High-throughput characterization of RNA using the BioPhase 8800 system

Zhichang Yang, Mario Pulido, Jane Luo, Tingting Li and Sahana Mollah
SCIEX, USA

Introduction

Significant research investment and technological innovation have made messenger ribonucleic acid (mRNA) a promising therapeutic tool in vaccine development and protein replacement therapy.[1] The development and quality control phases of producing therapeutic RNA-based molecules each require extensive characterization of the molecules produced. Accelerated analysis of characterization can save significant amount of time which is crucial for the fast RNA based vaccine development. Here, a multi-capillary capillary electrophoresis (CE) system, BioPhase 8800 system, is used to efficiently separate a wide range of RNA-based molecules by size and demonstrates the multiplex capability for RNA characterization.

The vaccine has been the most successful medical approach for preventing and controlling the spread of infectious diseases,[2] including the pandemic-causing severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Multiple Covid-19 vaccines have been authorized for emergency use, including mRNA vaccines that have been highly effective in controlling SARS-CoV-2. Relative to vaccines that utilize inactivated viruses, purified antigenic subunits of pathogens or DNA to induce immunity, mRNA vaccines offer advantages in safety, efficacy, development speed and large-scale production capability.[1]

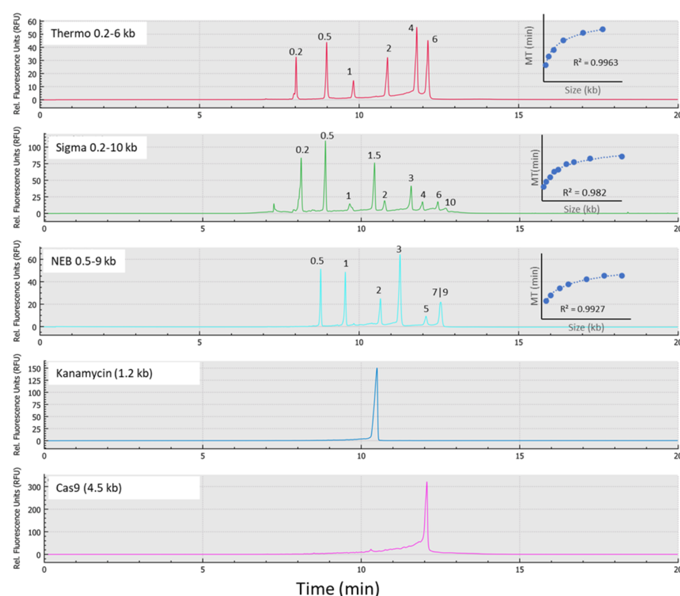

Figure 1. Electropherograms of 3 RNA ladders and 2 RNA standards analyzed in parallel by the BioPhase 8800 system. The insets show the regression analysis of MT against RNA marker size.

Promising results suggest that mRNA can stimulate immune responses to protect against both the influenza virus and Human Immunodeficiency Virus (HIV)[3-6], in addition to serving as an immunotherapy for some types of cancer.[7,8] However, the ubiquitous ribonucleases effect and inherent instability of RNA molecules can cause degradation of RNA structure during preparation, processing, formulation and long-term storage. Thus, it is crucial to characterize the size of therapeutic RNA molecules to ensure their quality, efficacy, safety and optimize the manufacturing process.[9] CE provides fast, robust and high-resolution separation of nucleic acids and can determine size, integrity and purity. The multi-capillary BioPhase 8800 system, which can analyze 8 samples in parallel with the reproducibility, accuracy and resolution required for RNA characterization, is therefore suitable for the high-throughput analyses needed for rapid vaccine development (Figure 1).

Key features

- Multi-capillary separation enables high-throughput screening of samples prepared using different conditions or concentrations of RNA standards
- The multiplex capability of the BioPhase 8800 system expedites RNA characterization, which is crucial for rapid RNA-based vaccine development
- Multi-capillary separation allows for fast linearity analysis with up to 8 concentration point analyzed in parallel.
- CE-LIF achieves high sensitivity and can detect RNA standards at pg/µL concentrations and impurities at < 1% solution concentration

In this technical note, we applied CE-LIF for RNA molecule analysis using the BioPhase 8800 system. Two representative RNA standards, Kanamycin Positive Control RNA (1.2 kb) and Cas9 mRNA (4.5 kb), were analyzed in parallel with 3 commercially available RNA ladders. Cas9 mRNA and the mRNA used in the Pfizer and Moderna vaccines for Covid-19 are similar in size.[10] The RNA size ladders range from 0.2 kb to 10 kb also covers the size range of various RNA-based therapeutical molecules.[1, 10, 11] Calibration curves for 2 RNA standards were analyzed and used to determine the assay linearity and sensitivity. We explored the stability of the RNA standards under different treatment conditions. Finally, we used CE for the impurity determination of the Cas9 mRNA by spiking in Kanamycin RNA at various concentrations.

Methods

Chemicals: Polyvinylpyrrolidone (PVP) (product #: 437190) and Transcript RNA Markers (product #: R7020) were from Sigma Aldrich (St. Louis, MO). Urea (product #: 29700), nuclease-free water (product #: AM9932), SYBR Green II (product #: S7564), and RNA 6000 Ladder (product #: AM7152) were from Thermo Fisher Scientific (Waltham, MA). Tris-Boric Acid-EDTA (TBE) 10x buffer (product #: 574795) was from Millipore (Burlington, MA). The Kanamycin Positive Control RNA (product #: C138A) was from Promega (Madison, WI) and the Cas9 mRNA (product #: L-7206-20) was from TriLink (San Diego, CA). The ssRNA Ladder (product #: N0362S) was from New England Biolab (Ipswich, MA), and the Sample Loading Solution (SLS) (product #: 608082) was from SCIEX (Framingham, MA). Acrodisc 25 mm Syringe Filters with 1.2 µm Versapor membrane (product #: 4488T) were purchased from PALL (Washington, NY).

Materials and instruments: The BioPhase bare fused silica (BFS) capillary cartridge (8 x 30 cm, part #: 5080121), sample and reagent plates (part # 5080311) and BioPhase 8800 system (part # 5083590F) were from SCIEX (Framingham, MA). LIF detection was used with 488 nm excitation and 520 nm emission wavelengths.

Software: The BioPhase analysis software package (version 1.0) was used for method and sequence creation, data acquisition and data processing.

PVP gel preparation: First, 1.325 g of PVP powder was dissolved in 60 mL of nuclease-free water and left on the benchtop at room temperature for 20 min. Once the PVP powder was fully dissolved, 24 g of urea was added to the solution and stirred slowly for 20 min to dissolve. When the solution reached room temperature, 10 mL of TBE 10x buffer was added into the solution. Nuclease-free water was then added to reach a final volume of 100 mL. The resulting gel solution was then filtered using a syringe filter with a 1.2 µm Versapor membrane prior to being stored at 2-8°C. Before use, the SYBR Green II solution was added to the PVP gel solution at a 2500:1, PVP gel/SYBR Green II concentration.

Sample preparation: The RNA ladders were diluted with 1:1, SLS/nuclease-free water (50% SLS solution) before analysis. After dilution, the samples were heated to 70 °C for 2 min by PCR thermal cycler and placed on ice for at least 5 min.

Calibration Curve: 500 ng/µL Kanamycin RNA and 1000 ng/µL Cas9 mRNA were firstly diluted with 50% SLS solution to 10 ng/µL and 20 ng/µL, respectively. Then a 2-fold serial dilution scheme was used to make 15 concentration points ranging from 0.6 pg/µL to 10 ng/µL for Kanamycin RNA and 1.2 pg/µL to 20 ng/µL for Cas9 mRNA.

Stability analysis: 500 ng/µL Kanamycin RNA and 1000 ng/µL Cas9 mRNA were firstly diluted with 50% SLS solution or nuclease-free water to 2.5 ng/µL and 5 ng/µL (200x dilution), respectively. Then, 50 µL aliquots of the diluted RNA samples were subjected to different treatments, including overnight storage at 4 °C, 10 min incubation at 80 °C, or freeze-thaw. For the freeze-thaw treatment, the diluted RNA samples were frozen on dry ice and then thawed at room temperature 5 times. After treatment, all samples were heated to 70 °C for 2 min by PCR thermal cycler and then placed on ice for at least 5 min.

Impurity analysis: Cas9 mRNA was first diluted to 5 ng/µL in 50% SLS solution. The diluted sample was then aliquoted into 50 µL per tube. Diluted Kanamycin RNA was then spiked into the Cas9 mRNA solution at concentrations including 2.5 ng/µL (2x), 0.625 ng/µL (8x), 0.313 ng/µL (16x), 0.156 ng/µL (32x), 0.0781 ng/µL (64x), 0.0391 ng/µL (128x).

Capillary gel electrophoresis on BioPhase 8800 system: The separations were accomplished using BFS capillaries filled with PVP gel buffer. The capillary conditioning and separation method conditions are shown in Figure 2, panels A and B, respectively. The capillary conditioning method was performed at the beginning of each sequence.

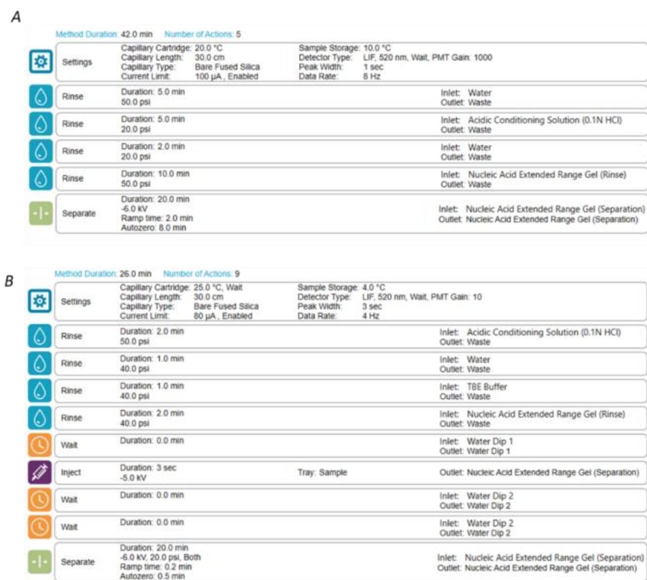

Figure 2. Screenshot of the conditioning and separation methods used on the BioPhase 8800 system. Panel A shows the detailed procedures for conditioning. Panel B shows the detailed procedures for separation.

Results and discussion

Simultaneous analysis of the 2 RNA standards and 3 RNA ladders: The multi-capillary environment of the BioPhase 8800 system enabled simultaneous analysis of the 2 RNA standards and 3 RNA ladders, as shown in Figure 1. This workflow was fast and highly efficient, requiring less than 15 minutes to achieve separation of the RNA molecules. The RNA markers were baseline resolved, except for the 7 kb and 9 kb markers for the ssRNA ladder (Figure 1, NEB). This result indicates that the PVP gel used for this analysis was more suitable to resolve small RNAs than large RNAs. This observation was confirmed by the good logarithmic fit of regression of migration time (MT) against RNA size, shown in the insets of the ladder electropherograms in Figure 1.

Based on the RNA ladders, the estimated sizes of the Kanamycin RNA and Cas9 mRNA were slightly larger than expected. This discrepancy might be explained by differences in the nucleic base composition of the ladders and RNA standards. The PVP and urea composition of the separation gel could be optimized to better resolve larger RNA sizes and improve accuracy for size determination. This workflow achieved simultaneous analysis of RNA standards and ladders from different vendors which would be beneficial for fast RNA size determination and identifying the most suitable RNA ladder to use.

Linearity analysis with 2 RNA standards: Kanamycin RNA and Cas9 mRNA at stock concentrations of 500 ng/µL and 1000 ng/µL were firstly diluted 10 ng/µL and 20 ng/µL, respectively. A 2-fold serial dilution was then performed. As shown in Figure 3, the peak intensities of both RNA standards exhibited good linearity as a function of concentration ($R^2 > 0.995$). The assay covered 3 orders of magnitude for both Kanamycin RNA (0.005 ng/µL-5 ng/µL) and Cas9 mRNA (0.005 ng/µL-10 ng/µL). The LOD was 0.005 ng/µL for both Kanamycin RNA and Cas9 mRNA with S/N ratios of 8.4 and 3.3, respectively. No intra-capillary signal normalization was performed. These results indicate that this workflow is a robust solution to run multiple concentration points simultaneously through the multi-capillary system. As multiple samples can be analyzed simultaneously and reliably, the parallel processing capability of the BioPhase 8800 system shortens method development time.

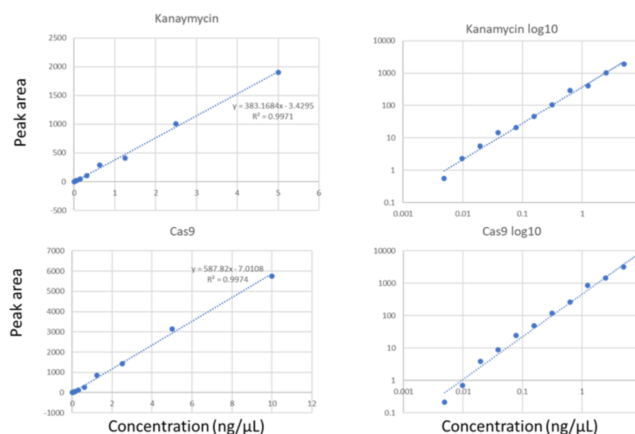

Figure 3. Linearity analysis of the 2 RNA standards. The graphs on the right were adjusted to a logarithmic scale (log10).

RNA analysis repeatability: The RNA 6000 ladder was used to determine instrumentation reliability. The RNA ladder was diluted from 150 µg/mL to 0.75 µg/mL (200x dilution) and aliquoted to 100 µL volumes into 8 wells of the sample plate. 6 consecutive injections were performed. Figure 4 shows an overlay of the 48 replicates. From the 48 injections in total, the relative standard deviation (RSD%) was calculated for both MT and corrected peak area % (CPA%) for the RNA 6000 ladder markers (Table 1).

Table 1. RSD% of MT and CPA for all RNA 6000 ladder markers.

	0.2kb	*0.5kb*	*1 kb*	*2 kb*	*4 kb*	*6kb*
CPA%	4.67	1.87	2.19	1.96	3.22	3.6
MT	*0.82*	0.85	0.92	0.94	0.94	0.93

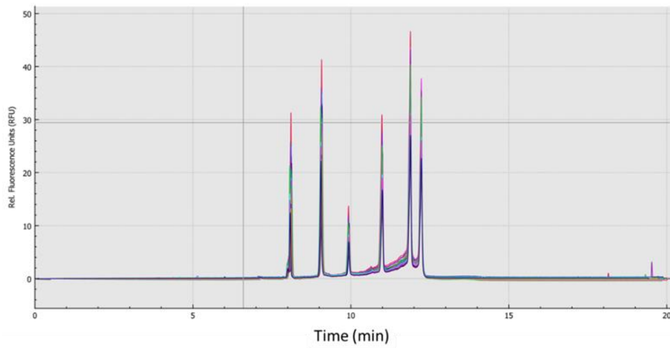

Figure 4. Repeatability analysis of RNA 6000 ladders using 48 replicates.

The RSD% is < 5% for CPA% and <1% for MT across all the RNA markers. These results indicate that the assay is highly reliable.

Fast assessment of different sample treatment conditions in a stability study: The Kanamycin RNA and Cas9 mRNA standards were treated in either water or 50% SLS solution to assess sample stability. Each sample type was subjected to a handling method, including overnight storage at 4 °C, 10 min incubation at 80 °C, or freeze-thaw. Figure 5 shows the electropherograms of the RNA standards subjected to different combinations of treatments. Notably, Cas9 mRNA is unstable in water (Figure 5: i-l), as severe degradation occurs even in the absence of a handling treatment. However, Cas9 mRNA appears to be stable in SLS solution (Figure 5: m, n, p). In contrast, Kanamycin RNA was stable in both water and SLS solution, with good peak intensity and consistent RNA profiles observed in both conditions without a handling treatment (Figure 5: d, h). The data indicate that heating the samples to 80 °C for 10 min was detrimental to the integrity of both RNA molecules (Figure 5: c, g, k o), while the freeze-thaw treatment reduced signal intensity compared to control (Figure 5: b **vs.** d, f **vs.** h, n **vs.** p). For Kanamycin RNA, overnight storage at 4 °C slightly reduced the signal compared to control (Figure 5: a **vs.** d, e **vs.** h).

In summary, the parallel processing capability of the BioPhase 8800 system allowed for fast determination of optimal sample handling conditions. These results indicate that Cas9 mRNA is best handled with SLS solution with no long-term heat treatment nor no freeze-thaw cycles. Kanamycin RNA is best handled with either water or SLS solution with no freeze-thaw cycles nor overnight storage at 4 °C.

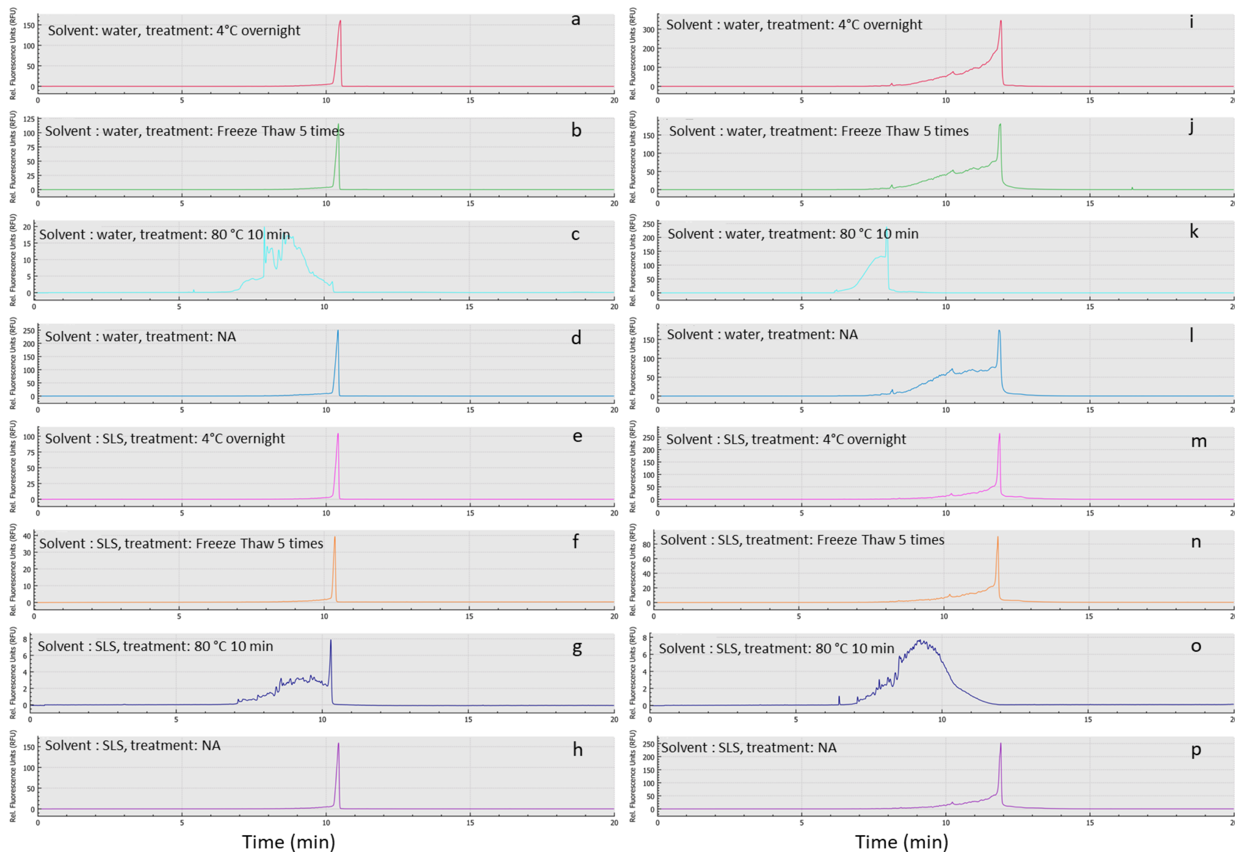

Figure 5. Electropherograms of Kanamycin RNA and Cas9 mRNA diluted and handled using different conditions. The left-side panels are results for Kanamycin RNA (a-h). The right-side panels are results for Cas9 mRNA (i-p).

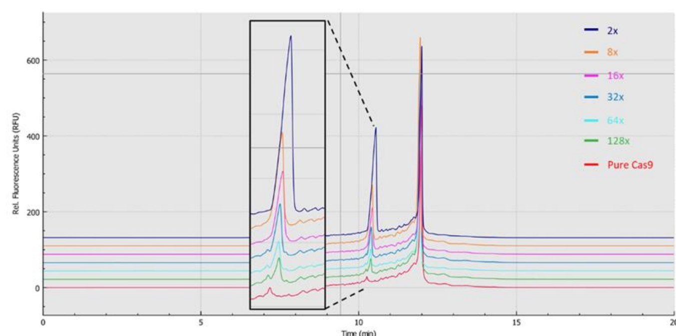

Figure 6. Electropherograms of Kanamycin RNA spiked into Cas9 mRNA at different concentrations.

Low-level impurity detection: Kanamycin RNA standard was spiked at different concentrations into the Cas9 mRNA sample. Briefly, 500 ng/µL Kanamycin RNA was diluted with 50% SLS solution to 125 ng/µL and then subjected to 2-fold serial dilutions. One µL of diluted Kanamycin RNA was spiked into 50 µL of the 5 ng/µL Cas9 mRNA solution. The Cas9 mRNA has endogenous impurities at small sizes, as indicated from the red trace shown in Figure 6. The spiked-in Kanamycin RNA can be detected and separated from other small size impurities, even at low (<1% of Cas9 mRNA concentration) concentration. These impurity tests were analyzed in parallel to expedite analysis. This result highlights the capability of the BioPhase 8800 system to detect low-level impurities during RNA analysis.

Conclusions

- The BioPhase 8800 system achieved fast and highly efficient analysis of different RNA standards and RNA ladders. It shows the potential for accelerating analysis and characterization of RNA for fast RNA-based vaccine development
- The calibration curves generated for Kanamycin RNA and Cas9 mRNA revealed good linearity over 3 orders of magnitude (R^2>0.995) with reliable detection at pg/µL concentrations
- Low inter-capillary variation afforded reliable analysis of different sample handling condition and concentration.
- The optimal handling conditions for Kanamycin RNA and Cas9 mRNA standards were explored and results indicated that Cas9 mRNA should be diluted with SLS solution. Heating and multiple cycles of freeze-thaw should be avoided when handling RNA molecules.
- The LIF detector of the BioPhase 8800 system has the sensitivity required to detect Kanamycin RNA spiked into a Cas9 mRNA sample at <1% solution concentration

References

1. Pardi N, et al. mRNA vaccines - a new era in vaccinology. Nat Rev Drug Discov. 2018 Apr;17(4):261-279. mRNA vaccines - a new era in vaccinology - PubMed (nih.gov)
2. Zhang C, et al. Advances in mRNA Vaccines for Infectious Diseases. Front Immunol. 2019 Mar 27; 10:594. Advances in mRNA Vaccines for Infectious Diseases - PubMed (nih.gov)
3. Kramps T, et al. Messenger RNA-based vaccines: progress, challenges, applications. Wiley Interdiscip Rev RNA. 2013 Nov-Dec;4(6):737-49. Messenger RNA-based vaccines: progress, challenges, applications - PubMed (nih.gov)
4. Deering RP, et al. Nucleic acid vaccines: prospects for non-viral delivery of mRNA vaccines. Expert Opin Drug Deliv. 2014 Jun;11(6):885-99. Nucleic acid vaccines: prospects for non-viral delivery of mRNA vaccines - PubMed (nih.gov)
5. Iavarone C, et al. Mechanism of action of mRNA-based vaccines. Expert Rev Vaccines. 2017 Sep;16(9):871-881. Mechanism of action of mRNA-based vaccines - PubMed (nih.gov)
6. Reichmuth AM, et al. mRNA vaccine delivery using lipid nanoparticles. Ther Deliv. 2016;7(5):319-34. mRNA vaccine delivery using lipid nanoparticles - PubMed (nih.gov)
7. Wang Y, et al. Systemic delivery of modified mRNA encoding herpes simplex virus 1 thymidine kinase for targeted cancer gene therapy. Mol Ther. 2013 Feb;21(2):358-67. Systemic delivery of modified mRNA encoding herpes simplex virus 1 thymidine kinase for targeted cancer gene therapy - PubMed (nih.gov)
8. Liu L, et al. Combination Immunotherapy of MUC1 mRNA Nano-vaccine and CTLA-4 Blockade Effectively Inhibits Growth of Triple Negative Breast Cancer. Mol Ther. 2018 Jan 3;26(1):45-55. Combination Immunotherapy of MUC1 mRNA Nano-vaccine and CTLA-4 Blockade Effectively Inhibits Growth of Triple Negative Breast Cancer - PubMed (nih.gov)
9. Lu T, et al. High-Resolution capillary electrophoresis separation of large RNA under non-aqueous conditions. J Chrom. A. 2020 May 10; 1618:460875. High-Resolution capillary electrophoresis separation of large RNA under non-aqueous conditions - PubMed (nih.gov)
10. Abu Abed OS. Gene therapy avenues and COVID-19 vaccines. Genes Immun. 2021 Jun;22(2):120-124. Gene therapy avenues and COVID-19 vaccines - PubMed (nih.gov)

11. Zhuang X, et al. mRNA Vaccines Encoding the HA Protein of Influenza a H1N1 Virus Delivered by Cationic Lipid Nanoparticles Induce Protective Immune Responses in Mice. Vaccines (Basel). 2020 Mar 10;8(1):123. mRNA Vaccines Encoding the HA Protein of Influenza A H1N1 Virus Delivered by Cationic Lipid Nanoparticles Induce Protective Immune Responses in Mice - PubMed (nih.gov)

The SCIEX clinical diagnostic portfolio is For In Vitro Diagnostic Use. Rx Only. Product(s) not available in all countries. For information on availability, please contact your local sales representative or refer to https://sciex.com/diagnostics. All other products are For Research Use Only. Not for use in Diagnostic Procedures.

Trademarks and/or registered trademarks mentioned herein, including associated logos, are the property of AB Sciex Pte. Ltd. or their respective owners in the United States and/or certain other countries (see sciex.com/trademarks).

© 2021 DH Tech. Dev. Pte. Ltd. RUO-MKT-02-14115-A.

Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
sciex.com

International Sales
For our office locations please call the division headquarters or refer to our website at
sciex.com/offices