PRECISION NANOSYSTEMS

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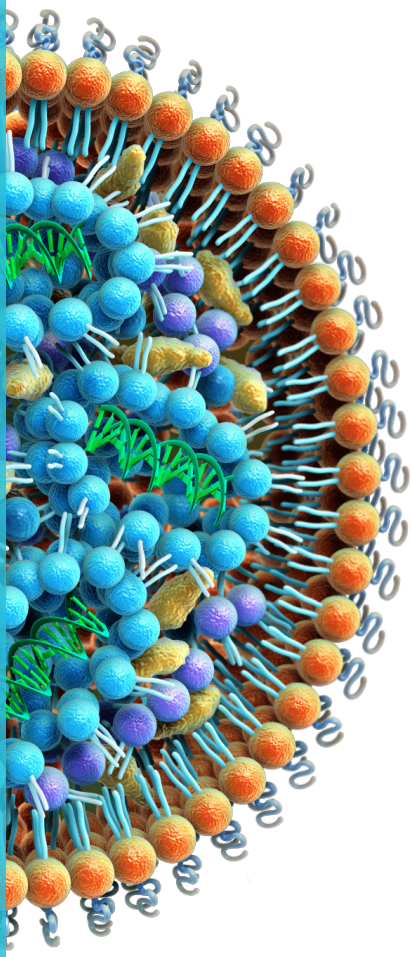
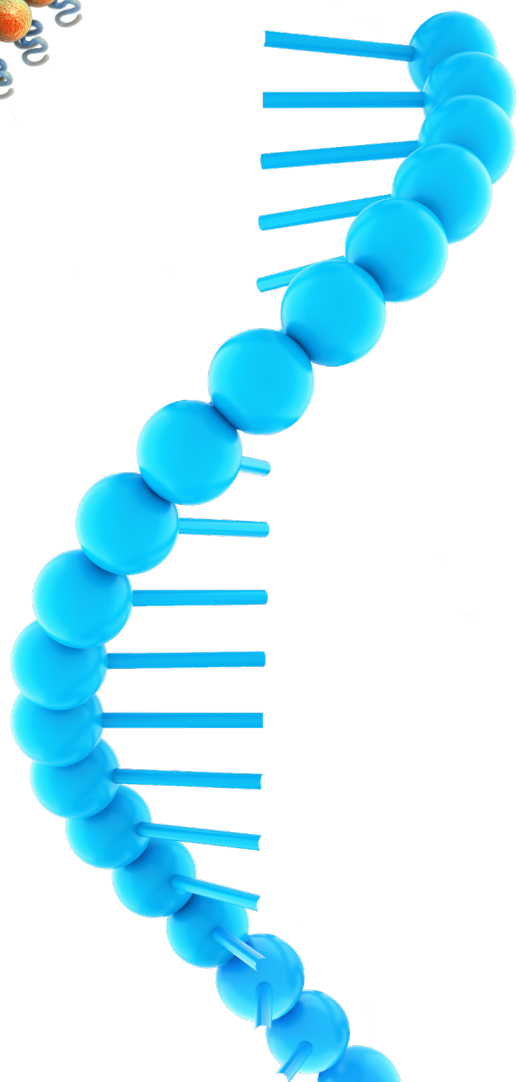
Developing a Scalable RNA-LNP Drug Product for Clinical Translation

Accelerating essential process development activities





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Document ID: ignite+-AN-0622

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Introduction

First identified in 2019, the severe acute respiratory syndrome coronavirus (SARS-CoV-2) is an infectious disease that has resulted in a global pandemic with an unprecedented human impact. As a result, a number of messenger RNA (mRNA)-lipid nanoparticle (LNP) vaccines have been developed: Comirnaty® by Pfizer[1] and Spikevax™ by Moderna[2]. These mRNA-LNP vaccines highlight the therapeutic benefits of genomic medicines deployed at scale. These nanomedicines have also clearly demonstrated the utility of LNP delivery systems to protect RNA from degradation and improve cellular uptake in target tissues. LNP-mediated delivery of RNA and other nucleic acids (NAs) to cells is not limited to vaccines for SARS-CoV-2, or other infectious diseases[1,3–6]. Rare diseases typified by deficiency in a single protein can be treated by the delivery and expression of an mRNA encoding the absent protein[7]. Conversely, diseases characterized by excess production of a given protein can be treated by delivery of short interfering RNA that reduces protein expression, as exemplified by the treatment of hereditary transthyretin-mediated amyloidosis by Onpattro®[7], the first approved RNA-LNP drug. 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 T cells for cancer therapy[8].

A common need for these RNA-LNP therapies is a highly compliant and scalable manufacturing process. The current state-of-the-art for large-scale production of RNA-LNPs can be divided into 4 key steps (***Figure 1A***): 1) production of RNA drug substance using *in vitro* transcription (IVT) from a DNA template, 2) formation of the RNA-LNP drug product by mixing an aqueous stream of RNA with lipids in ethanol and subsequent in-line dilution, 3) purification of the bulk drug product to remove ethanol using tangential flow filtration (TFF) and 4) sterile filtration and final fill-finish in vials.

The LNP encapsulation step requires controlled mixing of RNA and lipids and is among the most difficult unit operations to scale up to high throughput rates and large batch sizes. The mixing process is intrinsic to the drug product and impacts the physical properties, potency and toxicity of the drug[11]. Furthermore, it is cost-prohibitive and time consuming to do process development for RNA-LNPs at full scale. Precision NanoSystems has developed the NanoAssemblr® suite of instrumentation which utilize NxGen™ microfluidic mixing for homogeneous RNA-LNP production (***Figure 1B***). Specifically, the microfluidic channels were 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). The NanoAssemblr Ignite™ produces 1–20 mL of RNA-LNPs per run for preclinical formulation development and optimization; the NanoAssemblr Blaze™ and Blaze+™ can manufacture up to 10 L, which allows for upstream and downstream process development and scale-up; and the NanoAssemblr GMP System manufactures up to 50 L of RNA-LNPs at flow rates of up to 200 mL/min in accordance with current Good Manufacturing Practice (cGMP) guidelines (***Figure 1C***).

Precision NanoSystems has recently developed the new NanoAssemblr Ignite+ instrument which extends the capabilities of Ignite by enabling higher flow rates and larger volumes on the same simple benchtop system. The Ignite+ can manufacture RNA-LNPs at flow rates from 1–200 mL/min and batch sizes of up to 60 mL. This allows for small-scale evaluation of RNA-LNPs using the same critical process parameters (CPPs) that will be used for large-scale cGMP production. In addition, the increased batch size limit of up to 60 mL allows for sufficient material to enable the large-cohort animal studies, non-human primate studies, downstream processing protocol development and stability studies required for late-stage preclinical development.

In this work, we demonstrate the capabilities and workflow of the Ignite+ using two representative lipid compositions. First, we showed that 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine:cholesterol (POPC:Chol) liposomes produced using NxGen and NxGen 500 followed the predicted limit-size behavior in response to increasing flow rate and that

high-quality liposomes can be produced at up to 200 mL/min flow rates. Next, we selected a clinically relevant LNP composition to demonstrate how the Ignite+ can be used to accelerate process development of a self-amplifying RNA (saRNA)-LNP vaccine candidate for SARS-CoV-2. We established initial critical quality attributes (CQAs) for the vaccine candidate at low volume and low flow rate, and then stepwise increased flow rate and batch size to 200 mL/min and 40 mL, respectively. Finally, we demonstrated the utility of the Ignite+ to enable the testing of additional downstream unit operations earlier in the drug development journey. We provide examples to show how automated in-line dilution can be incorporated within the Ignite+ workflow to replace manual bulk dilution, and how to make the transition from small-scale dialysis or spin filters to tangential flow filtration (TFF) for post-formulation purification.

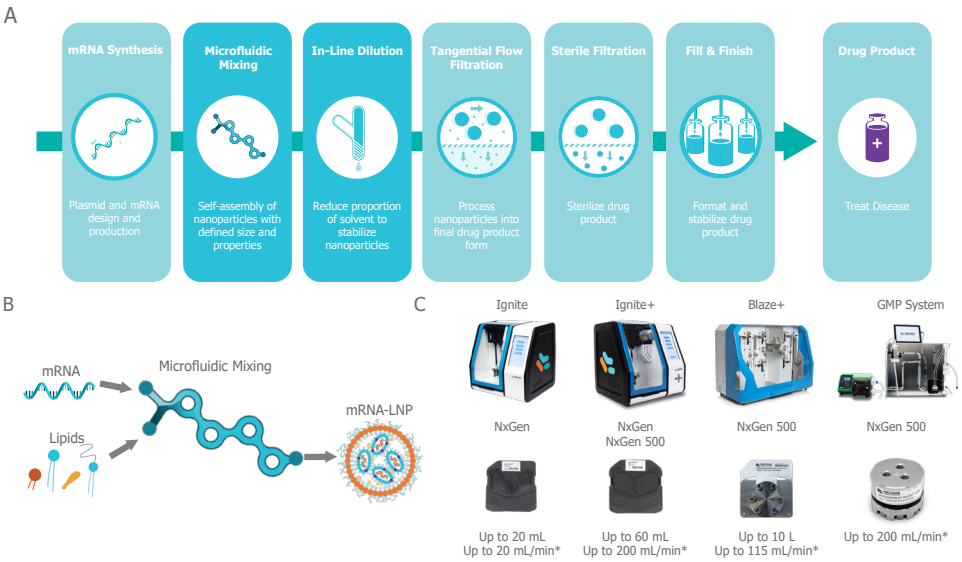


Figure 1. Large-scale production process for preparation of an RNA-LNP drug product using microfluidic mixing.
A) *Key steps for large-scale production of an RNA-LNP drug product.* ***B)*** *The generation of an mRNA-LNP drug product with a NxGen microfluidic mixer.* ***C)*** *The NanoAssemblr instrument series for microfluidic mixing. * Denotes pre-dilution flow rates.*

Materials & Methods

Materials & Consumables

NxGen cartridges	NxGen, Precision NanoSystems, NIN0061 NxGen Dilution, Precision NanoSystems, NIN0063 NxGen 500, Precision NanoSystems, 1001397 NxGen 500D, Precision NanoSystems, 1001399
RNA	Custom SARS-CoV-2 saRNA Construct 1 (~12,000 nt) and Construct 2 (~12,000 nt), Precision NanoSystems
Lipid reagents	Liposomes: POPC, NOF America, Coatsome MC-6081 Cholesterol, Millipore Sigma, C8667 Lipid Nanoparticles: Custom LNP Composition (Precision NanoSystems Ionizable Lipid, DSPC, Cholesterol, Stabilizer), Precision NanoSystems
Buffers	1x PBS, Corning, 21-031-CV Anhydrous ethanol, Commercial Alcohols; P016EAAN RNAse-free water, VWR, 02-0201-0500 Acidic buffer, Precision NanoSystems, proprietary Custom cryopreservation buffer, Precision NanoSystems
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

Filters	Amicon® Ultra-15 30kDa MWCO Centrifugal filter, Millipore Sigma, UFC903008 MidiKros 20 cm 300 K MPES 0.5 mm TFF filter, Repligen, D02-E300-05-N Acrodisc® Syringe filter 0.2 µm Super membrane, Pall, PN 4612
RNA quantification	Quant-iT™ RiboGreen® RNA Assay Kit, incl. 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, Thermo Fisher Scientific, 11965084 Paraformaldehyde, Millipore Sigma, 158127-5G Triton X-100, Thermo Fisher Scientific, HFH10 *In vivo* assay: BALB/c mice, Biological Procedures Unit at the University of Strathclyde, custom protocol SARS-CoV-2 total IgG ELISA, various vendors, custom protocol

Equipment

NanoAssemblr system	NanoAssemblr Ignite, NIN0001 NanoAssemblr Ignite+,1001413 NanoAssemblr Blaze, NIB0055 (All from Precision NanoSystems)
Tangential flow filtration system	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

Methods

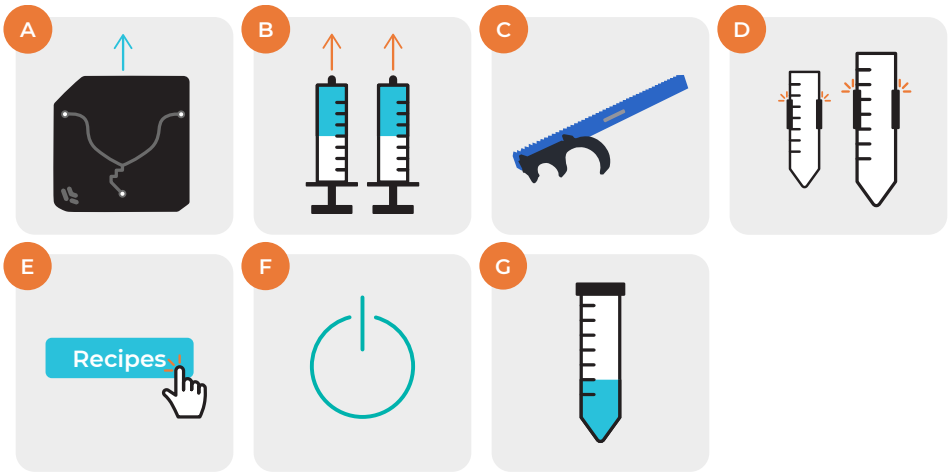


Figure 2. Operation of the NanoAssemblr Ignite+.
A) *Insert mixer into rotating block.* ***B)*** *Fill syringes with nucleic acid and lipid solutions and attach syringes to cartridge.* ***C)*** *Insert appropriately sized sample arm into instrument.* ***D)*** *Insert waste and sample vials.* ***E)*** *Select desired recipe.* ***F)*** *Start collection.* ***G)*** *Collect formulation.*

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. A NxGen Dilution or NxGen 500D cartridge was inserted into the rotating block of the NanoAssemblr Ignite+ (***Figure 2A***).
3. Two syringes were filled with the aqueous and organic phases. The organic syringe was attached to the right channel of the cartridge and the aqueous syringe was attached to the left channel. When mechanical in-line dilution was used, a syringe

was filled with 1X PBS and attached to the leftmost dilution channel while the aqueous syringe was attached to the center channel.

4. The correct sample arm was then inserted into the NanoAssemblr Ignite+ (***Figure 2C***). The appropriately sized waste and sample vials were then attached to this sample arm (***Figure 2D***).
5. The desired recipe was entered into the NanoAssemblr Ignite+ (***Figure 2E***).
6. A final check of the reagents, syringes, tubes and recipe was completed, then the formulation was started (***Figure 2F***).
7. The formulation was collected, and an aliquot was removed for further analytics (***Figure 2G***).
8. Liposome size and polydispersity were characterized by dynamic light scattering (DLS), with an expected size and polydispersity of 40 nm and 0.1 or lower, respectively. In a low-volume DLS cuvette, 200 µL of POPC:Chol formulation was combined with 200 µL of recently 0.22 µm-filtered 1X PBS.

B. Preparation of saRNA-LNPs using the NanoAssemblr Ignite+

1. The organic phase consisted of a Precision NanoSystems proprietary lipid composition in ethanol and the aqueous phase was a proprietary SARS-CoV-2 saRNA construct in weakly acidic buffer. The in-line diluent was 1X PBS.

   NOTE: the saRNA and saRNA aqueous phase was kept on ice whenever possible.
2. The NanoAssemblr Ignite+ was operated as described above (***Figure 2***).
3. Once formulation was complete (***Figure 2G***), an aliquot was removed as a 'post-formulation' sample for analytics.
4. The remainder of the formulation was diluted with additional 1X PBS for a total of 8X dilution. An aliquot was taken at this point as a 'post-bulk dilution' sample.

C. Preparation of saRNA-LNPs using the NanoAssemblr Blaze

1. The preparation of organic and aqueous phases was as described above.
2. The formulation of saRNA-LNP drug product using the NanoAssemblr Blaze was completed using the procedure described in the Blaze user guide (Document ID 1000513).

D1. saRNA-LNP downstream processing using centrifugal filters

1. The post-bulk diluted sample was transferred to an Amicon® Ultra-15 30kDa MWCO spin filter and centrifuged at 2500 xg and 4°C until the sample was concentrated 15 fold.
2. Precision NanoSystems proprietary cryopreservation buffer was then added to the spin filter and the sample was reconcentrated.
3. The saRNA-LNP sample was then sterile filtered with a 0.22 µm filter in a biosafety cabinet. The sample was then stored at -80°C up to 10 weeks.

D1. saRNA-LNP downstream processing using TFF

1. A 115 cm^2 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, 0.1 M NaOH, 0.01 M NaOAc, WFI and finally 1X PBS.

3. The post-bulk diluted sample was then concentrated to one fifteenth the original volume at a shear rate of 4000 s^{-1}.
4. Once concentrated, the sample was diafiltered with four volumes of cryopreservation buffer.
5. The sample was then sterile filtered using a 0.22 µm filter in a biosafety cabinet and then stored for up to 10 weeks at -80°C.

E. saRNA-LNP analytical characterization

1. LNP size and polydispersity were characterized throughout the production process by DLS, with an expected size of 60–90 nm and a polydispersity of less than 0.2. 20 µL of formulation was dispersed in 330 µL 1X PBS in a low-volume DLS cuvette.
2. RNA concentration was measured using RiboGreen® RNA reagent. This allowed for the calculation of the dilution needed to achieve the required concentration for dosing. Detailed protocols and accompanying calculation sheet are provided in the Appendix.
3. The lipid composition of the saRNA-LNPs was assayed with ultra-high performance-liquid-chromatography (UHPLC).
4. The saRNA integrity was assayed through capillary gel electrophoresis.

F. saRNA-LNP biological testing

1. Thymidine kinase-deficient Baby Hamster Kidney (BHK) 570 cells were plated in a 96-well plate (5k/well) and maintained in a humidified incubator at 37°C and 5% CO_2 for 2 days in Dulbecco's Modified Eagle Medium with 10% fetal calf serum before being transfected with a SARS-CoV-2 saRNA-LNP. Cells were incubated for a further 24 hours before being fixed (4% paraformaldehyde) and permeabilized (0.1% Triton X-100). Spike expression was detected with a commercial antibody linked to an AlexaFluor488 fluorophore. Total cells were imaged and quantified via a Cytation 7 high-throughput microscope.
2. *In vivo* immunization studies were performed in 7–9 week-old female BALB/c mice in accordance with University of Strathclyde procedures for the ethical treatment of animals. Tails bleeds were collected 2 days prior to immunization and on day 27. Mice were immunized by intramuscular (IM) injection of 50 µL of 8 µg/mL saRNA-LNPs on day 0 (prime) and day 28 (boost). On day 42, the mice were sacrificed via cardiac puncture. The blood samples were processed and sera collected via centrifugation. The total levels of SARS-CoV-2-specific IgG were assayed from the collected sera via an ELISA.

Results & Discussion

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

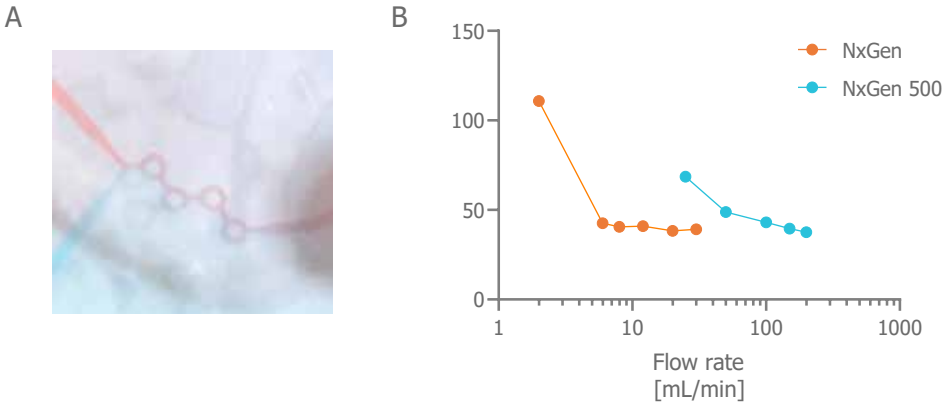


Figure 3. Controlled mixing using NxGen microfluidic technology allows for production of limit-size nanoparticles across a wide range of flow rates.
A) *Aqueous (blue coloring) and organic (red coloring) mixing in the NxGen mixer at an aqueous to organic flow rate ratio of 1:1 and total flow rate of 12 mL/min.* ***B)*** *POPC:Chol liposome limit-size curves at a flow rate ratio 3:1 on both the NxGen and NxGen 500.*

The quality of an RNA-LNP drug product is strongly influenced by the mixing process during the LNP encapsulation step. Therefore, a key process development goal in increasing RNA-LNP production throughput and scale is to ensure that critical quality attributes (CQAs), such as size and polydispersity, remain unchanged while critical process parameters (CPPs), such as flow rate, increase. The NxGen microfluidic technology has been designed to provide controlled mixing across a wide range of flow rates thereby ensuring that the same particles can be produced at both small and large scale. The standard NxGen mixer can produce RNA-LNPs at flow rates of up to 20 mL/min and is designed for use on the NanoAssemblr Ignite for small-scale research and development. To visualize the mixing within the NxGen mixer we constructed a mixer with a transparent viewing window that was compatible with a standard bright-field microscope (***Figure 3A***). Using this apparatus, the input channels for RNA (aqueous) and lipids (organic) and output channel for RNA-LNPs are visible along with four sequential toroidal mixers. Using artificial coloring, we show mixing of the input red and blue fluids which become a homogenous purple fluid at the output. The NxGen 500 has an enlarged geometry compared to NxGen and can produce RNA-LNPs at flow rates of up to 200 mL/min. Additionally, the NxGen 500 mixer is available on the NanoAssemblr Ignite+, Blaze and GMP System instruments to enable process development studies and large-scale manufacturing. Visualization experiments for NxGen 500 using the viewing apparatus exhibited similar mixing behavior (data not shown).

When establishing the CQAs and CPPs for a new RNA-LNP formulation, it is important to operate at a flow rate where so called "limit-size" LNPs are produced. Limit-size is the minimum size achievable for a specific composition of lipids and nucleic acids. An important property of limit-size is that once sufficiently high flow rates have been met, any further increase in flow rate produces the same size particles. In practice, operating above limit-size flow rates results in a process that produces RNA-LNPs with consistent CQAs while being tolerant of small perturbations in CPPs. Because limit-size is unique to each LNP formulation and RNA payload, typically ranging from 40 to 120 nm, a key activity during process development of a new formulation is to construct a limit-size curve whereby nanoparticles are prepared across a range of flow rates and their size is recorded.

We selected POPC:Chol liposomes as a representative formulation that demonstrates the concept of generating limit-size curves on the NxGen and NxGen 500 mixers. POPC:Chol liposomes are a well-studied formulation with a limit-size of 40 nm[11]. In addition, we have 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 (FFR) of 3:1 aqueous to organic and a range of total flow rates (TFR) appropriate to each mixer (***Figure 3B***). The POPC:Chol liposomes reached the predicted 40 nm limit-size at approximately 6 mL/min for the NxGen mixer and 80 mL/min for the larger geometry NxGen 500 mixer. This data illustrates how limit-size curves can be generated for a new formulation and how this information can be applied to select flow rates from mixer to mixer. Additionally, it can be noted that this experiment can be completed using only a minimal amount of reagent, allowing for significant savings to be realized. Furthermore, these results provide clear evidence that CQAs such as size can be preserved across the NxGen to NxGen 500 mixers while CPPs such as flow rate are increased.

The NanoAssemblr Ignite+ enables rapid, cost-effective process development of new RNA-LNP drugs

The new benchtop NanoAssemblr Ignite+ is enabled with both the NxGen and NxGen 500 mixers to allow for rapid, cost-effective process development of an RNA-LNP drug candidate at flow rates suitable for large-scale manufacturing.

To showcase these capabilities, we selected a COVID-19 vaccine model and a clinically relevant LNP composition encapsulating saRNA encoding for the SARS-CoV-2 pre-fusion

spike protein. We designed a study to compare the physicochemical and biological characteristics of SARS-CoV-2 saRNA-LNPs formulated at limit-size under four different Ignite+ experimental conditions useful in process development (***Table 1***). Firstly, preparation of RNA-LNPs at low flow (12 mL/min) and low volume (5 mL) on the NxGen mixer is useful for quick and cost-effective screening of new lipids and RNA constructs as well as small-cohort mouse studies. Secondly, extending the batch size at low flow with high volumes (40 mL) on NxGen provides larger quantities of material for lead candidates to enable further analytical characterization and larger cohort animal studies. Thirdly, transitioning to the NxGen 500 mixer at high flow (up to 200 mL/min) and low volume (5 mL) enables de-risking of the CPPs needed for large-scale manufacture in a small-scale, cost-effective test. Fourth and finally, production at high flow and high volume (40 mL) on NxGen 500 provides sufficient material for detailed analytical characterization, stability studies, development of TFF procedures and large animal studies using the same CPPs that can be implemented in a large-scale manufacturing process.

Table 1. *Experimental conditions of saRNA-LNPs prepared to test the capabilities of the NanoAssemblr Ignite+.*

	Low flow Low volume	Low flow High volume	High flow Low volume	High flow High volume
Aqueous phase	Precision NanoSystems custom saRNA Construct 1			
Organic phase	Precision NanoSystems custom lipids in ethanol			
Mixer	NxGen Dilution		NxGen 500D	
Total flow rate	12 mL/min		200 mL/min	
Formulation volume/mg saRNA mass	5 mL/ 0.325 mg	40 mL/ 2.5 mg	5 mL/ 0.325 mg	40 mL/ 2.5 mg
In-line dilution ratio (Buffer : Micromix volume)	0.85:1			
Flow rate ratio [Aq : Org]	3:1			
Downstream Processing	Centrifugal filter	TFF	Centrifugal filter	TFF

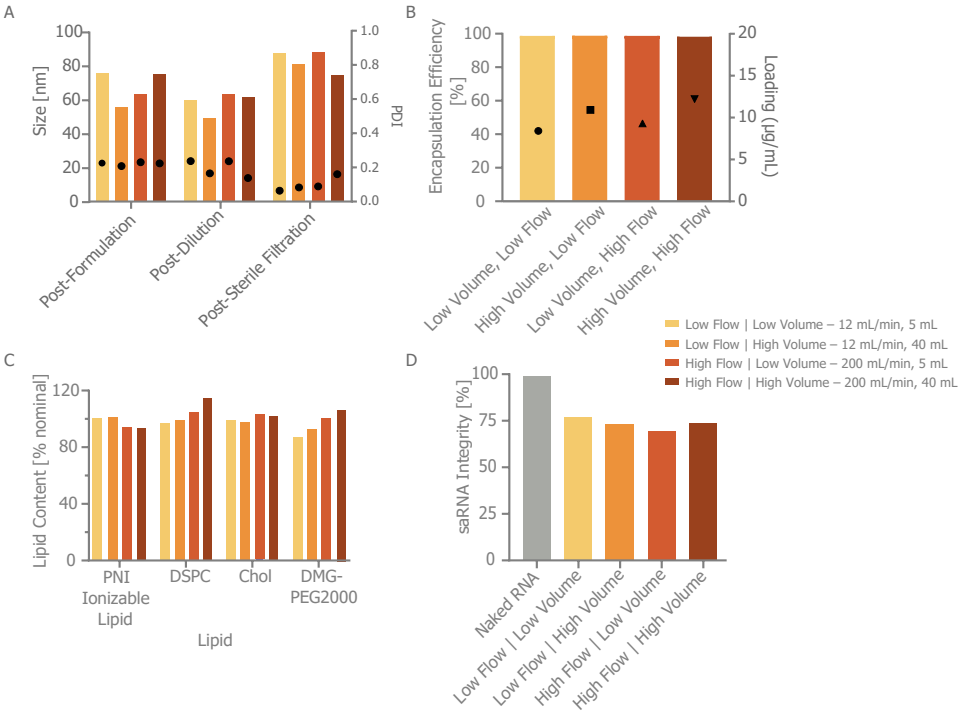


Figure 4. Physicochemical characterization of the four SARS-CoV-2 saRNA-LNPs prepared on the NanoAssemblr Ignite+.
A) *Size and polydispersity of in-process and finished saRNA-LNPs as determined by DLS.* ***B)*** *Encapsulation efficiency and saRNA loading of the saRNA-LNPs as determined by Ribogreen assay.* ***C)*** *Lipid content of saRNA-LNPs as expressed in mol % as determined by ultra-high performance liquid chromatography.* ***D)*** *saRNA integrity as assessed by capillary gel electrophoresis. Unless noted otherwise, all analytics were performed post-sterile filtration. Experimental details are described in* ***Table 1****.*

The four different saRNA-LNPs were prepared using the standard Ignite+ workflow followed by in-line dilution, centrifugal filtration or TFF and 0.22 µm sterile filtration (**Note:** see Case Study). The simple Ignite+ workflow and single-use NxGen mixers minimized hands-on time and eliminated cleaning, enabling the formulation and downstream processing of all four samples within one week by a single user.

Next, we used common analytical methods to characterize the four different saRNA-LNPs. Since the LNPs were formulated at limit-size flow rates appropriate for each mixer, the size and polydispersity were relatively uniform across test conditions (***Figure 4A***). Sizes varied from 75.0 to 87.4 nm and polydispersity varied from 0.07 and 0.16 post-sterile filtration. Similarly, using the RiboGreen assay we found equivalent encapsulation efficiency and saRNA loading across test conditions (***Figure 4B***). UHPLC was then used to assess if the lipid composition in the final RNA-LNPs matched that of the target input composition. Across the test conditions, the analysis showed the measured mol % divided by target mol % for all components was 100.2 % with a standard deviation of 6.3% (***Figure 4C***), demonstrating that the microfluidic mixing step, flow rate and volume have no impact on the ratio of lipids. The integrity of the saRNA encapsulated within the LNPs was then assessed with capillary gel electrophoresis. The results were similar across the test conditions with a range of saRNA integrities between 70.0% and 77.0% (***Figure 4D***), albeit values closely matched the naked controls. The slight difference in integrity between naked saRNA used as a control and the encapsulated saRNA can be attributed to the acidic formulation buffer, which degrades the saRNA over time. Collectively, this physicochemical analysis shows that CQAs are similar across the four saRNA-LNPs prepared over a range of flow rates and volumes.

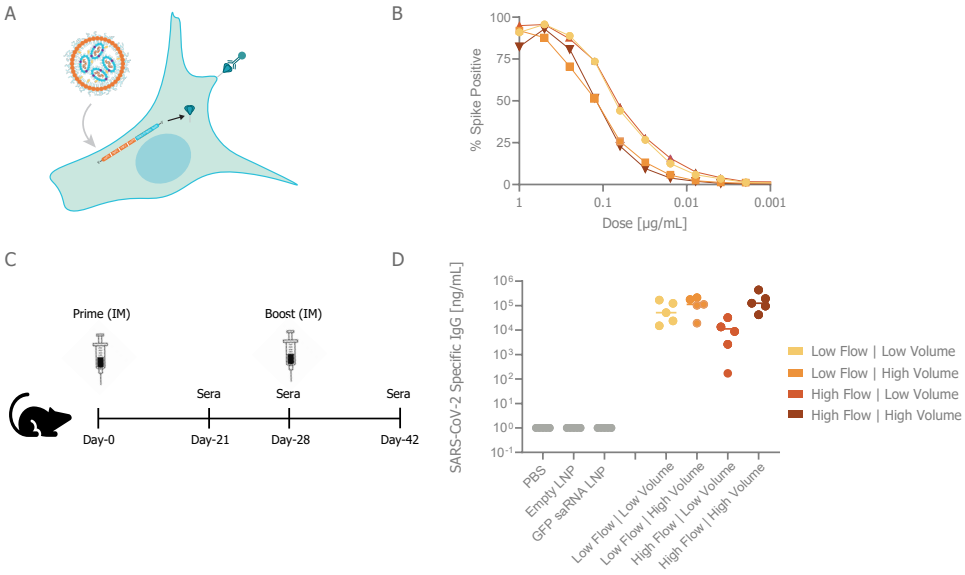


Figure 5. Expression of SARS-CoV-2 antigen in in vitro and in vivo models.
A) *Schematic of in vitro testing by transfection of BHK 570 cells. The cells were stained with an anti-spike conjugated AlexaFluor488 antibody for fluorescence imaging.* ***B)*** *% cells expressing SARS-CoV-2 Spike protein in BHK 570 cells as a function of saRNA dose for each of the four saRNA-LNPs.* ***C)*** *Schematic of in vivo immunization study design with initial and booster dose noted along with sera collection.* ***D)*** *SARS-CoV-2-specific IgG response in serum in BALB/c mice at day 42 post-injection for each of the four saRNA-LNPs.*

To evaluate biological activity, we tested the four SARS-CoV-2 saRNA-LNPs in both *in vitro* and *in vivo* COVID-19 vaccine models. BHK 570 cells were transfected with the saRNA-LNPs to determine the percentage of cells expressing the spike protein (***Figure 5A***). By analyzing the dose response, we determined that the EC50 values for the four saRNA-LNPs were within a 2-fold range from 0.06 to 0.12 µg/mL, indicating similar *in vitro* activity (***Figure 5B***). We then performed a 42 day (6 week) *in vivo* immunization study in BALB/c mice to compare the immune response to the four saRNA-LNPs. (***Figure 5C***). 0.4 µg of encapsulated saRNA was injected IM for both the prime and booster (day 28) doses. In sera samples collected at the terminal read (day 42), we found that SARS-CoV-2-specific IgG titers varied between 1.43×10^4 to 1.79×10^5 ng/mL (***Figure 5D***), indicating that all samples were able to elicit a strong immune response. One mouse in the high flow, low volume group was non-responsive to the immunization, with an IgG concentration of 100.4 ng/mL at day 42. Additionally, the lower titer level for this sample in general can be rationalized with insufficient waste volume being collected on an early prototype instrument. Recommended start waste volumes are described in Appendix B. The insufficient collection of start waste can result in LNPs formed from unsteady flow being included in the collected sample. Collectively, this data from the *in vitro* and *in vivo* assays suggests that all four saRNA-LNPs are biologically active and generate a robust pharmacodynamic effect.

Overall, the results from this study provide strong evidence for how the Ignite+ instrument and workflow can be used as a process development tool for new RNA-LNP drugs. The NxGen and NxGen 500 mixers enable the testing of a wide range of flow rates, allowing for rapid iteration of test conditions and de-risking of relevant clinical manufacturing parameters at a convenient and economical scale.

Ignite+ enables seamless transfer of RNA-LNP drugs to Blaze and GMP for large-scale process development and manufacturing for clinical trials

Table 2. *Experimental details comparing saRNA-LNPs produced on Ignite+ and Blaze.*

	Ignite+ using NxGen	Ignite+ using NxGen 500	Blaze using NxGen 500
Aqueous phase	Precision NanoSystems custom saRNA Construct 1		Precision NanoSystems custom saRNA Construct 2
Organic phase	Precision NanoSystems custom lipids in ethanol		
Mixer	NxGen Dilution	NxGen 500D	
Total flow rate	12 mL/min	200 mL/min	115 mL/min
Formulation volume/mg saRNA mass	40 mL/ 2.5 mg		
In-line dilution ratio (Buffer : Micromix volume)	0.85:1		3:1
Flow rate ratio [Aq : Org]	3:1		
Downstream processing	TFF		

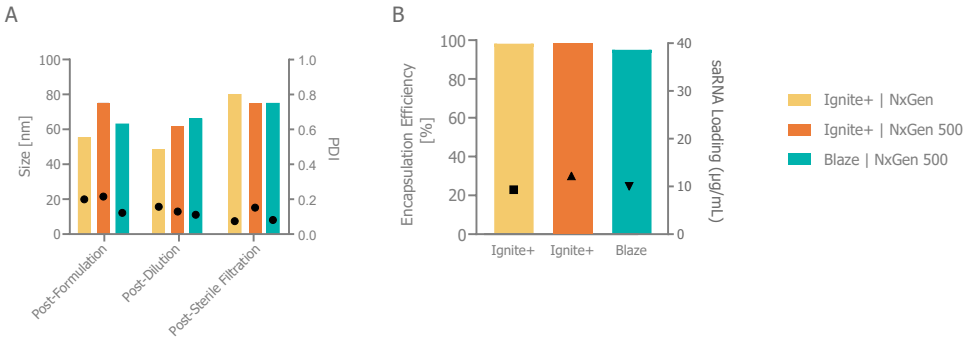


Figure 6. Physicochemical characterization of SARS-CoV-2 saRNA-LNPs prepared on the NanoAssemblr Ignite+ and Blaze.
A) *The size and polydispersity index (PDI) of saRNA-LNPs prepared on NanoAssemblr Ignite+ and Blaze instruments using NxGen and NxGen 500 dilution mixers.* ***B)*** *The encapsulation efficiency and saRNA loading of the above samples.*

The NanoAssemblr Ignite+ was designed to enable a seamless transfer of a small-scale RNA-LNP process to the NanoAssemblr Blaze and GMP System for high-throughput large-scale production. To demonstrate this concept, we compared SARS-CoV-2 saRNA-LNPs prepared on Ignite+ and Blaze using the NxGen 500 mixer with a 40 mL production volume, in-line dilution, TFF and sterile filtration (***Table 2***). For this comparison, we selected the maximum total flow rates of 200 mL/min and 115 mL/min for Ignite+ and Blaze, respectively, to match the CPPs typically recommended for large-volume production. Based on our knowledge of the limit-size behavior for this LNP composition we expected both flow rates to produce LNPs with comparable CQAs. Physicochemical characterization of the resulting in-process and final saRNA-LNPs showed similar sizes across Ignite+ and Blaze, with a range of ~50 to 80 nm and polydispersity indices of less than 0.2 (***Figure 6A***). saRNA encapsulation efficiency and concentration were equivalent for the Ignite+ and Blaze saRNA-LNPs, with greater than 95% encapsulation efficiency and 9 to 12 µg/mL saRNA, respectively. These results show how the production process for saRNA-LNPs on Ignite+ can be quickly transferred to Blaze without the need for additional process development. Since the same NxGen 500 microfluidic mixer is available across Ignite+, Blaze and GMP System, the high flow rates required for large-scale production can be de-risked quickly on Ignite+, and at a fraction of the raw material cost required for multi-liter production runs.

Conclusion

As development and interest in LNPs as a potent drug delivery modality for nucleic acid APIs continue to grow, the transition from bench to clinical scale production volumes will continue to gain additional importance. The NanoAssemblr Ignite+ can screen RNA-LNP formulations and produce lead RNA-LNP candidates in sufficient quantities for large animal studies, stability testing and extensive analytical characterization. The NanoAssemblr Ignite+ can also produce RNA-LNPs at the CPPs found on large clinical scale instruments, reducing process and formulation development when changing scales.

Case Study: De-risking downstream processing of RNA-LNP drugs using the NanoAssemblr Ignite+

After formulation of an RNA-LNP by microfluidic mixing, the LNP must be diluted with buffer and then purified to remove ethanol (***Figure 1A***). At scale, these activities are accomplished by mechanical in-line dilution and tangential flow filtration (TFF) unit operations. In this case study, we provide examples of how these unit operations can be investigated in a cost-effective manner with the NanoAssemblr Ignite+. We first show that by using the NxGen 500D mixer, we can investigate the transition from manual bulk dilution to in-line dilution. Secondly, we show how using the Ignite+ to generate 40 mL of RNA-LNPs in one batch allows for testing on larger TFF filters.

NxGen 500D cartridges enable the transition to in-line dilution

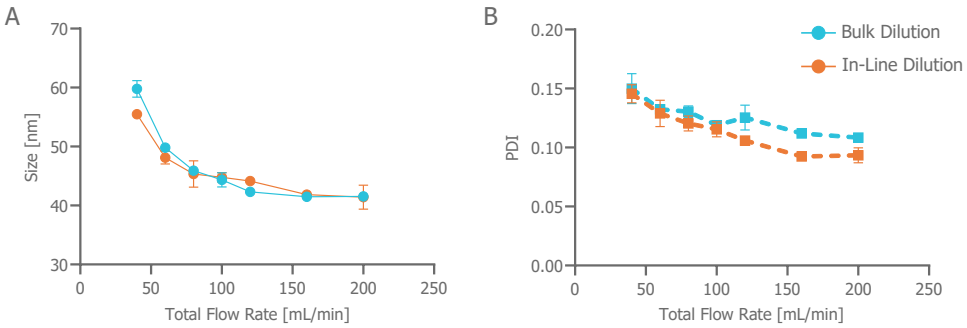


Figure 7. Bulk and in-line dilution of POPC:Chol liposome limit-size curves.
A) *Size of POPC:Chol liposomes prepared on the NxGen 500 mixer at a range of flow rates. Samples prepared with bulk dilution were diluted 2 times in 1X PBS. Those with in-line dilution were diluted by the same amount using a NxGen 500D cartridge.*
B) *The accompanying polydispersity of the samples shown in* ***A****.*

Dilution of the RNA-LNP drug product in buffer immediately after formulation is an essential step to improving LNP stability[14]. At the research scale, this is accomplished by pipetting or pouring the formulation into fresh buffer; however, at clinical scales this becomes untenable. A solution found on instruments such as the NanoAssemblr Blaze and GMP System is to introduce diluent buffer in-line immediately after formulation. The NanoAssemblr Ignite+ accomplishes this by including a channel for dilution on both the NxGen Dilution and NxGen 500D mixing cartridges.

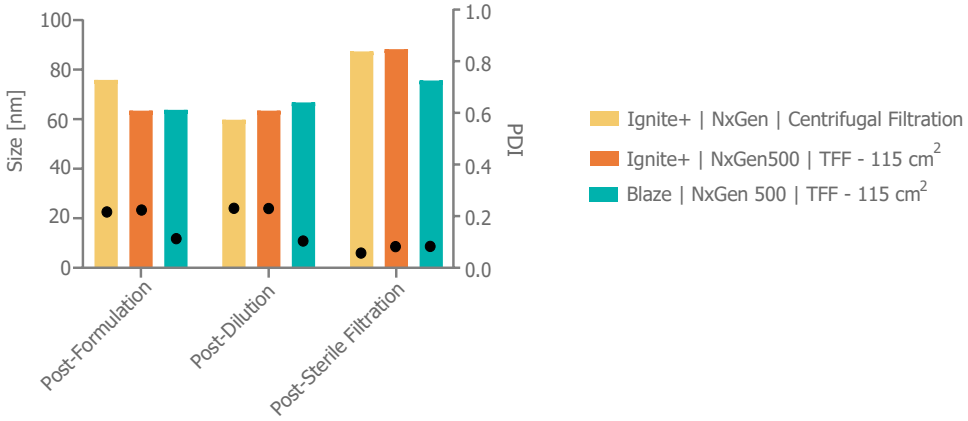
To demonstrate how this unit operation could be economically de-risked, we prepared POPC:Chol liposomes with either post-formulation bulk dilution in 1X PBS or in-line dilution using the NxGen 500 or NxGen 500D cartridges. Additionally, we screened a range of flow rates to see how the POPC:Chol liposomes responded at a range of CPPs. In the case of POPC:Chol liposomes, we see a robust tolerance of in-line dilution across the range of flow rates tested with respect to both size (***Figure 7A***) and polydispersity (***Figure 7B***). Since it is possible that not all formulations are compatible with in-line dilution, the ability of the Ignite+ to formulate at clinically relevant flow rates and millilitre volumes allows this testing to be accomplished quickly and economically.

Ignite+ de-risks the transition to tangential flow filtration

Table 1. *Experimental conditions of saRNA-LNPs prepared to test the capabilities of the NanoAssemblr Ignite+.*

	Ignite+ then centrifugal filtration	Ignite+ then TFF	Blaze then TFF
Aqueous phase	Precision NanoSystems custom saRNA Construct 1		Precision NanoSystems custom saRNA Construct 2
Organic phase	Precision NanoSystems custom lipids in ethanol		
Mixer	NxGen Dilution	NxGen 500D	
Total flow rate	12 mL/min	200 mL/min	115 mL/min
Formulation volume/mg saRNA mass	40 mL/ 2.5 mg		
In-line dilution ratio (Buffer : Micromix volume)	0.85:1		3:1
Flow rate ratio [Aq : Org]	3:1		
Downstream processing	TFF		

Figure 8. Downstream processing approaches for ethanol removal of saRNA-LNPs.
SARS-CoV-2 saRNA-LNPs were prepared on the NanoAssemblr Ignite+ and Blaze and purified with either centrifugal filters or TFF. Formulations prepared with the Ignite+ use Precision Nanosystems SARS-CoV-2 saRNA Construct 1, while samples prepared with the Blaze use Construct 2. Both constructs are of similar composition and length.



Removal of ethanol after formulation and buffer exchange to a storage buffer via tangential flow filtration (TFF) is an essential step in RNA-LNP drug product production (***Figure 1A***). TFF is a readily scalable unit operation as filter sizes are available with surface areas ranging from 10 cm^2 for exploratory investigations to >1 m^2 for clinical production. However, the transition from less scalable purification methods such as dialysis and centrifugal filters to TFF is not without cost and risk. The CPPs for the TFF process, such as filter material and construction, shear rate and target transmembrane pressure must all be determined and optimized. As well, not all formulations are inherently compatible with TFF. For example, we have previously found that an RNA-LNP drug product increased in size from 77 nm to 160 nm during TFF processing. When one of the structural lipids was substituted for another lipid, there was no increase in size during TFF processing[17]. It was concluded that the first formulation was sensitive to the shear forces within the TFF cartridge and formed a gel layer within the filter. The second formulation was tolerant of the shear forces and did not foul the filter. The transition to TFF is an essential activity that should be de-risked early in the process development process.

To demonstrate how the transition to TFF can be de-risked using Ignite+, we prepared a series of samples on the NanoAssemblr Ignite+ and Blaze that were purified with either TFF or centrifugal filtration (***Table 3***). When analyzed with DLS (***Figure 8***), the final saRNA-LNP drug product was between 88.3 and 76.0 nm with a PDI of 0.09 to 0.06 with no trends discernible with respect to mixer, instrument or downstream purification method. Similarly, no trends were observed with encapsulation efficiency which varied between 98.2 and 98.8 (data not shown). This experiment highlights the utility of the NanoAssemblr Ignite+ high-volume capacity. A single TFF experiment using a 115 cm^2 filter, or multiple experiments using smaller filters can be performed from a single 40 mL RNA-LNP production run, without the need to pool many smaller batches. This improves consistency, saves production time and removes the analytical burden needed for validating multiple batches.

Appendix A – Ribogreen Assay Protocol

Determining the mRNA 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 mRNA Encapsulation Efficiency

Additional Reagents/Disposables

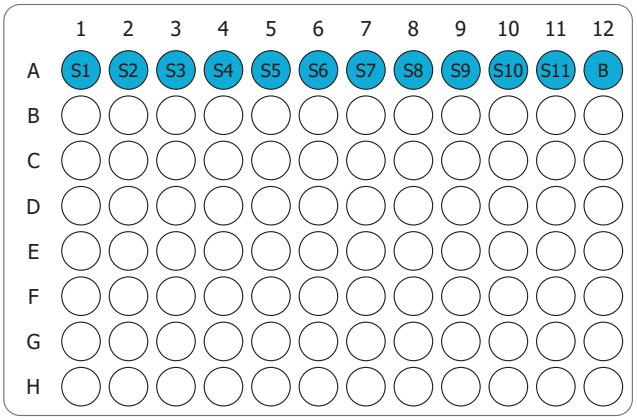
Description	Recommended Supplier
Invitrogen™ TE Buffer (20X), RNase-free	Thermo Fisher Scientific
Quant-iT™ Ribogreen® Assay Kit	Thermo Fisher Scientific
Triton™ X-100	Sigma Aldrich
RNase-Free Water	General Laboratory Supplier
RNase-Free Filter Pipette Tips (10, 20, 200 and 1000 µL)	General Laboratory 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

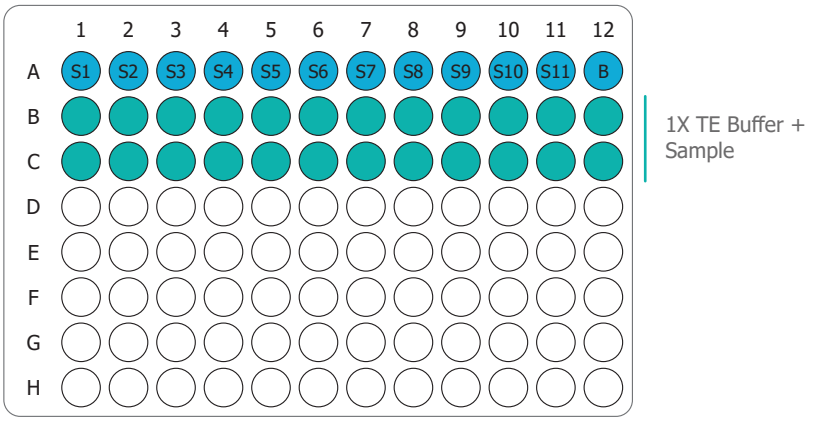
1. Prepare 1X TE buffer from 20X TE buffer by adding 10 mL of 20X TE buffer to 190 mL RNase-free water in a clear glass bottle. Shake the bottle to mix.
2. To 100 mL of prepared 1X TE buffer, add 2 mL of Triton X-100. Stir using a magnetic stirrer for 15 min. This solution is the Triton Buffer.
3. Pour 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 mRNA-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.



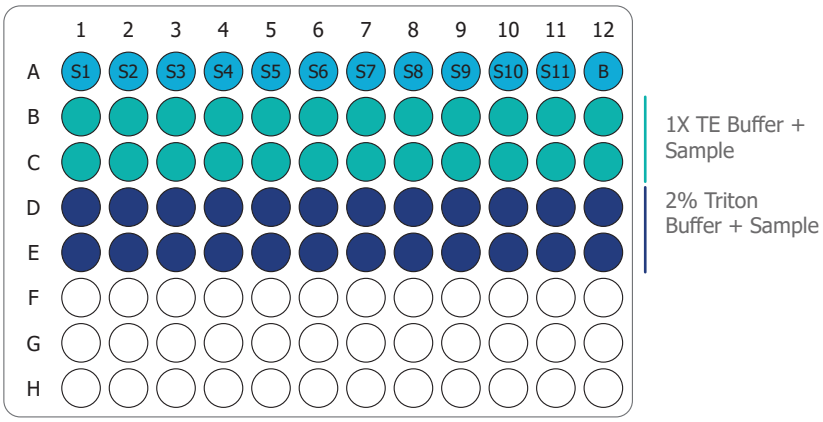
mRNA-LNP Sample Setup

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 mRNA-LNP sample (Rows B and C).
2. Add 50 µL of mRNA-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.



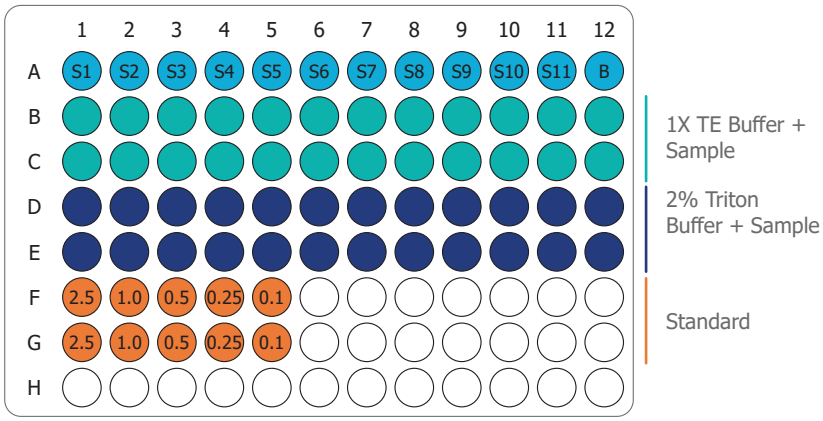
4. Add 50 µL of sample stock solution from Row A into the wells in Rows D and E.



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 mRNA-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 mRNA-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).

Appendix B – Start Waste Volumes

Syringe Size (mL)	Recommended Start Waste (mL)			
	Smaller Syringe	Same Size Syringe	Larger Syringe	Dilution Syringe
1	0.10	0.15	0.25	0.20
3	0.15	0.25	0.35	0.25
5	0.20	0.35	0.50	0.35
10	0.30	0.55	0.80	0.50
20	n/a			0.65
30	n/a			0.65

Example 1:

For a formulation with a 1 mL and a 3 mL syringe, the Start Waste value is calculated as follows:

- Smaller syringe, 1 mL:
- Larger syringe, 3 mL:
- Recommended start waste:

$$\begin{array}{r} 0.10 \\ +\ 0.35 \\ \hline =\ 0.45 \end{array}$$

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