PRECISION NANOSYSTEMS
now part of Cytiva

Strategies for Producing Clinical and Commercial RNA-LNP Drug Products

Standardizing scale-up and reducing time to market

Ian Johnston; Maria Kerin; Robert Young; Ashley Braun; Braeden MacDougall; Anastasia Lazic; Brian Ma; Leanna Yee; Kelsey Schwartz; Karen Markwick; Andrew Tapper; Logan Ingalls; Vinay Mayya, PhD; Emily Soon; Sijo Chemmannur, PhD; Andra St. Quintin; Jeff Farnese; Curtis Robin; Laura Ho; Pierrot Harvie, PhD; Suraj Abraham, PhD; Felix Yuen; Jeffery Lam, PhD; Pam Arstikaitis, PhD; Jay Paquette, PhD; Vicki Stronge, PhD; Samuel Clarke, PhD

Document ID: commercialformulationsystem-AN-OCT23

Precision NanoSystems ULC, Vancouver, BC, Canada

Introduction

The lipid nanoparticle (LNP) delivery technology underlying the prophylactic messenger RNA (mRNA) vaccines against SARS-CoV-2 infection has been clinically validated by the approval of Comirnaty® by Pfizer and Spikevax™ by Moderna[1-2]. This success is now driving interest in LNP-mediated delivery across a diverse range of new therapeutic approaches and targets. For infectious diseases, therapies for Zika virus, Influenza A, Rabies and plague are either in clinical trials or have appeared in literature[3-6]. For rare disease, applications such as protein replacement include delivery of mRNA that encodes for a missing or underexpressed protein ***(Figure 1, top)***. In addition, LNPs can be used to deliver gene-editing components such as CRISPR/Cas9 nucleases for permanent insertion or deletion of genes, which has applications for *in vivo* therapeutics as well as *ex vivo* engineering of allogeneic cell therapies for cancer therapy[7]. The RNA-LNP therapeutic market is expected to more than double by 2036 due to a healthy pipeline of new therapies: 22 RNA-LNP therapies are currently in phase 1; 16 are in phase 2/3; and 3 are currently marketed[8]. Despite this momentum, developing RNA-based vaccines and therapeutics still faces significant manufacturing challenges. First, RNA-LNP instrumentation must produce the same drug product at both the discovery and the commercial scales. Second, the manufacturing process upstream and downstream of the preparation of the RNA-LNP needs to be designed and de-risked to minimize process development time and accelerate timelines. Third and finally, the manufacturing process needs to be compliant with increasing regulatory requirements. In this application note, we discuss strategies for how NxGen™ mixing can solve these three key challenges.

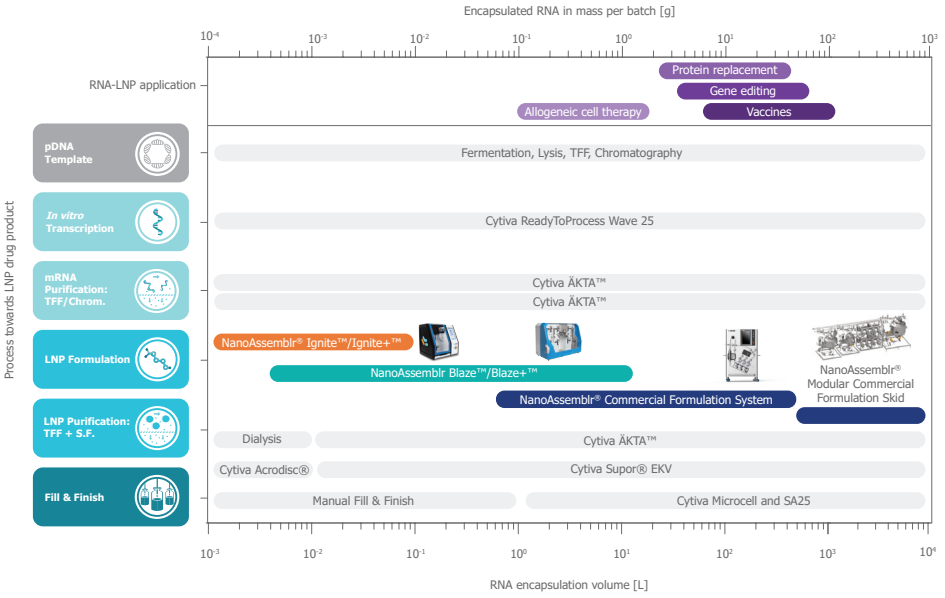


Figure 1. Overview of mRNA therapeutic applications and manufacturing workflow. Top) *Emerging and established applications of RNA-LNP therapies according to batch scale.* ***Left)*** *Key unit operations in preparing an RNA-LNP and representative instruments according to batch scale. The top scale is the mass of RNA encapsulated per batch on a log scale. The bottom scale is the equivalent RNA-LNP formulation volume, also on a log scale. Initialisms and abbreviations: TFF – tangential flow filtration, Chrom – chromatography, S.F. – sterile filtration.*

The production of an RNA-LNP therapy can be divided into six broad steps **(Figure 1, left)**: 1) production of pDNA template via fermentation, 2) *in vitro* transcription (IVT) reaction to generate the mRNA drug substance, 3) purification of the mRNA with chromatography and tangential flow filtration (TFF), 4) formation of the RNA-LNP drug product by mixing an aqueous stream of RNA with lipids in ethanol and subsequent in-line dilution, 5) removal of ethanol via TFF and sterile filtration, and 6) aseptic fill and finish into vials. The RNA encapsulation in an LNP in step 4 is among the most difficult unit operation to scale up to high throughput rates and large batch sizes, as this step requires highly controlled mixing of RNA and lipids. The mixing process is intrinsic to the drug product and impacts the physical properties, potency and toxicity of the drug. Furthermore, it is cost-prohibitive and time-consuming to do extensive process for development for RNA-LNPs at full scale.

As RNA-LNP drug products develop and move from discovery through process development to commercial production, adherence to regulatory requirements becomes increasingly important. In moving from late-stage process development to commercial manufacture, four regulatory requirements should be considered: 1) 100% ethanol is often used to dissolve lipids and is classified as a flammable liquid which can be ignited in air at ambient temperatures. Local regulations may require Hazardous Location (HazLoc) rated equipment to process a flammable liquid. Two HazLoc certifications are ATEX (ATmosphères EXplosibles) and IECEx (International Electrotechnical Commission System for Certification to Standards Relating to Equipment for Use in Explosive Atmospheres); 2) software supporting electronic records should be suitable for use in a regulated environment in a manner complying with FDA 21 CFR Part 11; 3) bioburden of the system needs to be controlled to support process validation, either with a clean-in-place (CIP) and subsequent validation or by using gamma-irradiated single-use technologies (SUTs); and 4) fluid-contacting materials should be assessed for extractables and leachables (E&L) using guidelines from the United States Pharmacopeia (USP) <665>.

Precision NanoSystems has developed the NanoAssemblr® suite of instrumentation which utilize NxGen mixing for RNA-LNP production. Specifically, the fluid channels were initially developed in two sizes, NxGen and NxGen 500, allowing for highly reproducible and scalable production of RNA-LNPs over a wide range of flow rates and volumes, overcoming key limitations of bulk methods (such as turbulent in-line mixing). All NxGen mixing cartridges are single-use, which reduces the risk of cross-contamination between batches. The NanoAssemblr® Ignite™ and Ignite+™ produce 1–60 mL of RNA-LNPs per run for preclinical formulation development, optimization, early process development, and working on initial process parameters for downstream process development. The NanoAssemblr Blaze™ and Blaze+™ can manufacture up to 10 L, which allows for further scale-up as well as upstream and downstream process development.

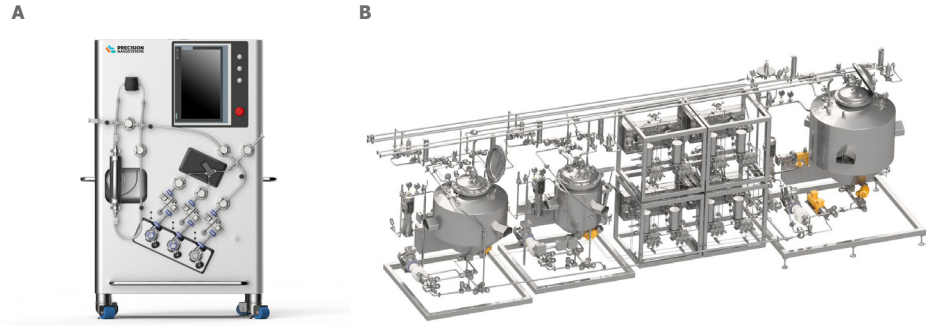


Figure 2. NanoAssemblr instruments from Precision NanoSystems for the preparation of commercial scale batches.
A) *The NanoAssemblr commercial formulation system.* ***B)*** *The NanoAssemblr modular commercial formulation skid.*

Precision NanoSystems has recently developed two systems with software that enables 21 CFR Part 11 compliance*, are ATEX and IECEx** rated, and that leverage the NxGen commercial cartridge 48 L/h for clinical and commercial RNA-LNP production. The NanoAssemblr commercial formulation system ***(Figure 2A)*** is designed to produce RNA-LNPs for clinical phases 1–3 and commercial production where the batch size is between 0.5 and 400 L. This system supports LNP flow rates between 6 and 48 L/h (100 and 800 mL/min) pre-dilution using a NxGen 500 mixer (marketed as the NxGen commercial cartridge 12 L/h) or the NxGen commercial cartridge 48 L/h as appropriate. To reduce turnover time between batches and eliminate cross-contamination between different workflows, the system uses a gamma-irradiated single-use fluid path that includes pumps, flow meters and a NxGen cartridge. The commercial formulation system supports both the NxGen 500 and the new NxGen commercial cartridge 48 L/h. The NanoAssemblr

**Pending third party audit*
***Pending final certification*

modular commercial formulation skid ***(Figure 2B)*** is designed to produce large commercial production batches between 400 L to 3200 L in volume for applications including phase three gene editing, protein replacement therapies, and pandemic response vaccines. This skid is configurable to user requirements and uses up to eight NxGen commercial cartridges simultaneously to produce up to 384 L/h of RNA-LNPs.

In this work, we demonstrate the capabilities of the commercial formulation system and modular commercial formulation skid, and the corresponding workflow that enables rapid progression from small-scale LNP production for discovery research through to large-scale manufacturing for commercial production. First, we show that POPC:Chol liposomes produced using the NxGen, NxGen 500 and NxGen commercial cartridge 48 L/h follow the predicted limit-size behavior in response to increasing flow rate and that high-quality liposomes can be produced at up to 48 L/h (800 mL/min) flow rates. To demonstrate scalability of NxGen technology, we next used a plasmid DNA as an mRNA surrogate and prepared batches of pDNA-LNPs of up to 50 L using the NxGen commercial cartridge 48 L/h. Finally, we selected a clinically relevant LNP composition to demonstrate how the commercial formulation system and modular commercial formulation skid can simplify the deployment of a self-amplifying mRNA (saRNA) LNP vaccine candidate for SARS-CoV-2. We establish critical quality attributes (CQAs) at low volumes and flow rates using the Ignite+ and stepwise increase the flow rate to 800 mL/min using the Blaze, commercial formulation system, and modular commercial formulation skid. We demonstrate equivalency in CQAs between systems and mixers using common physicochemical assays and by testing biological activity in *in vitro* cell lines and *in vivo* animal models.

Materials & Methods

Materials & Consumables

RNA	SARS-CoV-2 saRNA construct (~12,000 nt), Precision NanoSystems, custom composition
pDNA	pcDNA3.1+N-eGFP plasmid, Genscript™, custom synthesis
Lipid reagents	Liposomes: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), NOF America, Coatsome™ MC-6081 Cholesterol, Millipore Sigma, C8667 Lipid Nanoparticles: LNP lipid composition (Ionizable Lipid, DSPC, Cholesterol, Stabilizer), Precision NanoSystems, custom
Buffers	1X PBS, Corning, 21-031-CV Anhydrous ethanol, Commercial Alcohols; P016EAAN RNAse free water, VWR, 02-0201-0500 Acidic buffer, Precision NanoSystems, custom Cryopreservation buffer, Precision NanoSystems, custom
General consumables	15 mL Nunc™ Conical Sterile Tubes, Thermo Fisher Scientific, 339651 50 mL Nunc™ Conical Sterile Tubes, Thermo Fisher Scientific, 339652 100 mL Nalgene™ High-Speed Round-Bottom PPCO Centrifuge tube, Thermo Fisher Scientific, 3110-1000 Allegro™ 2D biocontainer, 5 L, Cytiva, 650-202P Allegro™ 2D biocontainer, 50 L, Cytiva, 650-202W
Filters	Amicon® Ultra-15 30kDa MWCO Centrifugal filter, Millipore Sigma, UFC903008 MidiKros™ 20 cm 300K mPES filter, Repligen, D02-E300-05-S Centramate 93 cm2 30 kD T-Series cassettes, Cytiva, DC030T01 Acrodisc® Syringe filter 0.2 µm Super membrane, Cytiva, PN 4612

RNA quantification	Quant-iT™ RiboGreen® RNA Assay Kit, including 20X TE Buffer, RNase-free, Thermo Fisher Scientific, R11490
Biological reagents	*In vitro* assay: BHK570 Cells, ATCC®, CRL10314™ SARS-CoV-2 Spike S1 Subunit Alexa Fluor®488-conjugated Antibody, bio-techne, FAB105403G Dulbecco's Modified Eagle Medium, ThermoFisher Scientific, 11965084 Paraformaldehyde, Millipore Sigma, 158127-5G Triton X-100, ThermoFisher Scientific, HFH10 *In vivo* assay: BALB/c mice, Private vendor, custom protocol SARS-CoV-2 total IgG ELISA, various vendors, custom protocol

Equipment

NanoAssemblr system and NxGen mixing cartridges	NanoAssemblr Ignite+, Precision NanoSystems, 1001413 NxGen Dilution, Precision NanoSystems, NIN0063 NxGen 500D, Precision NanoSystems, 1001399 NanoAssemblr Blaze, Precision NanoSystems, NIB0055 NxGen 500D, Precision NanoSystems, 1000461 NanoAssemblr commercial formulation system, Precision NanoSystems, 1002276 NxGen commercial manufacturing flow kit 12 L/h, Precision NanoSystems, 1002279 NxGen commercial manufacturing flow kit 48 L/h, Precision NanoSystems, 1002280 NanoAssemblr modular commercial formulation skid, Precision NanoSystems NxGen commercial cartridge 48 L/h, Precision NanoSystems, 1001396
Tangential flow filtration system	Centramate™ LV Holder, Cytiva, CM018LV KR2i, Spectrum Labs, 708-12295-000 KrosFlo™ KR Jr Pump, Spectrum Labs, 708-13683-000 KrosFlo™ Scale, Repligen, ACSS-20k
DLS	Zetasizer™ Nano, Malvern, ZEN1600
Fluorescent plate reader	Synergy™ H1, Biotek, SH1M
Live cell imaging	Cytation™ 7, Biotek, CYT7USN

A. Preparation and analysis of POPC:Chol liposomes

1. The organic phase consisted of a solution of 9.4 mg/mL POPC and 3.92 mg/mL cholesterol in ethanol. The aqueous phase was 1X phosphate buffer saline (PBS). Both phases were filtered through a 0.22 µm filter immediately before use.
2. Using a NxGen, NxGen 500, and NxGen commercial cartridge 48 L/h, POPC:Chol liposomes were formulated at a flow rate ratio of 3:1 aqueous to organic at a range of mixer-appropriate flow rates:
    a. NxGen: 2–20 mL/min
    b. NxGen 500: 25–200 mL/min
    c. NxGen commercial cartridge 48 L/h: 200–1000 mL/min
3. The POPC:Chol liposomes were immediately diluted 1:1 in 1X PBS and sized using DLS.

B. Preparation of pDNA- and saRNA-LNPs

1. The organic phase consisted of a Precision NanoSystems custom LNP lipid composition in ethanol and the aqueous phase was a mild acidic buffer containing the nucleic acid payload. The nucleic acid was either a plasmid encoding green

fluorescent protein or self-amplifying RNA encoding the SARS-CoV-2 spike protein. The in-line diluent was 1X PBS. These reagents were placed in system-appropriate containers: syringes for the Ignite+; conical flasks for the Blaze system; and bioprocess containers for the commercial formulation system and modular commercial formulation skid.

2. For the commercial formulation system and modular commercial formulation skid, additional ethanol and acidic buffer was prepared in bioprocess containers to prime and calibrate the systems.
3. Prior to preparation of the saRNA-LNP batches, all systems except for the Ignite+ system were cleaned-in-place by circulating $NaOH_{(aq)}$ and then rinsing with water for injection (WFI).
4. Batches of 5, 10, and 50 L of pDNA-LNPs were prepared using the NxGen commercial cartridge 48 L/h at an N/P ratio of 8 and a dilution ratio of 3:1.
5. Three batches of saRNA-LNPs were prepared on each system at a flow rate ratio of 3:1 with an N/P ratio of 8:
    a. For the Ignite+, 30 mL of saRNA-LNPs were prepared at total flow rates of 12, 115, and 200 mL/min with an in-line dilution ratio of 0.75:1 diluent to saRNA-LNP solution.
    b. For the Blaze, 30 mL of saRNA-LNPs were prepared at a total flow rate of 115 mL/min and an in-line dilution ratio of 2:1 diluent to sARNA-LNP solution.
    c. For the commercial formulation system, 100 mL of saRNA-LNPs were prepared at total flow rates of 200 mL/min using the commercial cartridge 12 L/h and at 800 mL/min using the NxGen commercial cartridge 48 L/h. Both conditions used an in-line dilution ratio of 3:1.
    d. For the modular commercial formulation skid, 150 mL of saRNA-LNPs were prepared at a total flow rate of 800 mL/min and an in-line dilution ratio of 3:1.

C. Downstream purification of saRNA-LNPs with the Cytiva Delta™ TFF cassette

1. TFF filter, reservoirs, pumps, pressure sensors and scales were set up according to the manufacturer's instructions.
2. The filter was conditioned by rinsing with WFI, $NaOH_{(aq)}$, $NaOAc_{(aq)}$, WFI and finally 1X PBS.
3. The bulk-diluted sample was then concentrated to one tenth the original volume.
4. Once concentrated, the sample was diafiltered with four volumes of Precision NanoSystems' cryopreservation buffer.
5. The sample was then sterile filtered using a 0.22 µm Cytiva Acrodisc® filter in a biosafety cabinet and diluted to 20 µg/mL encapsulated RNA concentration with additional cryopreservation buffer.
6. The samples were then stored at -80°C until needed.

D. Analytical characterization of saRNA-LNPs

1. LNP size and polydispersity were characterized throughout the production process by dynamic light scattering (DLS).
2. RNA concentration was measured using RiboGreen® RNA reagent *(Appendix 1)*.
3. The saRNA integrity was assayed through capillary gel electrophoresis.
4. saRNA-LNP morphology was assessed with cryogenic transmission electron microscopy (cryo-EM).

E. Biological testing of saRNA-LNPs

1. *In vitro* saRNA-LNP potency was assessed with a Baby Hamster Kidney (BHK) cell model where expression of the SARS-CoV-2 spike protein was quantitated by an anti-spike protein conjugated to an AlexaFluor488™ fluorophore.
2. *In vivo* saRNA-LNP immunogenicity was assessed in a BALB/c mouse model using a day 1 prime dose, day 28 booster dose, and day 42 terminal read schedule. The total levels of SARS-CoV-2 specific IgG were assayed from the collected sera via an ELISA.

Results & Discussion

NxGen mixing produces high-quality liposomes and LNPs across a wide range of flow rates

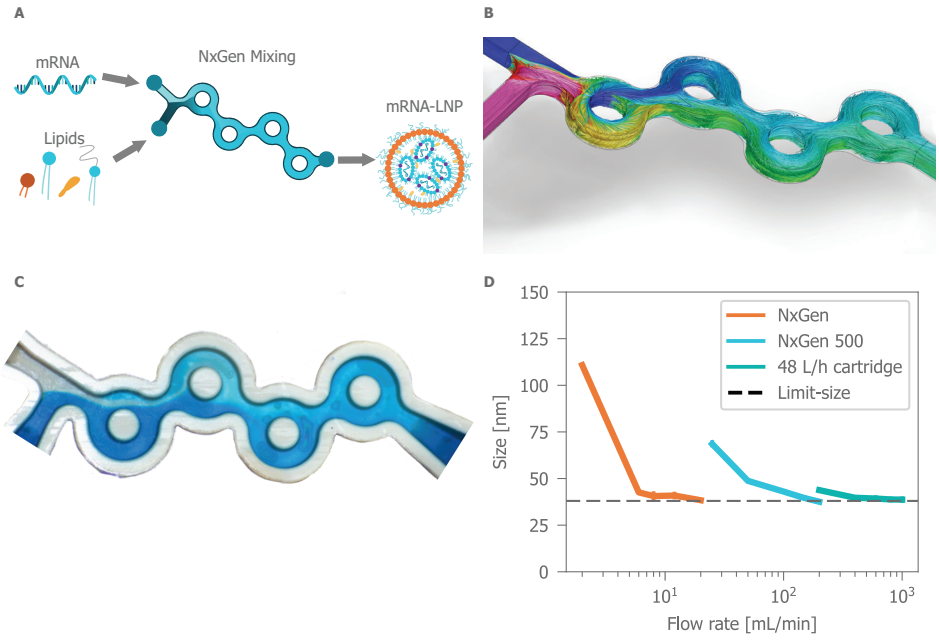


Figure 3. Controlled mixing using NxGen technology allows for production of limit-size nanoparticles across a wide range of flow rates.
A) *Diagram of the mixing process in NxGen mixers.* ***B)*** *Computational fluid dynamic simulation modeling the mixing of water (blue) and ethanol (pink) in the NxGen commercial cartridge 48 L/h.* ***C)*** *Aqueous (no dye) and organic (blue dye) mixing in a modified NxGen commercial cartridge 48 L/h at a flow rate ratio of 3:1 and a total flow rate of 48 L/min.* ***D)*** *The size of POPC:Chol liposomes prepared using the NxGen, NxGen 500 and NxGen commercial cartridge 48 L/h at a range of flow rates.*

The quality of an RNA-LNP drug product is strongly influenced by the mixing process during the encapsulation step. Therefore, a key process development goal in scaling RNA-LNP production is to ensure that critical quality attributes (CQAs), such as size and polydispersity index (PDI), remain unchanged while critical process parameters (CPPs), such as flow rate, are increased to meet the requirements for large batch sizes. The NxGen geometry comprises inlets for RNA and lipids, and four circular, toroidal mixers ***(Figure 3A)***. As fluid travels around the curve of the toroidal mixer, the fluids experience an outward centripetal force causing rotation of the fluid perpendicular to the channel. As this mechanism of mixing does not rely on fabrication of micron-scale features, the toroidal mixers of the NxGen geometry can be increased in size to allow for the high-rates required for large-scale production batches. The NxGen mixer can prepare RNA-LNPs up to 1.2 L/h while the increased size of the NxGen 500 and commercial cartridge 12 L/h can prepare RNA-LNPs up to 12 L/h. To meet the higher throughput necessary for large volume clinical and commercial production, we developed the NxGen commercial cartridge 48 L/h by further increasing the size of NxGen 500.

To verify the NxGen commercial cartridge 48 L/h design, we utilized computational fluid dynamics ***(Figure 3B)*** to model the mixing of water and ethanol in the larger mixer geometry. This analysis shows that complete mixing occurs by the end of the third of four toroidal mixers and is in good agreement with models for the smaller NxGen 500. To validate our computational models, we modified a NxGen commercial cartridge 48 L/h

with a transparent viewing window to enable direct viewing of the mixing fluids. Using this apparatus, we can visualize the mixing of the aqueous phase (clear and colorless fluid) with the organic phase (blue dyed fluid) to produce the fully mixed solution ***(Figure 3C)***. Experimentally, we see that the fluids are fully mixed by the end of the third toroid, suggesting that the computational models are accurately modeling the mixing within the NxGen commercial cartridge 48 L/h.

To test the NxGen commercial cartridge 48 L/h with an LNP formulation, we selected POPC:Chol liposomes as a representative formulation. POPC:Chol liposomes are a well-studied formulation with a limit-size of 40 nm. In past work, we found POPC:Chol liposomes to have a strong response to subtle changes in mixing and thus an ideal formulation to demonstrate controlled and consistent mixing. We prepared POPC:Chol liposomes using a flow-rate ratio (FRR) of 3:1 aqueous to organic and a range of total flow rates (TFR) appropriate to each mixing cartridge ***(Figure 3D)***. The POPC:Chol liposomes reached the predicted 40 nm limit-size at approximately 6 mL/min (0.36 L/h), 80 mL/min (4.8 L/h) and 400 mL/min (24 L/h) for the NxGen mixer, the NxGen 500 mixer, and the NxGen commercial cartridge 48 L/h, respectively. Above the limit-size TFR for NxGen, NxGen 500 and the NxGen commercial cartridge 48 L/h, the POPC:Chol liposomes had equivalent size and PDI, demonstrating how the CQAs for a representative formulation can be conserved while CPPs such as flow rate are increased stepwise over several orders of magnitude.

Figure 4. pDNA-LNPs prepared with the NxGen commercial cartridge 48 L/h in batches of up to 50 L in volume.
Size, polydispersity index and encapsulation efficiency of 3 pDNA-LNP formulations prepared using the NxGen commercial cartridge 48 L/h as a function of batch size (volume). Values are n = 1 for 5, 10 and 50 L batches while the 2 mL NxGen control sample is n =3.

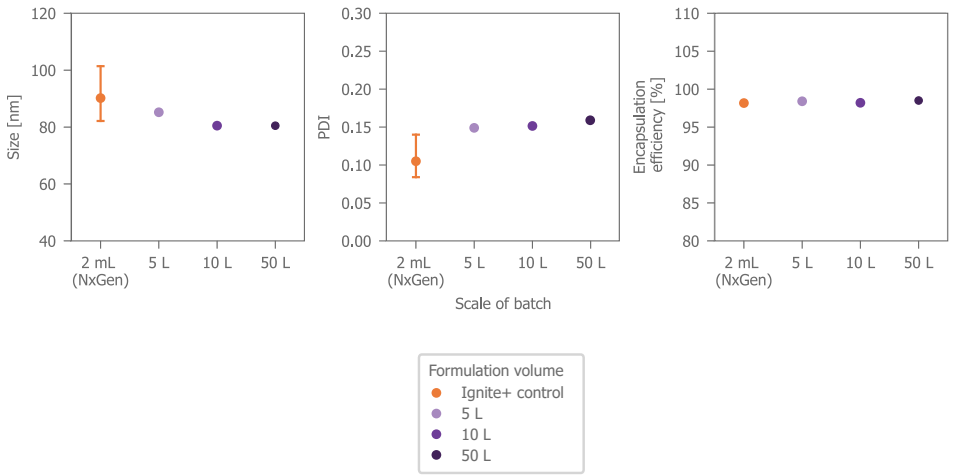


Table 1. Experimental conditions for the preparation of pDNA-LNPs to test the NxGen commercial cartridge 48 L/h.

Formulation volume [L]	Mixer	Flow rate [L/h]	Mass of encapsulated pDNA [g]
0.002	NxGen	0.72	$2.5x10^{-4}$
5	NxGen commercial cartridge 48 L/h	48	0.625
10	NxGen commercial cartridge 48 L/h	48	1.25
50	NxGen commercial cartridge 48 L/h	48	6.25

While POPC:Chol liposomes are appropriate for early-stage screening of small-scale process parameters, they do not contain a nucleic acid payload nor do they have the same lipid complexity of a typical RNA-LNP therapeutic. To demonstrate a more relevant formulation, we designed a four-component LNP composition using an ionizable lipid from Precision NanoSystems' lipid nanoparticle portfolio and a green fluorescent protein (GFP)-encoding plasmid DNA (pDNA) payload as an inexpensive surrogate for mRNA. Next, we formulated three batches of the pDNA-LNPs at 5, 10 and 50 L, using the NxGen commercial cartridge 48 L/h and assessed their physicochemical characteristics.

As a control, we also formulated pDNA-LNPs using a NxGen cartridge on the Ignite+. The pDNA-LNPs produced using the commercial cartridge were of high quality, with the size varying between 80 and 85 nm and a polydispersity index (PDI) below 0.2 in all cases ***(Figure 4)***. We also found similar encapsulation efficiencies of greater than 95% at all batch sizes. The physicochemical results of the pDNA-LNPs produced on the NxGen commercial cartridge 48 L/h were all consistent with the small-scale control on NxGen, providing further evidence for consistent mixing across mixers using an additional composition. The consistency in pDNA-LNPs at large scales between 5 and 50 L indicates stable LNP formulation, regardless of volume, highlighting how the NxGen commercial cartridge 48 L/h can be confidently used to manufacture the large batches needed for commercial production.

Scalable solutions for preparing RNA-LNP drug products

To meet the requirements of large-scale manufacturing for different drug products, the new commercial formulation system is compatible with both the NxGen commercial cartridge 12 L/h (which has identical mixing geometry to the existing NxGen 500 mixing cartridge) and NxGen commercial cartridge 48 L/h, while the new modular commercial formulation skid is compatible with the NxGen commercial cartridge 48 L/h. Because the underlying NxGen technology is designed to scale from the discovery stage to commercial manufacturing, small-scale processes established on Ignite and Blaze can be transferred to the commercial formulation system and modular commercial formulation skid for at-scale production with minimal process development time.

We selected a COVID-19 vaccine model to demonstrate how an RNA-LNP drug candidate can be designed, scaled-up, and produced using NxGen mixing on the new commercial formulation system and modular commercial formulation skid. We developed a lipid composition with an ionizable lipid from the Precision NanoSystems lipid nanoparticle portfolio, for optimal RNA delivery and potency in vaccine animal models, and a self-amplifying RNA (saRNA) encoding for the SARS-CoV-2 pre-fusion spike protein. We performed a study to compare the physicochemical and biological characteristics of SARS-CoV-2 saRNA-LNPs formulated with eight different system and mixer conditions which span flow rates and batch sizes relevant for early process development to commercial production. Initially, we established the limit-size flow rates and CQAs of the saRNA-LNP composition using the Ignite+ system. Next, we stepwise increased the flow rate and batch size of the formulation using the Blaze. Finally, we prepared saRNA-LNPs on the commercial formulation system and the modular commercial formulation skid using the NxGen 500 and the NxGen commercial cartridge 48 L/h where appropriate ***(Table 2)***. In all cases, the saRNA-LNPs were purified with a Cytiva Delta TFF cassette and sterile filtered with a Cytiva Acrodisc filter.

We selected experimental conditions that were representative of RNA-LNP manufacturing workflows. Instruments that require a clean-in-place protocol were prepared a day in advance by circulating sodium hydroxide and then rinsing with WFI. Instruments that use a single-use fluid path, such as the commercial formulation system, had a fresh fluid path installed the day of the RNA encapsulation step. Additionally, we prepared the TFF systems by sanitizing and rinsing the day before. We completed all unit operations from RNA encapsulation through to sterile filtration and freezing of samples in a single day with minimal hold times between steps. We completed the RNA encapsulation and all subsequent unit operations comfortably within a six-hour shift. In addition to added operator convenience, this structured approach to formulation removed any issues which may arise from variable hold-times between unit operations, improving consistency from batch-to-batch.

Table 2. Experimental conditions for preparation of saRNA-LNPs to test the capabilities of the commercial formulation system and modular commercial formulation skid.

Condition	NanoAssemblr system	NxGen mixer cartridge	Total flow rate [L/h]	Batch volume [mL]	RNA Encapsulated [mg]
1	Ignite+	NxGen	0.72	30	1.1
2	Ignite+	NxGen 500	6.9	30	1.1
3	Ignite+	NxGen 500	12	30	1.1
4	Blaze	NxGen 500	6.9	30	1.1
5	Commercial formulation system	NxGen commercial cartridge 12 L/h [Nxgen 500]	12	100	3.3
6	Commercial formulation system	NxGen commercial cartridge 48 L/h	48	100	3.3
7	Modular commercial formulation skid	NxGen commercial cartridge 48 L/h	48	150	5.0

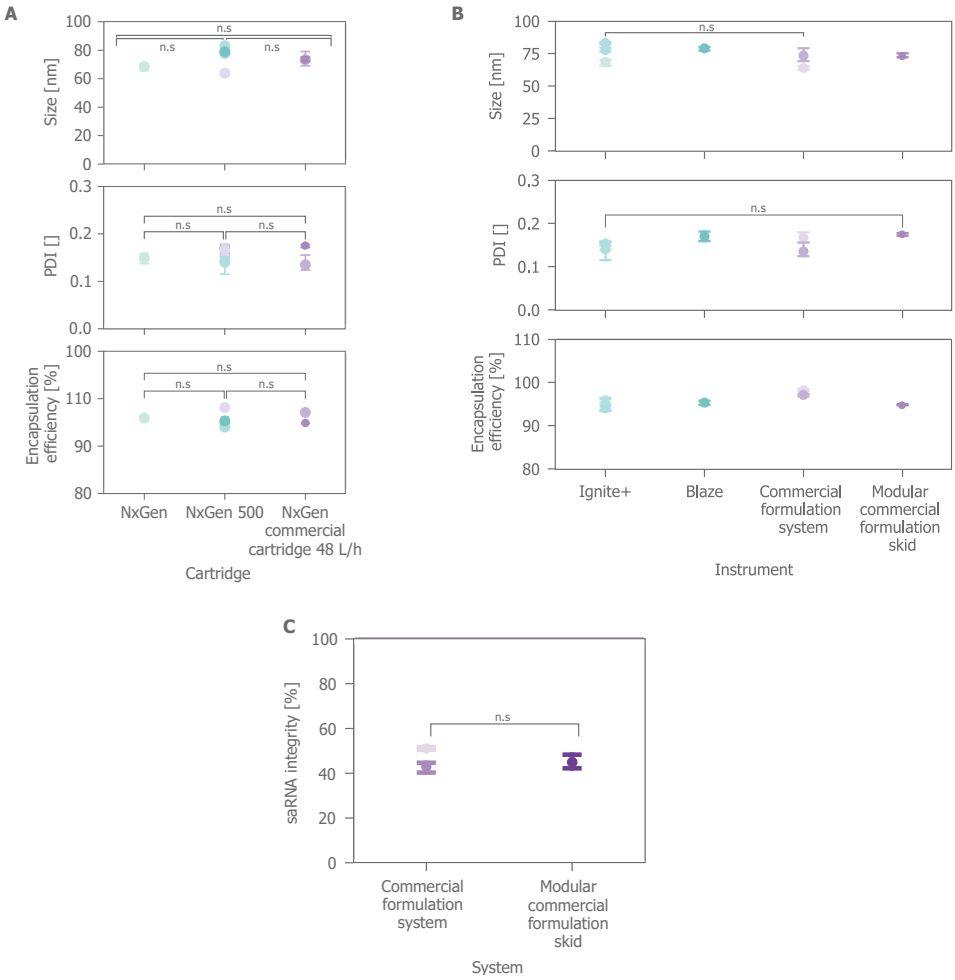


Figure 5. Physicochemical characterization of saRNA-LNPs prepared using NxGen technology. ***A)*** *Size, PDI and encapsulation efficiency as a function of NxGen mixer cartridge.* ***B)*** *Size, PDI, and encapsulation efficiency as a function of system used to prepare the saRNA-LNP.* ***C)*** *saRNA integrity of sterile-filtered samples as assessed by capillary electrophoresis.*

Next, we used common analytical methods to characterize the saRNA-LNPs generated under the eight system and mixer conditions. The saRNA-LNPs from all conditions were of high-quality and within the normal range for size, polydispersity index (PDI) and encapsulation efficiency. Size of the sterile-filtered particles varied from 60 to 86 nm with an average size of 73 nm, while the PDI was an average of 0.18 (SD=0.03). The encapsulation efficiency was above 93% in all cases. Examining the physicochemical data as a function of mixer ***(Figure 5A)*** or point in the saRNA-LNP formulation process (data not shown) shows high degrees of consistency between mixer and system platform. Pooling the data by mixer, a one-way analysis of variance (ANOVA) test found no significant (α=.05) difference in LNP size (p=.53), PDI (p=.31) or encapsulation

efficiency (p=.79) between the different mixers (pairwise comparisons are noted where appropriate). Analyzing the physicochemical properties as function of system again showed consistent results with respect to size and polydispersity ***(Figure 5B)***. Analyzing the physicochemical properties as functions of point in process and mixer type showed that while point in the process was often significant, there were no significant differences between mixer with respect to size (p =.57), PDI (p =.51) or encapsulation efficiency (p =.98) (data not shown). The increase in size throughout the process and decrease in polydispersity after sterile filtration are both expected and within the range of normal variation.

To better understand the structure of nucleic acid payload and lipid nanoparticle, we undertook cryogenic-electron microscopy (cryo-EM) and capillary electrophoresis (CE) measurements. Using cryo-EM, consistent saRNA-LNP morphology was observed in samples prepared by all system and mixer combinations (data not shown). The sizes of saRNA-LNPs analyzed from by cryo-EM were smaller compared to those obtained by DLS, with a mean difference of 8 nm (p =.028) by a paired t-test (data not shown). This can be rationalized by considering that DLS measures the hydration sphere around the RNA-LNP while cryo-EM does not. Next, we performed CE measurements to assess the integrity of the encapsulated saRNA. Comparing the commercial formulation system and modular commercial formulation skid, we found saRNA integrity varied between 40 and 53% ***(Figure 4C)***. A one-way ANOVA comparing system platforms showed no significant difference (α=.05, p =.50) suggesting the three different systems do not impact the integrity of the nucleic acid construct. Overall, we can conclude that saRNA-LNPs produced in this study have consistent CQAs with respect to size, PDI, and encapsulation efficiency regardless of system, NxGen mixing cartridge and flow rate.

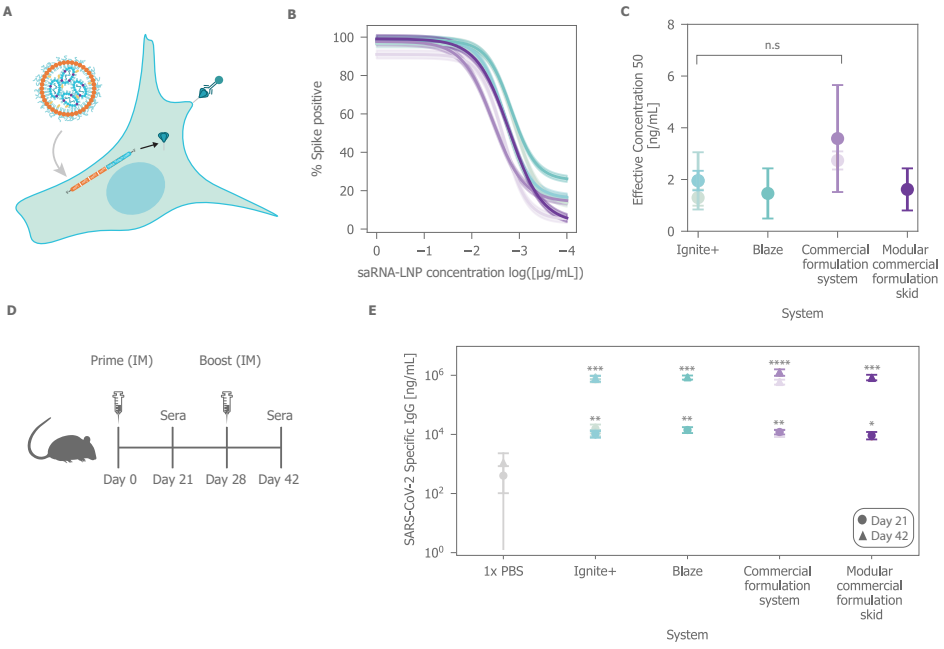


Figure 6. Expression of SARS-CoV-2 antigen and immune response for saRNA-LNPs prepared using NxGen technology. ***A)*** *Schematic of in vitro testing by transfection of BHK 570 cells. The cells were stained with an anti-spike conjugated AlexaFluor488 antibody for fluorescence imaging.* ***B)*** *Percentage of cells expressing SARS-CoV-2 spike protein in BHK 570 cells as a function of saRNA dose for each system and mixer condition with 95% confidence intervals in shaded areas.* ***C)*** *EC_{50} values plotted as functions of system. Error bars are 95% confidence intervals.* ***D)*** *Schematic of in vivo immunization study design with initial and booster dose noted along with sera collection.* ***E)*** *SARS-CoV-2 specific IgG response in serum from BALB/c mice at day 21 and 42 post-injection for each condition. Error bars are 1 standard deviation. 1X PBS versus instrument comparison p-value for a given time point using post-hoc Tukey test after one-way ANOVA (p≤.05: *, p≤.01: **, p≤.001: ***, p≤.0001: ****).*

To evaluate biological activity of the SARS-CoV-2 saRNA-LNP vaccine candidate, we tested the eight system and mixer conditions in both *in vitro* and *in vivo* COVID-19 assays. For *in vitro* testing, BHK 570 cells were transfected with decreasing amounts of saRNA-LNPs to determine the percentage of cells expressing the spike protein ***(Figure 6A)***. By analyzing the dose response ***(Figure 6B)***, we determined that the EC_{50} values for the different conditions varied between 1.3 ng/mL and 3.6 ng/mL, indicating consistent *in vitro* activity ***(Figure 6C)***. Next, we performed a 42 day (6 week) *in vivo* immunization study in BALB/c mice to compare the immune response of the saRNA-

LNPs generated under the eight conditions ***(Figure 6D)***. At day 0 and day 28, 1 µg of saRNA-LNPs was injected intramuscularly for the prime and booster, respectively. After 21 days, most saRNA-LNP conditions produced a statistically significant serum conversion compared to the 1X PBS control ***(Figure 6E)***. At the terminal end point of the study, serum conversion was more pronounced with titers ranging between $1.94x10^5$ to $2.83x10^6$ ng/mL SARS-CoV-2-specific IgG, indicating that all samples were able to elicit a strong immune response. An ANOVA test with *post-hoc* Tukey multiple comparisons test indicates that all instruments produce saRNA-LNPs that induce significant serum conversion in the mouse model (p=.0001). Additionally, there are no significant differences in *in vivo* potency between instrument conditions. Collectively, the data from the *in vitro* and *in vivo* assays suggests that all NanoAssemblr systems produce saRNA-LNPs that are biologically active and generate a robust and consistent pharmacodynamic effect.

Overall, the results from this study show consistent physicochemical and biological results from the SARS-CoV-2 saRNA-LNP vaccine candidate across different NanoAssemblr systems and mixers. We demonstrate how NxGen mixing enables small-scale testing and scale-up of RNA-LNP drugs on Ignite and Blaze, and subsequent at-scale production on the commercial formulation system and formulation skid with minimal process development time. The commercial formulation system and formulation skid enables high throughput and large-scale manufacturing of RNA-LNP drug products for different therapeutic applications and stages of clinical development.

Conclusion

As the field of RNA-LNP medicines continues to gain momentum, systems to encapsulate the RNA drug substance must be scalable and be compatible with larger upstream and downstream unit operations, all while meeting regulatory requirements. In this application note, we demonstrate how the NanoAssemblr commercial formulation system, modular commercial formulation skid, and NxGen commercial cartridge meet the requirements for commercial scale RNA encapsulation. First, using a representative LNP formulation with a series of increasing batch sizes, we demonstrate how CQAs such as size, PDI, and *in vitro* potency and *in vivo* immunogenicity are maintained while CPPs are stepwise increased from values at the discovery scale to the commercial production scale. Second, we show how saRNA-LNPs produced by the NanoAssemblr series of systems are easily processed in downstream unit operations such as TFF and sterile filtration. Third and finally, we described how the commercial formulation system and accompanying flow kit meet regulatory requirements for production of RNA-LNP bulk drug substance.

Appendix A – RiboGreen Assay Protocol

Determining the RNA encapsulation efficiency is necessary for accurate dosing of RNA-LNPs. If the RNA-LNP preparation protocol is followed as outlined in this guide, the RNA-LNPs are expected to be loaded with 100–120 µg/mL of RNA, with variation typically arising from changes in the final sample volume.

The recommended encapsulation efficiency protocol is as follows:

RiboGreen Assay for Determination of RNA Encapsulation Efficiency

Additional Reagents/Disposables

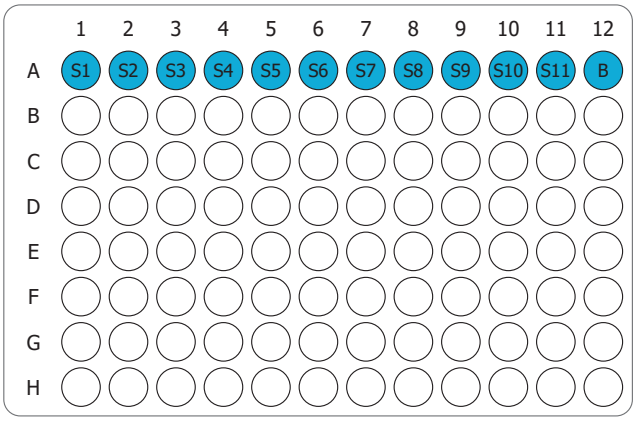
Description	Recommended Supplier
Invitrogen™ TE Buffer (20X), RNase-Free	Thermo Fisher Scientific
Quant-iT™ Ribogreen® Assay Kit	Thermo Fisher Scientific
Triton™ X-100	Sigma Aldrich
RNase-Free Water	General Laboratory Supplier
RNase-Free Filter Pipette Tips (10, 20, 200 and 1000 µL)	General Laboratory Supplier
Pipette Basins	General Laboratory Supplier
96-Well Black Plate	General Laboratory Supplier
Mg^{2+} / Ca^{2+} Free PBS 1x	General Laboratory Supplier
18 Gauge Needles	General Laboratory Supplier

Additional Equipment Required

Description	Recommended Supplier/Product
Plate Reader	Synergy™ H1 Biotek® Plate Reader
Multichannel Pipette (10 –300 µL)	General Laboratory Supplier
Micropipettes (10, 20, 200 and 1000 µL)	General Laboratory Supplier

Preparation of Sample Stock Solutions

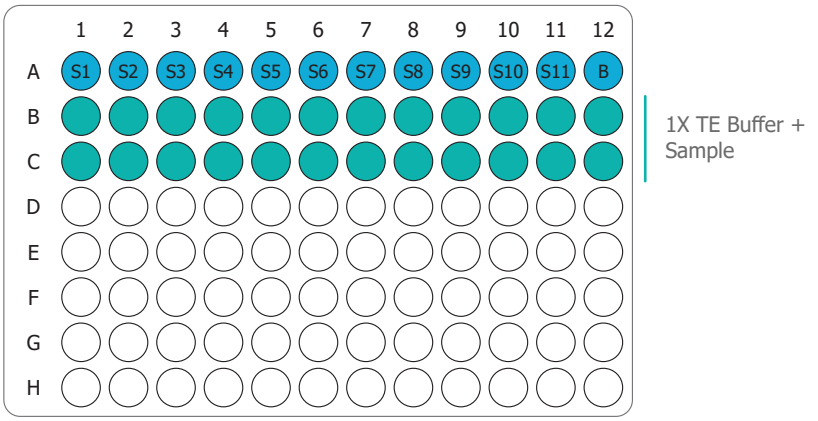
1. Prepare 1X TE buffer from 20X TE buffer by adding 10 mL of 20X TE buffer to 190 mL RNase-free water in a clear glass bottle. Shake the bottle to mix.
2. To 100 mL of prepared 1X TE buffer, add 2 mL of Triton X-100. Stir using a magnetic stirrer for 15 min. This solution is the Triton Buffer.
3. Pour the 1X TE buffer and Triton buffer in separate pipette basins.
4. In the top row of the 96-well plate (Row A), add 15 µL of RNA-LNP sample to these wells (S1-S11). Add 15 µL of PBS to the blank well (B).
5. Using a multi-channel pipette, add 1X TE buffer to Row A to make up the volume to 250 µL. Pipette to mix.



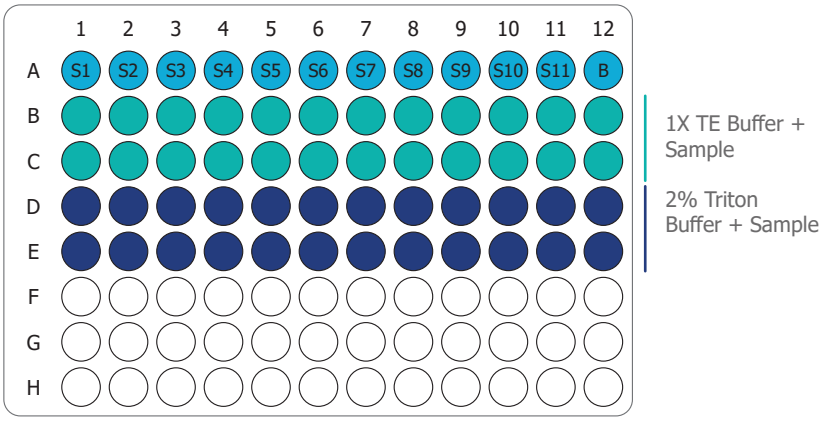
RNA-LNP Sample Setup

This assay is run in duplicate. It is recommended that liquid handling be done using a multi-channel pipette.

1. Add 50 µL of 1X TE buffer to the two wells directly below each RNA-LNP sample (Rows B and C).
2. Add 50 µL of RNA-LNP sample stock solution from Row A into the wells in Row B and C.
3. Add 50 µL of Triton buffer to the wells in Rows D and E below each sample.



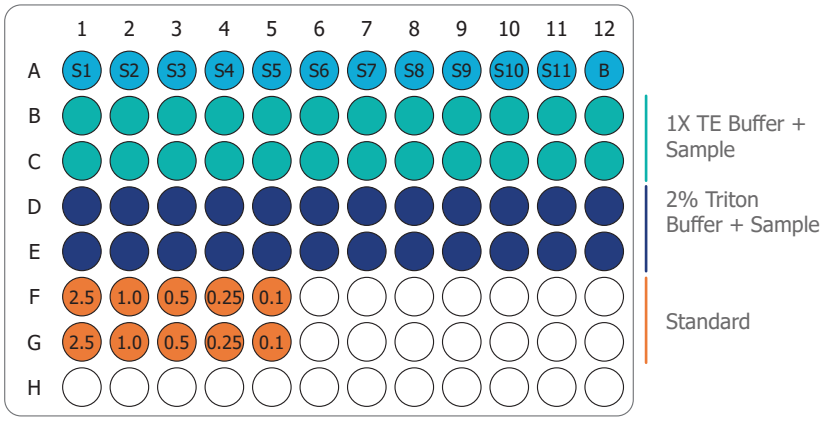
4. Add 50 µL of sample stock solution from Row A into the wells in Rows D and E.



RNA Standard Curve Setup

1. Setup a standard curve (in duplicate in rows F and G) as shown in the table below using the RNA Stock (20 µg/mL RNA), 1X TE Buffer and Triton Buffer.

Final RNA (µg/mL)	**RNA Stock Required** (µL)	**TE Buffer Required** (µL)	**Triton Buffer Required** (µL)	**Total Volume per Well** (µL)
2.5	25	25	50	100
1	10	40	50	100
0.5	5	45	50	100
0.25	2.5	47.5	50	100
0.1	1	49	50	100



2. Once the samples and standard curve are plated, incubate the plate at 37°C for 10 minutes to lyse the RNA-LNPs in the presence of Triton buffer.

Preparation of RiboGreen Solution

1. Sum the total number of sample wells and standard curve wells. Add 4 to this number, and multiply the total by 100. This is the total volume, in µL, of RiboGreen Solution needed for this assay.
2. In a 15 mL RNase-Free Falcon Tube, dilute the RiboGreen Reagent 1:100 into 1X TE buffer to the total volume calculated in the previous step.

   NOTE: For example, if 3000 µL of RiboGreen Solution is needed, add 30 µL of RiboGreen Reagent to 2970 µL of 1X TE buffer.
3. Vortex the RiboGreen Solution for 10 seconds to mix.

Addition of RiboGreen Solution and Sample Reading

1. Remove 96-well plate from 37°C incubator.
2. Add 100 µL of RiboGreen Solution to each well.
3. Pop any bubbles with a needle.
4. Read using fluorescent plate reader with the following settings:

Plate Reader Parameter

Excitation	485 nm
Emission	528 nm
Optics	Top Read
Gain	55
Read Height	8 mm

NOTE: The Gain and Read height will change depending on the instrument.

Sample Analysis

1. Enter each RNA-LNP sample and each Standard Curve sample into the RNA Quantification Workbook (PNI-WB-S9-001-INT). This sheet will calculate the encapsulation efficiency and mRNA concentration of each sample.

   NOTE: The RNA Quantification Workbook can be obtained by contacting your Field Application Scientist at Precision NanoSystems.

2. The second sheet on this workbook (Name: Plate Setup) gives the well numbers from which the O.D. values would be fed into the first sheet (Name: RNA Quantification).
3. The third sheet (Name: Dilution Factor Calculation) in the workbook gives the calculation to input the dilution factor values in column 'O' of the first sheet (Name: RNA Quantification).

References

1. L. R. Baden, *et. al.*, "Efficacy and Safety of the mRNA-1273 SARS-CoV-2 Vaccine," *N Engl J Med* , pp. 403-416, 2021.
2. Polack, F. P; *et. al.*, "Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine," *N Engl J Med*, vol. 383, pp. 2603-2615, 2020.
3. N. Pardi, *et. al.*, "Nucleoside-modified mRNA immunization elicits influenza virus hemagglutinin stalk-specific antibodies," *Nature Communications*, vol. 9, no. 3361, 2018.
4. Richner J. M.; *et. al.*, "Modified mRNA Vaccines Protect against Zika Virus Infection," *Cell*, vol. 10, pp. 1114-1125, 2017.
5. Alberer, M.; *et. al.*, "Safety and immunogenicity of a mRNA rabies vaccine in healthy adults: an open-label, non-randomised, prospective, first-in-human phase 1 clinical trial," *Lancet*, vol. 390, no. 10101, pp. 1511-1510, 2017.
6. Kon, E.; *et. al.*, "A single-dose F1-based mRNA-LNP vaccine provides protection against the lethal plague bacterium," *SCIENCE ADVANCES*, vol. 9, no. 10, 2023.
7. R. Geczy, "Genome Editing of Human Primary T Cells with Lipid Nanoparticles Gene edited CAR T," Precision NanoSystems ULC, Vancouver, BC, 2022.
8. M. Verma, I. Ozer, W. Xie, R. Gallagher, A. Teixeira and M. Choy, "The landscape for lipid-nanoparticle-based genomic medicines," *Nat Rev Drug Discov*, 2023.

Precision NanoSystems:
50 - 655 West Kent Ave. N.,
Vancouver, BC, V6P 6T7
Canada

Precision NanoSystems USA:
395 Oyster Point Boulevard, Suite 145
South San Francisco, CA, 94080
USA

phone: 1-888-618-0031
info@precision-nano.com

precisionnanosystems.com

RELATED MATERIAL

Visit our website regularly for the latest information, Application Notes, webinars and other scientific resources.

precisionnanosystems.com

Document ID: commercialformulationsystem-AN-OCT23