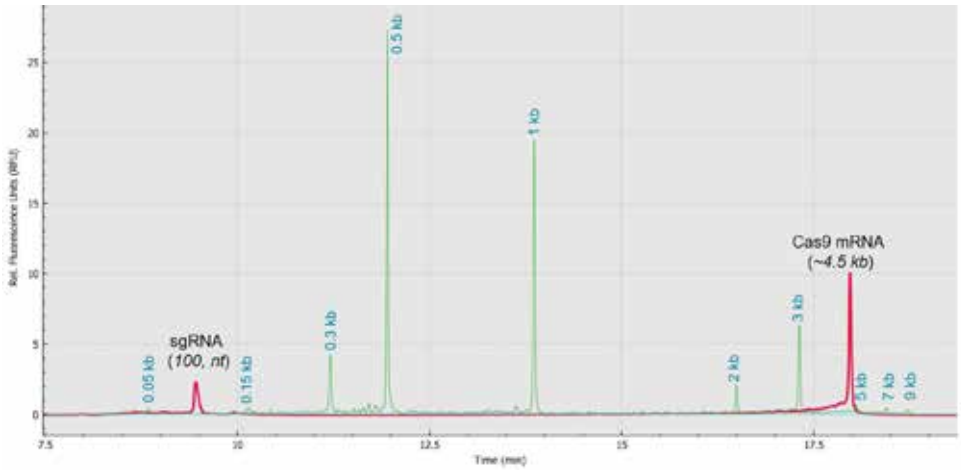


Figure 1. CRISPR/Cas9 system RNA profiling. Data acquired on the BioPhase 8800 system using the RNA 9000 Purity & Integrity Kit.

RNA vaccines and CRISPR-based gene editing are two recent breakthroughs used to combat SARS CoV-2 or potentially treat many genetic diseases, respectively.[1,2] The safety and efficacy of RNA vaccines and CRISPR-based gene editing products highly depend on the stability and purity of the nucleic acid in the final product.

In this technical note, we used the RNA 9000 Purity & Integrity Kit with the BioPhase 8800 system to characterize a chemically modified sgRNA targeting the human HPRT1 gene (100 bases) and the Cas9 mRNA (~4.5 kb). The integrity and purity of the sgRNA and Cas9 mRNA were assessed using capillary gel electrophoresis with laser-induced fluorescence (CGE-LIF).

This technical note highlights the efficiency of our optimized electro kinetic injection-based separation method developed for the RNA 9000 Purity & Integrity Kit and the BioPhase 8800 system.

Methods

Materials: The RNA 9000 Purity & Integrity Kit (PN: C48231) was from SCIEX (Framingham, MA) and contained the Nucleic Acid Extended Range Gel, SYBR™ Green II RNA Gel Stain*, Acid Wash (regenerating solution), CE-grade water, and the ssRNA Ladder (50 to 9,000 bases). Pre-assembled BioPhase BFS Capillary Cartridge (8 capillaries, 30 cm total length, PN: 5080121), the disposable BioPhase sample and reagent plates (PN: 5080311), and the sample loading solution (SLS, PN: 608082) were from SCIEX (Framingham, MA).

The CleanCap Cas9 mRNA (5moU) (PN: L-7206) was obtained from TriLink Biotechnologies (San Diego, CA). The Cas9 mRNA had an expected size of 4,521 bases, as characterized by agarose gel mobility and was shipped at a concentration of 1.0 mg/mL.

A proprietary crRNA sequence (20 bases) targeting the human HPRT1 gene (*NM-000194*) was fused by synthesis to the tracrRNA sequence (80 bases) at 10 nmol by Integrated DNA Technologies (IDT; Coralville, IA) to generate a long, sgRNA (100 nucleotides; 32,458.9 g/mole). This Alt-R CRISPR-Cas9 sgRNA product contained chemical modifications to increase the sgRNA stability, potency, and resistance against nuclease activity.[4]

Instrumentation: The multi-capillary and high throughput BioPhase 8800 system equipped with a laser induced fluorescence (LIF) detector was from SCIEX (Framingham, MA).

Sample dilution and storage: The Cas9 mRNA was stored at -80°C until the time of analysis. Working samples were diluted

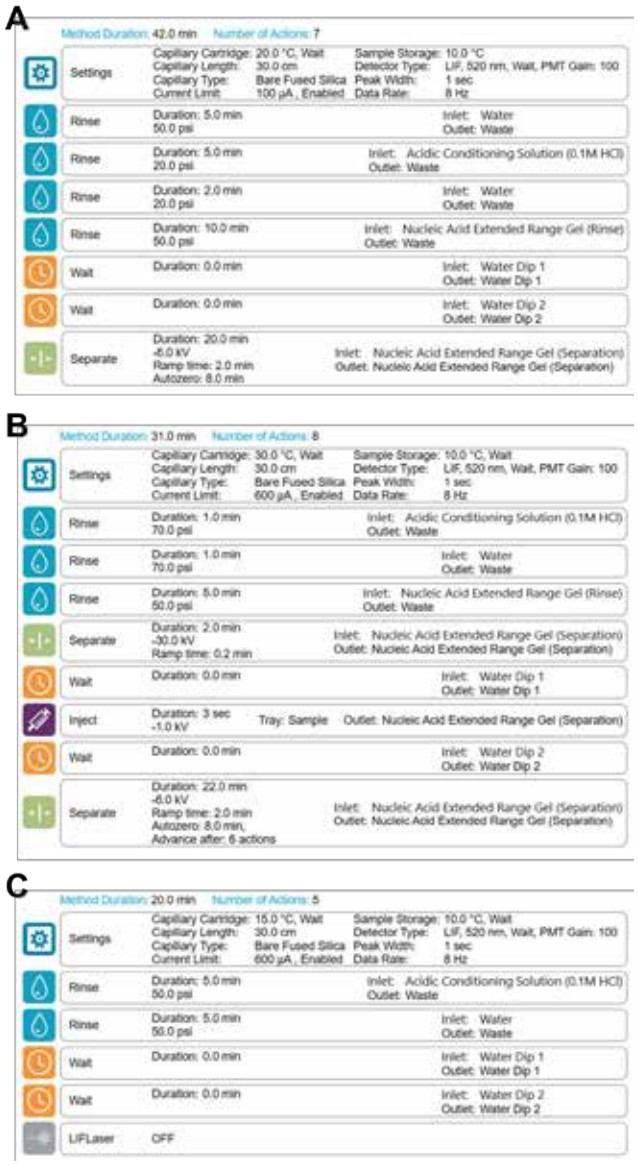


Figure 2. Methods implemented on the BioPhase 8800 system. Parameters are shown for the conditioning method (A), optimized separation method with a water-plug step prior to electrokinetic injection (B), and shutdown method (C).

with CE-grade water to 100 ng/µL in 20 µL aliquots from the main stock solution (1 mg/mL). The lyophilized sgRNA was resuspended at 3.246 µg/µL (100 µM), using a Tris-EDTA pH 7.5 solution containing 10mM Tris, 0.1mM EDTA, aliquoted into small volumes and stored at -80°C until the time of analysis. A 100 µL aliquot of sgRNA working solution was prepared by diluting the stock solution (3.25 µg/µL) to 100 ng/µL using CE-grade water.

Cas9 mRNA sample preparation: A 0.4 ng/µL sample solution was prepared for BioPhase 8800 analysis. To prepare the 0.4 ng/µL Cas9 mRNA analysis sample, first the Cas9 mRNA 100 ng/µL working solution was diluted tenfold (1 to 10) using CE-grade water, followed by mixing 2 µL of the tenfold Cas9 mRNA dilution with 48 µL of a 50:50 CE-grade water/SLS solution to yield a total volume of 50 µL.

sgRNA sample preparation: A 25 ng/µL sample solution was prepared for BioPhase 8800 analysis. To make the 25 ng/µL sgRNA analysis sample, 12.5 µL from the sgRNA working solution (100 ng/µL) were mixed with 37.5 µL of a 50:50 CE-grade water/SLS solution to yield a total volume of 50 µL.

Cas9 mRNA and sgRNA co-analysis: For the co-analysis of the Cas9 mRNA and the sgRNA, 2 µL of the tenfold Cas9 mRNA dilution and 12.5 µL from the sgRNA working solution (100 ng/µL) were mixed with 35.5 µL of a 50:50 CE-grade water/SLS solution to yield a total volume of 50 µL.

Cas9 mRNA sample load analysis: A 2 ng/µL, a 1.2 ng/µL, and 0.4 ng/µL sample solutions were prepared for BioPhase 8800 analysis. To make the 2 ng/µL Cas9 mRNA analysis sample, a 1 µL from the 100 ng/µL working solution was mixed with 49 µL of a 50:50 CE-grade water/SLS solution to yield a total volume of 50 µL. To prepare the 1.2 ng/µL Cas9 mRNA analysis sample, first the Cas9 mRNA 100 ng/µL working solution was diluted tenfold (1 to 10) using CE-grade water, followed by mixing 6 µL of the tenfold Cas9 mRNA dilution with 44 µL of a 50:50 CE-grade water/SLS solution to yield a total volume of 50 µL. The 0.4 ng/µL Cas9 mRNA analysis sample was prepared by mixing 2 µL of the tenfold Cas9 mRNA dilution with 48 µL of a 50:50 CE-grade water/SLS solution to yield a total volume of 50 µL.

After the above mixtures were prepared, they were denatured with heat using a thermal cycler at 70°C for 5 minutes, then immediately placed on ice until time of injection for CE analysis using the BioPhase 8800.

ssRNA ladder preparation: The ssRNA ladder was prepared according to the RNA 9000 Purity & Integrity Kit Application Guide. A 50 µL sample was denatured with heat using a thermal cycler at 70°C for 5 minutes and immediately placed on ice until time of injection for CE analysis. The number of prepared ssRNA ladder samples depended on the number of replicates needed.

CE separation method: Figure 2 illustrates the BioPhase 8800 system operation sequence for the conditioning (panel A), separation (panel B) and shut down (panel C) methods used for this study. For a detailed sample preparation workflow and

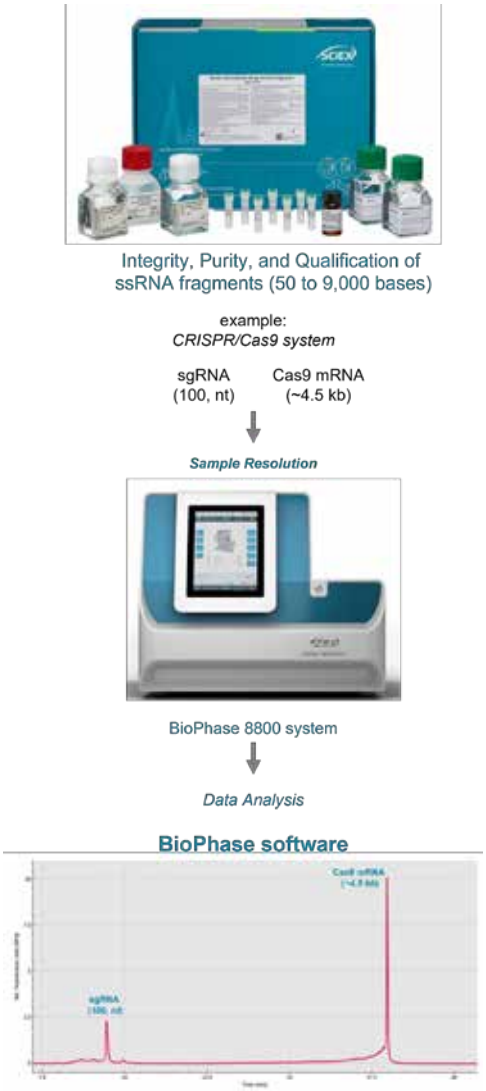


Figure 4. Workflow for simultaneous analysis the small (100 bases) and large (~4.5 kb) RNA fragments using the RNA 9000 Purity & Integrity Kit with the BioPhase 8800 system.

BioPhase 8800 operation refer to the RNA 9000 Purity & Integrity Kit Application Guide.[5]

Data processing: Unless indicated, peak analysis was performed by applying a 0.75% positive threshold with suspended integration from 0 to 8 minutes. The BioPhase software version 1.0 was used to calculate corrected peak area (CPA) and corrected peak area % (CPA%) for the main product, nucleic acid impurities, and higher molecular weight (HMW) products.

Results and discussion

The objective of this technical note was to demonstrate the ability of the RNA 9000 Purity & Integrity Kit from SCIEX to differentiate single-stranded RNA fragments of various sizes in the range of 50 to 9,000 bases with high-resolution. Figure 4 illustrates the workflow *for simultaneous analysis the small (100 bases) and large (~4.5 kb) RNA fragments* from the CRISPR/Cas9 system to showcase the robustness and timesaving sample analysis using *the RNA 9000 Purity & Integrity Kit with* the BioPhase 8800 system. Once samples were analyzed, data assessment was performed with the BioPhase software version 1.0. The representative electropherogram shown at the bottom of Figure 4, which was acquired on the BioPhase 8800 system demonstrates the simultaneous profiling of small and large RNA products used for gene editing applications.

An additional analytical asset provided in the RNA 9000 Purity & Integrity Kit is the ssRNA ladder that contains RNA fragments with lengths of 50, 150, 300, 500,1,000, 2,000, 3,000, 5,000, 7,000 and 9,000 bases. These RNA size markers, and shown in the following sections, provided the built-in technology for estimating the size of the main products and impurities.

CRISPR/Cas9 nucleic acid profiling, quantification and repeatability using the multi-capillary BioPhase 8800 system

HPRT1 sgRNA characterization

Figure 5A shows the overlay of 3 injections (red, green, and blue traces) of a sgRNA sample (100 bases). The pink trace shows the ssRNA ladder and corresponding sizes. The background signal acquired from an injection of water is indicated by the aqua trace. The sgRNA peaks appeared between the 50 bases and 150 bases ssRNA molecular markers. Figure 5B shows the overlay of the triplicate injections without including the RNA ladder. This analysis displayed a better visualization of the RNA fragment impurities and the RNA HMW products. Figure 5C shows the zoomed-in view of the region in front of the full-length sgRNA peak. The RNA fragmentation or impurities in this region were most likely from incomplete sgRNA synthesis such as n-1 species.

Table 1 shows the repeatability injections CPA% measurements from the same capillary attained on the multi-capillary BioPhase 8800 system for the nucleic acid products shown in Figure 5. Measurements were based on 3 consecutive injections of a sample preparation, as described in Methods. The nucleic acid impurities, main sgRNA product, and HMW content accounted for average CPA% values of 19.16%, 56.46%, and 24.39%,

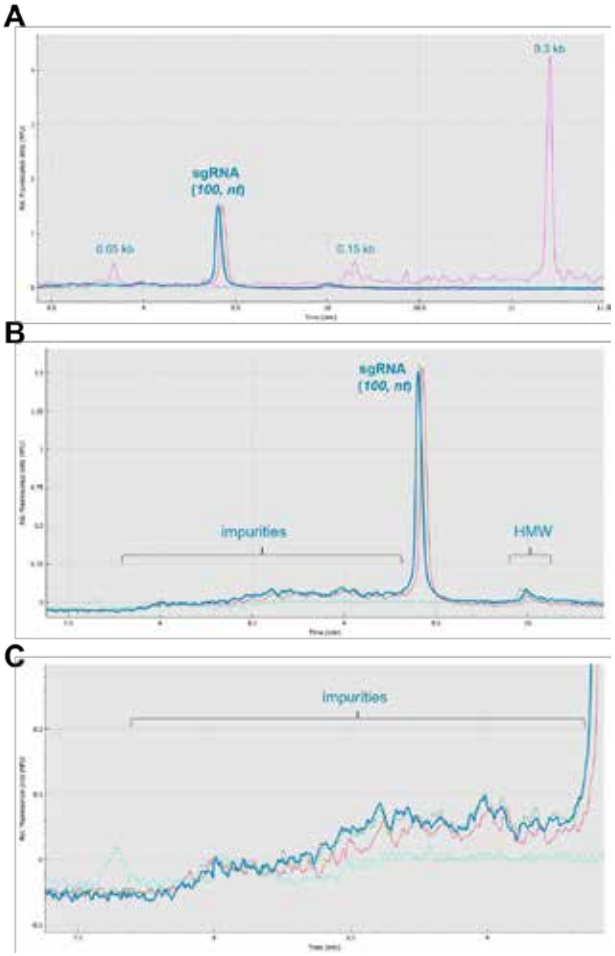


Figure 5. Overlaid electropherograms of HPRT1 sgRNA and ssRNA ladder profiling on the BioPhase 8800 system.

Table 1. Repeatability CPA% of major products in the sgRNA from analysis on the BioPhase 8800 system.

sgRNA (100 bases)	Impurity CPA%	Main product CPA%	HMW CPA%
Injection 1	*20.22%*	*58.50%*	*21.28%*
Injection 2	*18.14%*	*59.28%*	*22.58%*
Injection 3	*19.11%*	*51.59%*	*29.30%*
Average	***19.16%***	***56.46%***	***24.39%***
Standard Deviation	*0.85*	*3.46*	*3.51*
%CV	***4.44%***	***6.12%***	***14.41%***

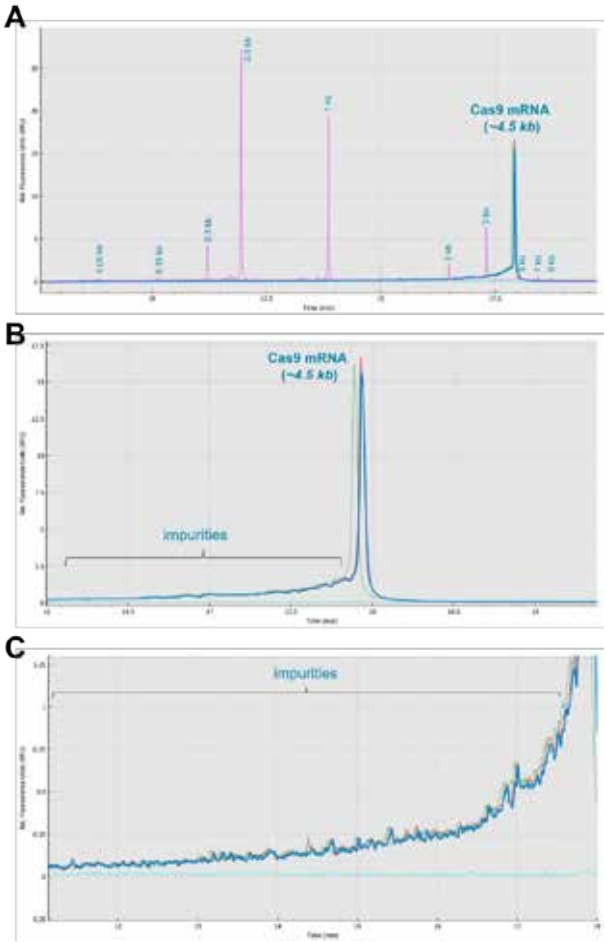


Figure 6. Cas9 mRNA profiling on the BioPhase 8800 system.

respectively. The relatively low standard deviation calculations for these measurements demonstrate the high repeatability performance of the RNA 9000 Purity & Integrity Kit on the BioPhase 8800 system. Demonstrating good injection repeatability with a calculated %CV for the main product of the sgRNA of 6.12%, suggesting the application of the BioPhase 8800 and the RNA Purity & Integrity Kit for assessment of sgRNA products.

Cas9 mRNA characterization

Figure 6A shows the overlay of 3 injections (red, green, and blue traces) of a Cas9 mRNA sample (~4.5 kb). The pink trace shows the ssRNA ladder and corresponding sizes. The Cas9 mRNA peaks appeared between the 3 kb and 5 kb ssRNA size markers.

Figure 6B shows the overlay of the triplicate injections without including the RNA ladder. This analysis displayed a better visualization of the RNA fragment impurities and the RNA HMW products. Figure 6C shows the zoomed-in view of the region in front of the full-length Cas9 mRNA peak. This analysis revealed the presence of fragmentation or impurities in this Cas9 mRNA sample, possibly from degradation of the full-length Cas9 mRNA.

Table 2 shows the repeatability CPA% measurements attained on the BioPhase 8800 system for the nucleic acid products shown in Figure 6. Measurements were based on 5 consecutive injections of a sample preparation, as described in Methods (first three injections shown in Figure 6 as representative injections). The nucleic acid impurities, main Cas9 mRNA product, and HMW content accounted for average CPA% values of 31.32%, 67.88%, and 0.80%, respectively. The relatively low standard deviation calculations for these measurements demonstrate the high repeatability performance of the RNA 9000 Purity & Integrity Kit on the BioPhase 8800 system. In summary, we calculated a %CV for the main product of the sgRNA sample to be an outstanding 1.91%, suggesting the application of the BioPhase 8800 and the RNA Purity & Integrity Kit for assessment of the Cas9 mRNA component of the CRISPR/Cas9 gene editing system.

Table 2. Repeatability CPA% of major products in the Cas9 mRNA from analysis on the BioPhase 8800.

Cas9 mRNA	Impurity CPA%	Main product CPA%	HMW CPA%
Injection 1	*33.64%*	***65.40%***	*0.96%*
Injection 2	*30.89%*	***69.11%***	*0.00%*
Injection 3	*31.09%*	***68.08%***	*0.83%*
Injection 4	*30.33%*	***68.71%***	*0.97%*
Injection 5	*30.66%*	***68.12%***	*1.22%*
Average	*31.32%*	***67.88%***	*0.80%*
Standard deviation	*1.19*	*1.30*	*0.42*
%CV	*3.79%*	***1.91%***	*52.46%*

Simultaneous Cas9 mRNA and sgRNA characterization

Figure 7A shows the overlay of 3 injections (red, green, and blue traces) of a sample containing Cas9 mRNA and the HPRT1 sgRNA. The pink trace shows the ssRNA ladder and corresponding sizes. The background signal acquired from an injection of water is indicated by the aqua trace. Consistent with our previous results, the Cas9 mRNA peaks appeared between the 3 kb and the 5 kb ssRNA molecular markers. The sgRNA peaks also appeared between the 50 bases and 150 bases ssRNA size markers as shown by the RNA profiling of the sgRNA alone. These results suggest that mixing the RNA

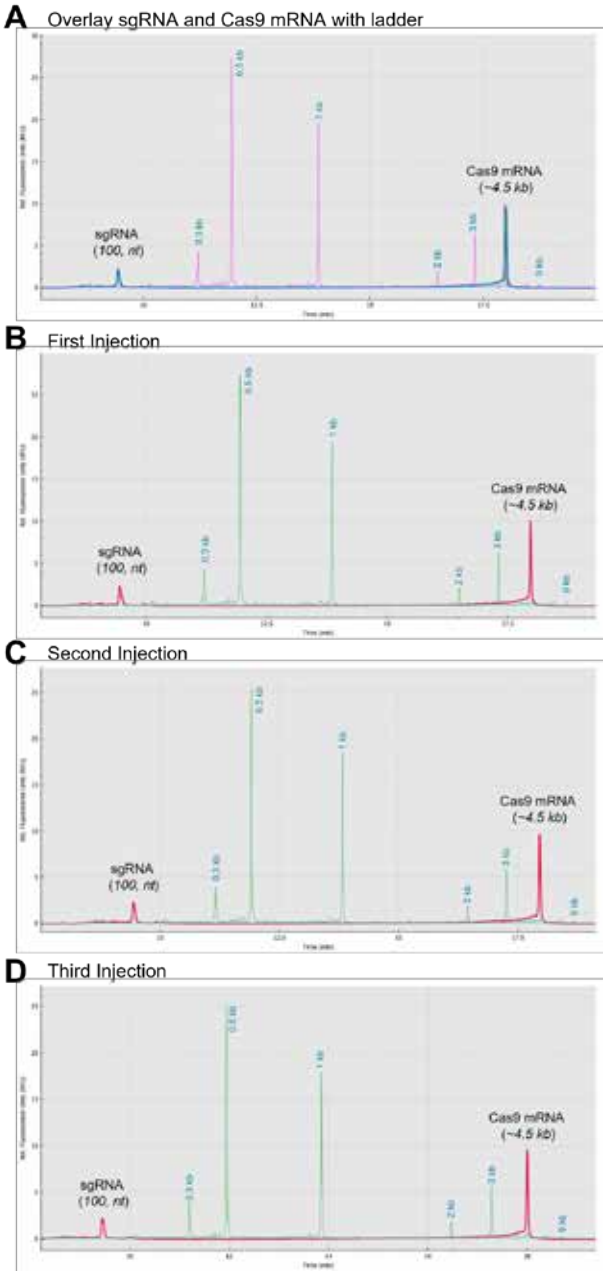


Figure 7. Simultaneous Cas9 mRNA and sgRNA profiling on the BioPhase 8800 system.

components of the CRISPR/Cas 9 gene editing system for purity and integrity co-characterization does not alter the structure or mobility of the Cas9 mRNA or the sgRNA during CGE-LIF analysis. However, prior to performing co-analysis of these RNA molecules, a comprehensive evaluation of these CRISPR/Cas9 gene editing system components should be performed as demonstrated in the sections analyzing the Cas9 mRNA and the sgRNA molecules on their own and without mixing these RNA components. A main advantage of the RNA 9000 Purity & Integrity Kit is the ability to resolve single stranded RNA fragments or products in the range of 50 bases to 9,000 kb, in this case the Cas9 mRNA and the sgRNA are excellent examples for the analytical applications of this Kit to accelerate the research and development pipeline or product release of RNA based therapeutics or vaccines.

Figures 7B through 7D show the individual RNA profiling of the overlayed injections as shown in Figure 7A. This extension into individual analysis of each injection from a sample containing mixed Cas9 mRNA and sgRNA demonstrates the reproducibility of CGE-LIF RNA profiling by using the BioPhase 8800 and the RNA 9000 Purity & Integrity Kit. Specifically, the red trace representing the mixed Cas9 mRNA and sgRNA sample, the green trace corresponding to the ssRNA ladder from the RNA 9000 Purity & Integrity Kit, and the aqua trace illustrating signal arising from water injection used as a negative control.

Table 3 summarizes the corrected peak area % of the Cas9 mRNA and the sgRNA mixed sample for each injection as shown in Figures 7B through 7D. This analysis involved setting up a 5.00% positive threshold to exclude RNA fragmentation or impurities in the sample mix. As a result, we obtained excellent reproducibility for the mixture containing 0.04 ng/uL of the Cas9 mRNA and 25 ng/uL of the sgRNA component. Specifically, an average Cas9 mRNA content of 59.35% and of the sgRNA of 40.65% were determine using the BioPhase software, respectively. A standard deviation value of 0.45 and %CV values of less than 2% were calculated based on this data set. Although, this analytical set up was not designed to reflect a specific stoichiometric relationship between the Cas9 mRNA and the sgRNA molecules, this analytical simulation demonstrates the applicability of the BioPhase 8800 system and the RNA 9000 Purity & Integrity Kit for monitoring or measuring pre-defined

Table 3. Repeatability CPA% of major products in the sgRNA and Cas9 mix on the BioPhase 8800 system.

Cas9 mRNA and sgRNA	sgRNA Main Product CPA%	Cas9 mRNA Main product CPA%	Total RNA Content CPA%
Injection 1	*40.27%*	*59.73%*	*100.00%*
Injection 2	*40.40%*	*59.60%*	*100.00%*
Injection 3	*41.29%*	*58.71%*	*100.00%*
Average	***40.65%***	***59.35%***	***100.00%***
Standard Deviation	*0.45*	*0.45*	*n/a*
%CV	***1.12%***	***0.76%***	*n/a*

molecular ratios among differently sized single stranded RNA products in the range of 50 bases to 9,000 kb.

RNA sample concentration and RNA stability: Key variables for evaluating RNA products and assay development

RNA sample concentration and peak shape

A powerful feature of the BioPhase 8800 is the accompanying software allowing in-depth peak analysis for single stranded RNA fragments. Among other peak measurements, peak symmetry can be automatically calculated to support the characterization of RNA samples used for CGE-LIF analysis. Specifically, we noticed that RNA concentration or amount of the same RNA molecule influences the peak symmetry. Figure 8A shows the overlay of the Cas9 mRNA molecule at 2.0 ng/uL (red trace), 1.2 ng/uL (green trace), and 0.4 ng/uL (blue trace). In contrast to the 2.0 ng/uL Cas9 mRNA sample, the 0.4 ng/uL Cas9 mRNA sample peak tailing seemed minimized. Specifically, we measured a peak symmetry of 1.45 for the 2.0 ng/uL Cas9 mRNA sample, a peak symmetry of 1.46 for the 1.2 ng/uL Cas9 mRNA sample, and a peak symmetry of 0.93 for the 0.4 ng/uL Cas9 mRNA sample. As a result, RNA sample concentration should be considered an important variable for assessing RNA stability and profiling. Figure 8B, shows a graphical analysis of the Cas9 mRNA samples and the corresponding corrected peak area values obtained by using the BioPhase software. Our analysis showed a linear trend with an R-squared value of 0.9919 for these representative injections. In summary, sample preparation techniques must use caution and interpretation of RNA peaks with varying peak fronting, peak tailing, peak ghosting, and assess whether the peaks are rounded or have a sharp at its crest.[6] Controlling for peak variation or peak shape-pattern could favor a robust assay development for final product qualification, release, or application of lifesaving technology.

Application of the RNA 9000 ladder for RNA stability studies

Due to naturally occurring RNA degradation events, because of physical or chemical processes, the integrity and purity profiling may vary for some RNA products. Nevertheless, and as previously cited, capillary gel electrophoresis is a frequently used technique that can give information about the size and the integrity of RNA products.[7] As a result, in this technical note, we demonstrate the application of the ssRNA ladder included in the RNA 9000 Purity & Integrity Kit as a potential molecular marker for stability assessment of RNA products used for CGE-LIF analysis. Figure 9 shows the corrected peak area measurements based on a series of injections obtained from RNA 9000 ladder analysis. The CPAs for the 1 kb and 3 kb RNA size markers were graphed using a box and whisker plot to assess the

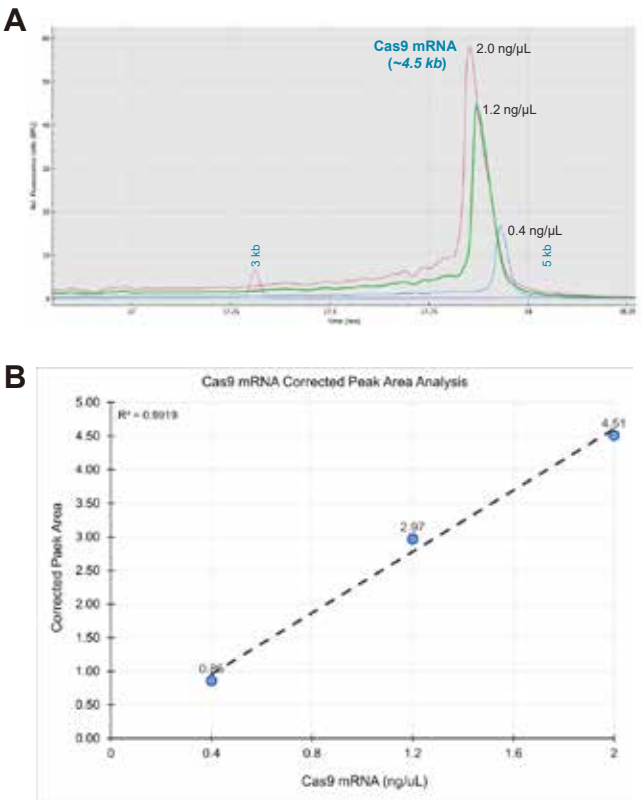


Figure 8. Cas9 mRNA purity profiling and corrected peak area (CPA) quantitation on the BioPhase 8800 system.

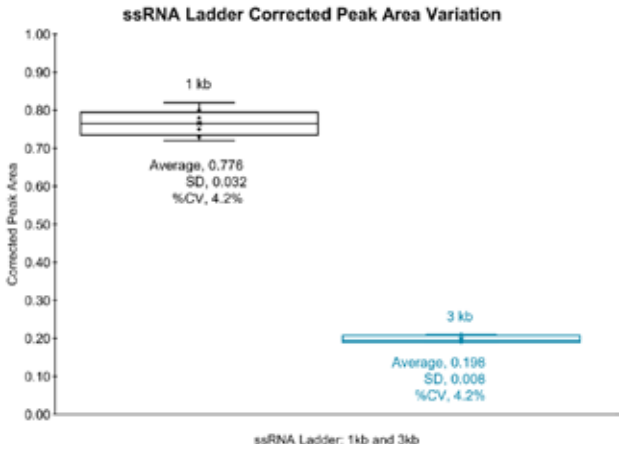


Figure 9. ssRNA stability profiling on the BioPhase 8800 system by laser-induced fluorescence (CGE-LIF).

injection variability of the RNA ladder during CGE-LIF analysis. Briefly, we calculated an average CPA of 0.78 for the 1 kb ssRNA marker with a %CV of less than 5%. The 3 kb ssRNA marker showed an average CPA of 0.20 with a %CV of less than

5%. These CPAs values obtained from a repeatability study of eight injections demonstrated the stability of the ssRNA markers, 1 kb and 3kb, respectively. These results suggest that the RNA 9000 ladder could serve as an analytical tool to assess the RNA stability of ssRNA products of varying sizes and different chemistries, as a result of varying nucleotide type content, or chemical modifications of RNA products.

Conclusions

- The RNA 9000 Purity & Integrity Kit provides a high-resolution separation solution that allows for impurity detection with excellent repeatability for small and large RNA products
- The RNA 9000 molecular weight ladder provides guidance for the qualification of differently sized RNA products
- Assay performance for the main expected product with %CVs <2% supports using CGE-LIF with the multiplexing capability of the BioPhase 8800 system for analytical characterization and large-scale manufacturing applications

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Addressing an unmet need: Developing a new method for purity analysis of large-sized RNA drugs

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This technical note outlines the development of a new method for assessing the purity of a 16 kilobase (kb) self-replicating RNA (srRNA), a type of messenger RNA (mRNA), using capillary gel electrophoresis with laser-induced fluorescence detection (CGE-LIF). This method aims to overcome the difficulties of characterizing large RNA molecules, such as srRNA or self-amplifying RNA (saRNA). Due to their self-replicative properties, srRNA and saRNA vaccines require lower doses than conventional RNA vaccines, thus reducing the burden of manufacturing and enabling faster pandemic responses.[1] However, these RNA molecules range from 9 to 16 kb in size. Traditional workflows, which are generally optimized for RNAs up to 6 or 9 kb, struggle with these large sizes. This technical note presents a new method developed by modifying the dsDNA 1000 gel and optimizing CGE-LIF conditions. These changes provide high-resolution separation (Figure 1) and reproducible purity analysis of a 16 kb srRNA. This development also paves the way for the characterization of other large-sized RNA vaccines and therapeutics.

Key features of the new method for analysis of large-sized RNA drugs

- **New method for purity analysis of a 16 kb srRNA:** Addresses the challenges in analyzing RNA up to 16 kb
- **High-resolution separation of the main product from impurities:** Enables reliable quantitation of srRNA purity
- **Comparable purity results with electrokinetic and pressure injections of the 16 kb srRNA:** Offers flexibility in srRNA analysis
- **Excellent assay repeatability with both electrokinetic and pressure injection modes:** Achieves relative standard deviations (RSDs) of <1.5% for migration time and corrected peak area percentage (CPA%) of the srRNA main product
- **Simple modification of a kit-based method with coated capillary:** Streamlines the operations and ensures consistent results

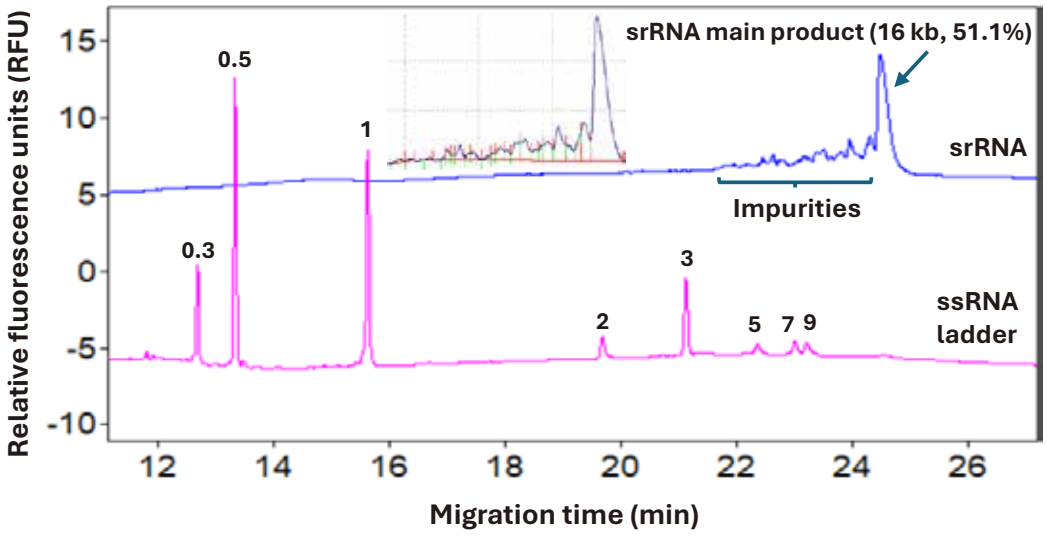


Figure 1: Purity analysis of a research-grade, 16 kb srRNA sample by CGE-LIF. High-resolution separation of the srRNA main product from its impurities was obtained using a denaturing dsDNA 1000 gel on the PA 800 Plus system. The sizes for the standards in the ssRNA ladder are labeled in kb. The purity of the srRNA main product was 51.1% for this sample. The inset shows the peak integration of the srRNA analysis.

Introduction

The success of the mRNA vaccine against the SARS-CoV-2 virus during the COVID-19 pandemic has led to rapid advancements in the RNA vaccine space. Currently, there are 3 major types of RNA vaccines: conventional, base-modified and self-amplifying. In addition to the sequence encoding the protein of interest, saRNA vaccines contain viral sequences encoding an RNA replicase, enabling it to make copies of itself following cellular uptake. Therefore, saRNA vaccines can be delivered at a lower dose than conventional mRNA vaccines to achieve similar levels of immune responses. The srRNA vaccines are derived from second-generation vectors with synthetic optimization.[2] Due to the requirement of including the coding sequences for the replicase, saRNA and srRNA vaccines have large sizes, ranging from 9 to over 16 kb. Current analytical methods that work well with 2 to 4 kb mRNA modalities have challenges in analyzing saRNA and srRNA vaccines. A recent publication described using CGE-LIF with a homebrew separation matrix to analyze a saRNA vaccine of 11.5 kb with some success.[3] However, this type of method faces challenges associated with variations inherent in using homebrew separation matrices. In this technical note, a new method was developed by modifying the dsDNA 1000 gel and optimizing CGE-LIF conditions, providing high-resolution separation and reproducible purity analysis of a 16 kb srRNA using kit-based reagents.

Methods

Materials: The dsDNA 1000 kit (P/N: 477410) containing the DNA capillary (100 µm ID, 65 cm), the dsDNA 1000 gel buffer and other components was from SCIEX (Framingham, MA). The RNA 9000 Purity & Integrity kit (P/N: C48231) containing the ssRNA ladder, SYBR™ Green II RNA gel stain* and other components was from SCIEX. Sample loading solution (SLS, P/N: 608082), Universal Vials (P/N: A62251), Universal Vial Caps (P/N: A62250), PCR Microvials (P/N: 144709), CE Grade water (P/N: C48034), and NanoVial (P/N: 5043467) were from SCIEX. The urea (P/N: 29700), Nuclease-free water (NFW, P/N: AM9932) and 5X Tris-borate-EDTA (TBE) buffer (P/N: J63487.K3) were from Thermo Fisher Scientific (Waltham, MA). The 0.2 µm syringe filter (P/N: 4612) was from PALL (Port Washington, NY). The LABQUAKE rotator (model: 400100) was from Barnstead International (Dubuque, IA). The research-grade, 16 kb srRNA was from Replicate Bioscience (San Diego, CA).

Cartridge assembly: The DNA capillary (P/N: 477477) was installed per instructions on the kit insert (P/N: 726412) in the dsDNA 1000 kit. The total capillary length was 40.2 cm, with a length of 30 cm to the detection window. Since the inner wall of the DNA capillary is coated, the cartridge assembly should be carried out promptly. When the cartridge assembly is complete, the capillary ends must be immersed in liquid (water or buffer) to prevent the coating from drying out.

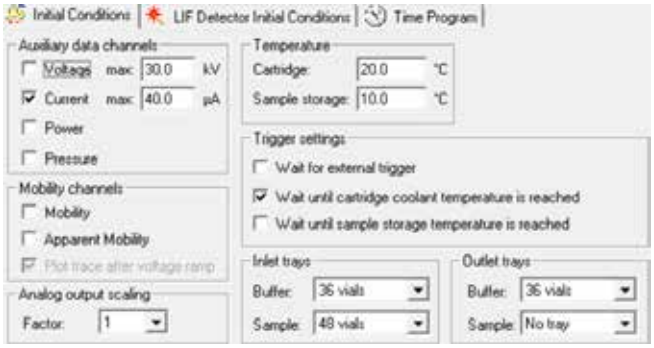
LIF calibration: To ensure a consistent response of the LIF detector throughout this study, the LIF detector was calibrated using the LIF Calibration Wizard and Performance test mix (P/N: 726022), following the instructions in the LIFluor EnhanCE user's guide (P/N: 725824). The target relative fluorescence units (RFU) value was set at 62. This calibration was done whenever the capillary, LIF detector or laser was changed.

General conditions for CGE-LIF analysis of RNA: To minimize sample degradation, all samples, NFW and buffers were placed on ice before use. Filter tips were used for all liquid transfer steps.

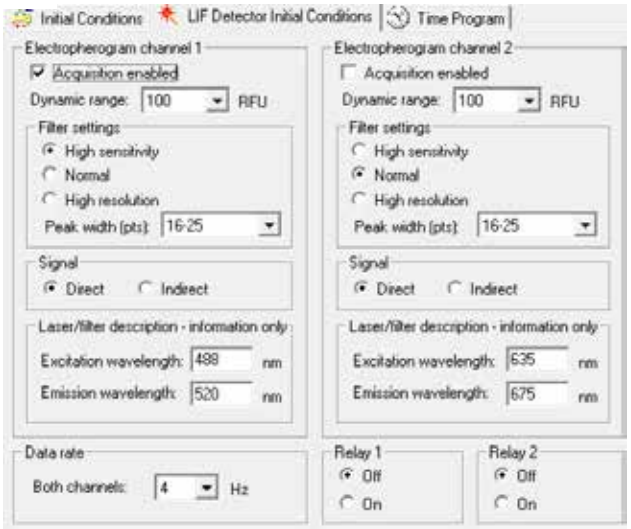
Preparation of the denaturing dsDNA 1000 gel: A 100 mL stock of 8M urea solution was made by dissolving 48.1 grams of urea in deionized water without heat. The solution was filtered with a 0.2 µm syringe filter. The dsDNA 1000 gel was rehydrated by adding 20 mL of 8M urea to a bottle of the lyophilized dsDNA 1000 gel. After the gel bottle was securely capped, it was attached to a rotator and rotated in a cold room for about 72 hours. A solution of 1X TBE and 5.73M urea (1X TBE/5.73M urea solution) was made by adding 800 µL of the 5X TBE, 2865 µL of 8M urea and 335 µL of NFW in a 50 mL tube, inverting it gently 10 times and filtering it with a 0.2 µm syringe filter. The 8M urea solution, the 1X TBE/5.73M urea solution and the rehydrated dsDNA 1000 gel can be stored in small aliquots at 2–8°C for 1 month. Before sample analysis, the rehydrated dsDNA 1000 gel and the 1X TBE/5.73M urea solution were warmed to room temperature. The final gel (the denaturing dsDNA 1000 gel) was made by diluting the rehydrated dsDNA 1000 gel 4-fold with the 1X TBE/5.73M urea solution in a 50 mL tube and then filtering it with a 0.2

µm syringe filter. The denaturing dsDNA 1000 gel contained 6.3M urea and 1X TBE. Finally, 24 µL of the SYBR Green II RNA gel stain was added to 6 mL of the denaturing dsDNA 1000 gel in a 50 mL tube, mixed well by inverting 20 times and then transferred to the universal vials.

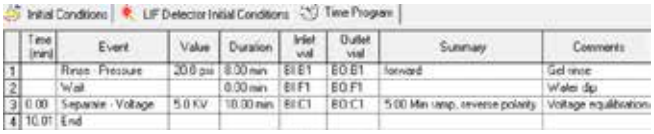
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B



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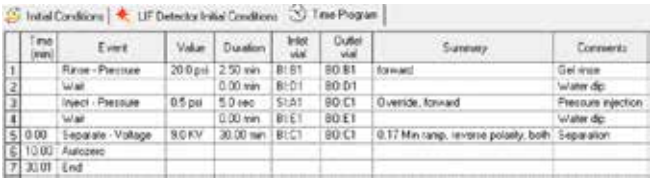
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2		Wait		0.00 min	B:F1	B0:F1		Water dip
3	0.00	Separate - Voltage	5.0 KV	10.00 min	B:C1	B0:C1	5.00 Min ramp, reverse polarity	Voltage equilibration
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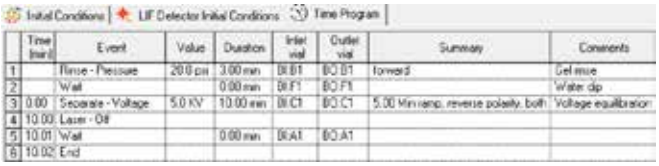
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2		Wait		0.00 min	B:D1	B0:D1		Water dip
3		Inject - Voltage	1.0 KV	2.0 sec	S:A1	B0:C1	Override, reverse polarity	Electrokinetic injection
4		Wait		0.00 min	B:E1	B0:E1		Water dip
5	0.00	Separate - Voltage	9.0 KV	30.00 min	B:C1	B0:C1	0.17 Min ramp, reverse polarity, both	Separation
6	10.00	Autozero						
7	30.01	End						

E



	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1		Rinse - Pressure	20.0 psi	2.50 min	B:B1	B0:B1	forward	Gel rinse
2		Wait		0.00 min	B:D1	B0:D1		Water dip
3		Inject - Pressure	0.5 psi	5.0 sec	S:A1	B0:C1	Override, forward	Pressure injection
4		Wait		0.00 min	B:E1	B0:E1		Water dip
5	0.00	Separate - Voltage	9.0 KV	30.00 min	B:C1	B0:C1	0.17 Min ramp, reverse polarity, both	Separation
6	10.00	Autozero						
7	30.01	End						

F



	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1		Rinse - Pressure	20.0 psi	3.00 min	B:B1	B0:B1	forward	Gel rinse
2		Wait		0.00 min	B:F1	B0:F1		Water dip
3	0.00	Separate - Voltage	5.0 KV	10.00 min	B:C1	B0:C1	5.00 Min ramp, reverse polarity, both	Voltage equilibration
4	10.00	Laser - Off						
5	10.01	Wait		0.00 min	B:A1	B0:A1		
6	10.02	End						

Figure 2: Parameter settings used in this study. Settings are shown for the initial conditions (A), LIF detection initial conditions (B), time programs for conditioning (C), separation with electrokinetic injection (D), separation with pressure injection (E) and shutdown (F).

Sample preparation: In a 200 µL thin-walled PCR tube, 2.5 µL of the ssRNA ladder from the RNA 9000 Purity & Integrity kit was mixed with 100 µL of SLS, heated at 70°C for 5 minutes, then quickly chilled in an ice water bath for at least 5 min. Fifty µL was transferred to a microvial for CE analysis. For srRNA, 2 µL of stock 1 mg/mL srRNA was added to 98 µL of SLS (final concentration: 20 ng/µL) in a 200 µL thin-walled PCR tube. The mixture was heated at 65°C for 5 minutes or the indicated amount of time (Table 1). After chilling in an ice water bath for at least 5 min, 50 µL was transferred to a microvial for CE analysis. Alternatively, 15 µL was transferred to a nanovial for CE analysis.

Preparation of buffer trays: The buffer trays were set up according to vial positions in the time programs shown in Figure 2. Each water vial was filled with 1.5 mL of CE Grade water, each waste vial was filled with 1 mL of CE Grade water and each gel vial was filled with 1.5 mL of the denaturing dsDNA 1000 gel. Three sets of water dip vials were set up to ensure minimal sample carryover and better repeatability.

Instrument and software: A PA 800 Plus system equipped with LIF detection and a 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 v10.2.

Instrument setup: The "Initial Conditions" and "LIF Detector Initial Conditions" were set up as indicated in Figures 2A and 2B. The same initial conditions were used for all 4 methods: capillary conditioning, separation with electrokinetic

injection, separation with pressure injection, and shut down, except the capillary temperature was set to 15°C for shut down. The time programs for the 4 methods are shown in Figures 2C–2F.

Results and discussion

Optimization of the gel formulation: The dsDNA 1000 gel has been widely used for over 2 decades for sizing and purity analysis of dsDNA by CE under native conditions. Its popular applications include sizing analysis of linearized plasmid DNA, restriction profiling for plasmid integrity and analysis of host cell DNA in influenza and respiratory syncytial virus vaccines.[4-5] This technical note explored its utility in analyzing large-sized single-stranded RNA. Due to the large size (16 kb) of the srRNA sample, the regular dsDNA 1000 gel needed to be diluted to achieve a realistic separation time. In addition, since large-sized RNAs, such as srRNA, contain higher-order structures under native conditions, a denaturant must be added to the gel to ensure the srRNA sample remains in the unfolded conformation during separation. Initial method development efforts included rehydrating the dsDNA 1000 gel in 8M urea instead of deionized water. This gel was diluted 3- or 4-fold so that the final formulation contained 7.4M urea and 1X TBE. The 4-fold dilution produced a slightly better resolution with a slightly faster migration time than the 3-fold dilution (data not shown). Further optimization included a titration of the urea concentration. When urea concentration was below 6M, an inconsistent peak profile with low resolution was observed, possibly due to a combination of changes in gel viscosity and refolding of the srRNA sample during separation (data not shown). In contrast, peak tailing appeared when the urea concentration was too high. As shown in Figure 3, when the urea concentration was reduced from 7.4M to 6.3M, the peak shape significantly improved. A concurrent increase in resolution was also observed. Therefore, the 6.3M urea concentration was used for later experiments. The final gel formulation, containing 1X TBE, 6.3M urea and a 4-fold dilution of the rehydrated dsDNA 1000 gel, was designated as the denaturing dsDNA 1000 gel.

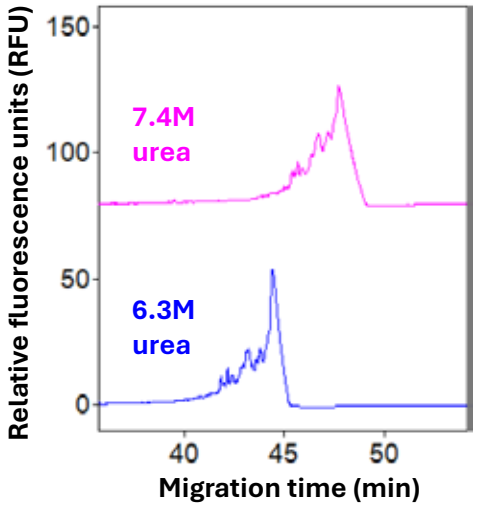


Figure 3: Optimization of the urea concentration in the gel formulation. The srRNA sample was analyzed using rehydrated dsDNA 1000 gel containing either 7.4M (pink trace) or 6.3M (blue trace) urea. The sample was introduced at 2 kV for 2 seconds. The separation voltage was 5 kV.

Selection of optimal separation voltage and sample injection conditions: Manufacturers in cell and gene therapy desire faster, high-resolution analytical methods. Decreasing urea concentration or increasing the capillary temperature to 30°C during separation improved migration time at the cost of reduced resolution (data not shown). Therefore, further optimization was performed by testing different separation voltage and sample injection conditions. As indicated in Figure 4, increasing the separation voltage from 5 kV to 7 kV and then to 9 kV gradually reduced the migration time of the srRNA main product peak from 44 minutes to 25 minutes. The peak shape was also improved, and better resolution was achieved between the srRNA main product peak and impurities. When the sample injection voltage was reduced from 2 kV to 1 kV, the peak shape and resolution were further improved. In summary, injecting the srRNA sample at 1 kV for 2 seconds and separating at 9 kV produced the best resolution between the srRNA main product peak and impurities.

Optimization of sample treatment conditions: Since the srRNA is prone to degradation, the heat treatment to unfold the higher-order structure must be gentle and effective. A quick time course experiment was done to test different incubation times at 65°C and their impact on the srRNA analysis. As shown in Table 1, the purity decreased when the incubation time was longer than 5 minutes. At 0 and 2 minutes of incubation time, the peak shape and resolution were not as consistent as at 5 minutes of incubation time (data not shown). Therefore, 5 minutes of incubation at 65°C was the optimal sample treatment condition.

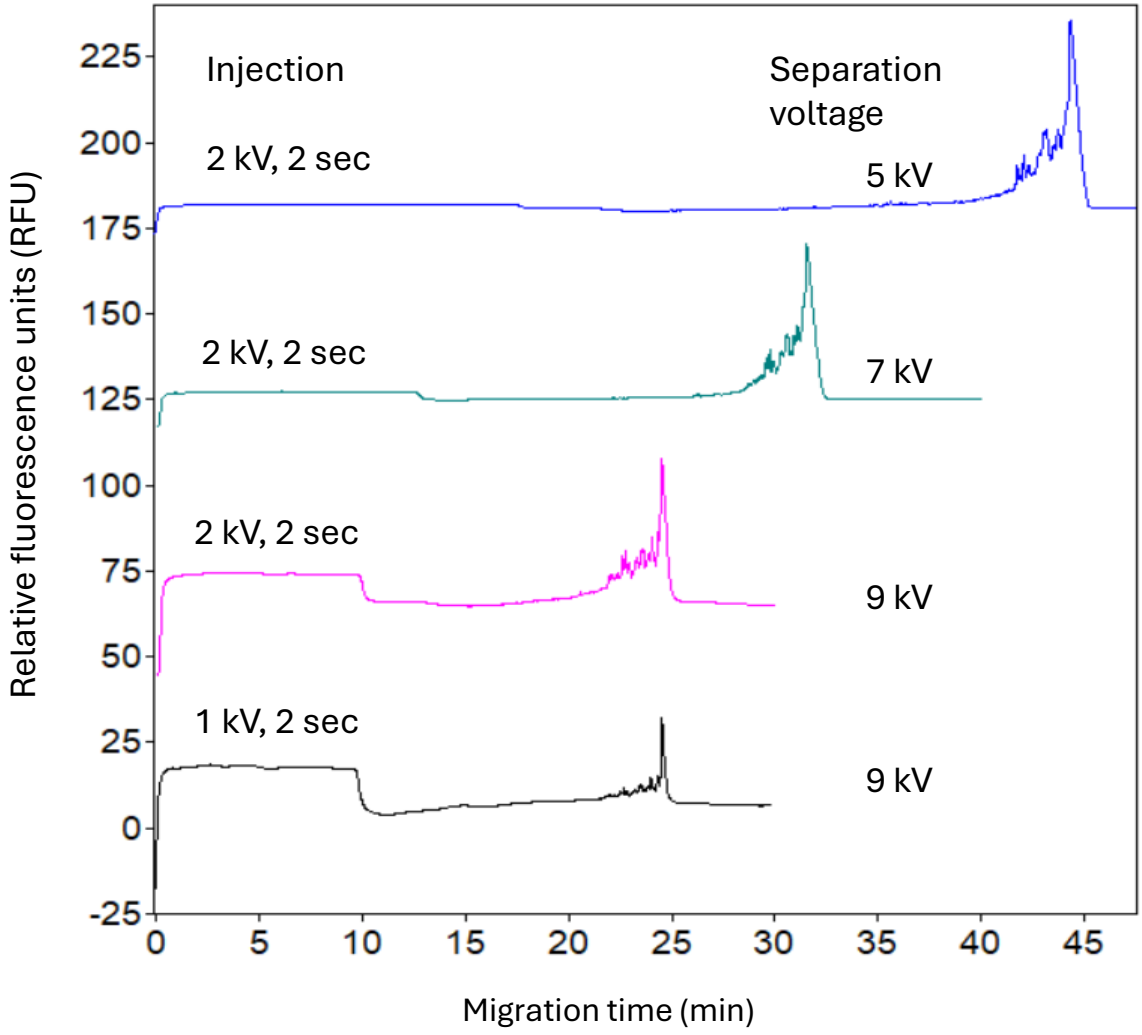


Figure 4: Optimization of separation voltage and sample injection time. The srRNA sample was separated using the denaturing dsDNA 1000 gel on the PA 800 Plus system at different separation voltages or injection conditions. The optimal conditions to achieve the highest resolution were injecting at 1 kV for 2 seconds and separating at 9 kV.

Table 1: Optimization of sample treatment conditions. The srRNA sample was heated at 65°C for indicated amounts of time and quickly chilled in an ice-water bath before analysis on the PA 800 Plus system. The purity of the srRNA sample was measured using the CPA% of the main product peak.

Incubation time at 65°C (minutes)	0	2	5	8	14
Purity of the srRNA sample (CPA%)	50.0%	50.8%	50.3%	48.3%	35.8%

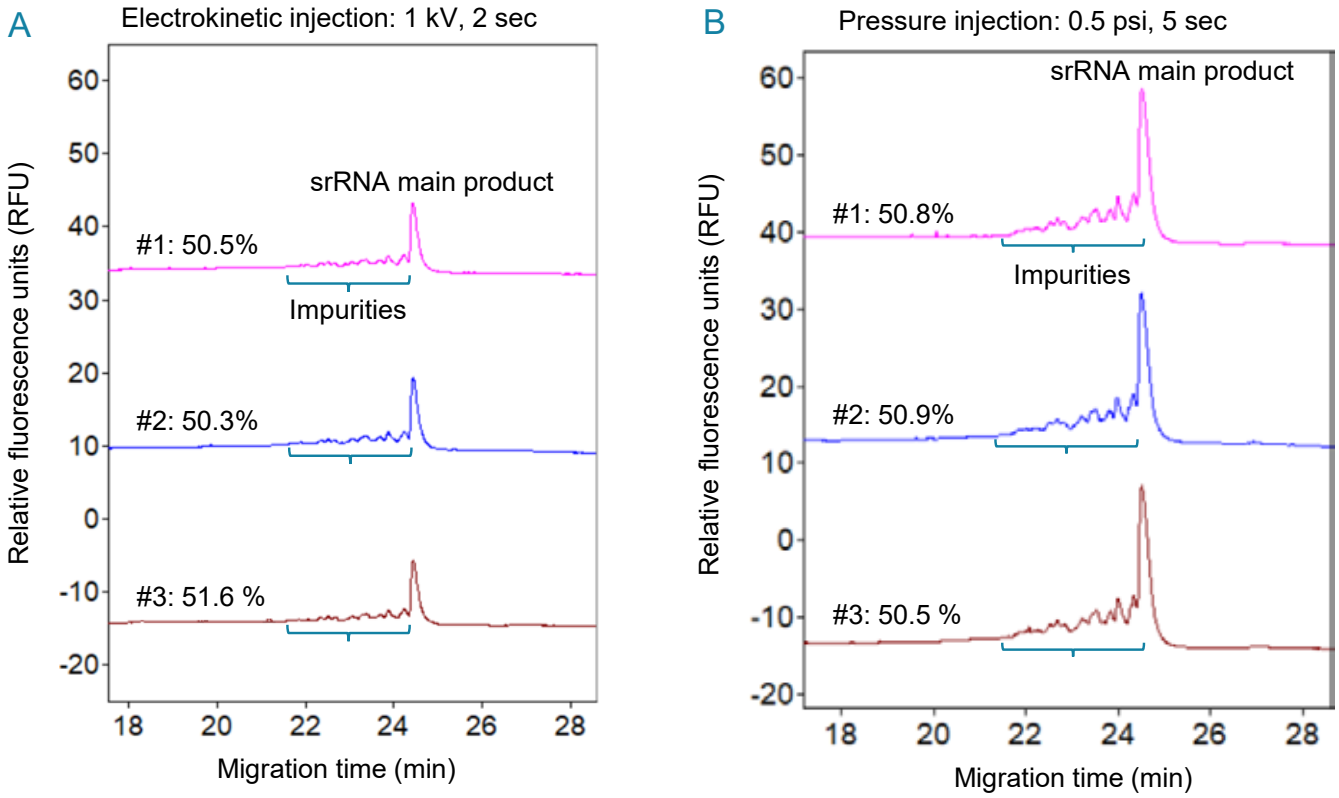


Figure 5: Excellent assay repeatability of srRNA analysis by CGE-LIF with electrokinetic (A) and pressure (B) injections. The srRNA sample was injected from the same sample vial for 3 consecutive injections by either electrokinetic (1 kV, 2 seconds) or pressure (0.5 psi, 5 seconds) injection and analyzed on the PA 800 Plus system with a neutral capillary cartridge and the denaturing dsDNA 1000 gel. The bracket shows the impurities. The srRNA purity, determined as the CPA% of the main product, is indicated above each electropherogram.

High-resolution purity analysis of the srRNA under optimized conditions: The electropherogram at the top of Figure 1 was obtained with the srRNA sample using the optimized conditions. The srRNA sample was treated at 65°C for 5 minutes before injection at 1 kV for 2 seconds and separation at 9 kV by CGE-LIF in the denaturing dsDNA 1000 gel. The srRNA main product was well separated from the impurities, facilitating data analysis for purity assessment. This separation quality was an advantage, as the impurities in the same sample separated by agarose gel electrophoresis were detected as smears that were poorly separated from the main product (data not shown). The CPA% of the main product was 51.1% by CGE-LIF. This purity level was consistent with the expectations for a research-grade srRNA sample. The lower trace in Figure 1 displays the electropherogram obtained with the ssRNA ladder, showing the high-resolution separation of the standard fragments, except for the 7 kb and 9 kb standards. Based on our experience, the resolution between these 2 standard fragments did not closely correlate to the resolution between the srRNA main product and its impurity peaks. The optimal conditions were selected solely based on results obtained with the srRNA sample.

Excellent assay repeatability of srRNA analysis by CGE-LIF with electrokinetic and pressure injections: Assay repeatability was tested by 3 consecutive injections at 1 kV for 2 seconds from the same srRNA sample vial. Results in Figure 5A showed consistent peak profiles between all 3 injections. The migration times of the srRNA main product were identical in the 3 injections and the CPA% values for the main product were consistent for the 3 injections (Table 2),

demonstrating excellent assay repeatability. Furthermore, another repeatability test was performed by 3 consecutive pressure injections at 0.5 psi for 5 seconds from the same srRNA sample vial. Results summarized in Figure 5B and Table 2 displayed consistent peak profiles, migration times and CPA% values of the srRNA main product, showcasing excellent assay repeatability with pressure injection. The signal obtained with pressure injection at 0.5 psi for 5 seconds was higher than that obtained with electrokinetic injections at 1 kV for 2 seconds of the same srRNA sample, possibly due to different amounts of the sample injected under the 2 conditions. However, the purity obtained from both injection modes was comparable. Therefore, users have the flexibility to choose either injection mode based on the characteristics of their samples. For example, pressure injection might be more suitable for in-process development samples than electrokinetic injection because a consistent amount of sample can be injected by pressure injection regardless of sample ionic strength. Furthermore, various sample stacking techniques can be explored when higher sensitivity is needed.

Table 2: Summary of repeatability results obtained with 3 injections of the same srRNA sample by electrokinetic injection (EKI) or pressure injection (PI). Migration time (Mt) is listed in minutes (min). CPA% and %RSD are listed in percentage (%).

	Mt_EKI (min)	CPA%_EKI	Mt_PI (min)	CPA%_PI
Injection #1	24.44	50.5	24.48	50.5
Injection #2	24.44	50.3	24.48	50.9
Injection #3	24.44	51.6	24.49	50.8
Average	24.44	50.8	24.48	50.7
%RSD	0.00	1.4	0.02	0.4

Conclusions

- A new CGE-LIF method was developed for the purity analysis of a 16 kb srRNA on the PA 800 Plus system, offering new tools to address challenges in analyzing large-sized RNA vaccines and therapeutics
- An optimized gel formulation suitable for analysis of a 16 kb srRNA was developed by a simple modification of a kit-based gel, enabling consistent assessment of srRNA quality
- Similar results were obtained with electrokinetic and pressure injections, providing flexibility in srRNA analysis
- High assay repeatability was demonstrated with RSDs in migration time and CPA% of <1.5% for the main srRNA product in multiple injections

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BioPhase™ 8800 系统进行不同长度环状RNA疫苗的高通量纯度分析

High-throughput purity analysis of CircRNA vaccine with different size by BioPhase™ 8800 system

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关键词： BioPhase™ 8800 系统；环状RNA；前体线性RNA；未成环RNA

1. 前言

环状RNA（CircRNA）以稳定性、免疫原性低，半衰期长、无需加帽和加尾等优势吸引多家疫苗企业纷纷布局。毛细管电泳技术可有效分离CircRNA疫苗产品中CircRNA、前体线性RNA（Precursor RNA）、未成环RNA（Nicked RNA），是CircRNA疫苗质量控制的有力分析工具[1]。作为作为高通量的毛细管电泳分析系统，BioPhase™8800系统可在一个系统中通过8根平行的毛细管同时进行检测分析，以8倍的通量获得与PA 800 Plus同质量的数据，为生物工艺、分析开发和QA/QC提供高通量分析。

本技术说明利用BioPhase™8800系统对CircRNA及产品相关杂质进行了分离和纯度分析，1）方法对不同长度的CircRNA样品（700 nt-3600 nt）均可实现分离分析，且CircRNA长度越长，相应的Precursor RNA 与CircRNA分离度越大，Precursor RNA与Nicked RNA分离度越小；2）方法可同时进行8个样品的检测分析，6小时可完成96个样品的检测，平均每个样品用时为3.9 min，显著提高了大量样本的分析检测效率；3）方法可准确反映不同纯化程度样品中CircRNA的纯度变化，以高效评价CircRNA疫苗的环化效率、纯化工艺、产品质量等。该方案可更快速地评估样品性质，加快产品从研发到上市的时间。

2. 仪器及方法

BioPhase™ 8800系统，配备激光诱导荧光检测器（LIF，激发波长488nm，发射波长520nm），BioPhase软件1.2.20（SCIEX）。用于BioPhase™ 8800系统的熔融石英毛细管卡盒 8×30 cm（SCIEX PN 5080121）；BioPhase™ 8800系统启动盘套装（SCIEX PN 5080311），包含4块96孔样品盘、4块缓冲液盘、8块出口盘；



图1. BioPhase™ 8800 系统分离方法时间程序

BioPhase™ 8800 系统分离方法时间程序见图1，31 min内可完成8个样品的检测，平均每个样品的分析时间为3.9 min。

3. 试剂及样品

RNA 9000 Purity & Integrity Kit（SCIEX, PN C48231），具体包含物如表1，SLS溶剂（SCIEX, PN 608082）。背景电解质配置：5 mL Nucleic Acid Extended Range Gel与10 µL SYBR™ Green II RNA Gel Stain混匀，即得。

表1. RNA 9000 Purity & Integrity Kit

Component	Quantity
Acid Wash/Regenerating Solution (0.1N HCl)	100 mL
CE Grade Water	140 mL
LIF Performance Test Mixture	20 mL
Nucleic Acid Extended Range Gel	140 mL
SYBR™ Green II RNA Gel Stain	0.11 mL
ssRNA Ladder (0.05 kB to 9 kB)	70 µL

CircRNA疫苗相关样品由某疫苗企业提供，一组不同纯化阶段的CircRNA样品（样品1：未纯化；样品2：纯化后，长度均为1765 nt），相应的Precursor RNA对照品（长度2005 nt）。不同长度的CircRNA样品（样品A：744 nt；样品B：1765 nt；样品 C，3589 nt）。初始浓度不等，均使用去SLS试剂稀释至1 ng/µL左右。

4. 结果

使用同一长度（1765 nt）、不同纯化阶段的样品考察CircRNA与其他杂质的分离，根据对照品迁移时间对样品中Nicked RNA、Precursor RNA进行定性，CircRNA出峰最晚[1]，结果见图2，CircRNA与Precursor RNA等杂质可实现良好的分离。通过BioPhase软件对样品中各峰进行积分，并以CircRNA校正峰面积占总峰面积的百分比表示纯度，未纯化和纯化后的CircRNA样品的纯度分别为71.45%，92.44%。

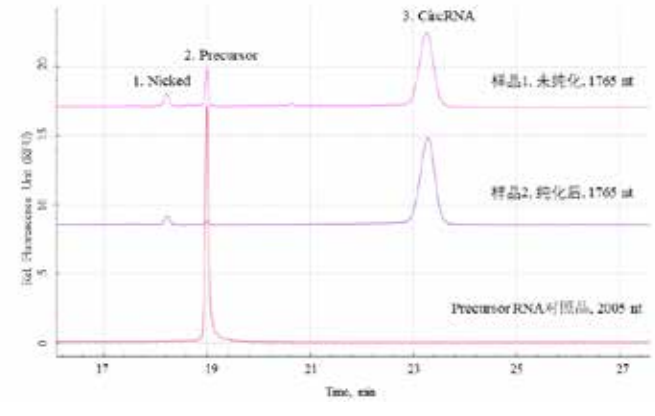


图2. BioPhase™ 8800 系统分离分析不同纯化阶段的CircRNA样品

利用CGE-LIF方法考察了三个不同长度（744 nt，1765 nt，3589 nt）的样品中，CircRNA与其他杂质的分离情况，见图3，峰1、3、5分别为744 nt，1765 nt，3589 nt的precursor RNA峰，峰2、4、6分别为744 nt，1765 nt，3589 nt的CircRNA峰（鉴定数据未展示）。对于Precursor RNA与CircRNA 的分离， CircRNA环状构象体积起到主要的分离作用，由于长度越长，环状构象体积越大，因此其与线性RNA的分离度也越大；对于Precursor RNA与Nicked RNA的分离，由于两者都是线性的结构，其分离度规律为长度越长，分离度越小。各峰的分离度统计结果见表2。

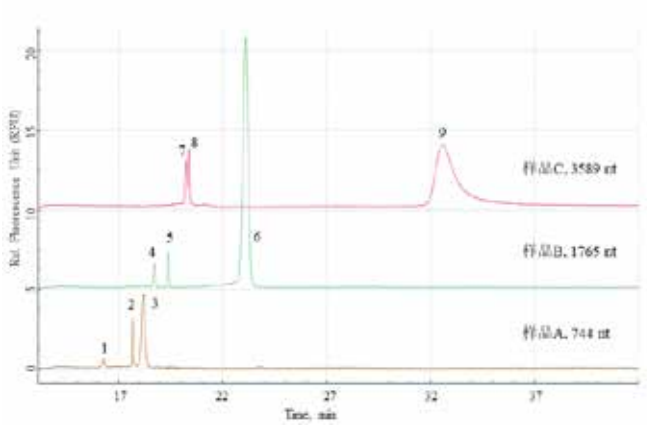


图3. BioPhase™ 8800 系统分离分析不同长度的CircRNA样品。样品A：744 snt；样品B：1765 nt；样品C：3589 nt；1、4、7号峰：Nicked RNA；2、5、8号峰：Precursor RNA；3、6、9号峰：CircRNA

表2. 不同纯化阶段、不同长度的CircRNA样品分析结果

样品类型	长度 (CircRNA)	MT (CircRNA)	纯度/ CAP% (CircRNA)	R (Precursor 与 CircRNA)	R (Precursor 与 Nicked)
样品1-未纯化	1765 nt	23.47	71.45	13.63	5.17
样品2-纯化后	1765 nt	23.47	92.44	13.54	5.21
样品A	744 nt	18.48	61.06	2.11	9.78
样品B	1765 nt	23.47	75.34	13.66	5.16
样品C	3589 nt	32.69	72.12	18.10	0.98

注：MT：迁移时间；CAP%：校正峰面积百分比，即纯度；R：分离度

5. 结论

本文介绍了利用BioPhase™ 8800 系统对CircRNA疫苗及产品相关杂质进行了高通量的分离分析，该方法可实现CircRNA与Precursor RNA、Nicked RNA等杂质的良好分离，准确反映不同纯化阶段样品中CircRNA的纯度变化；该方法可作为平台方法，对不同长度CircRNA样品均可实现良好的分离；方法通量高，可同时进行8个样品的检测，6小时可完成96个样品的检测。因此，BioPhase™ 8800 系统可作为CircRNA疫苗研发和质控过程中的高效分析工具。

6. 参考文献

[1] SCIEX Tech Note：CGE-LIF方法对环状RNA疫苗的纯度分析（RUO-MKT-02-15386-ZH-B）

CGE-LIF方法对较长（4000 nt以上）环状RNA的纯度分析

Purity analysis of longer (above 4000 nt) CircRNA by the CGE-LIF method

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Key words: 毛细管凝胶电泳；激光诱导荧光检测器；环状RNA

1. 前言

与mRNA 相比，环状 RNA（CircRNA）具有独特性质且具有潜在治疗应用价值，在基因传递、蛋白表达和疫苗开发方面展现出了巨大的应用潜力，已成为一种颇具前景的替代mRNA的选择，受到了广泛关注。为确保CircRNA产品的安全性和有效性，纯度为质量控制中需要密切监测的关键质量属性。

毛细管凝胶电泳匹配激光诱导荧光检测器（CGE-LIF）在核酸分析中具有独特的优势，SCIEX RNA 9000 kit可实现4000nt以下的CircRNA及产品相关杂质Precursor RNA、Nicked RNA的有效分离[1-2]。但对于长度为4000 nt以上的CircRNA，峰型展宽比较严重，不利于准确积分，本文通过优化dsDNA 1000 kit的凝胶及分离条件实现了4000 nt以上CircRNA的良好分离并准确的纯度定量。CGE-LIF方法有望为 CircRNA 产品的质量控制和技术评估提供指导，推动高质量 CircRNA 产品的开发。

2. 仪器和试剂

2.1 试剂及样品

dsDNA 1000 Gel Buffer (lyophilized，PN477628)；DNA 毛细管，65 cm, 100 um ID（PN 477628）；SYBR Gold Nucleic Acid (Invitrogen S11494)；10 ×TBE溶液（Sigma Aldrich, PNT4323）；Sample Loading Solution (SLS)（PN 608082）；尿素（分析纯，Amresco，PN 0568）。背景电解质配置：

1、向dsDNA 1000 Gel Buffer瓶中加入20 mL 8M尿素溶液，室温下搅拌24 h，充分溶解。命名为Gel Buffer-1。

2、将SLS：10×TBE=9:1混合，然后此溶液：Gel Buffer-1=1:1混合均匀，命名为Gel Buffer-2 。

3、按照每10 mL Gel Buffer-2：1 µL SYBR Gold nucleic acid 的比例向缓冲溶液中加入染料，充分混合均匀，并超声去除气泡。此溶液作为本实验的背景电解质。

CircRNA样品由某疫苗企业提供，均使用SLS试剂稀释至10 ng/µL左右。

2.2 仪器及方法

SCIEX PA 800 Plus药物分析系统，匹配激光诱导荧光检测器（LIF）。DNA涂层毛细管：30/40.2 cm（有效/总长度），100 µm内径；LIF检测器激发波长：488 nm，发射波长：520 nm。样品室温度：10 ℃；毛细管温度： 20 ℃；分离时间程序见图1。样品进样前、采集分析完成后可使用0.1 M盐酸、水清洁毛细管。

3. 结果

通过在dsDNA 1000 Kit的凝胶基础上进行优化，可实现4000 nt以上的CircRNA的有效分离及准确的纯度定量，Nicked RNA与Precursor RNA因长度太长分辨率受限，因此合并积分，结果见图2，4750 nt、6600 nt、9680 nt的CircRNA样品纯度分别为48.55%，42.78%。38.01%。另外，由图2可看出，无论是CircRNA，还是Nicked RNA与Precursor RNA，长度越长，出峰时间越慢，这与4000 nt以下CircRNA的实验发现一致[2]。

	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary
1		Rinse - Pressure	20.0 psi	5.00 min	BI:B1	BO:B1	forward
2		Wait		0.00 min	BI:D1	BO:D1	
3		Inject - Pressure	0.5 psi	10.0 sec	SI:A1	BO:B1	Override, forward
4		Inject - Pressure	0.5 psi	5.0 sec	BI:B1	BO:B1	No override, forward
5		Wait		0.00 min	BI:E1	BO:E1	
6	0.00	Separate - Voltage	7.8 KV	60.00 min	BI:C1	BO:C1	1.00 Min ramp, reverse polarity, both
7	1.00	Autozero					
8	60.00	End					
9							

图1. CGE-LIF分析4000 nt以上CircRNA的分离时间程序

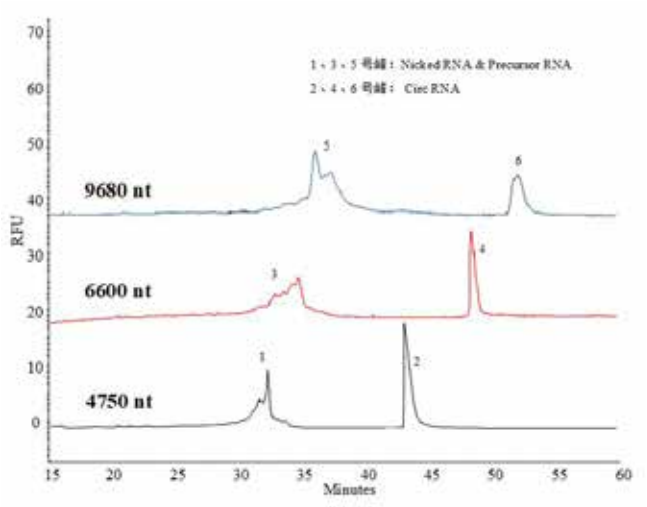


图2. CGE-LIF方法分析4000 nt以上的 CircRNA及产品相关杂质

4. 结论

本文通过调整dsDNA 1000 kit的凝胶及分离条件实现了4000 nt以上的CircRNA的良好分离和纯度分析。CGE-LIF方法有望为CircRNA 疫苗的质量控制和技术评估提供指导，从而推动高质量CircRNA 疫苗的开发。

5. 参考文献

[1] SCIEX Tech Note：CGE-LIF方法对环状RNA疫苗的纯度分析（RUO-MKT-02-15386-ZH-B）

[2] SCIEX Tech Note：BioPhase 8800 系统进行不同长度环状RNA疫苗的高通量纯度分析（RUO-MKT-02-15467-ZH-B）

Accurate and robust mRNA-LNP encapsulation efficiency analysis by capillary gel electrophoresis with laser-induced fluorescence detection (CGE-LIF)

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This technical note demonstrates high accuracy and robustness in determining the encapsulation efficiency of mRNA-lipid nanoparticles (mRNA-LNPs) by capillary gel electrophoresis with laser-induced fluorescence detection (CGE-LIF). Combining the multi-capillary BioPhase 8800 system and the ready-to-use RNA 9000 Purity & Integrity kit enables efficient and accurate encapsulation efficiency determination of mRNA-LNPs.

Key features of encapsulation efficiency determination of mRNA-LNP by CGE-LIF

- **High assay accuracy and excellent robustness of mRNA-LNP encapsulation efficiency determination by CGE-LIF**
- **High linearity of calibration curves with a coefficient of determination (r^2) of > 0.995**
- **Exceptional assay accuracy at 400 to 600 µg/mL, a typical total mRNA range for a vaccine product**
- **Excellent assay accuracy at 10 to 50 µg/mL free mRNA concentration range**
- **Excellent sample injection reproducibility with a coefficient of variation (CV) at 3.5% or better**
- **The multi-capillary capability of the BioPhase 8800 system enables rapid generation of calibration curves**
- **The ready-to-use RNA 9000 Purity & Integrity kit helps simplify operation and minimize user error**

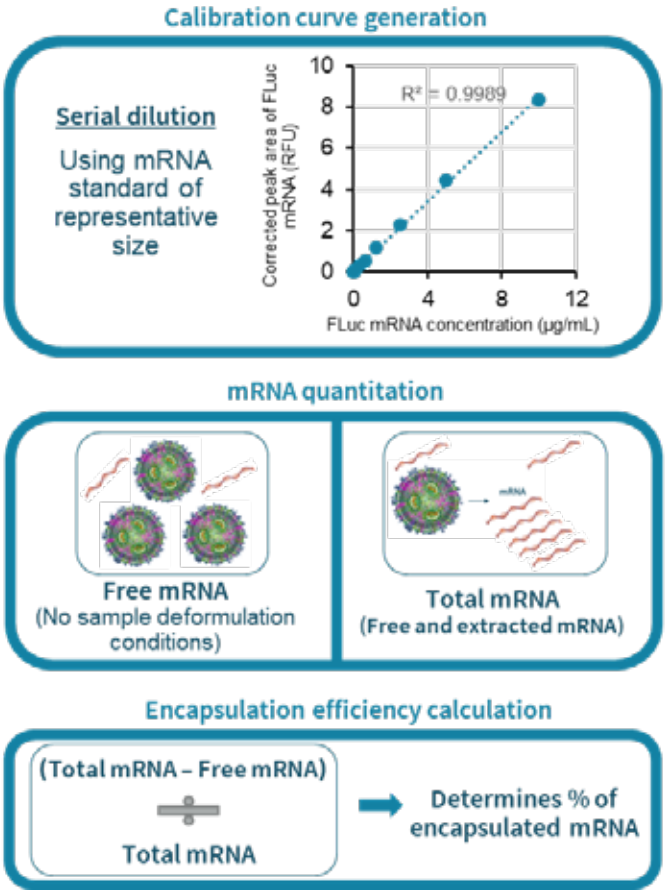


Figure 1: Workflow to determine the encapsulation efficiency of mRNA-LNP by CGE-LIF. A calibration curve was generated with serially diluted mRNA standards such as the FLuc mRNA with known concentrations in the presence of Tris EDTA buffer or Triton X-100 as described in the Methods section. The amounts of the free mRNA in the non-treated mRNA-LNP sample and the total mRNA in the deformulated mRNA-LNP sample were determined using the corresponding calibration curve. The amount of encapsulated mRNA was calculated by subtracting the amount of free mRNA from the total mRNA. Encapsulation efficiency was determined by dividing the amount of encapsulated mRNA by the amount of total mRNA.

Introduction

The mRNA-LNPs are gaining growing prominence in the realm of biotechnological research and pharmaceutical applications, including their pivotal role in areas like vaccine development and next-generation therapies.[1] Determining encapsulation efficiency is a critical quality attribute (CQA) for characterizing mRNA-LNPs. The RiboGreen dye test is a frequently employed method for assessing encapsulation efficiency, but it cannot differentiate full-length mRNA from degraded species. In a technical note published recently, a new method for mRNA-LNP encapsulation efficiency determination by CGE-LIF was achieved with the BioPhase 8800 system and the RNA 9000 Purity & Integrity kit.[2] In the current technical note, the assay accuracy and robustness of encapsulation efficiency determination by CGE-LIF were assessed with two sets of carefully designed experiments. One was used to evaluate assay accuracy for free mRNA determination, the other for total mRNA determination. Results demonstrated high assay accuracy and excellent robustness of mRNA-LNPs by CGE-LIF.

Methods

Materials: The RNA 9000 Purity & Integrity kit (P/N: C48231) was from SCIEX (Framingham, MA) and contained Nucleic Acid Extended Range Gel, SYBR™ Green II RNA Gel Stain*, Acid Wash/Regenerating Solution, CE Grade Water and ssRNA Ladder. The BioPhase BFS capillary cartridge - 8 x 30 cm (P/N: 5080121), BioPhase sample and reagent plates (4,4,8) (P/N: 5080311) and sample loading solution (SLS, or formamide, P/N: 608082) were from SCIEX. The 0.2 µm syringe filter (P/N: 4612) was from PALL (Port Washington, NY). Rainin LTS filter tips were from Mettler Toledo (Oakland, CA). Nuclease-free water (NFW, P/N: AM9932), Surfact-Amps X-100 (10% (v/v) (Triton X-100, P/N: 28314), RNA storage solution (P/N: AM7000, 1mM sodium citrate), 10x Tris EDTA (TE) buffer (P/N: 75834) and 10x phosphate buffered saline (PBS, P/N: AM9624) were obtained from Thermo Fisher Scientific (Waltham, MA). Sucrose (P/N: S9378) was from Sigma-Aldrich (St. Louis, MO). The firefly luciferase (FLuc) mRNA (P/N: L-7602, 1929 nucleotides) was from TriLink BioTechnologies (San Diego, CA). The FLuc mRNA contains a 5' Cap and a 3' poly A tail, mimicking an mRNA drug substance. The mRNA-LNPs and corresponding empty LNP samples were formulated and provided by Acuitas Therapeutics Inc. (Vancouver, Canada).

Instrument and software: A BioPhase 8800 system with UV/LIF (P/N: 5089278) was from SCIEX. The excitation wavelength for the LIF detector was 488 nm and the emission wavelength was 520 nm. Data acquisition and analysis were performed using BioPhase 8800 software v1.2 from SCIEX. The Nanodrop 2000 spectrophotometer (P/N: ND2000) was from Thermo Fisher Scientific (Waltham, MA).

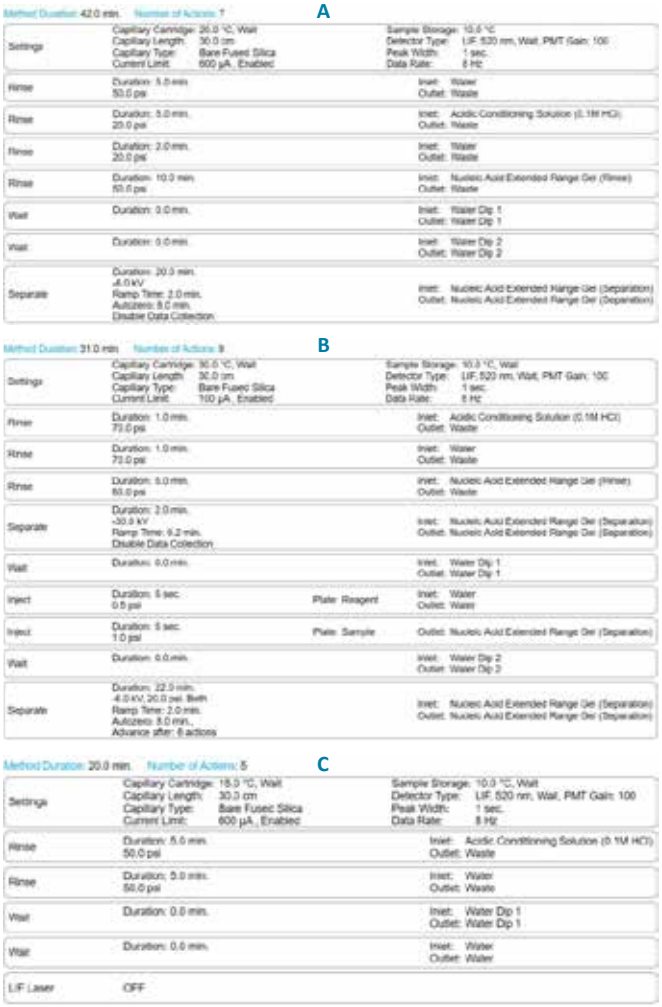


Figure 2. Parameter settings for the conditioning (A), separation (B) and shutdown (C) methods.

General conditions for CGE-LIF analysis of mRNA: To minimize sample degradation, all samples, NFW and buffers were placed on ice before use. Filter tips were used for all liquid transfer steps. The amount of Nucleic Acid Extended Range Gel needed for each experiment was removed from the gel bottle in the kit and warmed to room temperature on the laboratory bench for about 30 minutes. The gel was filtered using the 0.2 µm syringe

filter. The SYBR™ Green II RNA Gel Stain was thawed at room temperature in the dark and then added to the filtered gel at 500-fold dilution. Buffer and sample plates were prepared based on the experimental design and the plate map generated by the BioPhase 8800 software.[3]

Preparation of solutions and buffers: The 0.4% Triton X-100 solution was prepared by adding 60 µL of 10% Triton X-100 to 1440 µL of NFW in a 2-mL Eppendorf tube. The solution was mixed well by carefully inverting the tube at least 20 times and avoiding bubbles. The TE diluent buffer containing 1x TE, 1/5x 300mM sucrose in PBS and 1/5x 1mM sodium citrate (SC) was prepared by mixing 100 µL of 10x TE buffer, 200 µL of 300mM sucrose in PBS, 200 µL of 1mM SC and 500 µL of NFW. The Triton diluent buffer was made by mixing 40 µL of 300mM sucrose in PBS, 40 µL of 1mM SC, 500 µL of 0.4% Triton solution and 420 µL of NFW in a 1.5-mL Eppendorf tube. After a quick vortex and a quick spin, the diluent buffers were kept on ice until use.

Assay accuracy test in determining free mRNA: As shown in Figure 3, this experiment was designed to test 3 FLuc mRNA concentrations (10 µg/mL, 30 µg/mL and 50 µg/mL) in the presence of empty LNP to determine the assay accuracy in determining the free mRNA that is not associated with the LNP. This value is used in the calculation of mRNA encapsulation efficiency. For a 500 µg/mL mRNA-LNP drug product, 10 µg/mL, 30 µg/mL and 50 µg/mL free mRNA would correlate to 98%, 94% and 90% encapsulation efficiency. The FLuc mRNA standard (1 mg/mL) and the empty LNP samples were stored at -80°C until analysis. First, the FLuc mRNA standard was thawed on ice and diluted with 1mM SC to a working solution at 100 µg/mL concentration. An aliquot was removed to verify the FLuc mRNA concentration using the NanoDrop spectrophotometer. For generating the calibration curve, the FLuc mRNA working solution was first diluted 2.5 fold to 40 µg/mL by mixing 100 µL of the FLuc working solution with 25 µL of 10 x TE buffer, 50 µL of 300mM sucrose in PBS and 75 µL of NFW. After a brief vortex and a quick spin, an aliquot of 120 µL was removed from this mixture and serially diluted two-fold with the TE diluent buffer to yield an 8-point dilution series. 50 µL was transferred from each dilution point to a well on the sample plate before CGE-LIF separation using the methods described in Figure 2. For the accuracy test, the FLuc mRNA working solution was diluted to 10 µg/mL, 30 µg/mL and 50 µg/mL with 1mM SC. Then, 36 µL of each of these diluted FLuc mRNA solutions was mixed with 36 µL of empty LNP in 300mM sucrose in PBS, 18 µL of 10 x TE and 90 µL of NFW to obtain a total volume of 180 µL. After a brief vortex and a quick spin, each mixture was transferred to 3 wells on the sample plate at 50 µL per well before CGE-LIF separation using the methods described in Figure 2. Each diluted FLuc mRNA sample for the calibration curve and each mixture of FLuc mRNA and the empty LNP had the same buffer composition as the TE diluent buffer. There was no empty LNP in samples for generating the calibration curve.

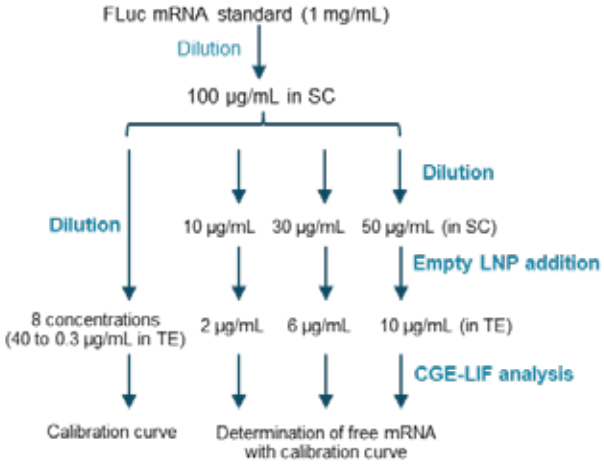


Figure 3. Experimental design for assay accuracy test in determining free mRNA. The FLuc mRNA standard was diluted to 10 µg/mL, 30 µg/mL and 50 µg/mL, mixed with empty LNP with buffer composition adjusted to be the same as the TE diluent buffer before CE analysis. The actual concentration of FLuc mRNA in each sample was determined using the calibration curve and compared with the nominal concentration. For a 500 µg/mL mRNA-LNP drug product, 10 µg/mL, 30 µg/mL and 50 µg/mL free mRNA would correlate to 98%, 94% and 90% encapsulation efficiency.

Assay accuracy test in determining total mRNA: As shown in Figure 4, this experiment was designed to test 3 FLuc mRNA concentrations (400 µg/mL, 500 µg/mL and 600 µg/mL) in the presence of the empty LNP to evaluate the assay accuracy in determining the total mRNA in an mRNA-LNP sample. The 400 µg/mL, 500 µg/mL and 600 µg/mL concentrations would cover a total mRNA range of ± 20% for a vaccine product at 500 µg/mL mRNA. For generating the calibration curve, the FLuc mRNA standard at 1 mg/mL was first diluted 25 fold to 40 µg/mL by mixing 5 µL of the FLuc mRNA standard with 62.5 µL of 0.4% Triton X-100, 5 µL of 300mM sucrose in PBS and 52.5 µL of NFW. After a brief vortex and a quick spin, an aliquot of 60 µL was removed from this mixture and serially diluted two-fold with the Triton diluent buffer to yield an 8-point dilution series. After a quick vortex and spin, 50 µL from each dilution point was mixed with 50 µL SLS, heated for 5 minutes at 70°C in a thermal cycler, and immediately chilled in an ice-water bath for ≥ 5 minutes.

Finally, 50 µL was transferred from each sample to a well on the sample plate before CGE-LIF separation using the methods described in Figure 2. For the accuracy test, the FLuc mRNA standard was first diluted to 800 µg/mL with 1mM SC, then further diluted to 400 µg/mL, 500 µg/mL and 600 µg/mL with 1mM SC. Then, 3.6 µL of each of these diluted FLuc mRNA solutions was mixed with 3.6 µL of empty LNP in 300mM sucrose in PBS, and 37.8 µL of NFW to obtain a total volume of 45 µL. After a brief vortex and a quick spin, 45 µL of 0.4% Triton X-100 was added to each mixture, and the sample was incubated at room temperature for 20 minutes. Then, 90 µL of SLS was added to each sample. After a brief vortex and a quick spin, each sample was transferred to 3 PCR tubes at 55 µL/tube, heated for 5 minutes at 70°C using a thermal cycler, and immediately chilled in an ice-water bath for at least 5 minutes. Finally, 50 µL was transferred from each PCR tube to a well on the sample plate before CGE-LIF separation using the methods described in Figure 2. There was no empty LNP in samples for generating the calibration curve.

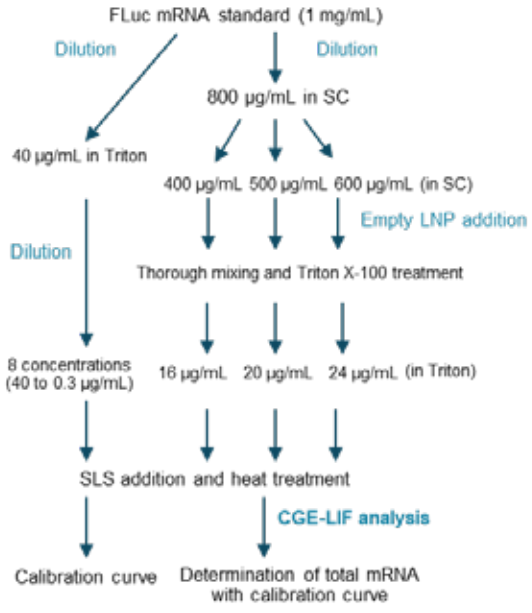


Figure 4. Experimental design for assay accuracy test in determining total mRNA. The FLuc standard mRNA was diluted to 400 µg/mL, 500 µg/mL and 600 µg/mL, mixed with empty LNP before treatment with Triton X-100. The buffer composition for each sample was adjusted to be the same as the Triton diluent buffer before CE analysis. The actual concentration of FLuc mRNA in each sample was determined using the calibration curve and compared with the nominal concentration. The 400 µg/mL, 500 µg/mL and 600 µg/mL concentrations would be equivalent to a total mRNA range of ± 20% for a vaccine product at 500 µg/mL mRNA.

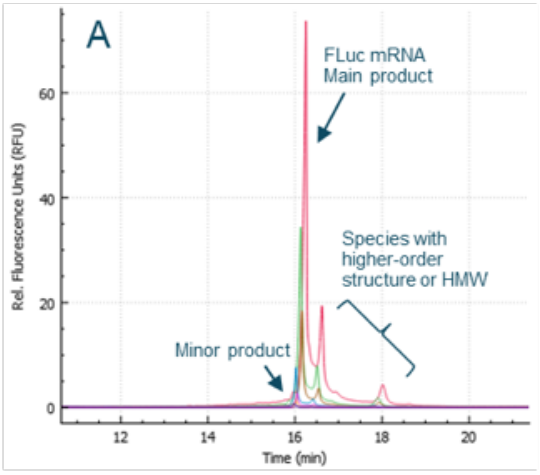
Data processing: Results were analyzed using the BioPhase 8800 software. The Optimizer feature on the Integration tab was enabled to automatically select a best-fit analysis based on a minimum signal-to-noise ratio of 10. Minor adjustments were made for peak integration.

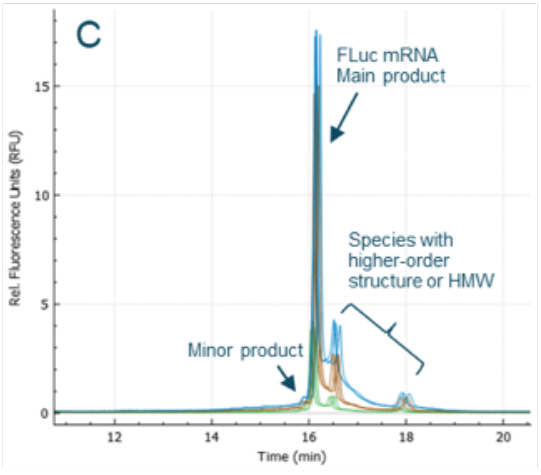
Workflow for mRNA-LNP encapsulation efficiency determination by CGE-LIF

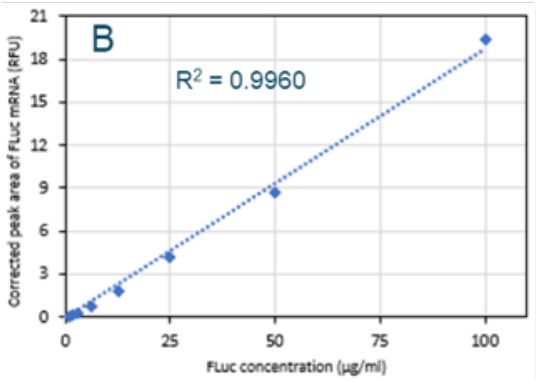
Figure 1 depicts the workflow for the determination of the encapsulation efficiency of mRNA-LNP by CGE-LIF. First, a calibration curve was generated with serially diluted mRNA standards such as the FLuc mRNA with known concentrations in the presence of either the TE buffer or the Triton X-100 as described in the Methods section. Then, the amounts of free mRNA in the non-treated mRNA-LNP sample and the total mRNA in the deformulated mRNA-LNP sample were determined using the corresponding calibration curve. The amount of the encapsulated mRNA was calculated by subtracting the amount of free mRNA from the total mRNA. Encapsulation efficiency was determined by dividing the amount of encapsulated mRNA by the amount of total mRNA. As described in a technical note previously, there is a much lower amount of free mRNA than the total mRNA in an mRNA-LNP sample. [2] Therefore, the mRNA-LNP sample was diluted 5-fold and 50-fold to measure the free and total mRNA, respectively. [2]

Accurate determination of free mRNA in mRNA-LNP

Since measuring free mRNA requires minimizing the disruption of mRNA-LNPs, samples were not treated with any surfactant or formamide and were not heat-treated before CE analysis. An experiment was designed to test the accuracy of free mRNA determination by CGE-LIF, as illustrated in Figure 3. The FLuc mRNA standard was diluted to 10 µg/mL, 30 µg/mL and 50 µg/mL, and mixed with empty LNP, which was diluted 5-fold before CE analysis to simulate the analysis of free mRNA in mRNA-LNP samples. The actual concentration of FLuc mRNA in each sample was determined using the calibration curve and compared with the nominal concentration. For a 500 µg/mL mRNA-LNP drug product, 10 µg/mL, 30 µg/mL and 50 µg/mL free mRNA would correlate to 98%, 94% and 90% encapsulation efficiency. Results are summarized in Figure 5. Panel A shows an overlay of electropherograms obtained with serially diluted FLuc mRNA standards in the TE diluent buffer. Signal intensity increased proportionally with higher FLuc mRNA concentrations. Panel B shows the calibration curve of FLuc mRNA corrected peak area as a function of FLuc mRNA concentration over a







D

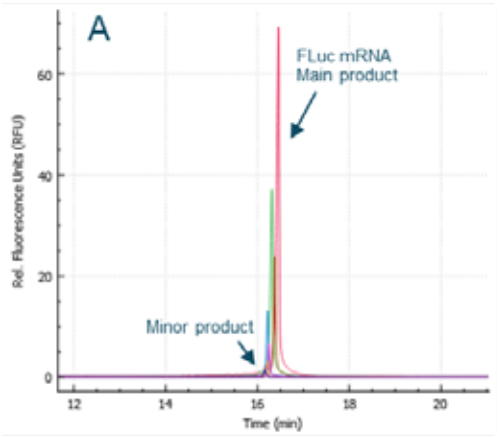
Nominal (µg/mL)	Measured (µg/mL)					Recovery
	#1	#2	#3	Mean	CV	
10	8.1	8.2	8.1	8.1	0.8%	81%
30	31.5	30.1	29.8	30.5	3.0%	102%
50	53.3	51.0	49.7	51.3	3.5%	103%

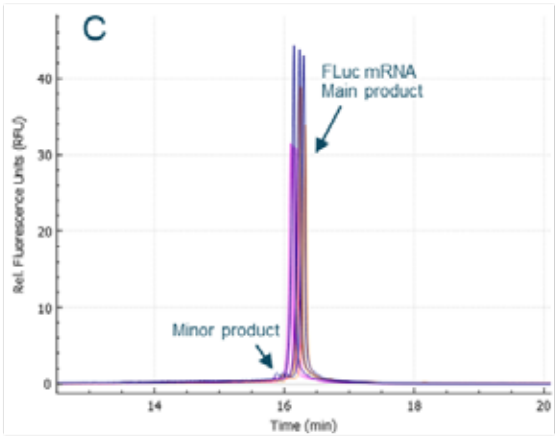
Figure 5. Assay accuracy test results in determining free mRNA. Panel A shows an overlay of electropherograms obtained with serially diluted FLuc mRNA standard in TE diluent buffer. Panel B is the calibration curve of the corrected peak area to the FLuc concentration. Panel C is an overlay of electropherograms from FLuc mRNA samples at concentrations of 10 µg/mL (green), 30 µg/mL (brown) and 50 µg/mL (blue) in the presence of empty LNP. Panel D tabulates the measured FLuc mRNA concentrations and the recovery relative to the nominal concentrations. HMW: high molecular weight species. #1, #2 and #3 represent three different injections of the same sample.

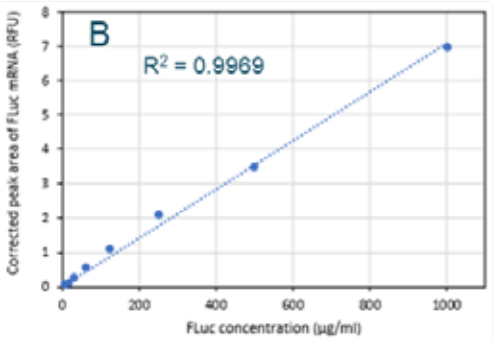
dynamic range spanning 2.1 orders of magnitude. The R^2 value was 0.9960, indicating an excellent linear correlation between the observed signal and mRNA standard concentration. Panel C is an overlay of electropherograms from FLuc mRNA samples at concentrations of 10 µg/mL (green), 30 µg/mL (brown) and 50 µg/mL (blue) in the presence of empty LNP. Each sample was injected from 3 different wells. Since the samples were not treated with surfactant, formamide and heat, results in both panel A and panel C display the typical peak profiles for untreated samples. Species containing higher-order structures or of high molecular weight (HMW) are present in addition to the FLuc mRNA main product and minor product. Panel D tabulates the measured FLuc mRNA concentrations and the recovery relative to the nominal concentrations. Results from the three injections for each sample were very consistent with a CV of 3.5% or lower for all three samples. To determine the recovery, the average measured concentrations for each sample were divided by their respective nominal values. The recovery at 30 µg/mL and 50 µg/mL mRNA concentrations were 102% and 103%, demonstrating excellent accuracy at these two concentrations. The recovery at 10 µg/mL was 81%, indicating good accuracy given the low concentration of the sample. The 19% error observed at 10 µg/mL would result in a calculated encapsulation efficiency of 98.4% compared to the nominal 98.0% for a 500 µg/mL mRNA-LNP drug product. Therefore, the impact of this error is negligible on the calculated encapsulation efficiency.

Accurate determination of total mRNA in mRNA-LNP

To test the accuracy of total mRNA determination by CE, an experiment was designed, as depicted in Figure 4. The FLuc mRNA standard was diluted to 400 µg/mL, 500 µg/mL and 600 µg/mL, mixed with empty LNP, and then treated with 0.2% Triton X-100 to simulate extraction of mRNA from different mRNA-LNP samples. The empty LNP was diluted 50-fold before CE analysis to simulate the analysis of total mRNA in mRNA-LNP samples. The actual concentration of FLuc mRNA in each sample was determined using the calibration curve and compared with the nominal concentration. The 400 µg/mL, 500 µg/mL and 600 µg/mL concentrations would be equivalent to a total mRNA range of ± 20% for a vaccine product at 500 µg/mL mRNA. Results are summarized in Figure 6. Panel A shows an overlay of electropherograms obtained with serially diluted FLuc mRNA standard in Triton diluent buffer. Signal strength rose in direct correlation with increasing FLuc mRNA concentrations. Panel B displays the calibration curve plotting the FLuc mRNA corrected peak area against the FLuc mRNA concentration across a linear dynamic range spanning 2.1 orders of magnitude. The R^2 value of 0.9969 demonstrates a strong linear correlation between the observed signal and mRNA standard concentration. Panel C presents an overlay of electropherograms for the FLuc mRNA samples at concentrations of 400 µg/mL (purple), 500 µg/mL (orange) and 600 µg/mL (blue) when mixed with empty LNP. Each sample was injected from 3 different wells. Because the samples underwent heat treatment in the presence of formamide, both panel A and panel C exhibit characteristic peak profiles for treated samples, featuring a prominent main product peak for FLuc mRNA and a small minor product peak. Panel D presents a table listing the







D

Nominal (µg/mL)	Measured (µg/mL)					Recovery
	#1	#2	#3	Mean	CV	
400	387.3	390.1	381.7	386.4	1.1%	97%
500	497.2	488.7	480.3	488.7	1.7%	98%
600	574.7	580.3	570.4	575.1	0.9%	96%

Figure 6. Assay accuracy test results in determining total mRNA. Panel A shows an overlay of electropherograms obtained with serially diluted FLuc mRNA standard in Triton diluent buffer. Panel B is the calibration curve of the corrected peak area to the FLuc concentration. Panel C is an overlay of electropherograms from FLuc mRNA samples at concentrations of 400 µg/mL (purple), 500 µg/mL (orange) and 600 µg/mL (blue) in the presence of empty LNP. Panel D tabulates the measured FLuc mRNA concentrations and the recovery relative to the nominal concentrations. #1, #2 and #3 represent three different injections of the same sample.

measured FLuc mRNA concentrations alongside their recovery compared to the nominal concentrations. The results of the three injections for each sample exhibited remarkable consistency, with a CV of less than 2% across all three samples. The mean measured concentration for each sample was divided by the nominal value to compute the recovery. At all three mRNA concentrations, the recovery ranged between 96% and 98%, demonstrating exceptional accuracy.

Accurate and robust determination of mRNA-LNP encapsulation efficiency by CGE-LIF

The measured values of free mRNA at 10, 30 and 50 µg/mL and total mRNA at 400, 500 and 600 µg/mL were used to calculate the mRNA-LNP encapsulation efficiency for 9 scenarios. The measured EE% values were compared with the nominal EE% values. As listed in the 3 tables in Figure 7, the recovery was within 1% across all 9 scenarios, demonstrating exceptional assay accuracy and robustness. The error of 19% seen at the 10 µg/mL level for free RNA has no significant impact on the calculation of the encapsulation efficiency.

400 µg/mL total mRNA			
Free mRNA	Nominal (EE%)	Measured (EE%)	Recovery
10 µg/mL	97.5%	97.9%	100.4%
30 µg/mL	92.5%	92.1%	99.6%
50 µg/mL	87.5%	86.7%	99.1%

500 µg/mL total mRNA			
Free mRNA	Nominal (EE%)	Measured (EE%)	Recovery
10 µg/mL	98.0%	98.3%	100.3%
30 µg/mL	94.0%	93.8%	99.8%
50 µg/mL	90.0%	89.5%	99.4%

600 µg/mL total mRNA			
Free mRNA	Nominal (EE%)	Measured (EE%)	Recovery
10 µg/mL	98.3%	98.6%	100.3%
30 µg/mL	95.0%	94.7%	99.7%
50 µg/mL	91.7%	91.1%	99.4%

Figure 7. Assay accuracy of mRNA-LNP encapsulation efficiency (EE%) determination by CGE-LIF. Two sets of experiments were designed to assess assay accuracy: one for free mRNA determination and the other for total mRNA determination. Free mRNA was tested at three different concentrations: 10 µg/mL, 30 µg/mL and 50 µg/mL while total mRNA was tested at 400 µg/mL, 500 µg/mL and 600 µg/mL. Each table summarizes the nominal EE%, the measured EE% and the recovery. In all 9 scenarios, the recovery was within 1%, demonstrating exceptional assay accuracy.

Conclusions

- High assay accuracy and robustness of the mRNA-LNP encapsulation efficiency determination were achieved by CGE-LIF
- Highly linear calibration curves with a coefficient of determination (r2) of > 0.995 were obtained
- Exceptional assay accuracy at 400 to 600 µg/mL, covering a total mRNA range of ± 20% for a vaccine product at 500 µg/mL mRNA, was demonstrated
- Excellent assay accuracy at 10 to 50 µg/mL free mRNA concentration range was obtained
- Good sample injection reproducibility was obtained with a CV of 3.5% or better
- Calibration curves were quickly generated on the multi-capillary BioPhase 8800 system

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2. A new approach to determine encapsulation efficiency of mRNA-lipid nanoparticles (mRNA-LNP) by capillary gel electrophoresis with laser-induced fluorescence detection. **SCIEX technical note. MKT-28376-A.**
3. RNA 9000 Purity & Integrity kit for the BioPhase 8800 system, **application guide, RUO-IDV-05-13438-B.**

毛细管凝胶电泳方法用于mRNA疫苗中宿主残留DNA(HCD)的分布分析

Integrity analysis of bivalent mRNA vaccine by capillary gel electrophoresis

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Hongmei Tang, Xiaoxia Zhang, Hongxu Chen, Ji Luo, Bingjie Liu

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SCIEX, China;

Key words: Capillary Gel Electrophoresis, mRNA vaccine, HCD, Integrity

1. 引言

用传代细胞株生产的生物制品, 其终产品中通常会存在宿主细胞残留 DNA(HCD)。由于HCD具有潜在的安全风险对病毒感染性、致瘤性、免疫原性以及致突变性，残留的HCD已得到生物制药公司和监管机构的广泛关注。在过去的几十年里，不同国家的监管机构相继发布了生物样本中残留HCD片段的可接受量等相关指南，同时各国药典也提供数种经典的检测方法，建立合适的宿主细胞残留DNA检测方法有助于监测生产工艺, 确保生物制品的安全性；目前美国食品药品监督管理局(FDA)和中国国家药品监督管理局(NMPA)均在基因治疗新产品生产指导类型文件中明确指出HCD的片段要小于200 bp，残留量小于10 ng/剂。为了满足监管机构对mRNA疫苗中HCD的产品特性、功能及安全性的严格评估要求，必须对多个关键质量属性(CQA)进行细致监控。定量聚合酶链反应(QPCR)方法是常用于HCD片段分析的方法，但是QPCR方法需要先扩增，不能检测非特异性片段；与定量聚合酶链反应(QPCR)相比，毛细管凝胶电泳法无需预先对HCD片段进行扩增和标记，就能够检测样本中所有大小的残留HCD片段，从而有效避免了Q-PCR仅能检测特定片段的缺点，并且分析速度更快。其中Shen等人率先进行了标记使用SYBR Green I对DNA进行染色，并利用毛细管凝胶电泳-激光诱导荧光(CGE-LIF)技术分析MDCK细胞来源的流感病毒疫苗和vero细胞来源的呼吸道合胞病毒疫苗中残留HCD片段的大小分布[1]。Gao等人采用CGE-LIF方法对重组蛋白类新冠疫苗[2]，慢病毒[3]，狂犬疫苗中[4]的HCD进行了分析；Wang等人采用CGE-LIF方法已完成腺相关病毒中的HCD 分布分析[5]，并优化了前处理方案。此外，激光诱导荧光检测器(LIF)具有极高的灵敏度，因此能够为样品中残留碎片的分布分析提供更准确的结果。

在本研究中，采用SCIEX公司的20 kb DNA Plasmid and Linear Kit试剂盒结合LIF检测器建立了一种CGE-LIF方法分析含有双链DNA基因组的mRNA疫苗产品中残留HCD片段的尺寸分布。在本研究中，毛细管凝胶电泳-激光诱导荧光(CGE-LIF)能够根据片段尺寸的差异实现核酸的分离，并成功用于残留HCD片段的尺寸分布分析。

2. 试剂及方法

2.1 试剂和样品

试剂：DNA 20 kb Plasmid and Linear Kit (PN 5311708) 试剂盒包含：DNA 20 kb Plasmid and Linear gel(SCIEX, 5312283)；DNA 20 kb Plasmid and Linear conditioning solution(SCIEX，5312283) ; DNA 20 kb Plasmid and Linear sample buffer(SCIEX, 5312283) ; CE Grade water(SCIEX, C48034) ; 0.1N HCl (Acid wash/regenerating solution) (SCIEX, 5312349); SYBR™ Gold Nucleic Acid gel stain ; DNA 20 kb Plasmid test mix ; 200 bp marker(需要基因合成公司合成)。

样品：经过QIAquick试剂盒(QIAGEN, PN 28104)提取纯化后的HCD样品，浓度约30 ng/μL。前处理流程图如下图1所示；

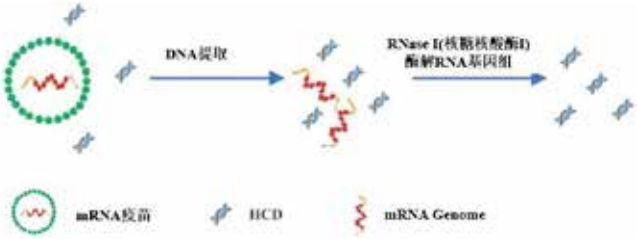


图1. mRNA疫苗中HCD纯化流程示意图

2.2 样品及前处理

配制背景凝胶缓冲液：取80 µL SYBRTM Green Ⅱ RNA Gel Stain溶于20 mL DNA 20 kb Plasmid and Linear gel 中，涡旋混匀备用。

样品溶液制备：使用已用去离子水进行3倍稀释的TE溶液将样品稀释到约1 ng/µL，涡旋混匀作为待测溶液。

200 bp marker溶液制备：使用已用去离子水进行3倍稀释的TE溶液将200 bp marker稀释到约0.001 ng/µL，涡旋混匀作为外标法待测溶液。

样品与200 bp marker混合液：取100 µL 2 ng/µL的样品溶液与100 µL 0.002 ng/µL的200 bp marker溶液涡旋混合均匀作为待测溶液，使得最终溶液中样品浓度为1 ng/µL，200 bp marker浓度为0.001 ng/µL(内标溶液)。

2.3 仪器及方法设置

采用SCIEX PA 800 plus药物分析系统，匹配LIF检测器，采集频率：4 Hz，激发波长488 nm，发射波长520 nm；熔融石英毛细管：50 µm内径，20/30.2 cm (有效/总长度)；毛细管温度：22 ℃；样品室温度：10 ℃；电压进样条件：-8 kV，20 s；分离条件：-9 kV，15 min。

毛细管预处理：新毛细管运行前，需要先进行condition动态涂层；每次序列运行开始时，依次用0.1 N HCl(70 psi, 1 min)、去离子水(70 psi, 1 min)和凝胶缓冲液(70 psi, 10 min)冲洗毛细管。进样前后各沾洗毛细管两端一次，然后用电进样方式(-8 kV, 20 s)进样。分离程序为-9 kV，15 min，并在分离时施加两端20 psi的气压。

3. 结果与分析

3.1 CGE方法分离结果

图2为HCD样品分离电泳谱图，本文通过两种方式进行定位；一种是外标法，200 bp marker单独运行用于定位样品中大于200 bp的片段；另一种是内标法，样品与200 bp marker混合对样品中大于200 bp的片段进行定位。从分离结果可以看出作为外标加入的200 bp marker与混合样品中的200 bp marker出峰位置一致，定位准确；样品中PK#5,6是大于200 bp的片段，校正峰面积比例为8.1%。

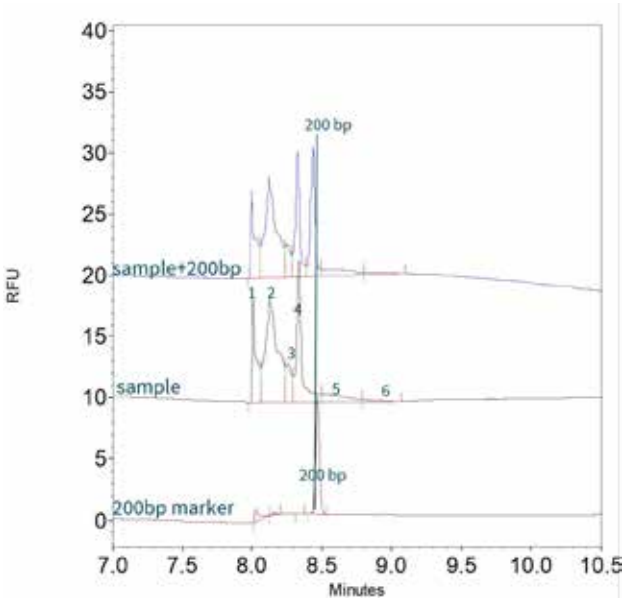


图2. mRNA疫苗中HCD分布结果

4. 结论

本文借助于PA 800 plus生物制药分析系统以及SCIEX的DNA 20 kb Plasmid and Linear Kit 试剂盒对mRNA疫苗中HCD进行分析，主要结论如下：

1. 采用 CGE-LIF方法搭配DNA 20 kb Plasmid and Linear Kit 试剂盒，提供了一种能够方便快捷的方案用于mRNA疫苗中HCD片段的分布分析，并能够准确的对样品中大于200 bp片段进行定位。
2. 激光诱导荧光检测器(LIF)具有极高的灵敏度，能够为样品中残留碎片的分布分析提供更准确的结果。

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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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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。

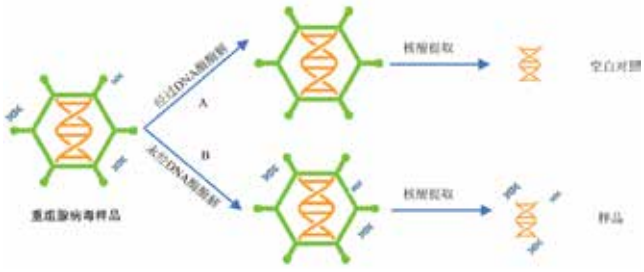


图2. 重组腺病毒疫苗HCD分布分析样品前处理流程。A）空白对照；B）样品；

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

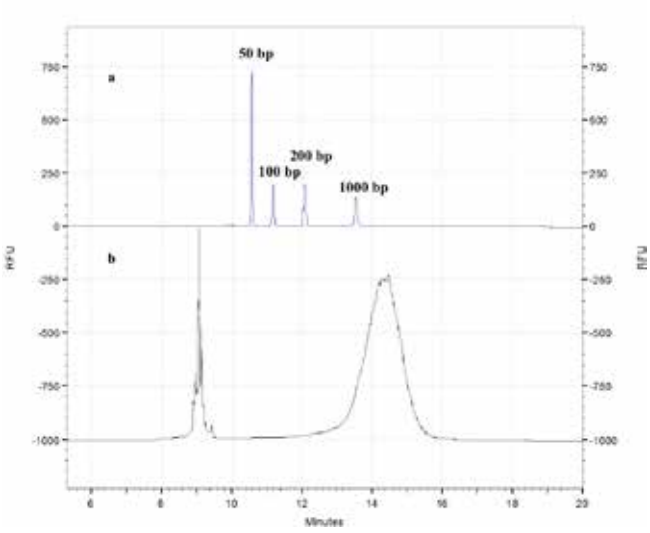


图3. CGE-LIF 法对重组腺病毒的HCD片段大小分析。a, marker（50 bp,100 bp, 200 bp, 1000 bp）；b, 重组腺病毒疫苗样品（未经DNA酶处理）；

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。

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CGE-LIF方法分析不同工艺阶段狂犬疫苗中宿主残留DNA的片段大小分布

Size distribution analysis of residual host cell DNA in rabies vaccines at different process by CGE-LIF

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Key words: CGE-LIF，HCD片段大小分布，狂犬疫苗

1. 引言

宿主细胞残留DNA（HCD）存在制瘤性和感染性，目前其残留问题受到了生物制品企业和监管机构的广泛关注。现有研究表明，HCD可能引发致病的功能基因至少在200 bp以上，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。由此可见，一种简便快捷的用于基因细胞治疗产品中的HCD分布分析的方法是必要。目前基于激光诱导荧光检测器的毛细管凝胶电泳方法（CGE-LIF）已经应用多种基因细胞治疗产品的检测，如慢病毒、腺病毒等[1,2]。

HCD的片段大小分布检测对于基因工程类的疫苗也很重要。狂犬疫苗原液的生产工艺中通常为狂犬病病毒固定毒株接种Vero细胞，经过病毒培养，收获病毒液，再经灭活、浓缩、纯化等工艺，制成疫苗原液。在整个过程中，HCD是一种可能存在于最终的产品中的工艺相关杂质，需要进行考察。

本文以狂犬疫苗不同工艺阶段的样品为例，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），对其宿主细胞残留DNA进行片段大小分布检测。



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）。dsDNA 1000试剂盒（SCIEX公司 PN 477410）内含DNA 涂层毛细管（100 μm内径，PN 477477）、dsDNA 1000固体凝胶（PN 477628）；SYBR Gold荧光染料购自Thermo Fisher（Carlsbad, CA，USA）；10× Tris Borate-EDTA（TBE）缓冲液购自Sigma Aldrich（St Louis, MO，USA）；100 bp、200 bp、500 bp、1000 bp的DNA标准品由中检院提供。

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12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

狂犬疫苗不同工艺阶段的样品由中检院提供，按照工艺先后顺序，分别为收获液、超滤浓缩液、灭活前液、灭活液、原液，如图2所示。不同阶段样品均利用湖州申科HCD磁珠法提取试剂盒进行提取，由于狂犬疫苗的基因组是RNA，因此提取后的样品经过RNA酶处理去除掉基因组，处理后各个样品及DNA标准品混合物的浓度如表1。

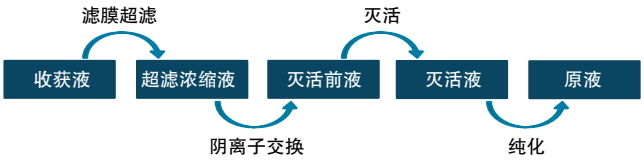


图2. 不同阶段的狂犬疫苗样品

表1. 样品及DNA标准品混合物的浓度

序号	名称	核酸浓度（nanodrop测定）
1	收获液	4.70 ng/μL
2	超滤浓缩液	6.05 ng/μL
3	灭活前液	7.35 ng/μL
4	灭活液	6.95 ng/μL
5	原液	5.20 ng/μL
6	DNA标准品混合物	单独标准品为0.20 ng/μL

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：-9 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；-9 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

本文对狂犬疫苗不同生产阶段样品中的核酸提取物进行了CGE-LIF分析，结果如图3所示。结果显示，收获液、超滤浓缩液中含有分布较宽的HCD，且超滤浓缩液经过了滤膜超滤，其HCD的含量明显高于收获液；灭活前液、灭活液和原液经过了阴离子交换、柱层析的工艺，其HCD的分布范围明显缩小，但是结果中还是能检测到少量HCD。对于原液中的HCD，需要考察其分布范围。对比100、200、500、1000 bp的DNA标准品，可以发现原液中的HCD分布均小于200 bp。而另外四个阶段的样品中灭活前液和灭活液中检测到的HCD均已小于200 bp，而收获液和超滤浓缩液均含有大于200 bp的核酸信号，说明纯化工艺中阴离子交换步骤去除掉大部分的HCD，尤其是可有效去除大于200 bp的HCD。

通过CGE-LIF的方法，可以快速准确的判定狂犬疫苗样品中HCD的分布情况，进而可以帮助用户更好的对工艺的优化和产品的质控。

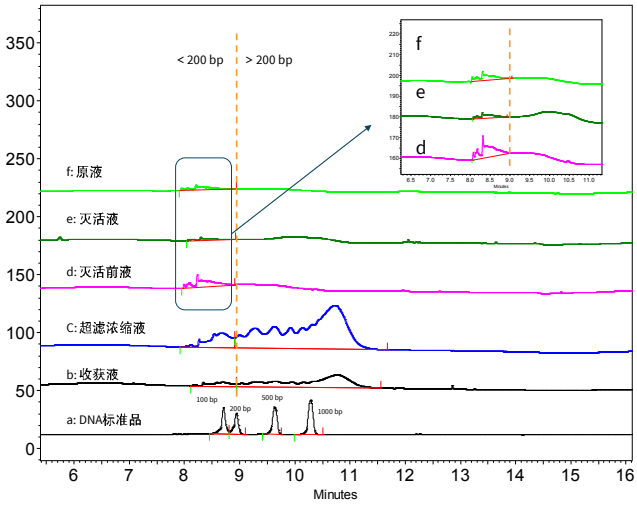


图3. CGE-LIF 法对DNA标准品混合物及不同阶段狂犬疫苗样品中的HCD片段分析。a. DNA标准品混合物（100、200、500、1000）、b. 收获液、c. 超滤浓缩液、d. 灭活前液、e. 灭活液、f. 原液。

4. 结论

毛细管凝胶电泳（CGE-LIF）方法分离HCD片段大小分布的应用具有如下特点：

- **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[3]，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

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2. SCIEX Tech Note：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（RUO-MKT-02-13912-ZH-A）
3. SCIEX Tech Note：CE-LIF方法分析宿主细胞残留DNA的片段分布（ RUO-MKT-02-9259-ZH-A）

CGE-LIF方法对重组蛋白类新冠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 protein vaccine by CGE-LIF

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关键词： PA800 Plus药物分析系统，毛细管凝胶电泳，重组蛋白疫苗，宿主残留DNA分布

1. 引言

新冠疫情爆发以来，已有多种类型的疫苗成功完成临床申报，用于预防新冠病毒的感染，包括灭活疫苗、腺病毒载体疫苗、重组蛋白疫苗、核酸疫苗等。其中重组蛋白疫苗是利用基因工程进行生产，将编码抗原（如新冠病毒表面蛋白S）的DNA插入大肠杆菌/酵母菌、昆虫-杆状病毒、哺乳动物细胞等体系中进行表达，然后将表达的抗原进行纯化制备成疫苗。在生产中，宿主细胞残留DNA（Host Cell Residual DNA，HCD）是一种可能存在于最终的产品中的工艺相关杂质，而HCD的含量与分布可能存在制瘤性和感染性等问题，受到了行业及法规机构的关注。现有研究表明，HCD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。目前对于很多基于宿主细胞培养的生物制品，都需要监控HCD的片段分布及含量，如美国食品药品监督管理局（FDA）和中国国家药品监督管理局（NMPA）均在基因治疗新产品生产指导类型文件中明确指出HCD的片段要小于200 bp。而重组蛋白疫苗同样存在HCD残留的可能性，因此需要评估终产品中HCD片段的大小分布情况。

本文以重组S蛋白疫苗为例，提取产品中的核酸物质，利用SCIEX PA800 Plus药物分析系统匹配激光诱导荧光检测器（LIF）（图1），对宿主细胞残留DNA进行片段分布检测。



图1. PA800 Plus药物分析系统

2. 试剂及样品

2.1 试剂耗材

dsDNA 1000试剂盒自SCIEX（Brea, CA, USA），试剂盒中包含DNA涂层毛细管（100 µm内径）和核酸分析使用的凝胶缓冲液；SYBR Gold 荧光染料、200 bp的marker购自Thermo Fisher（Carlsbad, CA, USA）；10× Tris Borate-EDTA（TBE）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方法分析使用。

2.2 样品及前处理

重组S蛋白疫苗由某疫苗企业提供，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：

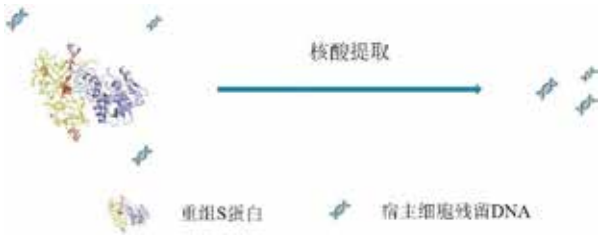


图2. 重组S蛋白疫苗HCD分布分析样品前处理流程

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为11 ng/µL。

3. 仪器及方法

PA800 Plus药物分析系统，匹配激光诱导荧光检测器（SCIEX）。

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 ℃；检测波长：激发波长488 nm，发射波长520nm。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。

4. 结果与分析

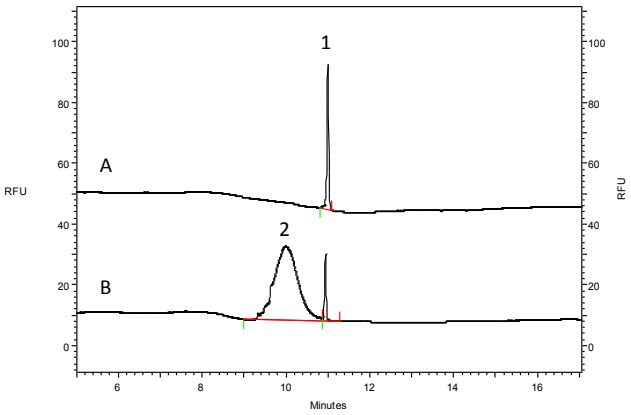


图3. CGE-LIF 法对重组S蛋白疫苗的HCD片段大小分析。
A：DNA marker 200 bp，B：重组S蛋白样品核酸提取物与DNA marker混合物；峰1为200 bp DNA marker，峰2为HCD片段。

利用CGE-LIF方法，通过与marker的对比，可准确判定重组S蛋白疫苗的HCD的片段大小是否大于200 bp，从而确定样品是否符合格。如图3为重组S蛋白疫苗中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均在在200 bp以下，并未发现含有长度大于200 bp的HCD片段。CGE-LIF方法可快速准确的判定重组S蛋白疫苗中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。

参考文献

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

毛细管电泳法对宫颈癌疫苗HPV纯度和等电点的分析

Analysis of purity and isoelectric points of HPV by capillary electrophoresis

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Keywords： HPV; purity; isoelectric points; capillary electrophoresis;

1. 前言

宫颈癌疫苗（HPV）由称为类病毒颗粒（VLP）的空蛋白质壳制备而成，HPV双价疫苗和四价疫苗已广泛使用，最近在几个国家已经批准使用九价HPV疫苗。随着HPV疫苗的市场化，其性能鉴定对于患者的健康至关重要。一般来说，一批新疫苗在推向市场之前，其异质性、杂质含量和活性等都应该进行彻底的探讨。目前用于HPV疫苗分析的技术有十二烷基硫酸钠-聚丙烯酰胺凝胶电泳（SDS-PAGE）、蛋白质印迹法（Western blot）、酶联免疫吸附测定（ELISA）等方法，但这些方法都是半定量的，比较费时。毛细管电泳（CE）以其自动化程度高、定量准确和高速高效等特点，有效地取代了传统的SDS-PAGE方法，被广泛应用于生物制药领域进行生物制品的质量控制。

SCIEX依托强大的研发实力和其高效的毛细管电泳平台，创新研发了基于分子量差异分析的SDS-MW分子量分析的试剂盒（SCIEX, PN.390953）以及基于等电点差异分析的cIEF试剂盒（SCIEX, PN. A80976），在单一平台上可以实现对疫苗的纯度和等电点分析。本文展示了采用SDS-MW分析试剂盒和cIEF分析试剂盒方法对HPV纯度和等电点的分析。

2. 试剂和耗材

2.1 实验仪器

本实验采用的仪器为PA 800 Plus药物分析系统（SCIEX, PN. A66527），HPV疫苗纯度分析需用PDA检测器，检测波长220 nm，数据采集频率为4 Hz，样品储藏温度和毛细管温度分别为20 ℃和25 ℃；HPV等电点的分析需用UV检测器（PN. 144733），配备280 nm滤光片（PN. 144439），数据采集频率为2 Hz，样品储藏温度和毛细管温度分别为10 ℃和20 ℃。

2.2 实验试剂

2.2.1 HPV纯度鉴定所需试剂及耗材

SCIEX SDS-MW试剂盒包含内容详见表1。

表1. SDS-MW试剂盒包含的内容。

货号	项目
338451	50 µm ID Capillary
A30341	SDS Gel Buffer
A26487	10 kD Marker
A22196	MW Marker
	SDS Sample Buffer
	0.1 N NaOH
	0.1 N HCl

其他试剂和耗材：2-巯基乙醇（Sigma-Aldrich, PN. M7154）、水浴锅（37 ℃-100 ℃）或加热模块、Centricon YM-10超滤管（Millipore, PN. 4205）、封口膜、超声震荡器、涡旋仪、移液器和枪头、微量离心管、离心管、通用瓶及瓶盖、200 µL样品管、去离子水（Millipore）。

2.2.2 等电点测定所需试剂及耗材

SCIEX cIEF试剂盒（PN. A80976）包含内容详见表2.

表2. cIEF试剂盒包含的内容。

货号	成分
477441	中性涂层毛细管, 50 µm ID × 45 cm
477497	cIEF Gel (Polymer solution)
A58481[a]	cIEF Peptide Marker, pI 10.0
A58481[a]	cIEF Peptide Marker, pI 9.5
A58481[a]	cIEF Peptide Marker, pI 7.0
A58481[a]	cIEF Peptide Marker, pI 5.5
A58481[a]	cIEF Peptide Marker, pI 4.1
477427	eCap 50 mM Tris buffer at pH 8.0

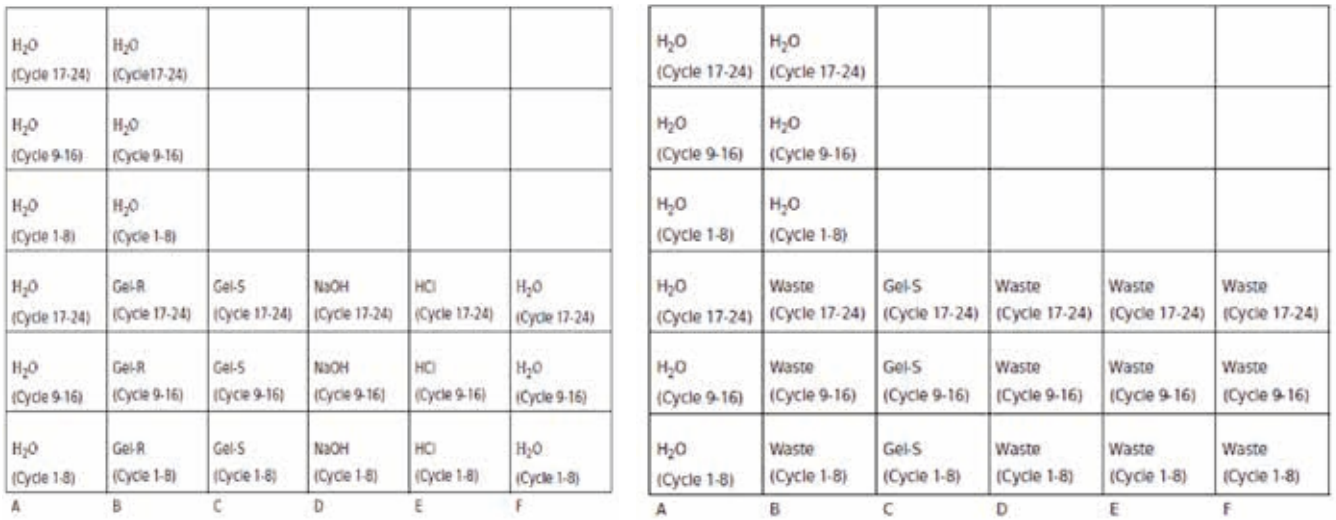
其他试剂和耗材：精氨酸（Sigma-Aldrich, PN. A5006）、尿素（Sigma-Aldrich, PN. U0631）、亚氨基二乙酸（Sigma-Aldrich, PN. 220000）、Pharmalyte 3-10 carrier ampholytes（CE Healthcare, PN. 17-0456-01）、冰醋酸（Sigma-Aldrich, PN. 537020）、磷酸（85%）（Sigma-Aldrich, PN. 345245）、氢氧化钠（1 M）（Sigma-Aldrich, PN. 72082），移液器和枪头、涡旋混合器、微量离心机、去离子水、分析天平、5 µm孔径滤膜、0.25 µm孔径滤膜、一次性注射器、一次性10 mL和50 mL塑料管、10 mL和50 mL容量瓶、0.5 mL离心管、通用瓶（PN. A62251）、瓶盖（PN. A62250）、微量样品管（PN. 144709）

2.3样品处理方法

2.3.1 HPV纯度分析样品处理方法

用样品缓冲液溶解稀释样品，使HPV样品的终浓度在0.2-2 mg/mL之间，总体积为95 µL。加入2 µL内标，5 µL巯基乙醇溶液，盖好瓶盖，用封口膜封好，并充分混合，100 ℃水浴中加热3 min，室温冷却5 min，准备进样。

缓冲液托盘的放置按照图1所示，分析方法包括毛细管预平衡、分离以及关机，具体参数设置详见图2-4.



入口托盘　　出口托盘

图1. CE-SDS缓冲液托盘的放置。

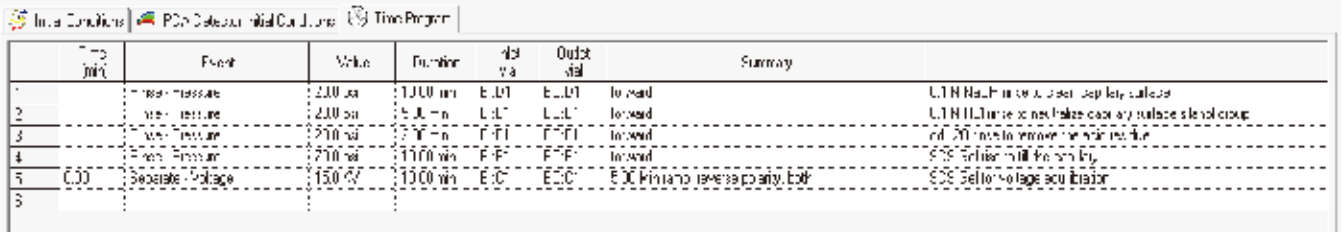


图2. CE-SDS预平衡的时间程序表。

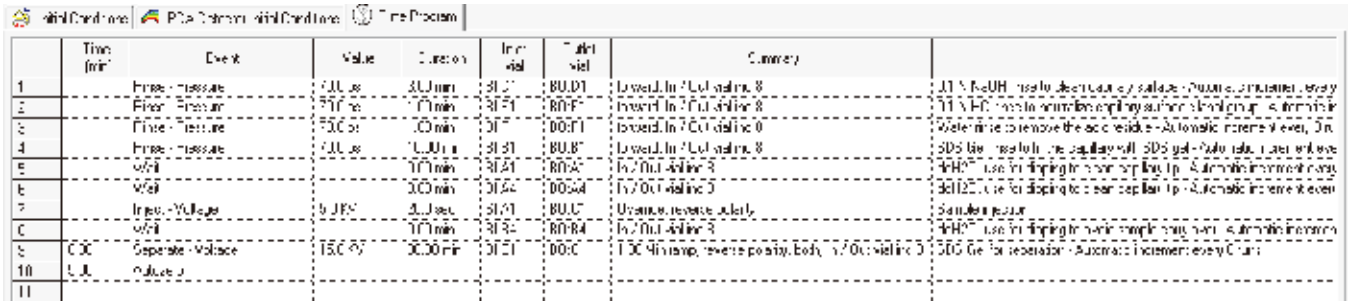


图3. CE-SDS分离的时间程序表。

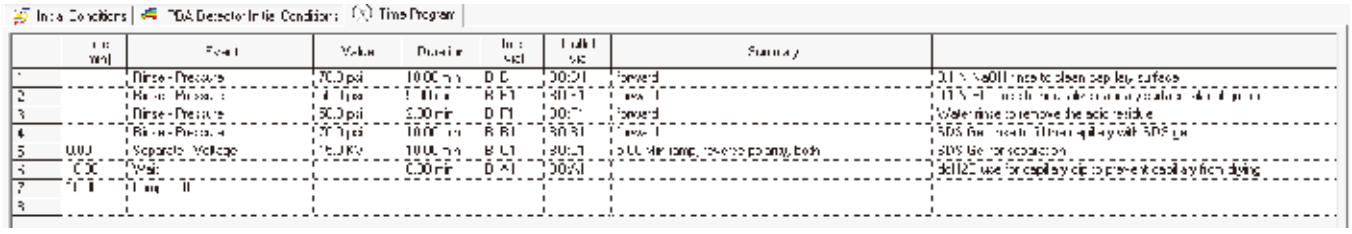


图4. CE-SDS关机的时间程序表。

2.3.2 HPV等电点分析样品处理方法

缓冲液配置：阳极液（200 mM 磷酸）、阴极液（300 mM氢氧化钠）、化学迁移液（350 mM醋酸）、阴极稳定液（500 mM精氨酸）、阳极稳定液（200 mM亚氨基二乙酸）、尿素溶液（4.3 M尿素）、3 M Urea-cIEF Gel等；

样品配置：当分析一个样品是，先将下列试剂混合到0.5 mL离心管中：200 µL 3 M 尿素-cIEF胶、12.0 µL 3-10两性电解质、20.0 µL阴极稳定剂、2.0 µL阳极稳定剂和2.0 µL每个pI标准品。当分析很多样品的时候，SCIEX推荐预先配置样品预混液（master mix）。master mix可简化样品配置并且使误差最小化。当准备用于cIEF分析的蛋白样品时，取240 µL master mix与10 µL蛋白样品混合，蛋白样品的浓度在5 mg/mL-10 mg/mL之间，并且蛋白样品的盐浓度要低于50 mM，充分涡旋cIEF样品，将200 µL样品转移至内插管中，低速离心除去气泡后，将内插管放入样品瓶中并用蓝色盖子盖好，放入样品托盘中，最后将样品托盘放入仪器。

HPV等电点测定实验缓冲液托盘按照图5放置，分析方法包括预平衡、分离以及关机，具体参数设置详见图6-8.

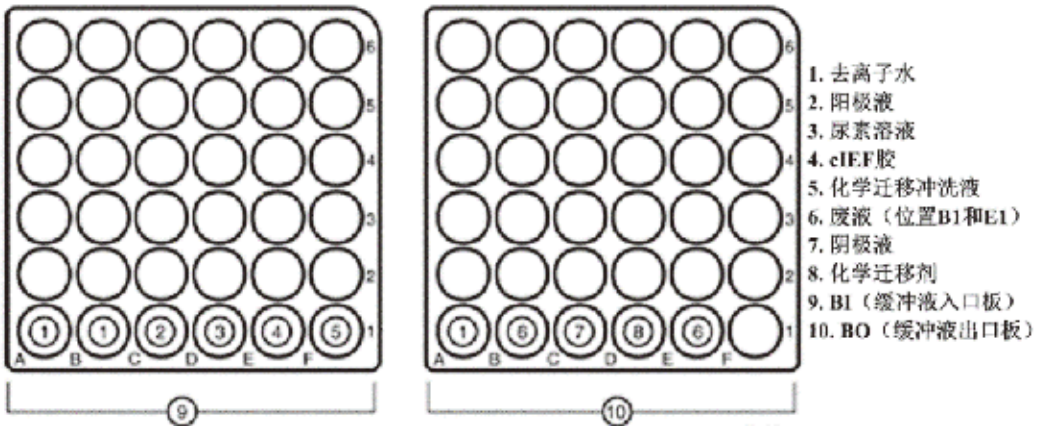


图5. cIEF缓冲液托盘的放置。

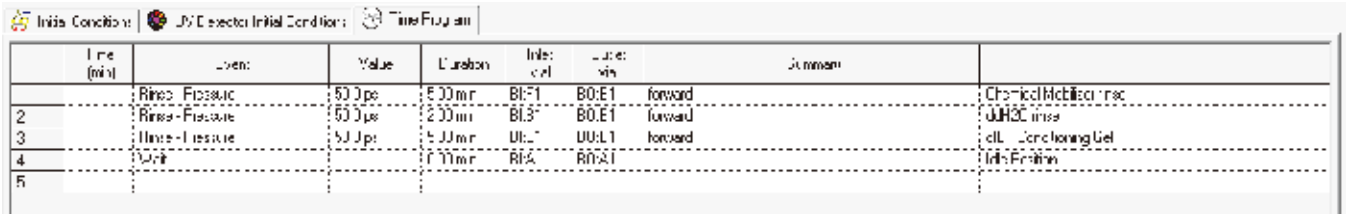


图6. cIEF毛细管预平衡的时间程序表。

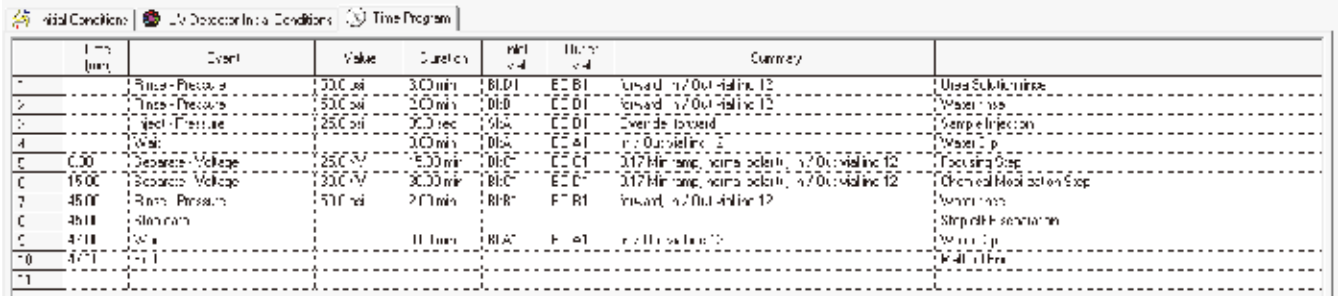


图7. cIEF分离的时间程序表。

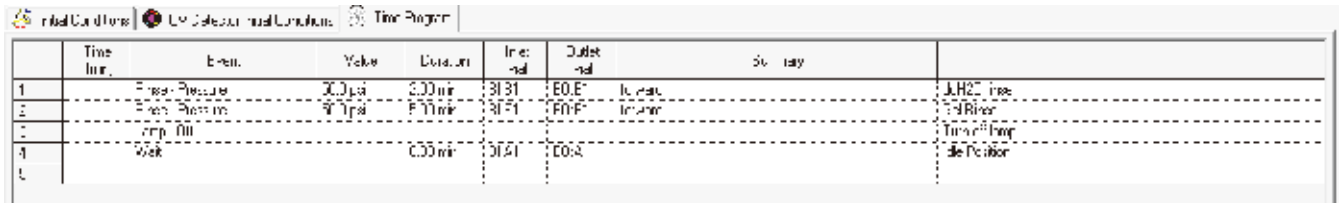


图8. cIEF关机的时间程序表。

3. 分析与讨论

HPV疫苗经巯基乙醇处理后，得到纯度分析结果如图9所示。使用SDS-MW分子量检测试剂盒可以有效地实现HPV疫苗与其他杂质之间的分离，进而准确实现对HPV疫苗纯度的分析，该方法具有非常好的重复性。此外，通过cIEF实验，可以实现对HPV疫苗电荷异质性的分析和等电点的测定，此方法也显示出了良好的重现性，如图10所示。

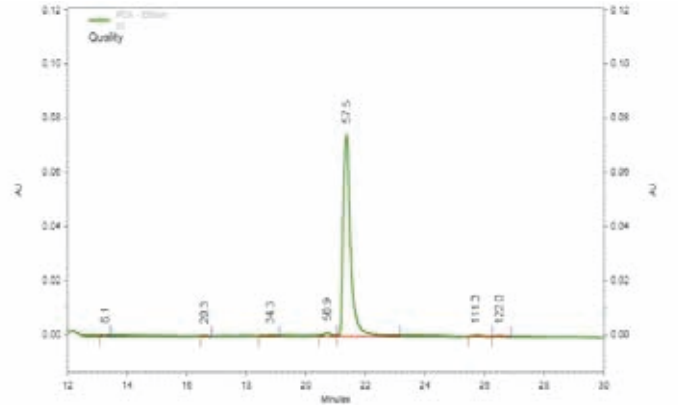


图9. HPV疫苗的CE-SDS纯度分析谱图及校正峰面积。

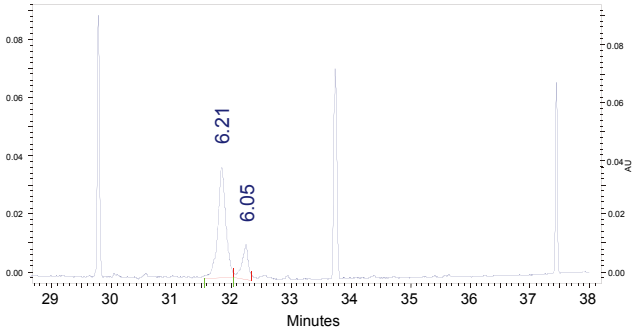


图10. HPV疫苗cIEF的检测结果图。

4. 结论

基于SCIEX稳定的毛细管电泳平台，结合其性能优良的SDS-MW纯度分析试剂盒和cIEF分析试剂盒，可以实现对HPV疫苗纯度和电荷异质性的有效分析和质量监控。cIEF方法是目前能够对宫颈癌疫苗HPV完整颗粒等电点分析的极佳方法。

CE-SDS方法对百日咳疫苗相关毒素的纯度分析

Purity analysis of pertussis vaccine-related toxins by CE-SDS method

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Key word: CE-SDS；无细胞百日咳疫苗；百日咳黏附素；百日咳毒素；丝状血凝素

1. 前言

百日咳是由百日咳杆菌引起的一种小儿常见的急性呼吸道传染病，该传染病的首选疫苗类型为无细胞百日咳疫苗（Acellular Pertussis Vaccine, aP），主要采用柱层析工艺纯化无细胞百日咳疫苗的各毒力因子，再经过不同比例进行混合制成。这种由几种毒素组成的疫苗诱发副作用的可能性更小，产品更加安全，且各个成分明确、质量更易控制，易于工艺放大，因此逐步取代了全细胞百日咳疫苗（Whole-Cell Pertussis Vaccine, wP）。

无细胞百日咳疫苗主要由三种蛋白质毒素组成：百日咳黏附素（pertactin, PRN）能够介导对宿主细胞的黏附，可以通过加热或酸处理从外膜脱落，分子量约为 69 kDa；百日咳毒素（pertussis toxin, PT）由S1 ~ S5这5种亚基组成，具有典型的AB型细菌外毒素结构，是百日咳杆菌的主要致病因子，分子质量约为117 kDa；丝状血凝素（filamentous hemagglutinin, FHA）是百日咳杆菌的主要黏附因子，存在于百日咳杆菌膜表面，分子量约为220 kDa。

随着百日咳疫苗的发展，对于各毒素在原液阶段的纯度分析成为生产企业和法规机构重点关注的质量属性。目前多采用十二烷基硫酸钠-聚丙烯酰胺凝胶电泳法（sodium dodecyl sulfate-polyacrylamide gel electrophoresis, SDS-PAGE）进行各毒素的纯度表征，但该方法需要制胶、染色、脱色等繁琐的人工操作，且具有耗时长，重复性差，只能进行半定量等局限性。毛细管凝胶电泳-十二烷基硫酸钠法（capillary electrophoresis-sodium dodecyl sulfate, CE-SDS）方法的出现，解决了传统SDS-PAGE面临的问题，具有快速、自动化程度高、分辨率高、定量准确、在线检测、重复性好等优势，已成为蛋白质药物纯度分析的最优方法。本文分别以PRN、PT、FHA的原液为代表，建立了CE-SDS纯度分析的方法，用于评估研发阶段的工艺及生产阶段的产品质量。

2. 仪器及方法

毛细管电泳仪PA800 Plus，配备二极管阵列检测器（PDA，波长220 nm）。毛细管长度：20 cm/30 cm（有效/总长），进样条件：-5kV，20 sec；分离电压：-15 kV，30 min；毛细管温度25℃，样品室温度25℃，毛细管的预处理、针与针间冲洗程序：使用碱性冲洗液在70 psi压力下冲洗3 min，酸性冲洗液70 psi冲洗1 min，去离子水70 psi冲洗1 min，背景电解质70 psi冲洗5 min。

2.1 试剂及样品

IgG 分子大小变异体分析试剂盒（SCIEX，A10663），包含50 μm内径熔融石英毛细管、SDS凝胶缓冲液、SDS样品缓冲液（pH 9.0）、0.1 M氢氧化钠、0.1 M盐酸。β-巯基乙醇（Sigma，PN M7154或M6250）、10 kD超滤离心管（Millipore，UFC5010BK）。

百日咳3种毒素样品由相关疫苗企业提供，初始浓度不等，均使用10 kD超滤管将百日咳黏附素、百日咳毒素和丝状血凝素进行超滤并置换为样品溶剂，浓度调整至3 mg/mL，该条件下样品中的盐浓度小于50 mM。各取上述浓度的样品40 μL，分别添加SDS样品缓冲液（pH 9.0）60 μL、2-巯基乙醇5 μL，涡旋混匀，100℃孵育3分钟。冷却至室温后2000 rpm离心2 min，再将处理好的样品转移至样品瓶中，进行电泳分析。 样品溶剂同样进行与样品一致的还原处理，作为空白对照。

3. 结果与讨论

3.1 三种毒素标准品的电泳结果

将已知纯度较高的各毒素样品作为标准品进行分析，考察各毒素分离度及方法的特异性，结果如图1，百日咳黏附素呈现一个主峰，主峰纯度为98.51%；百日咳毒素中共检测到5个峰，分别为百日咳毒素的五种亚基S1、S2、S3、S4、S5，五个峰之间的分离度均大于1.70，以5个峰的总和代表百日咳毒素的纯度，结果为100%；丝状血凝素中共检测到2个明显的峰（P1、P2）及降解的碎片杂质，两个峰之间的分离度为1.36，以2个峰的总和代表丝状血凝素的纯度，结果为95.74%。空白对照中均无与三种毒素的相关峰，证明该方法对于三种毒素的分离及杂质分析具有特异性。

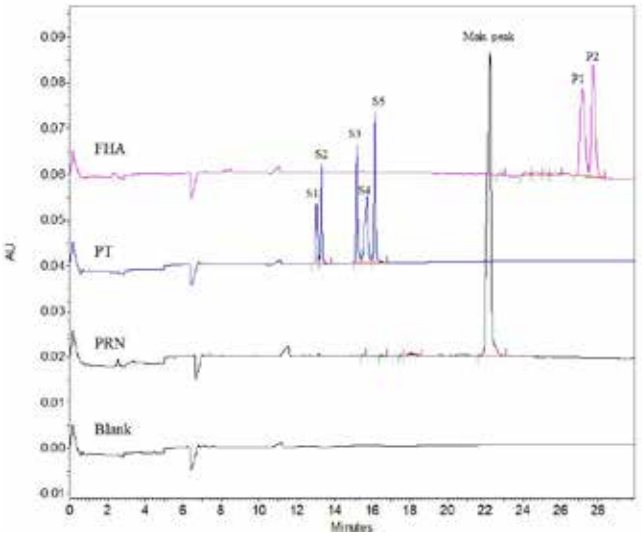


图1. 空白对照及各毒素标准品的CE-SDS电泳图
（PRN：百日咳黏附素；PT：百日咳毒素；FHA：丝状血凝素）

3.2 三种毒素不同批次的CE-SDS结果

使用该方法对三种毒素、每种3个批次的样品（命名为毒素的缩写+1、2、3）进行分析，考察方法的普适性，三种毒素的不同样品电泳结果如图2，纯度结果见表1。百日咳毒素和丝状血凝素的样品中，各亚基分离度良好，主峰比例或纯度有较大差异（各亚基比例未展示）。百日咳黏附素样品除了主峰外，还可观察到为不同程度的杂质峰。说明该方法对各类样品具有良好的普适性，可作为各毒素实际样品的纯度质控。

表1. 各毒素不同样品的纯度（Purity）

PT			FHA			PRN		
PT1	PT2	PT3	FHA1	FHA2	FHA3	PRN1	PRN2	PRN3
100%	100%	100%	42.50%	92.12%	89.55%	92.22%	98.52%	95.98%

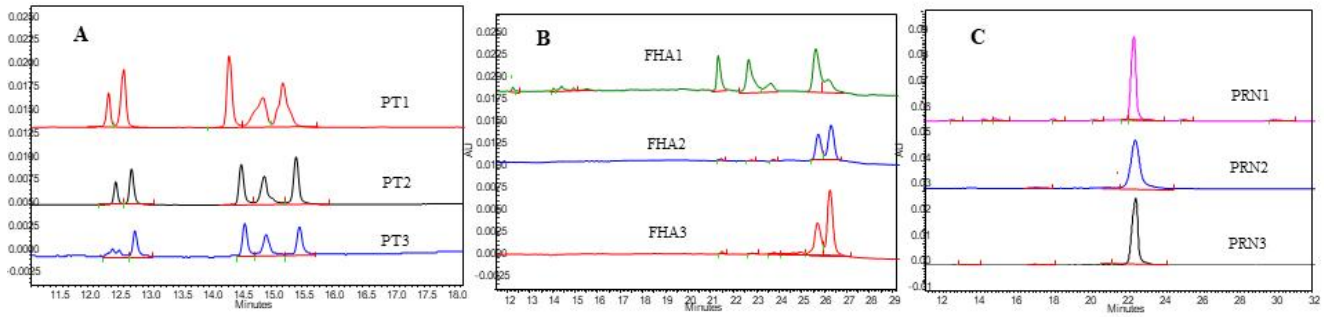


图2. 各毒素不同样品的CE-SDS电泳图（A：PT，百日咳毒素；B：FHA，丝状血凝素；C：PRN，百日咳黏附素）

4. 结论

本文建立了CE-SDS方法分析百日咳疫苗中的三种毒素因子：百日咳黏附素、百日咳毒素、百日咳丝状血凝素的的纯度，通过对标准品、不同工艺和生产阶段的多批次样品进行考察，证明了方法具有良好的特异性和普适性，能够有效评估百日咳不同毒力因子的纯度，可作为平台化方法指导企业建立合理的质量标准，以用于考察研发阶段的生产工艺与工业化生产阶段的质量控制。

毛细管胶束电动色谱法对多糖蛋白结合疫苗中游离载体蛋白的检测

Determination of free carrier proteins in polysaccharide-protein conjugate vaccines by capillary micellar electrokinetic chromatography

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Key words: Polysaccharide-protein conjugate vaccine; Free carrier proteins; Capillary micellar electrokinetic chromatography; Quantitative determination;

1、前言

多糖蛋白结合疫苗是一类以细菌多糖与蛋白质偶联的疫苗，其优点主要表现在：能激发2岁以下儿童、老年人和免疫缺陷者体内产生有效的免疫应答，并产生记忆性B细胞；诱生的保护性抗体主要为IgG，较多糖疫苗诱生的IgM抗体（在人体内可激起IgG2）更有效，且能维持较长时间；采用其他病原微生物的蛋白成分作为载体蛋白时，能起到联合免疫的效果。

在多糖蛋白结合疫苗中，蛋白作为载体，增强了多糖的免疫原性。多糖蛋白结合疫苗中常用的载体蛋白包括：破伤风类毒素（Tt）、白喉类毒素（Dt）、白喉类毒素无毒变异蛋白（CRM197）、B群脑膜炎球菌外膜蛋白（MenB-OMP）、流感嗜血杆菌 D 蛋白、绿脓杆菌外毒素 A（PEA）等[1]。游离载体蛋白的含量是疫苗质量控制的重要指标，也是反映疫苗质量的主要参数之一[2]。多糖蛋白结合疫苗中的游离载体蛋白含量超过规定的限度，会影响疫苗的免疫原性，并增加副反应发生率，因此 WHO 推荐在细菌多糖蛋白结合疫苗中游离载体蛋白含量应小于总蛋白（游离载体蛋白及多糖蛋白结合物中蛋白之和）的 5%[3]。游离载体蛋白检测的现有方法有层析柱法、十二烷基硫酸钠-聚丙烯酰胺凝胶电泳（SDS-PAGE）法和分子排阻色谱法，其中层析柱法耗时、分辨率低、灵敏度低，不适合企业大批量样品的检测需求；SDS-PAGE法分辨率低，定量准确度差；分子排阻色谱法背压高、分辨率差、色谱柱寿命短。相比而言，毛细管电泳法分辨率高、定量准确、简单快速、毛细管寿命长是多糖蛋白结合疫苗中游离载体蛋白定量的最佳方法。

目前，已有多种多糖蛋白结合疫苗获准上市，如13价肺炎链球菌多糖蛋白结合疫苗（PCV13）、b型流行性感冒嗜血杆菌（Hib）多糖蛋白结合疫苗、单/2/4价脑膜炎球菌多糖蛋白结合疫苗等。本文以13价肺炎链球菌多糖蛋白结合疫苗原液中两种常用的载体蛋白CRM197和Tt为例，开发了毛细管胶束电动色谱法（MEKC）对这两种载体蛋白及其结合物的分离，并考察了对这两种载体蛋白进行定量分析的线性和灵敏度。

2、仪器和试剂

2.1、仪器

毛细管电泳仪（PA800 Plus生物制药分析系统，匹配UV检测器，Sciex）；离心机（Biofuge，Thermo）。

2.2、试剂

四硼酸钠（229946-100G，Sigma-Aldrich）；十二烷基硫酸钠（74255-250G，Sigma-Aldrich）；氢氧化钠（795429-500G，Sigma-Aldrich）；二次去离子水（Millipore）。

背景电解质（BGE）：15 mL 50 mmol/L四硼酸钠，2.5 mL 200 mmol/L SDS，2.5 mL二次去离子水新鲜混合配置。

2.3、样品

CRM197、单价多糖蛋白（CRM197）结合疫苗原液（样品1）、Tt、单价多糖蛋白（Tt）结合疫苗原液（样品2）均由某疫苗生产厂家提供。其浓度分别为2.0 mg/mL、0.8 mg/mL、4.5 mg/mL、0.5 mg/mL。

3、CE方法

毛细管：熔融石英毛细管，50/60 cm（有效/总长度），75 μm内径；进样：0.5 psi，10 s；分离电压：30 kV；分离时间：30 分钟；毛细管温度：20 ℃；样品室温度：20 ℃；检测波长：200 nm。

新毛细管的预平衡： 0.1 mol/L NaOH，20 psi冲洗10 分钟；二次去离子水20 psi冲洗10分钟；背景电解质20 psi冲洗10 分钟；30 kV电压分离10 分钟。

每针之间的冲洗：0.1 mol/L NaOH ，20 psi冲洗5 分钟；二次去离子水，20 psi冲洗5分钟；背景电解质，20 psi冲洗5 分钟。

4、结果与分析

4.1、MEKC方法对载体蛋白CRM197的分离与定量

4.1.1、MEKC方法对CRM197与多糖蛋白结合疫苗的分离

使用MEKC方法对多糖蛋白结合疫苗样品1中的游离载体蛋白进行分离（图1a）。电泳图显示在7分钟（峰1）和11.6分钟（峰2）获得两个可基线分离的主峰。通过添加载体蛋白CRM197标准品（图1b），可判断峰2为载体蛋白CRM197，而峰1为多糖蛋白结合疫苗。

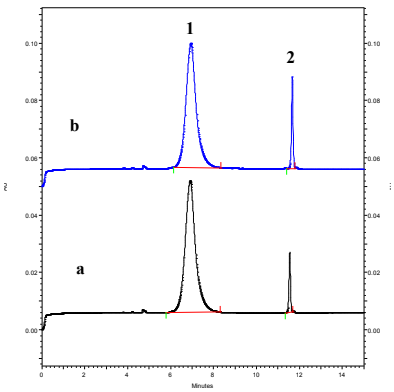


图1. MEKC法对载体蛋白CRM197和多糖蛋白结合疫苗分离的电泳图。a：疫苗样品1，b：疫苗样品1添加2 μL 2.0 mg/mL的CRM197。电泳条件：见第3节。

4.1.2、CRM197检测的线性、定量限 (LOQ) 及检出限 (LOD)

将2.0 mg/mL CRM197梯度稀释至250.0、125.0、62.5、32.2、15.6、7.8 μg/mL。使用MEKC法分离不同浓度的CRM197标准品（电泳图见2a），根据校准峰面积与浓度制定标准曲线，线性相关系数为0.999（图2b）。继续稀释CRM197至4.0 μg/mL，可达到CRM197的检出限（如图3）。根据LOQ=3×LOD，可得CRM197的定量限LOQ为12.0 μg/mL。

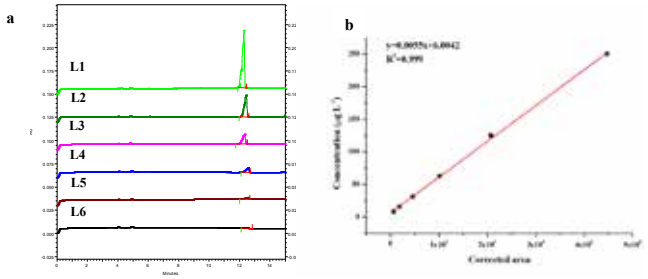


图2a. MEKC法分析不同浓度CRM197的电泳图。样品：CRM197，浓度由L1-L6为250.0、125.0、62.5、32.2、15.6、7.8 μg/mL；电泳方法：见第3节；b.根据校准峰面积及浓度制定的校准曲线。

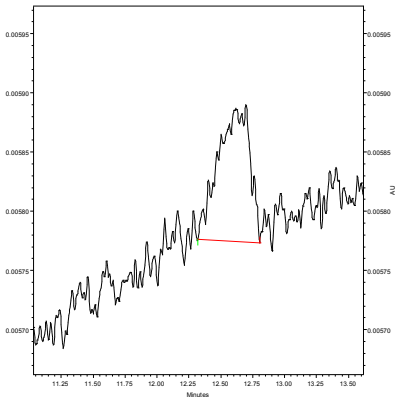


图3. MEKC法分析CRM197的电泳图。样品：CRM197；浓度：4.0 μg/mL；电泳方法：见第3节。

4.2、MEKC方法对载体蛋白Tt的分离与定量

4.2.1、MEKC方法对Tt与多糖蛋白结合疫苗的分离

使用MEKC方法对多糖蛋白结合疫苗样品2中的游离载体蛋白进行分离（图4a）。电泳图显示在6.2分钟（峰1）和11.7 分钟（峰2）获得两个可基线分离的主峰。通过添加载体蛋白Tt标准品（图4b），可判断峰2为载体蛋白Tt，而峰1为多糖蛋白结合疫苗。

4.2.2、Tt检测的线性、定量限 (LOQ)及检出限 (LOD)

将4.5 mg/mL Tt梯度稀释至281.2、140.6、70.3、35.2、17.6、8.8 μg/mL。使用MEKC法分离不同浓度的Tt标准品（电泳图见5a），根据校准峰面积与浓度制定标准曲线，线性相关系数为

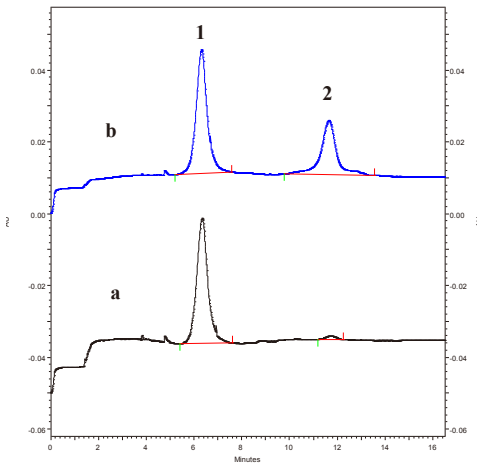


图4. MEKC法对载体蛋白Tt和多糖蛋白结合疫苗分离的电泳图。
a：疫苗样品2， b：疫苗样品2添加5 µL 4.5 mg/mL的Tt；方法详见第3节。

0.999（图5b）。继续稀释Tt至5.0 µg/mL，可达到Tt的检出限（如图6）。根据LOQ=3×LOD，可得Tt的定量限LOQ为15.0 µg/mL。

5、结论

本文成功地建立了MEKC方法用于肺炎多糖蛋白结合疫苗中两种游离载体蛋白CRM197和Tt的检测。载体蛋白及其对应多糖蛋白结合疫苗可以实现很好的分离。载体蛋白CRM197线性范围为7.8-250.0 µg/mL，线性指数R^2为0.999，LOD为4.0 µg/mL，LOQ为12.0 µg/mL。载体蛋白Tt的线性范围 为8.8-281.2 µg/mL，线性指数R^2为0.993，LOD为 5.0 µg/mL，LOQ为15 µg/mL。MEKC方法对肺炎多糖蛋白结合疫苗中这两种游离载体蛋白的检出限低，可满足多糖蛋白结合疫苗原液中游离载体蛋白含量低于总蛋白5%的标准（以原液浓度为100 µg/mL计算）。本实验结果表明，MEKC法测定多糖蛋白结合疫苗原液中游离载体蛋白分离度高、简单、快速，可适用于单价多糖蛋白结合疫苗原液中游离载体蛋白的检测。

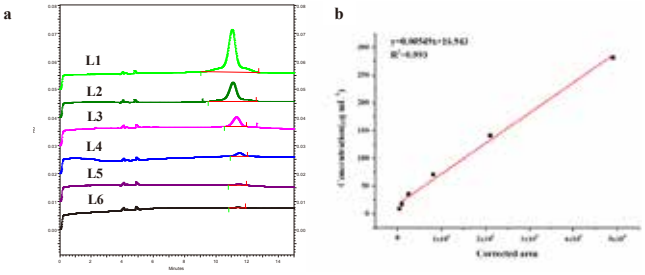


图5a. MEKC法分析不同浓度Tt的电泳图。样品：Tt，浓度由L1-L6为281.2、140.6、70.3、35.2、17.6、8.8 µg/mL；电泳方法：见第3节；
b.根据校准峰面积及浓度制定的校准曲线。

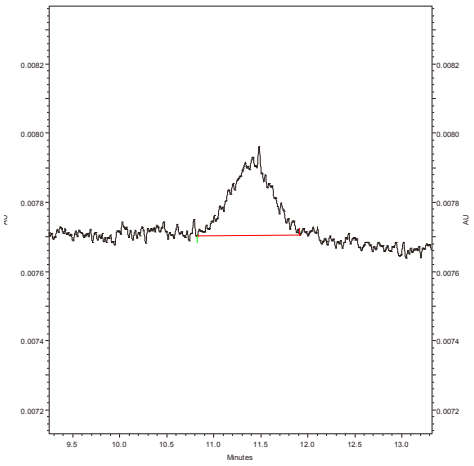


图6. MEKC法分析Tt的电泳图。样品：Tt；浓度：5.0 ug/mL；电泳方法：见第3节。

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