

# 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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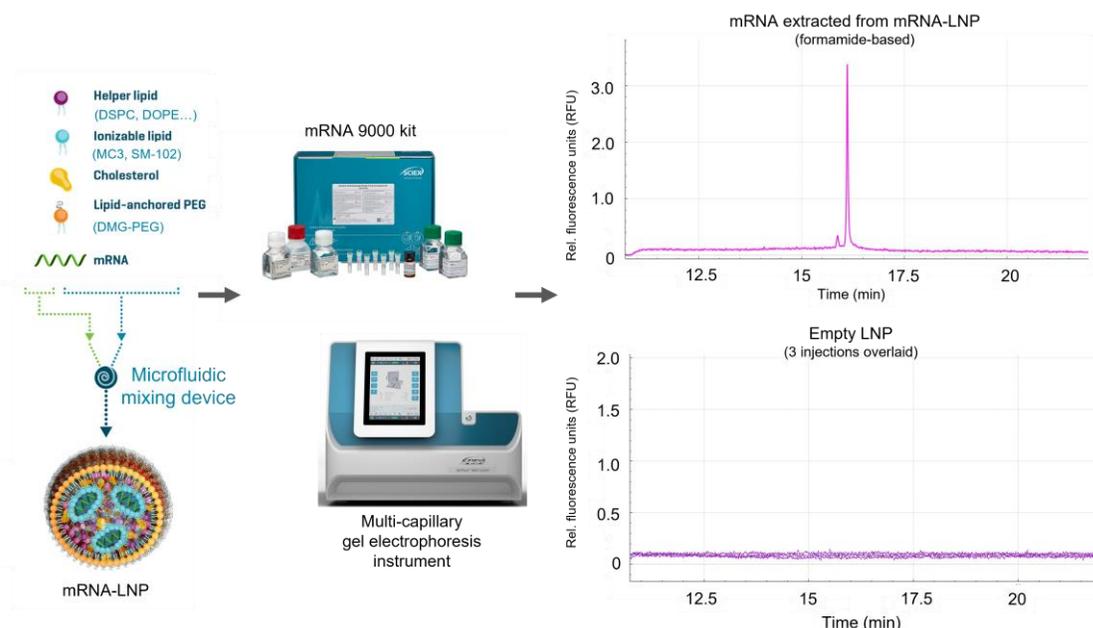
The use of lipid nanoparticles (LNPs) to deliver mRNA products against SARS-CoV-2 during the COVID-19 pandemic demonstrated the power and efficacy of this drug-delivery device for RNA-based vaccines.<sup>1</sup> In addition to vaccine development, the application of mRNA-LNPs has expanded widely into 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), lower limit of quantification (LLOQ) and upper limit of detection (ULOD), and to guide the identification of low-level impurities or nucleic acid fragmentations associated with manufacturing mRNA-LNPs.<sup>2</sup>

### 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 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.

## 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.

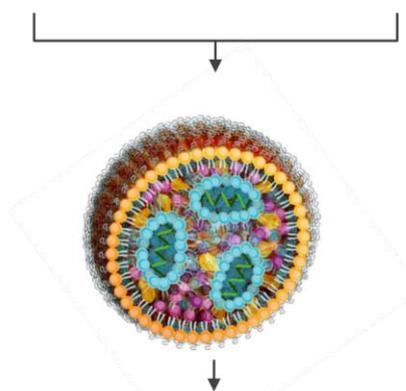
Non-denaturing mRNA-LNP sample preparation conditions involved mixing 10 µL of the respective mRNA-LNP sample with 20 µL of 0.3% Triton X-100 in CE-grade water. Samples were incubated at room temperature for 20 minutes and then heated for 5 minutes at 70°C using a thermal cycler. Samples were chilled 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  $6.10 \times 10^{-6}$  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.<sup>3</sup> 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

## mRNA-LNP optimization and analysis

MC3-ionizable lipid      SM-102-ionizable lipid



Nucleic acid extraction  
for integrity and stability analysis

Triton-X100

Triton-X100  
and formamide



Multi-capillary  
gel electrophoresis system  
BioPhase 8800 system

- ✓ mRNA-LNP nucleic acid stability & purity analysis
- ✓ CGE-LIF based RNA quantification
- ✓ Automated RNA size determination
- ✓ High-resolution data for RNA fragments ranging from 50 to 9,000 bases

**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.

**A**

Method Duration: 42.0 min Number of Actions: 7

|          |  |  |
|----------|--|--|
| Settings | Capillary Cartridge: 20.0 °C, Wait<br>Capillary Length: 30.0 cm<br>Capillary Type: Bare Fused Silica<br>Current Limit: 100 µA, Enabled | Sample Storage: 10.0 °C<br>Detector Type: LIF, 520 nm, Wait, PMT Gain: 100<br>Peak Width: 1 sec<br>Data Rate: 8 Hz |
| Rinse    | Duration: 5.0 min<br>50.0 psi  | Inlet: Water<br>Outlet: Waste  |
| Rinse    | Duration: 5.0 min<br>20.0 psi  | Inlet: Acidic Conditioning Solution (0.1M HCl)<br>Outlet: Waste  |
| Rinse    | Duration: 2.0 min<br>20.0 psi  | Inlet: Water<br>Outlet: Waste  |
| Rinse    | Duration: 10.0 min<br>50.0 psi   | Inlet: Nucleic Acid Extended Range Gel (Rinse)<br>Outlet: Waste  |
| Wait     | Duration: 0.0 min  | Inlet: Water Dip 1<br>Outlet: Water Dip 1  |
| Wait     | Duration: 0.0 min  | Inlet: Water Dip 2<br>Outlet: Water Dip 2  |
| Separate | Duration: 20.0 min<br>-6.0 kV<br>Ramp time: 2.0 min<br>Autozero: 8.0 min   | Inlet: Nucleic Acid Extended Range Gel (Separation)<br>Outlet: Nucleic Acid Extended Range Gel (Separation)        |

**B**

Method Duration: 31.0 min Number of Actions: 8

|          |  |  |
|----------|--|--|
| Settings | Capillary Cartridge: 30.0 °C, Wait<br>Capillary Length: 30.0 cm<br>Capillary Type: Bare Fused Silica<br>Current Limit: 600 µA, Enabled | Sample Storage: 10.0 °C, Wait<br>Detector Type: LIF, 520 nm, Wait, PMT Gain: 100<br>Peak Width: 1 sec<br>Data Rate: 8 Hz |
| Rinse    | Duration: 1.0 min<br>70.0 psi  | Inlet: Acidic Conditioning Solution (0.1M HCl)<br>Outlet: Waste  |
| Rinse    | Duration: 1.0 min<br>70.0 psi  | Inlet: Water<br>Outlet: Waste  |
| Rinse    | Duration: 5.0 min<br>50.0 psi  | Inlet: Nucleic Acid Extended Range Gel (Rinse)<br>Outlet: Waste  |
| Separate | Duration: 2.0 min<br>-30.0 kV<br>Ramp time: 0.2 min  | Inlet: Nucleic Acid Extended Range Gel (Separation)<br>Outlet: Nucleic Acid Extended Range Gel (Separation)              |
| Wait     | Duration: 0.0 min  | Inlet: Water Dip 1<br>Outlet: Water Dip 1  |
| Inject   | Duration: 3 sec<br>-1.0 kV   | Tray: Sample<br>Outlet: Nucleic Acid Extended Range Gel (Separation)   |
| Wait     | Duration: 0.0 min  | Inlet: Water Dip 2<br>Outlet: Water Dip 2  |
| Separate | Duration: 22.0 min<br>-6.0 kV<br>Ramp time: 2.0 min<br>Autozero: 8.0 min<br>Advance after: 6 actions                                   | Inlet: Nucleic Acid Extended Range Gel (Separation)<br>Outlet: Nucleic Acid Extended Range Gel (Separation)              |

**C**

Method Duration: 20.0 min Number of Actions: 5

|          |  |  |
|----------|--|--|
| Settings | Capillary Cartridge: 15.0 °C, Wait<br>Capillary Length: 30.0 cm<br>Capillary Type: Bare Fused Silica<br>Current Limit: 600 µA, Enabled | Sample Storage: 10.0 °C, Wait<br>Detector Type: LIF, 520 nm, Wait, PMT Gain: 100<br>Peak Width: 1 sec<br>Data Rate: 8 Hz |
| Rinse    | Duration: 5.0 min<br>50.0 psi  | Inlet: Acidic Conditioning Solution (0.1M HCl)<br>Outlet: Waste  |
| Rinse    | Duration: 5.0 min<br>50.0 psi  | Inlet: Water<br>Outlet: Waste  |
| Wait     | Duration: 0.0 min  | Inlet: Water Dip 1<br>Outlet: Water Dip 1  |
| Wait     | Duration: 0.0 min  | Inlet: Water Dip 2<br>Outlet: Water Dip 2  |
| LIFLaser | OFF  |  |

**Figure 3. Methods implemented on the BioPhase 8800 system.** (A) Parameters are shown for the conditioning method, (B) the optimized separation method with a water-plug step prior to 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.

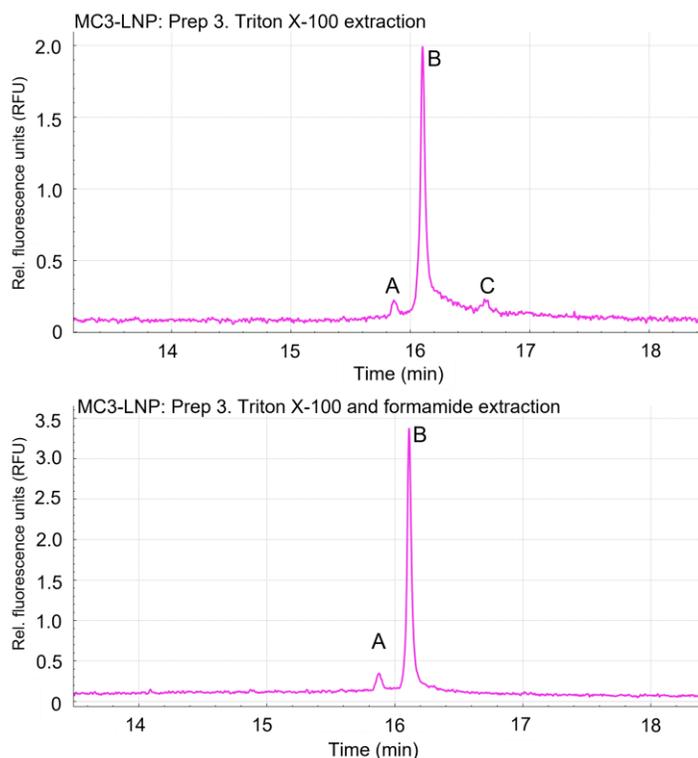
## Results and discussion

### 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.<sup>2</sup>

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.<sup>4</sup> Additional studies have indicated that RNA can form lipid complexes that are detectable by mass spectrometry.<sup>5</sup> 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.

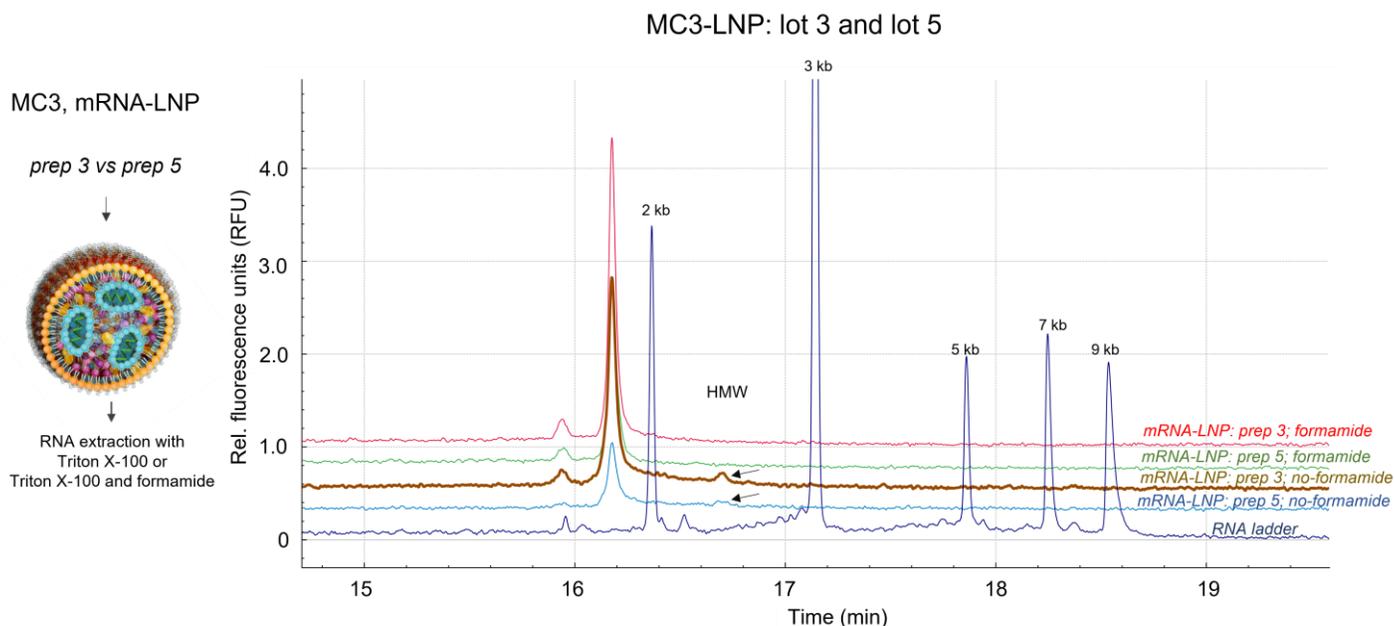
The expected size for the main RNA product was 1.929 kb. The electropherograms for prep 3 and prep 5 of the mRNA-LNP indicated that the main nucleic acid products eluted before the 2 kb marker. These results suggest that the integrity of the mRNA was maintained during mRNA-LNP synthesis. These results 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**

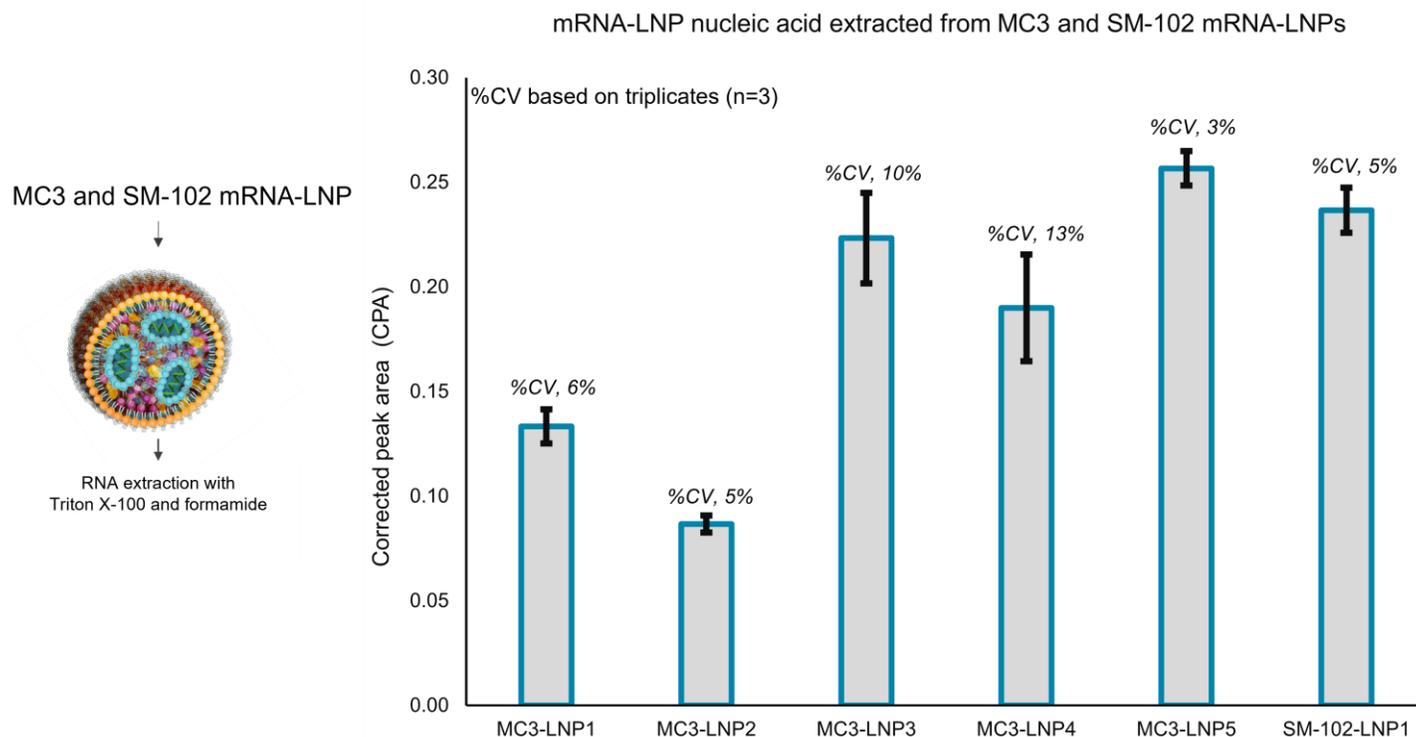
Five differently loaded MC3-based LNPs and a loaded SM-102 mRNA-LNP formulation, containing 50% MC3 or SM-102, 38.5% cholesterol, 10% DSPC and 1.5% DMG-PEG and differing in the initial amount of mRNA for encapsulation, were analyzed by

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.<sup>1</sup> 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.

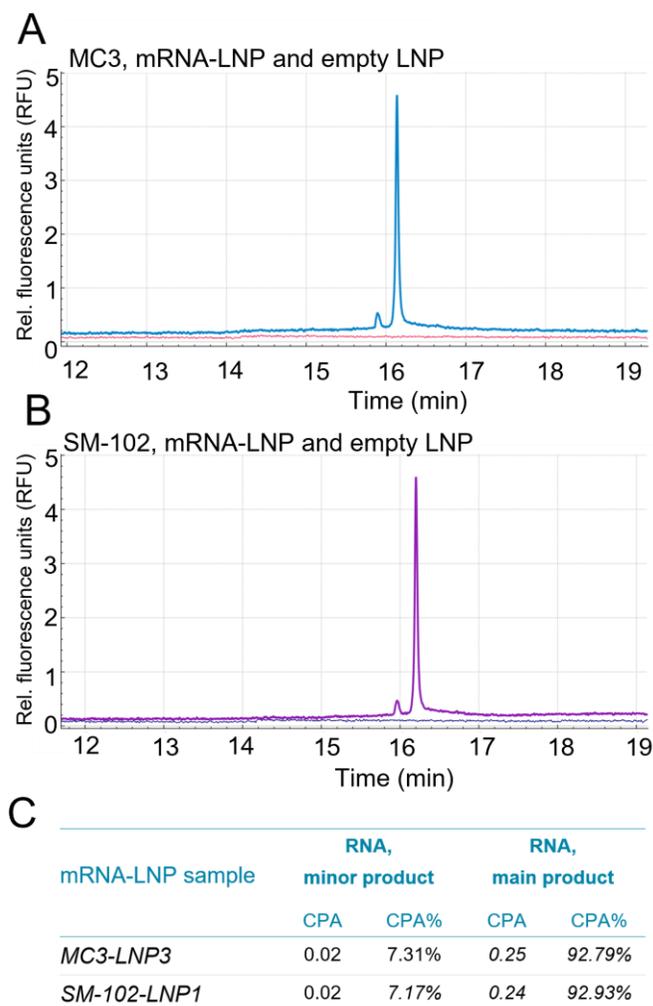
In summary, the use of the BioPhase 8800 system and the ready-to-use RNA 9000 Purity & Integrity Analysis kit demonstrated consistency in the mRNA extracted from mRNA-LNPs made of different ionizable lipids. These analyses can therefore be beneficial for the mRNA-LNP field by expanding on the applications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

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

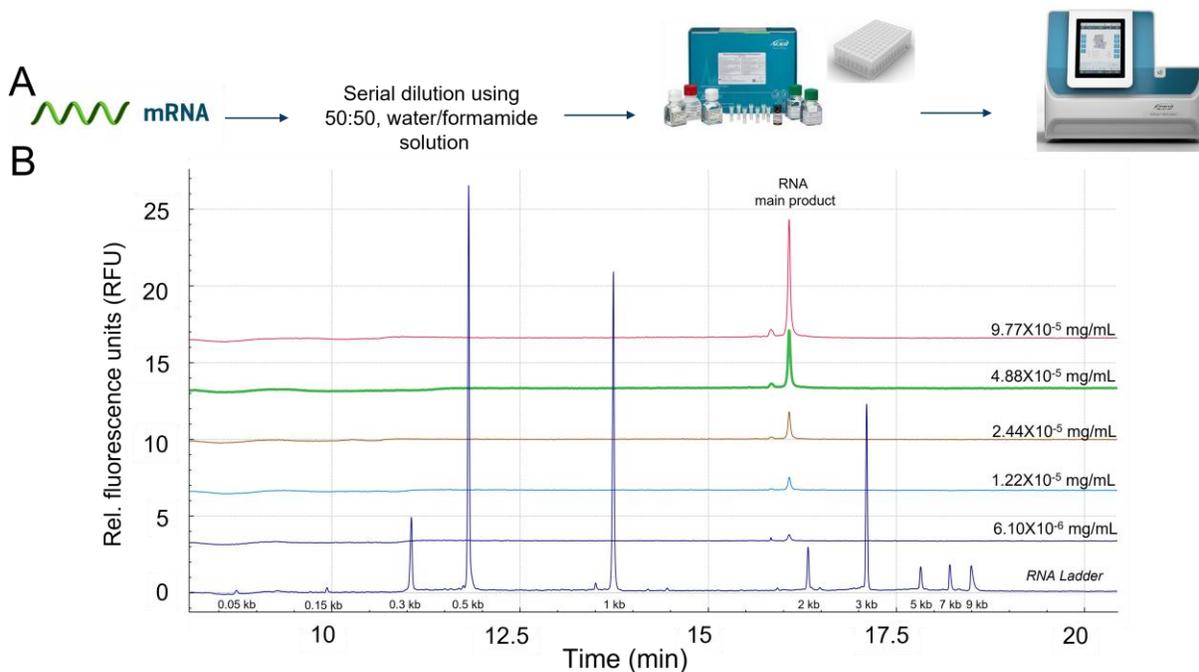
The free mRNA product (1.929 kb) used to load the different mRNA-LNP formulations was used to generate a linearity curve in a water-formamide matrix. The lipid compositions and mixing ratios of mRNA-LNP formulations are diverse and therefore it can be challenging to replicate specific matrix compositions. 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  $6.10 \times 10^{-6}$  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.

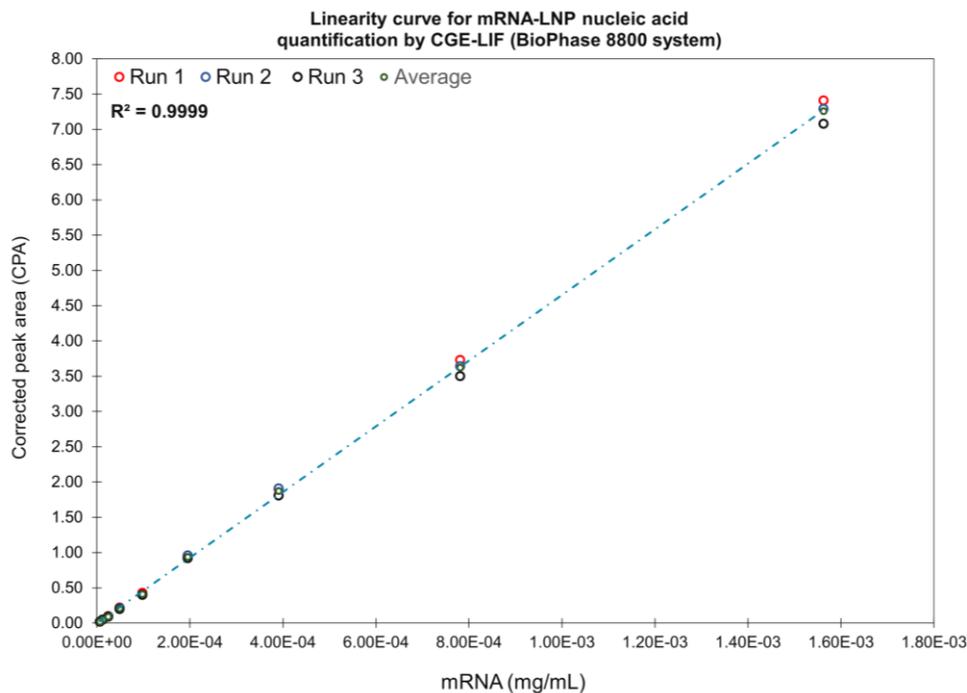
In summary, this study produced a 9-point linearity curve that spanned approximately 2.5 orders of magnitude ( $R^2 = 0.9999$ ). The linear plotting and regression modeling were based on the average corrected peak area produced from 3 injections. The open circles indicated the specific corrected peak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



**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 overlaid 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  $6.10 \times 10^{-6}$  mg/mL, the LLOQ was  $1.22 \times 10^{-5}$  mg/mL and the ULOD was  $1.56 \times 10^{-3}$  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  $6.10 \times 10^{-6}$  mg/mL, an LLOQ of  $1.22 \times 10^{-5}$  mg/mL and a ULOD of  $1.56 \times 10^{-3}$  mg/mL

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