APPLICATION NOTE

High Sensitivity & High Throughput Stability Assessment of mRNA in Solution & Lyophilized Solids using the LabChip® GXII Touch™ System – a microfluidic electrophoresis-based analyzer

LabChip® GXII Touch™ System

AUTHORS

Caio H. N. Barros,[1] Elizabeth M. Topp,[1,2] Ilse Villmann[3]

[1]Formulation and Stability Laboratory, NIBRT – National Institute for Bioprocessing Research and Training, Foster Avenue, Mount Merrion, Blackrock, Dublin, Ireland

[2]Department of Industrial and Physical Pharmacy, College of Pharmacy, Purdue University, West Lafayette, Indiana 47907-2091, United States

[3]PerkinElmer, 940 Winter St, Waltham, MA 02451

INTRODUCTION

The COVID-19 pandemic has demonstrated the applicability of mRNA vaccines to protect against infectious diseases. These unprecedented circumstances have also dictated the need to evaluate mRNA stability as initial COVID vaccines had to be distributed at ultracold temperatures. Since then, efforts to develop room temperature stable mRNA formulations have intensified. Dried powder forms of the vaccines show promise for improved shelf stability, and lyophilization (freeze-drying) is among the methods being considered to produce these materials.[1]

Several analytical methods have been used to assess mRNA integrity, but most are intended for applications in biological systems and lack the precision needed to address mRNA stability and degradation mechanisms ex vivo. The simplest assays for nucleic acid purity use UV spectroscopy (A260 and A260/A280) which cannot differentiate intact strands from strand fragments. Functional characterization of mRNA via in vitro translation experiments or reverse transcription via quantitative polymerase chain reaction (RT-qPCR) is also possible, but these techniques are time-consuming, often imprecise, and cannot detect chemical modifications.[2] Chromatographic methods have also been developed using different types of column chemistry, (reverse phase,[3] ion exchange,[4] size exclusion[5]), although these advances have not kept pace with the development of mRNA-based therapies. Capillary electrophoresis systems such as the LabChip® GXII Touch™ HT System address many of the disadvantages and limitations of the other methods. Analyses are high-throughput, fast, and generate less waste than HPLC based assays. The levels of precision, resolution and sensitivity are also greater than the other methods.[6] Moreover, the resulting electropherograms can provide insights into degradation mechanisms from the study of the shape and intensity of native mRNA and fragment peaks.

Here, we show the potential of the PerkinElmer LabChip GXII Touch HT microfluidics technology for assessing the rate and extent of degradation of mRNA in solution and lyophilized formulations. The sensitivity that can be achieved enables the analysis of formulations with mRNA concentrations as low as 15 ng/µl. Since the first vaccines for COVID contained doses of 0.3 mL, at mRNA concentrations to the order of 100 ng/µl, low limits of detection are needed to accurately monitor degradation kinetics when there is 50-90% loss, with corresponding mRNA concentrations as low as 10 ng/ µL. Additionally, the LabChip GX Touch System allows the analysis of up to 24 (low-throughput) or 96 samples (high-throughput) samples per run in a short period with little hands on time. We also show how this system can be useful for the analysis of mRNA encapsulated in lipid nanoparticles (LNPs).

PerkinElmer®
For the Better

Materials

Analyses were carried out in a PerkinElmer LabChip GXII Touch system using the RNA Assay. The High Sensitivity workflow of this assay was performed in all cases. Messenger RNA (mRNA) was prepared using the plasmid pKT305 (AddGene) after linearization with restriction enzyme AscII (Thermo Fisher® FastDigest™ SgsI restriction enzyme) as the DNA template. In vitro transcription (IVT) was carried out using Jena Bioscience mRNA synthesis kit (m6ATP), part number RNT-120-S. Lipids were purchased from Sinopeg. Lyophilization was performed in a Telstar® LyoBeta™ Mini benchtop freeze-dryer.

Methods

The concentration of mRNA obtained by IVT was determined using Thermo Fisher® NanoDrop® analysis and adjusted to 15 µg/mL with mannitol 5% (w/v) solution in RNase-free water. The resulting mixture served as the solution mRNA formulation. To prepare lyophilized mRNA formulations, 18 µL of the solution formulation was added to 2 mL glass lyophilization vials (total of 13 vials) and then lyophilized in the freeze-dryer. The freeze-drying cycle consisted of freezing for 6 h at -40°C, followed by primary drying for 26 h at -35°C and 0.130 mbar, and secondary drying for 8 h at 25°C at the lowest possible pressure permitted by the instrument. The two formulations (solution and lyophilized) were then incubated for 12 h at either 20°C or 50°C. Sample aliquots were retrieved every 2 hours in triplicate and immediately frozen to -20°C to suppress further degradation. Lyophilized formulations were solubilized in RNase-free water to the pre-lyophilization volume. The samples were then analyzed using the LabChip High

Sensitivity RNA Assay

Electropherograms were used to calculate the area of the main mRNA peak and degradation was determined as loss in area of this peak over time. The smear area was determined to provide information on the mRNA degradation products. To normalize any possible concentration deviations among samples, main peak and smear quantity were calculated as a percent of analyte area in the electropherogram. The smear region (100-1700 nt) and main mRNA peak (1700-2600 nt) were defined somewhat arbitrarily based on the shape of the main peak, to allow comparison among samples. Figure 1 shows a schematic of the methods used.

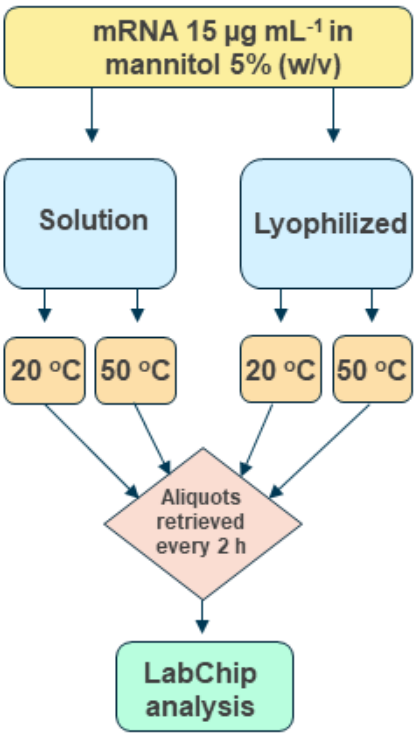

Figure 1: *Flowchart of mRNA stability studies.*

mRNA containing lipid nanoparticles (mRNA-LNPs) were prepared using a NanoAssemblr® Ignite™ system (Precision Nanosystems). The lipids used for the encapsulation of mRNA were (4- hydroxybutyl) azanediyl)bis (hexane-6,1-diyl)bis(2-hexyldecanoate), 1,2-distearoyl-sn-glycero- 3-phosphocholine (DSPC), cholesterol and 2- [(polyethylene glycol)-2000]-N,N ditetradecylacetamide at a molar lipid ratio of 46.3:9.4:42.7:1.6. Initial mRNA concentration of 0.35 mg/mL, final mRNA concentration of 0.04 mg/mL, N/P ratio of 6 and flow rate of 20 mL/min were employed. For buffer exchange after mRNA-LNP preparation, samples were centrifuged in spin filters with a 10 kDa MWCO, and PBS buffer at pH 7.4 was slowly introduced in the suspension after each centrifugation cycle (approximately 3-4 cycles were needed). Nanoparticle size was determined using Dynamic Light Scattering (VASCO KIN™, Corduan Technologies) and encapsulation efficiency was determined using the Invitrogen® Quant-it™ RiboGreen RNA Assay kit as described elsewhere.[7] Optimization of mRNA-LNP sample preparation for LabChip analysis was performed according to Table 1 and based on the existing procedure that has been reported.[8] In all cases, 6 µL of mRNA-LNP suspension was mixed with 20 µL of Brij™-58 10% (w/v) in formamide and 3 µL of sample buffer 10x concentrated.

Table 1: *Optimization of conditions for mRNA-LNP sample preparation*

Sample	Condition
1	No heating and 4°C for 5 min, then addition of surfactant solution and sample buffer
2	70°C for 2 min and 4°C for 5 min, then addition of surfactant solution and sample buffer
3	70°C for 10 min and 4°C for 5 min, then addition of surfactant solution and sample buffer
4	Addition of surfactant solution and sample buffer. No heating, then 4°C for 5 min
5	Addition of surfactant solution and sample buffer, then 70°C for 2 min and 4°C for 5 min
6	Addition of surfactant solution and sample buffer, then 70°C for 10 min and 4°C for 5 min

Results & Discussion

The mRNA electropherogram is characterized by a main peak corresponding to the native strand and a smear assigned to degradation products (Figure 2). The degradation products are of smaller molecular weight than native mRNA, and probably result from phosphodiester bond cleavage at several sites throughout the mRNA molecule. The low limit of detection of the LabChip RNA assay (~5 ng/µL) and the low sample volume requirements allow rapid replicate measurements of the same sample, enabling the precise determination of the degradation profile. In this case, the presence of a sugar excipient, which is common in mRNA vaccine formulations,[2] did not affect the analysis.

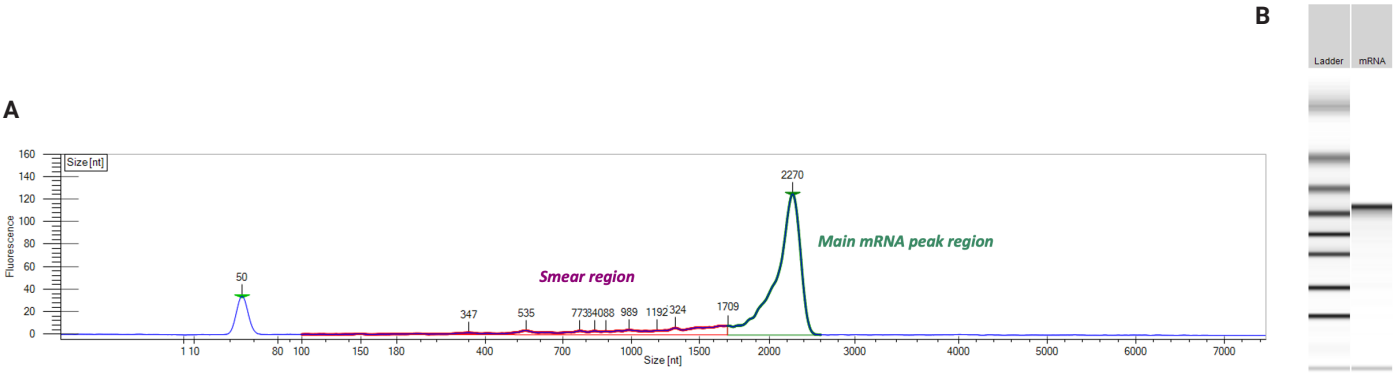

Figure 2: *Electropherogram of a control sample (t=0 h, no stress conditions) of luciferase-encoding mRNA with the main mRNA peak region (1700-2600 nt) and smear region (100-1700 nt) manually defined (A); corresponding gel image generated from the electropherogram (B).*

Electropherograms of the same sample at different incubation times shows a decrease in the area of the mRNA main peak and an increase in the area of the degradation products, here termed the "smear area." Figure 3 shows a comparison of the electropherograms of the lyophilized mRNA formulation after two hours of incubation (t=2 h) and at the end of the incubation period (t=12 h). The electropherograms demonstrate mRNA degradation over the course of the assay as a visible increase in signal of the smear area. After 2 h, a shoulder is observed (~ 1950 nt) corresponding to a smaller mRNA fragment. At 12 h, a peak in this region (1934 nt) is the largest contributor to the main mRNA peak. For a more quantitative analysis, the LabChip GXII Touch Reviewer software can be used to define the areas of interest and determine the percentage of the area occupied by these regions of interest normalized by the total area of the electropherogram.

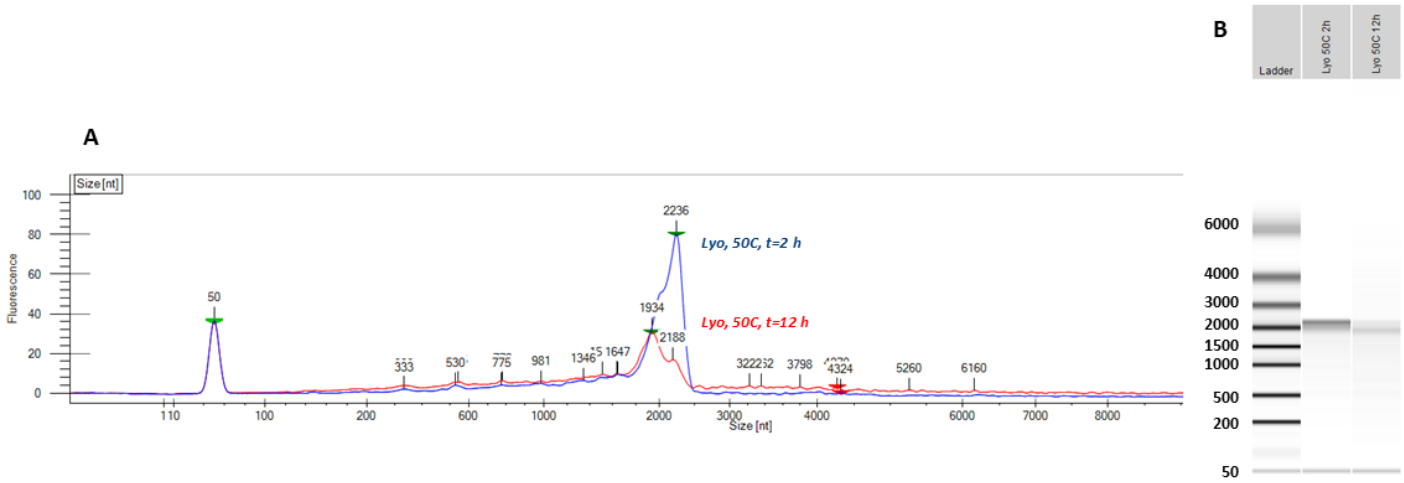

Figure 3: *Electropherograms of lyophilized sample incubated at 50°C after 2 h (blue) and after 12 h (red). Noticeably, the main mRNA peak has its area decreased whereas the smear area slightly increases after the incubation period (A); corresponding gel image generated from the electropherogram (B)*

Using the electropherograms for individual samples, degradation profiles were plotted showing the loss of the mRNA main peak area with time in solution and solid formulations at the two incubation temperatures tested (Figure 4). As expected, formulations incubated at 50°C showed greater degradation than those incubated at 20°C. Specifically, after 12 h at 50°C, ~60% native mRNA in solution and ~45% for lyophilized formulations remained, while there was no significant degradation at 20°C for either formulation type (Figure 4). Interestingly, degradation was greatest in lyophilized formulations at 50°C, suggesting that removing moisture does not decrease the extent of degradation under these conditions. The shapes of the 50°C degradation profiles in solution and lyophilized solids are also noticeably different: whereas the peak area in the solution formulation decreases approximately linearly, the lyophilized formulation shows a more complex pattern comprised of an initial linear decay (t=0 h to t=4h), followed by a brief stabilization or slower decay (t=4 h to t=8h) and then another phase of more rapid degradation (t=8 h to t=12 h).

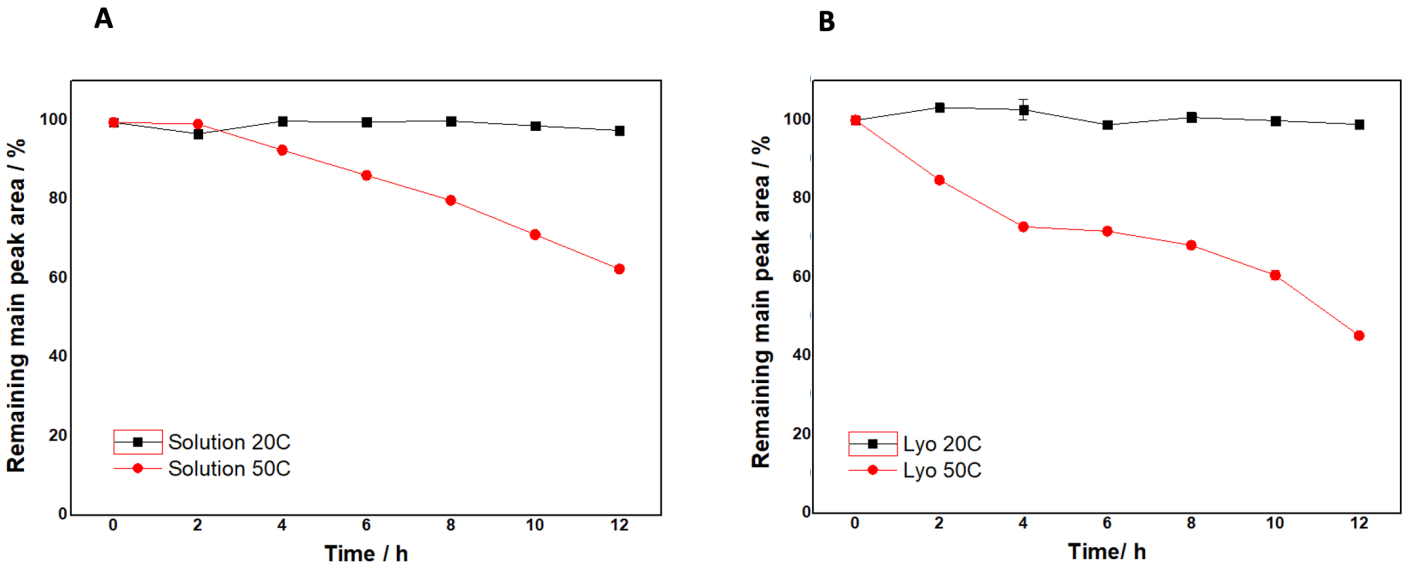

Figure 4: *Degradation profile of mRNA in terms of decrease of main peak area after LabChip GXII Touch Reviewer software analysis in solution (A) and lyophilized formulation (B) (n = 3, error bars are standard errors of the mean).*

Analysis of the smear area confirms the pattern observed. Figure 5 shows the increase in the smear area after 12 h for each of the conditions tested. Only formulations incubated at 50°C had a detectable percentage increase.

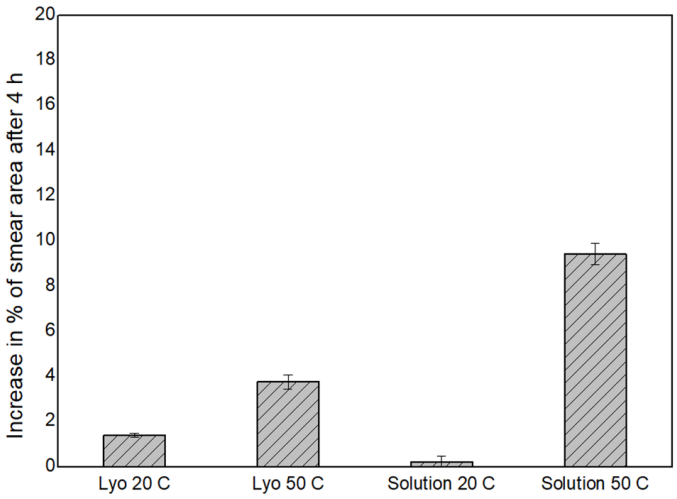

Figure 5: *Increase in smear area after 12 h of incubation (n = 3, error bars are standard errors of the mean).*

Formulations containing mRNA for intramuscular administration are delivered via a lipid nanoparticle (LNP) carrier, which has the function of encapsulating, protecting and transporting the mRNA to its final destination within the organism. The LNP usually consists of four different types of lipids, and therefore the sample preparation of mRNA-LNPs complexes must go through an extra step in which the interactions between lipids and mRNA are destroyed and the mRNA strand goes from condensed and encapsulated to dissociated and unordered. A mix of an adequate surfactant (here, Brij™-58) and formamide is added to the sample to accomplish this. Figure 6 shows electropherograms and gel images of a mRNA-LNP sample which went through different sample preparation procedures. Different incubation times and orders of addition of components were tested in order to better elucidate the best practices of sample preparation for mRNA-LNPs. The mRNA-LNP sample used here has an average particle size of 86 ± 1 nm and an encapsulation efficiency after buffer exchange of 75.2 ± 0.6 %.

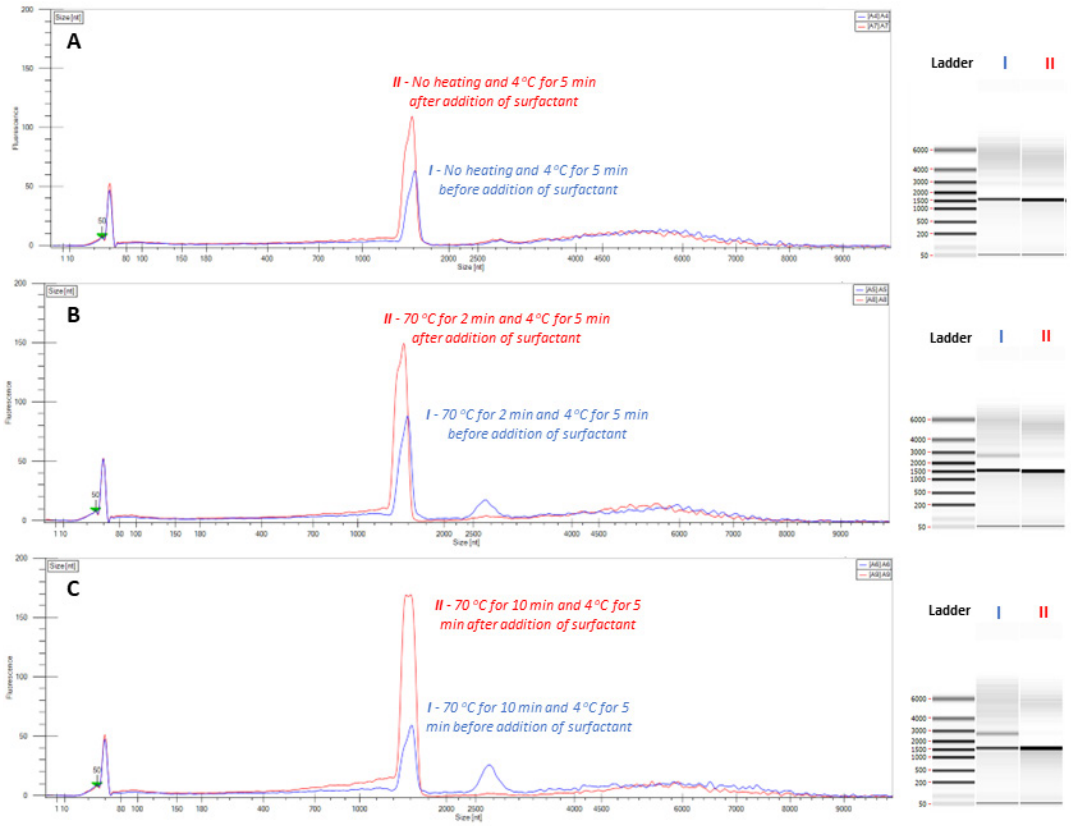

Figure 6:*Electropherograms of mRNA-LNP samples with no heating during sample preparation (A), with heating at 70°C for 2 minutes (B) and heating at 70°C for 10 minutes (C).*

The mRNA peak was sensitive to the sample preparation procedures. Overall, the samples which were subjected to incubation at 70°C in the presence of the surfactant/formamide mixture resulted in more intense mRNA peaks and lower content of higher molecular weight species (both the prominent peak in the region of 2500-3000 nt and the broad, low intensity peak situated between 4000-8000 nt). The duration of the heating step at 70°C also had a significant influence on the shape of the electropherogram, with the longest the incubation time showing the highest quality mRNA peak (i.e., higher intensity and less presence of larger aggregates/species). Incubation times longer than 10 min did not show any further improvements (data not shown). Nevertheless, this effect is only seen for samples exposed to temperature treatment in the presence of the surfactant/formamide solution.

Conclusion

This case study demonstrates the ability of the LabChip® GXII Touch™ system to assess the stability of low concentration mRNA formulations at high throughput. The integrity of mRNA was assessed by monitoring the area percent of the main mRNA peak, which led to the elucidation of the degradation profiles of solution and solid formulations. Additionally, this work gives insights on the sample preparation step of mRNA-LNPs, which is critical for acquiring a good quality electropherogram. A quick and inexpensive extra step is sufficient to improve mRNA signal intensity, hinder the presence of larger complexes and provide reliable identification of the encapsulated mRNA. These capabilities are especially relevant as the “mRNA revolution” moves beyond the initial COVID vaccines toward new products for the prevention and/or treatment of other illnesses. Rapid, reliable analytical methods will be crucial in the development and manufacturing of these new medications.

References

1. *Muramatsu, H., et al. Lyophilization provides long-term stability for a lipid nanoparticle-formulated, nucleoside-modified mRNA vaccine. Molecular Therapeutics 30 (2022) 1941-1951.*
2. *Schoenmaker L., et al. mRNA-lipid nanoparticle COVID-19 vaccines: Structure and stability. International Journal of Pharmaceutics 601 (2021) 120586.*
3. *Issa W., et al. Methods for HPLC analysis. International patent WO 2019/036685 AI. 21 Feb. 2019.*
4. *Thayer J. R., et al. Identification of RNA linkage isomers by anion exchange purification with electrospray ionization mass spectrometry of automatically desalted phosphodiesterase-II digests. Analytical Biochemistry 399 (2010) 110-117.*
5. *Spivak V.B., et al. Analysis of mRNA heterogeneity and stability. International Patent WO 2014/144711 AI. 18 Sept. 2014.*
6. *Kahle J., et al. The next generation of capillary electrophoresis instruments: Performance of CE-SDS protein analysis. Electrophoresis 39 (2018) 311-325.*
7. *Roces, C. B., et al. Manufacturing considerations for the development of lipid nanoparticles using microfluidics. Pharmaceutics 12 (2020) 1095.*
8. *Raffaelle, J., et al. Development of a microchip capillary electrophoresis method for determination of the purity and integrity of mRNA in lipid nanoparticles vaccines. Electrophoresis 0 (2021) 1-6.*

Learn More

LabChip® GX Touch™ Nucleic Acid Analyzer

LabChip® GXII Touch™ Protein Characterization System

PerkinElmer, Inc.
940 Winter Street
Waltham, MA 02451 USA
P: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com

PerkinElmer®

For a complete listing of our global offices, visit www.perkinelmer.com/ContactUs

AG012207_26_AP PKI