A new approach to determine encapsulation efficiency of mRNA-lipid nanoparticles (mRNA-LNP) by capillary gel electrophoresis with laser-induced fluorescence detection

Featuring the BioPhase 8800 system and the RNA 9000 Purity & Integrity kit

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This technical note demonstrates a new workflow to determine the encapsulation efficiency of 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.

The mRNA-LNP is becoming increasingly significant in biotechnological research and pharmaceutical applications, such as vaccines and next-generation therapies.[1] Encapsulation efficiency determination is a critical quality attribute (CQA) for characterizing mRNA-LNPs. The RiboGreen dye test is commonly used to determine encapsulation efficiency. Although this test is a simple test, it cannot differentiate full-length mRNA from degraded species.[1] Additionally, the use of Triton X-100 introduces background noise and excipients in certain LNP formulations might interfere with the binding of the RiboGreen dye to mRNA molecules. In this technical note, a new method (Figure 1) for mRNA-LNP encapsulation efficiency determination by CGE-LIF was achieved with the BioPhase 8800 system and the RNA 9000 Purity & Integrity kit. The high-resolution

separation of the intact mRNA from impurities and other species by CGE-LIF provides insights into the mRNA sample quality, enabling accurate encapsulation efficiency determination.

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

- The multi-capillary capability of the BioPhase 8800 system enables fast optimization of sample preparation conditions and rapid generation of a calibration curve
- The high sensitivity and high resolving power of CGE-LIF enable accurate encapsulation efficiency determination with a 2.7 log dynamic range using a single concentration of the dye
- A novel CE-based workflow to determine encapsulation efficiency ensures efficient mRNA-LNP CQA analysis
- The same workflow provides encapsulation efficiency and sizing information about impurities, which can give insight into the mRNA quality
- The ready-to-use RNA 9000 Purity & Integrity kit helps simplify operation and minimize user error
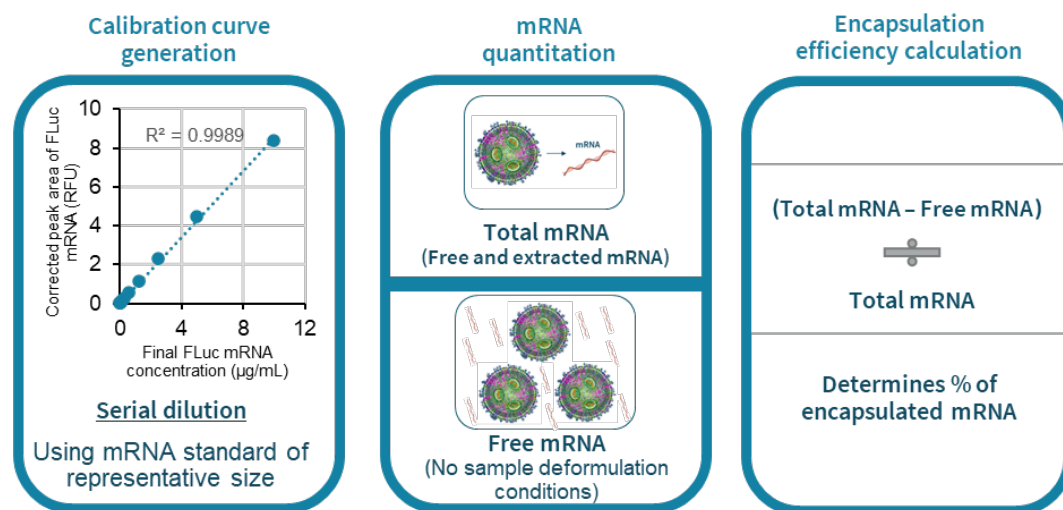

Figure 1. Workflow to determine the encapsulation efficiency of mRNA-LNP by CGE-LIF. A calibration curve was generated with serially diluted FLuc mRNA samples with known concentrations, as described in the Methods section. The amounts of total mRNA in the deformulated mRNA-LNP sample and free mRNA in the non-treated mRNA-LNP sample were determined using the 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.

Materials and 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 (0.05 kB to 9 kB). 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% Triton X-100 or Triton, P/N: 28314), RNA storage solution (P/N: AM7000) 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 a therapeutic mRNA vaccine. The mRNA-LNP samples encapsulating the FLuc mRNA and containing the ionizable lipid ALC-0315 were from 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.

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 plates and sample plates were prepared based on the experimental design and the plate map generated by the BioPhase 8800 software.

ssRNA Ladder preparation: The ssRNA Ladder was prepared according to the RNA 9000 Purity & Integrity kit Application Guide.[2] A 50 μL sample was denatured with heat using a thermal cycler at 70°C for 5 minutes and immediately placed on ice until the time of CE analysis.

Sample preparation for analysis of free FLuc mRNA outside of mRNA-LNP: The mRNA-LNP samples were stored at -80°C

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

until the time of analysis. For capillary gel electrophoresis laser-induced fluorescence (CGE-LIF) analysis, the mRNA-LNP samples were diluted with NFW at different dilution factors. For 5-fold dilution, 25 μL of the mRNA-LNP was mixed with 100 μL of NFW. For 25-fold dilution, 5 μL of the mRNA-LNP was mixed with 120 μL of NFW. For 125-fold dilution, 5 μL of the 5-fold diluted mRNA-LNP was mixed with 120 μL of NFW. All diluted mRNA-LNP samples were vortexed briefly and 55 μL was transferred from each sample to a well on the sample plate. CGE-LIF separation was then performed using the methods described in Figure 2.

Sample preparation for analysis of total FLuc mRNA in mRNA-LNP: The mRNA-LNP samples were diluted 2-fold by mixing 10 μL of the mRNA-LNPs with 10 μL of 300mM sucrose in PBS. Then, 3 μL of the diluted mRNA-LNP samples were combined with 31.5 μL of NFW, 37.5 μL of 0.4% Triton solution and 3 μL of RNA storage solution. The 0.4% Triton solution was

prepared by diluting the 10% Triton with NFW. After a brief vortex and a quick spin, the FLuc mRNA was released from LNPs by incubating the mixture at room temperature for 20 minutes. Next, 75 µL of formamide was added to the mixture to yield a 100-fold dilution of the main stock. The samples were then 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, 55 µL was transferred from each sample to a well on the sample plate before CGE-LIF separation using the methods described in Figure 2.

Preparation of the mRNA standard diluent buffer: The diluent buffer was made by mixing 20 µL of 300mM sucrose in PBS, 20 µL of RNA storage solution, 500 µL of 0.4% Triton solution and 460 µL of NFW. After a quick vortex and a quick spin, the diluent buffer was kept on ice until use.

Sample preparation for generating the calibration curve with the FLuc mRNA standard: The 1 mg/mL FLuc mRNA standard was initially diluted 2-fold by mixing 10 µL of the FLuc mRNA standard with 10 µL of RNA storage solution. Next, 3 µL of the 2-fold diluted FLuc mRNA standard solution was mixed with 31.5 µL of NFW, 37.5 µL of 0.4% Triton solution and 3 µL of 300mM sucrose in PBS. The total volume of the solution was 75 µL. After a brief vortex and a quick spin, an aliquot of 37.5 µL was removed from this mixture and serially diluted 2-fold with the diluent buffer to yield a 10-point dilution series. After a brief vortex and a quick spin, the mixture was incubated at room temperature for 20 minutes. Then, 37.5 µL of formamide was added to each mRNA standard solution, except for the last dilution point, to which 75 µL of formamide was added. The FLuc

mRNA standards were then 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, 55 µL was transferred from each sample to a well on the sample plate before CGE-LIF separation using the methods described in Figure 2.

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.

Optimization of sample preparation conditions for analysis of free mRNA outside of mRNA-LNP

Multiple sample treatment conditions were explored to analyze free mRNA outside of mRNA-LNP. Initially, the mRNA-LNP sample was diluted 100-fold with either NFW or 50% formamide in NFW, heated at 70°C for 5 minutes, chilled on ice and finally separated by CGE-LIF. Results in Figure 3 show that a higher FLuc signal was detected when the sample was diluted with 50% formamide compared to with NFW, indicating that 50% formamide caused the release of some FLuc mRNA from the mRNA-LNPs. In a separate experiment, the mRNA-LNP sample was diluted 5-fold with NFW. Then, the diluted sample was either loaded directly for CGE-LIF analysis or heated at 70°C for 5 minutes and chilled on ice before separation by CGE-LIF. As shown in the top panel of Figure 4, the unheated sample contained species with higher-order structures or HMW (high molecular weight) in addition to the FLuc mRNA main product
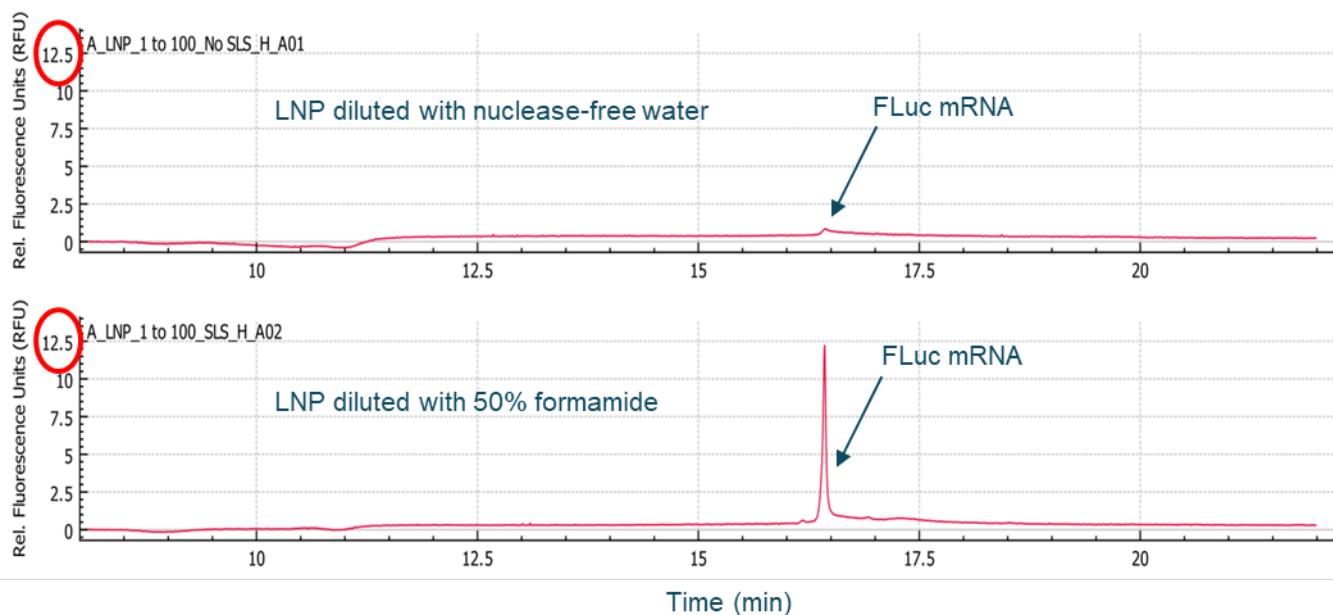

Figure 3. Treatment with 50% formamide releases some mRNA from mRNA-LNP samples. The mRNA-LNP sample was diluted 100-fold with either NFW or 50% formamide in NFW, heated at 70°C for 5 minutes and chilled on ice before separation by CGE-LIF.
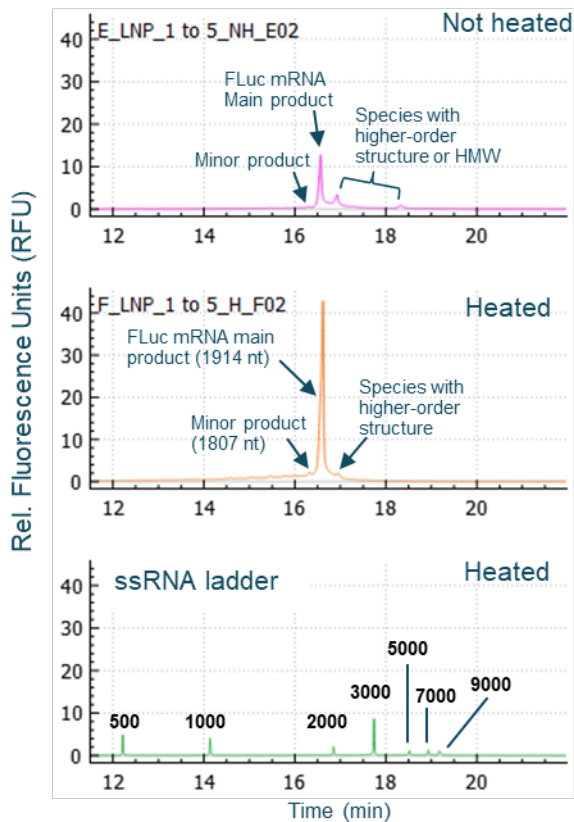
Figure 4. Effects of heat treatment of the mRNA-LNP sample. The mRNA-LNP sample was diluted 5-fold with NFW. Then, the diluted sample was either loaded directly for CGE-LIF analysis or heated at 70°C for 5 minutes and chilled on ice before separation by CGE-LIF.

and the minor product peaks. The mRNA molecules spontaneously fold into various secondary structures through intramolecular base pairing. Furthermore, mRNA molecules can form aggregates or multimers. Heating the mRNA sample helps to unfold these secondary structures and break up the aggregates or multimers, leading to a better peak shape and the disappearance of the HMW species, as shown in the middle panel of Figure 4. The sizes of the FLuc mRNA main and minor products were estimated as 1914 nucleotides (nt) and 1807 nt respectively using the ssRNA Ladder from the same run (bottom panel, Figure 4). The increased FLuc mRNA signal in the heated sample compared to the unheated sample suggests that some FLuc mRNA might be released by heating the diluted mRNA-LNP sample. Therefore, in further experiments to analyze the free mRNA, the mRNA-LNP samples were not mixed with formamide and were not heated before CGE-LIF analysis.

Figure 5 shows the results obtained when the mRNA-LNP sample was diluted 5-, 25- and 125-fold with NFW before CGE-LIF analysis. An increased FLuc mRNA signal was obtained when the sample was diluted less as indicated in the left panel. The middle panel shows that a consistent peak profile was obtained at different dilutions. The right panel shows the FLuc mRNA corrected peak area as a function of relative sample concentration in a linear plot. The R^2 value was 0.9990, demonstrating excellent linear correlation between the free FLuc mRNA signal and sample concentration.
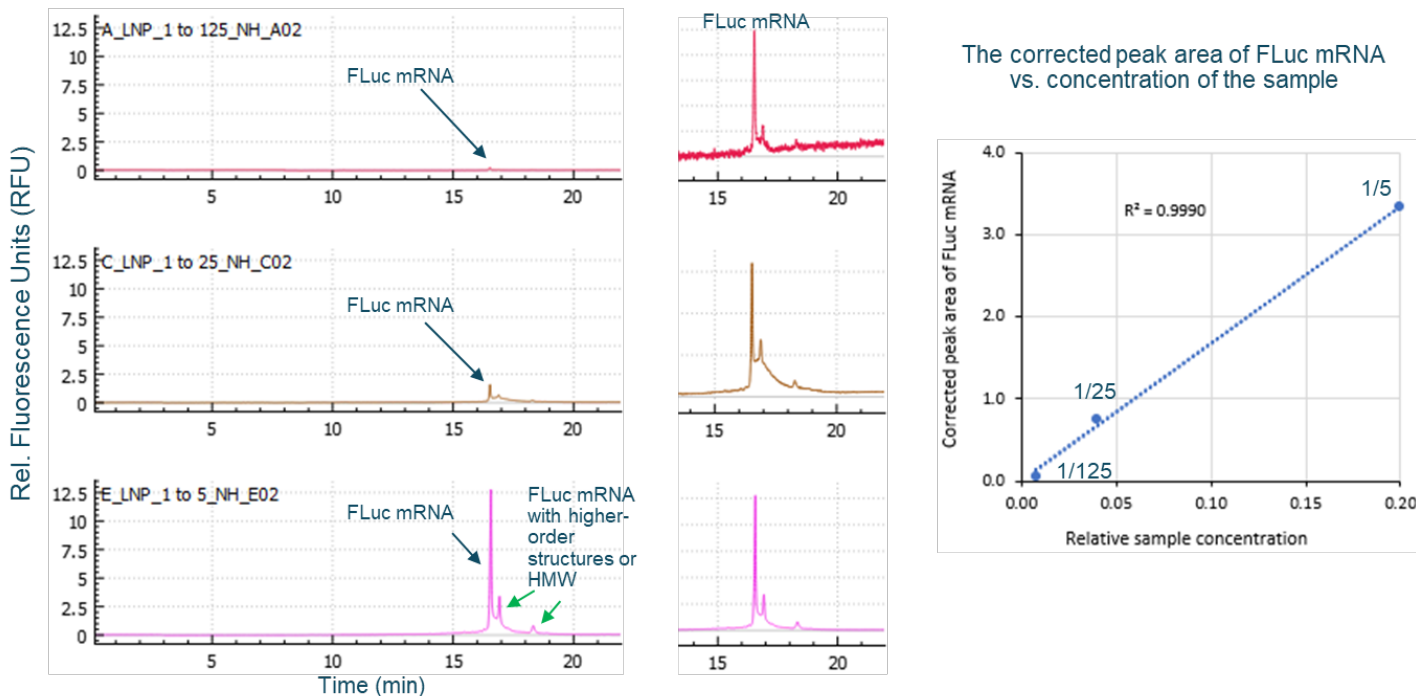

Figure 5.   Analysis of free mRNA at different sample dilutions. The mRNA-LNP sample was diluted 5-, 25- and 125-fold with NFW before CGE-LIF analysis (left). The middle panel shows a zoomed-in view of the FLuc peaks. The right panel shows a linear plot of the FLuc mRNA corrected peak area as a function of relative sample concentration.

Optimization of sample preparation conditions for the deformulation of mRNA-LNP samples

To analyze the encapsulated mRNA, the mRNA must be released from the mRNA-LNPs. A titration experiment was carried out by incubating the mRNA-LNP sample at different concentrations of Triton for 20 minutes at room temperature, then diluting it with an equal volume of formamide and heating before CGE-LIF analysis. Samples prepared from all conditions were analyzed in a single separation run on the BioPhase 8800 system. As shown in Figure 6, 0.2% Triton was sufficient to deformulate the mRNA-LNPs. Any FLuc mRNA detected at 0% Triton can be attributed to the effects of formamide and heat. The mRNA detected in samples treated with Triton is the total amount of mRNA that contains both the free mRNA and the encapsulated mRNA. It is worth noting that the optimal concentration of Triton might differ for mRNA-LNPs with different lipid formulations.

Generation of a calibration curve with the FLuc mRNA standard

A calibration curve was generated by serial dilution of the FLuc mRNA standard, as described in the Methods section. The left panel of Figure 7 shows the overlay of electropherograms obtained from serially diluted FLuc mRNA standard. The right panel shows the FLuc mRNA corrected peak area as a function of concentration over a linear dynamic range spanning 2.7 orders of magnitude. The R^2 value was 0.9989, indicating an excellent linear correlation between the observed signal and mRNA standard concentration. The limit of detection (LOD) was 20 ng/mL and the limit of quantitation (LOQ) was 40 ng/mL.
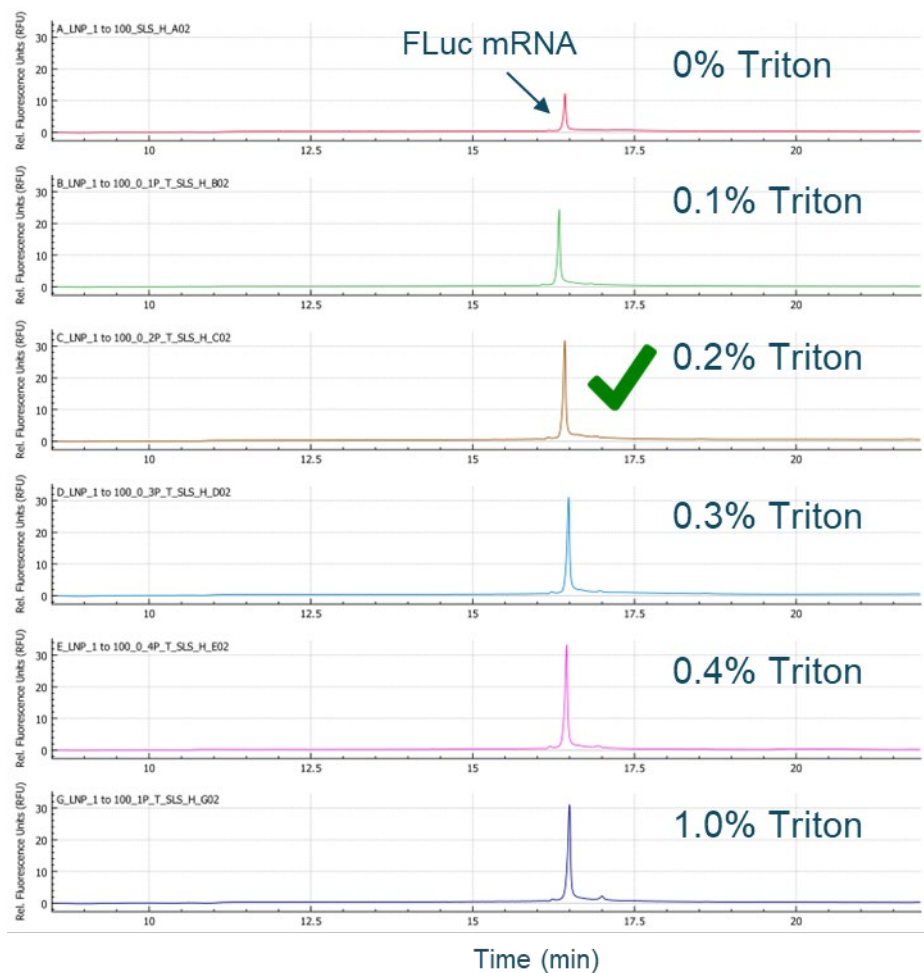

Figure 6. Optimization of the Triton concentration for deformulation of mRNA-LNP. The mRNA-LNP sample was diluted 50-fold with solutions containing different Triton concentrations and incubated for 20 minutes at room temperature, then diluted with an equal volume of formamide and heated before CGE-LIF analysis. Samples prepared under all conditions were analyzed in the same separation run on the BioPhase 8800 system.
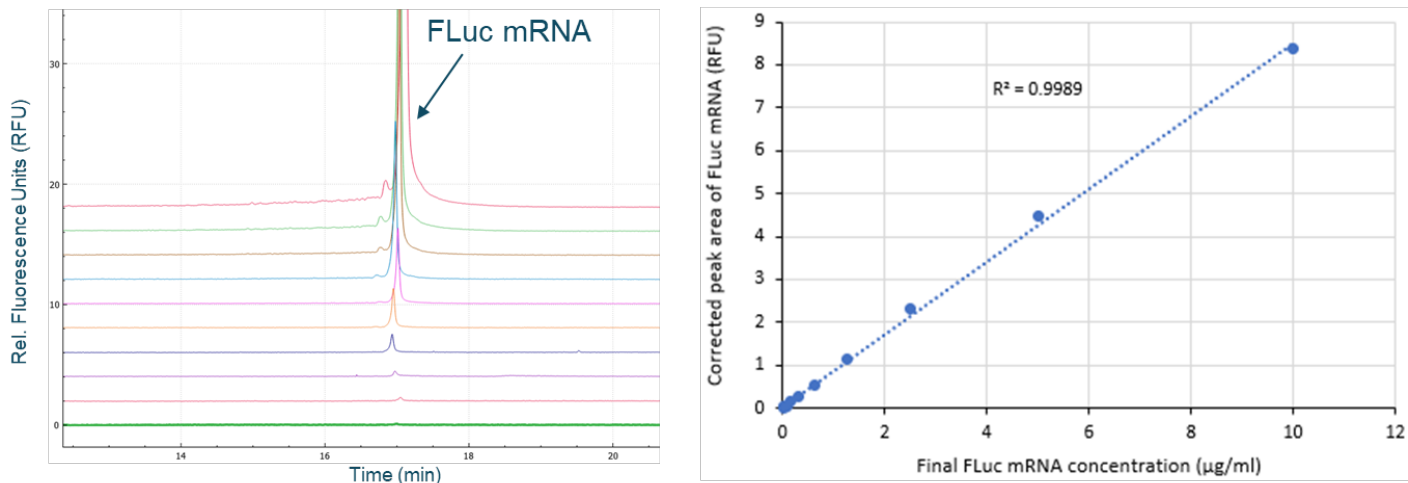
Figure 7. Calibration curve generation using FLuc mRNA standard. The left panel shows the overlay of electropherograms obtained with serially diluted FLuc mRNA samples. The right panel shows the mRNA corrected peak area as a function of FLuc mRNA concentration. The R^2 value was 0.9989, indicating an excellent linear correlation between the observed signal and the mRNA standard concentration.
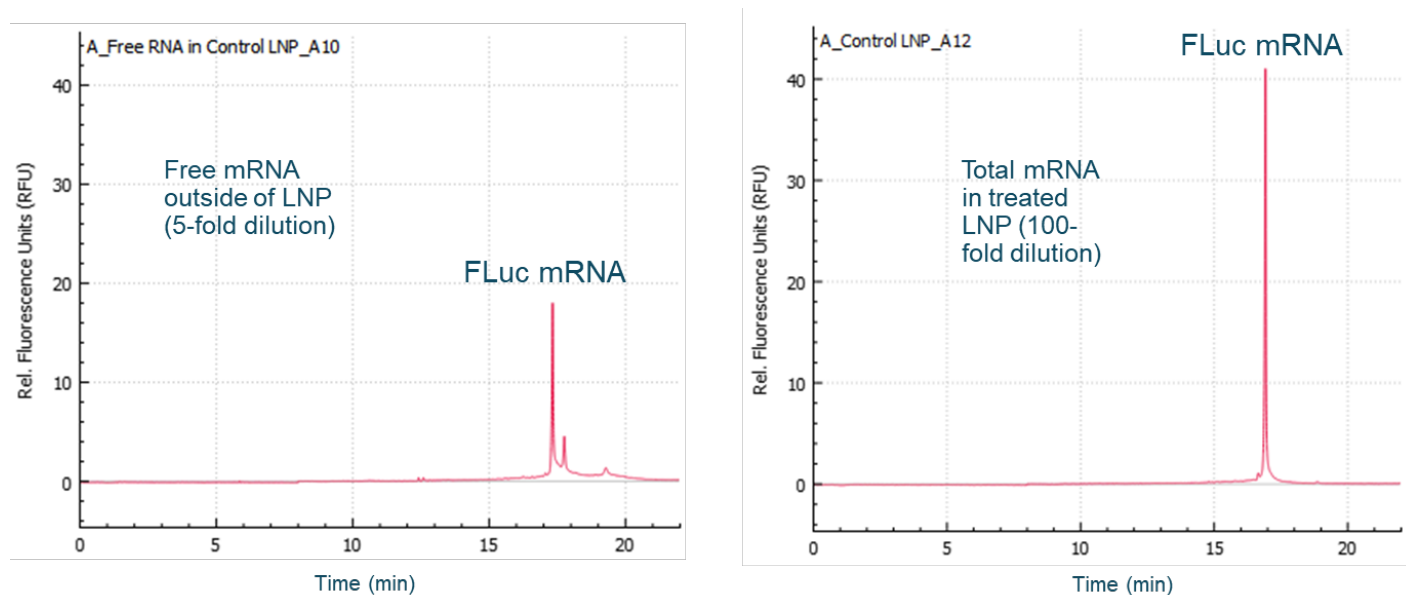

Figure 8. Representative electropherograms of free and total mRNA from the mRNA-LNP samples. In the left panel, the mRNA-LNP sample was diluted 5-fold with NFW without heat treatment before CGE-LIF analysis. In the right panel, the mRNA-LNP sample was deformulated in the presence of 0.2% Triton at room temperature for 20 minutes, then mixed with an equal volume of formamide, heated and chilled on ice before CGE-LIF analysis.

Determination of mRNA-LNP encapsulation efficiency

The generated calibration curve was used to calculate the amount of free mRNA outside the LNP and the total amount of mRNA from the same mRNA-LNP sample. Figure 8 shows representative electropherograms for free and total mRNA from the same mRNA-LNP sample. Free mRNA was detected in the untreated LNP sample at 21 µg/mL and included the main product, minor product and species with higher-order structure or HMW. The total mRNA in the Triton-treated LNP sample was 436 µg/mL. The amount of encapsulated mRNA was calculated by subtracting the free mRNA from the total mRNA as 415 µg/mL. The encapsulation efficiency was determined by dividing the amount of encapsulated mRNA by the total amount of mRNA, times 100. The encapsulation efficiency for the mRNA-LNP sample tested was 95%. The encapsulation efficiency for the same mRNA-LNP sample determined by the RiboGreen dye test was 92%. These results indicate that the encapsulation

efficiency determined by the CGE-LIF method correlates well with the encapsulation efficiency determined by the traditionally used RiboGreen dye test.

With the RiboGreen dye test, 2 standard curves are often prepared with Triton and TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA).[3] The standard curve generated using the Triton buffer is used to determine the total amount of mRNA in an mRNA-LNP sample, whereas the standard curve generated with the TE buffer is used to determine the amount of free mRNA. To verify whether 2 standard curves are needed to determine encapsulation efficiency by CGE-LIF, 2 standard curves were generated with TE buffer and Triton. Using the method with 2 standard curves (Figure 9) indicated that 27 µg/mL of free mRNA and 473 µg/mL total mRNA were contained in the same sample previously analyzed with a single calibration curve (Figure 8). The encapsulation efficiency was 94%, consistent with the encapsulation efficiencies calculated using a single standard curve (95%) or by the RiboGreen dye test (92%).
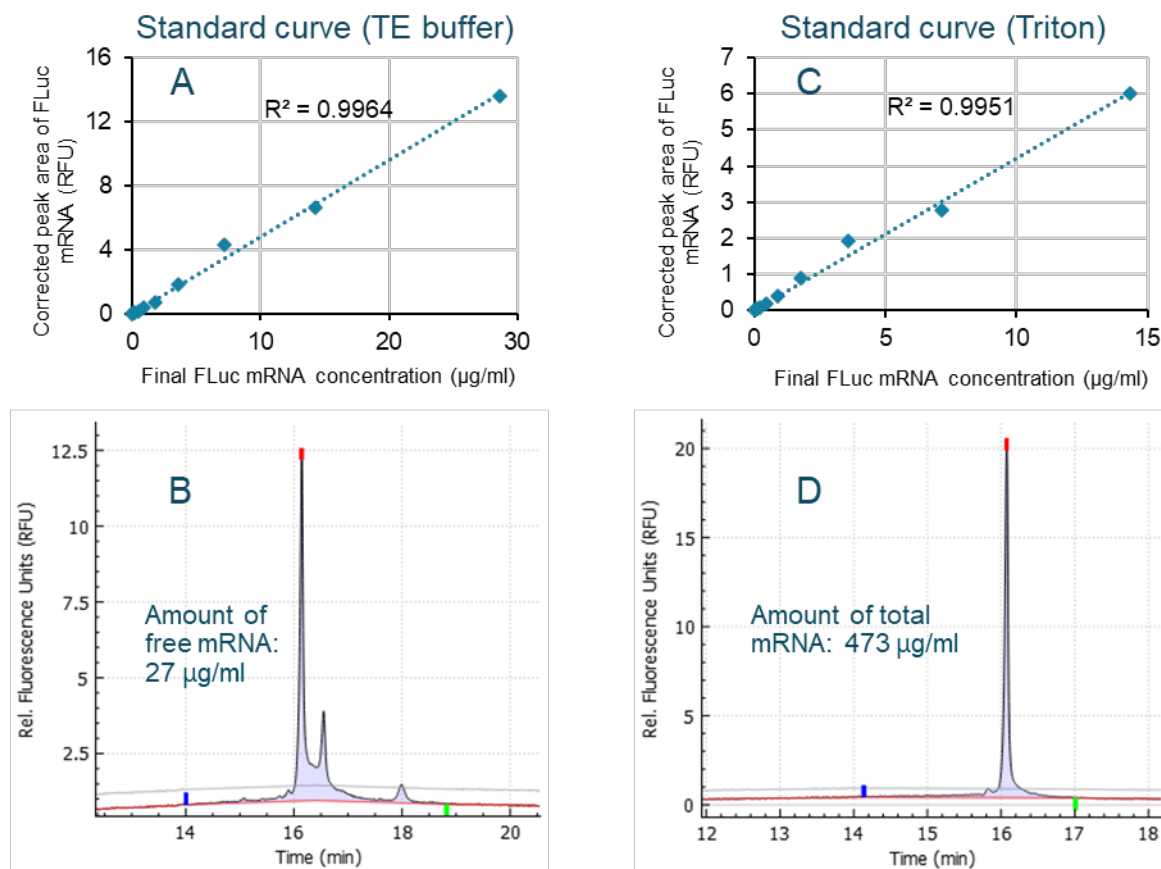

Figure 9. Determination of the free and total mRNA from mRNA-LNP using 2 standard curves. For this experiment, the concentration of the FLuc mRNA stock solution was confirmed as 715 µg/mL by Nanodrop and it was not diluted 2-fold with RNA storage solution before serial dilutions. The standard curve shown in panel A was generated by serially diluting the FLuc mRNA standard in TE buffer. The free mRNA was determined by diluting the mRNA-LNP sample 5-fold in the TE buffer before CE analysis (panel B). The amount of free mRNA was determined as 27 µg/mL based on the standard curve in panel A. The standard curve shown in panel C was built by serially diluting the FLuc mRNA standard in the diluent buffer containing 0.2% Triton. The amount of total mRNA (panel D) in the mRNA-LNP sample was determined as 473 µg/mL based on the standard curve in panel C.

In summary, this technical note demonstrates the use of CGE-LIF to determine the mRNA encapsulation efficiency of mRNA-LNPs. A standard curve was generated using serially diluted FLuc mRNA standards and then used to calculate the amounts of free and total mRNA present in an mRNA-LNP sample (Figure 1). The results generated using CGE-LIF and a single standard calibration curve were verified using 2 additional methods. Both the common RiboGreen dye test and a second CGE-LIF method that used 2 calibration curves provided results consistent with those generated using CGE-LIF and a single calibration curve. Together, these results demonstrate the utility of the BioPhase 8800 system and RNA 9000 Purity & Integrity kit to streamline the determination of LNP encapsulation efficiency by CGE-LIF.

Conclusions

- Sample preparation conditions were optimized for the analysis of free mRNA outside of mRNA-LNP and total mRNA in deformulated mRNA-LNP samples

- A simplified workflow for RNA extraction from mRNA-LNPs using Triton and formamide showed compatibility with the BioPhase 8800 system and the RNA 9000 Purity & Integrity kit

- Detection of unencapsulated mRNA from untreated, diluted samples coupled to analysis of mRNA from deformulated LNPs enabled a robust encapsulation efficiency determination

- The encapsulation efficiency determined by CGE-LIF was comparable to that obtained by the commonly used RiboGreen dye test

- The same workflow can provide information about both the encapsulation efficiency and the size of the main product and its impurities

Patent pending

References

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