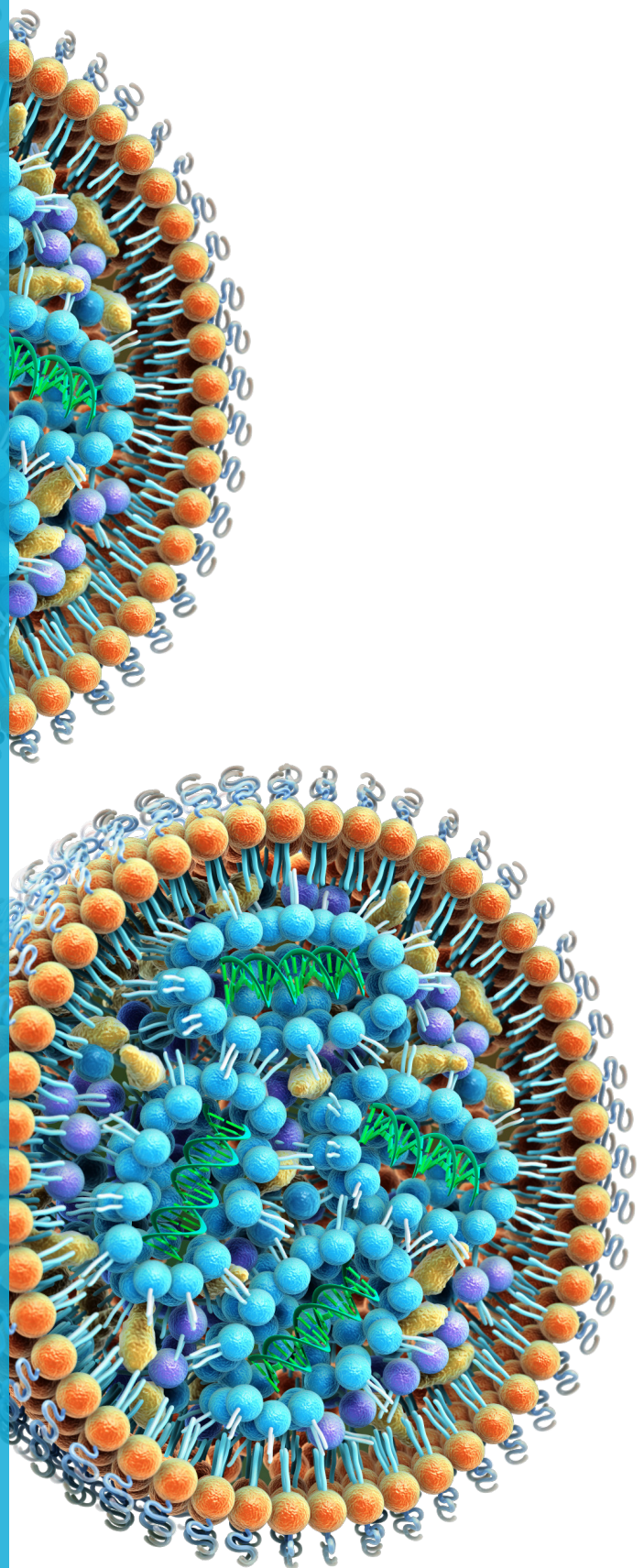


# mRNA Lipid Nano- particles

Robust low-volume  
production for screening  
high-value nanoparticle  
materials



Chelsea Cayabyab, Andrew Brown, Grace Tharmarajah,  
Anitha Thomas

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Precision NanoSystems Inc, Vancouver, BC, Canada

## Abstract

In 2018, the FDA approval of Patisiran, a lipid nanoparticle (LNP) formulation and the first small interfering RNA therapeutic to receive FDA approval, established LNPs as the premier technology for non-viral RNA delivery. Concurrently, the NanoAssemblr® platform, which harnesses microfluidic mixing, has been demonstrated as a simple, robust and scalable production method for LNPs encapsulating various types of nucleic acids with near 100% encapsulation efficiencies. LNPs provide a versatile option for gene knockdown or gene expression studies *in vitro* and *in vivo*. The NanoAssemblr Spark™ exploits an additional advantage of microfluidic mixing: microliter-scale formulation that conserves high-value materials such as novel lipids and mRNA. This unique combination of ultra-low volume formulations with a rapid, simple and reproducible process makes Spark an ideal platform for screening and early preclinical development of mRNA-LNP formulations. Effective screening programs can significantly narrow the parameter space for developing and optimizing next-generation delivery technologies and nanoparticle therapeutics.

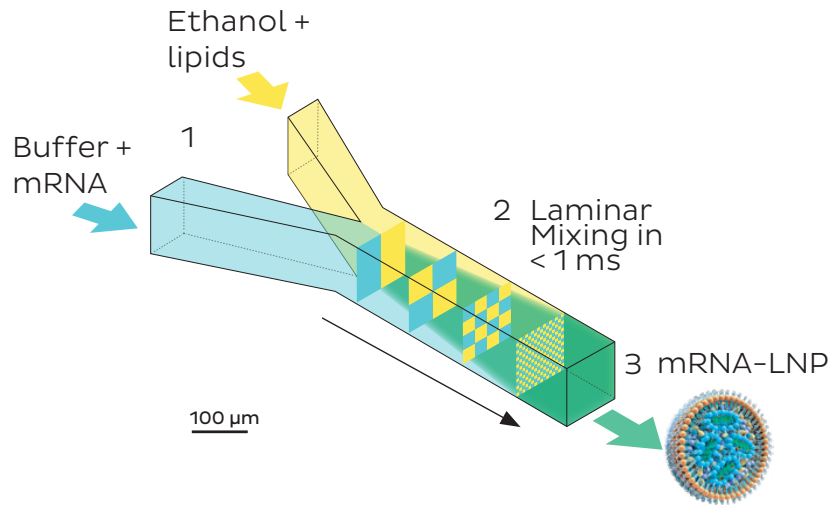
## Introduction

Screening an array of active pharmaceutical ingredients, excipients and formulation parameters in early nanomedicine discovery and development enables more focused and efficient development in later stages by narrowing the parameter space. To this end, fast, reproducible nanoparticle production at low volume is needed to ensure observed differences in the properties or activity of the formulation can be attributed to controlled changes in composition or conditions, which informs rational design of nanomedicines. Furthermore, achieving this at low volumes minimizes the use of API and excipients, which at the discovery stage, may be limited in availability, expensive to acquire or laborious to produce.

In particular, active ingredients used in the genetic manipulation of cells such as small interfering RNA (siRNA), guide RNA (gRNA), messenger RNA (mRNA) or plasmids are either scarce or very expensive. While non-viral nucleic acid delivery systems are enabling revolutionary treatments such as mRNA vaccines<sup>1,2</sup>, immuno-oncology<sup>3</sup>, targeted oncology<sup>4-6</sup>, CRISPR/Cas9 gene editing<sup>7-9</sup>, and the treatment of rare diseases<sup>10,11</sup>, there remains a substantial need for improving the fundamental understanding of nucleic acid delivery systems and for further innovation to improve the quality and performance of gene-delivery nanoparticles. Specifically, further innovation of nanoparticle excipients used to encapsulate, protect, and deliver these payloads into diseased cells is necessary to advance the field. These excipients are, by their innovative nature, largely unavailable at large, low-cost commodity scales.

So, there is an unmet need in the field for robust and reproducible low volume production of nanoparticles containing genetic payloads appropriate for researchers in the discovery space. For instance, minimum volumes for T-tube mixing are on the order of 10 mL when only microliters are required for *in vitro* screening. For this reason, researchers are using crude pipette-mixing methods to perform bottom-up nanoprecipitation of complex nucleic acid-nanoparticle formulations. Mixing with pipettes offers little control, is operator-

**Figure 1.) Microfluidic mixing technology for manufacturing nanoparticles:** An organic solvent containing dissolved lipids and an aqueous solution containing nucleic acids are injected into the two inlet channels of the NanoAssemblr cartridge. Under laminar flow, the two solutions do not immediately mix, but microscopic features engineered into the channel cause the two fluids to intermingle in a controlled and reproducible way, where molecules interact with each other by diffusion. Within 1 millisecond, the two fluids are completely mixed, causing a change in solvent polarity that triggers the homogenous self-assembly of nanoparticles loaded with nucleic acids.

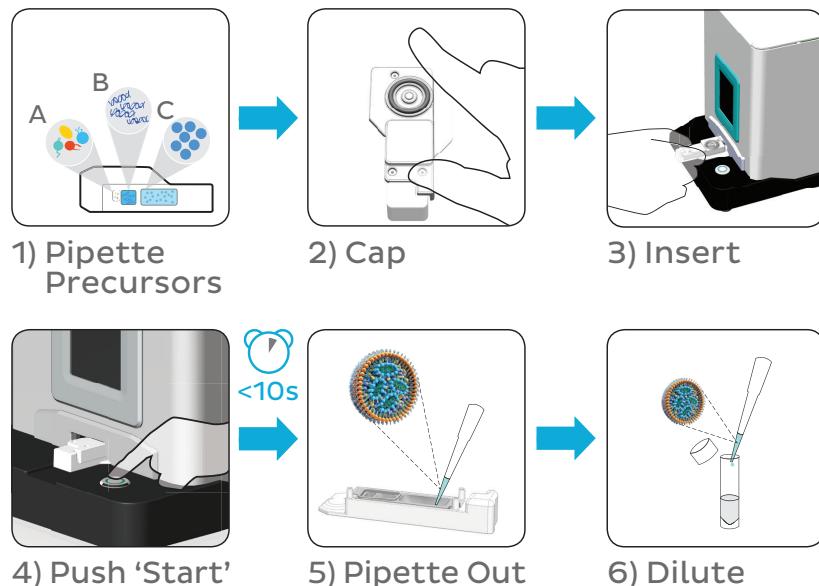


dependent, and mixing conditions that dictate the properties of resulting nanoparticles cannot be repeated with fidelity. Hence, nanoparticle attributes and payload protection vary, resulting in high variability in functional activity studies. This hampers the innovation in this field. Furthermore, once lead formulations are identified, the production conditions have to be scalable such that formulations have the potential to advance through pre-clinical development and clinical manufacturing.

Time-invariant conditions afforded by NanoAssemblr mixers ensures the conditions of self-assembly remain consistent throughout a single formulation and between individual formulations. This allows formulations to be scaled in volume across several orders of magnitude to suit various stages of development. **Figure 1** describes the process of mixing in a NanoAssemblr microfluidic mixer in more detail.

Here, we describe the NanoAssemblr Spark for the controlled and reproducible manufacturing of nucleic acid-containing lipid nanoparticles (LNPs) at volumes ranging from 100 to 250  $\mu\text{L}$ . The Spark realizes the advantages of microfluidic mixing in a format that allows consistent formulation at microliter scales in seconds (**Figure 2**). The resulting formulation can be applied directly to cells in culture. This makes Spark ideal for rapidly producing numerous formulations for in vitro testing while conserving rare or costly materials. Consistent conditions allows any observable differences in the outcomes to be

**Figure 2.) The NanoAssemblr Spark™ workflow for producing nanoparticle systems:** Step 1 – Precursor solutions were pipetted into the wells of the Spark microfluidic cartridge as follows: a) 12  $\mu\text{L}$  of lipid mix in ethanol, b) 36  $\mu\text{L}$  of mRNA in acetate buffer, c) 48  $\mu\text{L}$  of PBS. Step 2 – The cartridge cap was fitted, Step 3 – The cartridge is inserted into the Spark instrument. Setting 2 was selected via the touch screen interface. Step 4 – The “Start” button was depressed to begin mixing. Mixing takes ~3s. Step 5 – The resulting mRNA-LNP suspension is pipetted out of the collection well. Step 6 – The LNP suspension (96  $\mu\text{L}$ ) is mixed with 96  $\mu\text{L}$  of dilution buffer in a microcentrifuge tube.



attributed to the differences in formulations to inform rational design of gene-delivery vehicles.

Similar lipid delivery systems have been well described in the literature for the delivery of siRNA, mRNA and plasmid payloads<sup>12-15</sup>. We demonstrated how mRNA-LNP formulations can be screened by systematically varying LNP composition, reagent concentrations, and the N/P ratio (the ratio between cationic amines in the lipid excipient and the anionic phosphates on mRNA). We also demonstrated how the Spark can be used for encapsulating various lengths of mRNA with consistent results. In all, we demonstrated the utility of the Spark as an efficient screening platform that allows a large number of different mRNA-LNP formulations to be rapidly produced using sub-milligram quantities of both API and nanoparticle excipients at a scale well suited for downstream physical characterization or in vitro functional screening.

## Materials & Methods

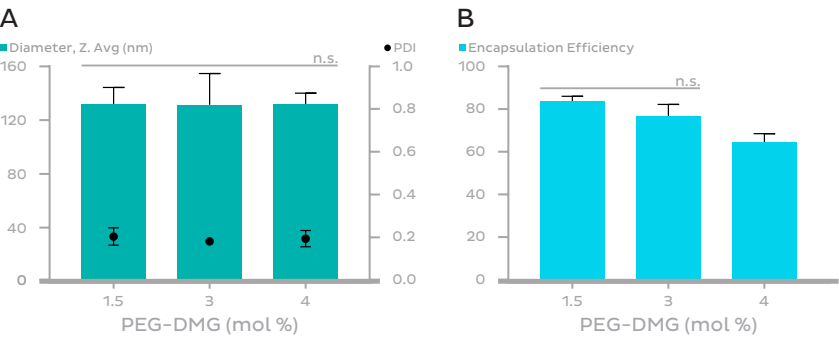
### Solution preparation:

Messenger RNA (TriLink Biotechnologies) was diluted using sodium acetate buffer, pH4 (Sigma Aldrich) to a final buffer strength of 100 mM and to the required mRNA concentration. Stock solutions of cationic lipid (CL), 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC, Avanti Polar lipids), Cholesterol (CHOL, Sigma Aldrich) and PEG-lipid (NOF America Corp) in ethanol were mixed at a mole fraction of 50:10:40-x:x respectively. Here, x is the mole fraction of PEG-lipid and was varied between 1.5 to 5%, substituting for cholesterol. Cationic lipids (CL) were one of 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP, Avanti Polar Lipids), 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA, Avanti Polar Lipids), or a proprietary ionizable cationic lipid (PNI-ILa).

### Ultra-low volume LNP preparation:

LNP samples were prepared as indicated in the Spark workflow (**Figure 2**) 12 µL of 35 – 65 mM lipid mix (to explore the effect of lipid concentration), and 36 µL of mRNA solution containing 10 – 25 µg of nucleic acids (as required by the N/P ratio) in 100 mM sodium acetate buffer were pipetted into the first and second wells, respectively, of the Spark microfluidic cartridge (Precision NanoSystems, Vancouver, Canada). 48 µL Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free PBS

**Figure 3.) Ultra low volume screening of mRNA LNP formulations for lipid-PEG amounts.** Figure shows the effect of different PEG content on the size and PDI (A), and encapsulation efficiency (B) of mRNA-LNP formulations using the NanoAssemblr Spark by changing the mole percentage of PEG (2000) -DMG. Values represent the mean from triplicate formulations. Error bars represent the standard deviation. Means grouped by horizontal bars were not significantly different ( $P > 0.05$  by ANOVA (A) and Tukey's multiple comparison test (B)). Error bars not drawn when error is less than the size of the symbol.



### Formulation Parameters

Lipid Composition	PNI-ILa:DSPC:Cholesterol:PEG-DMG 50:10:40-x:x   x = 1.5, 3, 5 mol%
Initial Lipid Concentration	50 mM
mRNA	GFP (996 nt), 0.025 mg
N/P	4
Organic Solvent	Ethanol
Aqueous Phase	0.695 mg/mL mRNA in 100 mM sodium acetate
Instrument Setting	2
Downstream Processing	4x dilution with nuclease-free PBS



(Corning) at pH 7.4 was pipetted into the collection well (well 3). To initiate microfluidic mixing of lipid and mRNA solutions, the loaded cartridge was capped, and inserted into the NanoAssemblr Spark (Precision NanoSystems, Vancouver, Canada) and the formulation was run with the Spark setting at "3". The resulting mRNA LNPs were immediately diluted on-chip in the collection well. The contents of the collection well were then immediately transferred to a microcentrifuge tube containing 96  $\mu\text{L}$  of  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free PBS at pH 7.4. All samples were prepared in triplicate.

#### Analysis of LNPs:

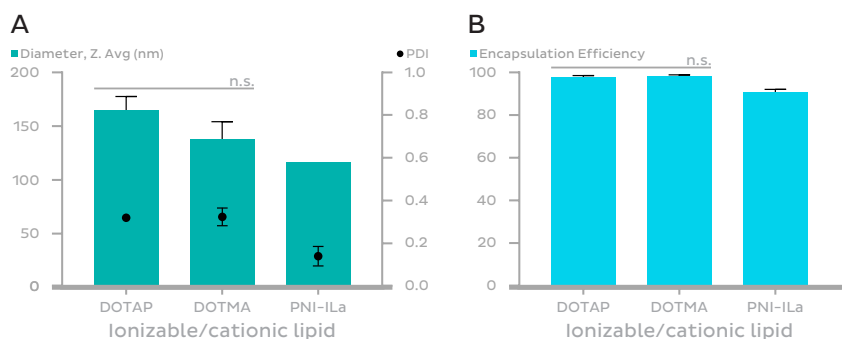
Encapsulation efficiency (EE%) was measured using a fluorescence plate-based assay employing the Ribogreen reagent (Invitrogen) as per PNI Ribogreen assay protocol<sup>16</sup>. This assay measures the quantity of mRNA in samples with intact LNPs to determine the quantity of unencapsulated RNA as well as in LNP samples disrupted by triton X-100 (Sigma Aldrich) to measure the total RNA. EE% is calculated as the difference between the total RNA and the unencapsulated RNA divided by the total RNA.

Size and polydispersity index (PDI) were determined by dynamic light scattering using a Zetasizer Nano ZS (Malvern Panalytical, UK). 10  $\mu\text{L}$  of LNP suspension was transferred to a low-volume cuvette containing 300  $\mu\text{L}$  of  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free PBS at pH 7.4. Refractive index was set to 1.14 and temperature was set to 25°C.

## Results

The effect of PEG content was examined by comparing samples containing 1.5, 3 and 4 mol% PEG lipid (**Figure 3**). PEG content did not have a statistically significant impact on size. The formulation PNI-ILa:DSPC:Cholesterol:PEG-DMG (50:10:38.5:1.5 mol%) had the highest encapsulation efficiency, whereas there was a statistically significant reduction in EE% at 4 mol% PEG-lipid.

The suitability of three cationic lipids for producing mRNA-LNPs was determined by comparing EE% and particle size (**Figure 4**). Permanently cationic lipids DOTAP and DOTMA were compared to PNI's proprietary ionizable cationic lipid (PNI-ILa). N/P ratio, concentrations of reagents and the formulation process were held constant. PNI-ILa produced significantly smaller LNPs with the lowest PDI.



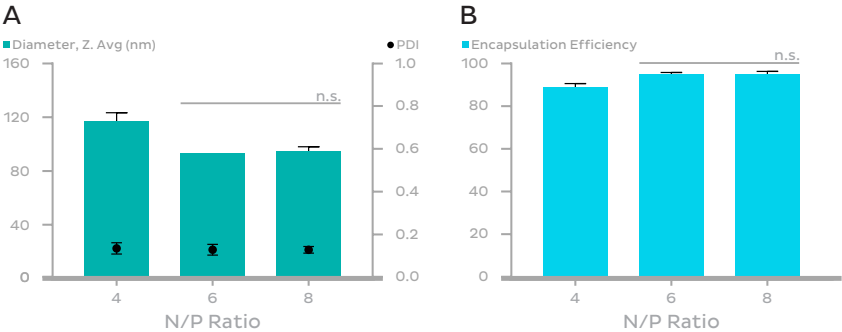
**Figure 4.) Screening different cationic/ionizable lipids in low-volume mRNA-LNP formulations.** Figure shows effect of different cationic/ionizable lipids on size and PDI (A) and encapsulation efficiency (B) of mRNA LNP formulations. Encapsulation efficiencies over 90% were achieved. Formulations were prepared in triplicate. Values represent the mean and error bars, the standard deviation. Means grouped by horizontal bars were not significantly different ( $P > 0.05$  by Tukey's multiple comparison test). Error bars not drawn when error is less than the size of the symbol.

#### Formulation Parameters

Lipid Composition	CL:DSPC:Cholesterol:PEG-DMG (50:10:38.5:1.5mol%) CL = DOTAP (18:1 TAP) or DOTMA or PNI-ILa
Initial Lipid Concentration	50 mM
mRNA	GFP (996 nt), 0.025 mg
N/P	4
Organic Solvent	Ethanol
Aqueous Phase	0.695 mg/mL mRNA in 100 mM sodium acetate
Instrument Setting	2
Downstream Processing	4x dilution with nuclease-free PBS

**Figure 5.) Systematic screening of N/P ratios with low-volume mRNA-LNP formulations.** Increasing N/P ratios were tested. Size and PDI (A) were measured using dynamic light scattering and encapsulation efficiency (B) was measured by modified ribogreen assay. Formulations were prepared in triplicate. Values represent the mean and error bars, the standard deviation. Means grouped by the horizontal bars were not significantly different ( $P>0.05$  by Tukey's multiple comparison test). Error bars not drawn when error is less than the size of the symbol.

The N/P ratio has been shown to affect siRNA-LNP quality and efficacy. To determine the effect of N/P ratio on mRNA LNPs, three N/P ratios were tested: 4:1, 6:1 and 8:1 (**Figure 5**). At 4:1 the particle size is larger with a slight decrease in EE% compared to 6:1 and 8:1. At N/P of both 6:1 and 8:1 similar size, PDI and EE% were achieved suggesting that an asymptotic limit has been reached.

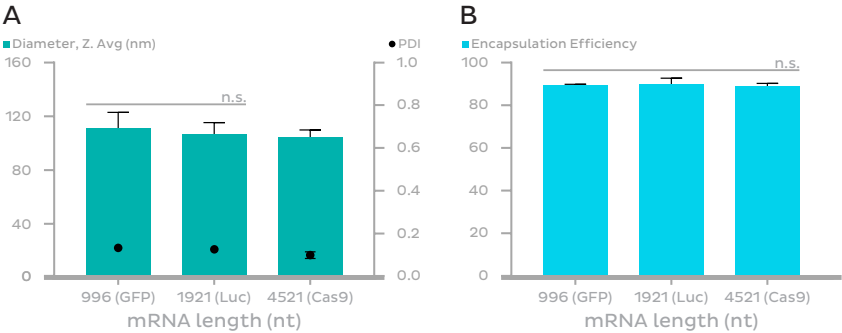


**Formulation Parameters**

	N/P		
	4	6	8
Lipid Composition	PNI-ILa:DSPC:Cholesterol:PEG-DMG (50:10:38.5:1.5 mol%)		
Initial Lipid Concentration	50 mM		
mRNA	GFP (996 nt), 0.025 mg	GFP (996 nt), 0.0167 mg	GFP (996 nt), 0.0125 mg
Organic Solvent	Ethanol		
Aqueous Phase	0.695 mg/mL mRNA in 100 mM sodium acetate	0.464 mg/mL mRNA in 100 mM sodium acetate	0.347 mg/mL mRNA in 100 mM sodium acetate
Instrument Setting	2		
Downstream Processing	4x dilution with nuclease-free PBS		

Three mRNAs with different lengths were encapsulated to explore the effect of RNA length. These were: GFP mRNA containing 996 nt, Luc mRNA containing 1921 nt and Cas9 mRNA containing 4521 nt. Across all three mRNAs EE% of 90% were achieved with consistent size and PDI, indicating that mRNA length does not impact key physical attributes of the LNPs (**Figure 6**).

**Figure 6.) High encapsulation efficiency achieved for mRNAs independent of mRNA length.** Size and PDI (A) was measured by dynamic light scattering, and encapsulation efficiency (B) was measured by modified ribogreen assay. Formulations were prepared in triplicate. Values represent the mean and error bars, the standard deviation. Means grouped by horizontal bars were not significantly different ( $P>0.05$  by Tukey's multiple comparison test (A) and ANOVA (B)). Error bars not drawn when error is less than the size of the symbol.

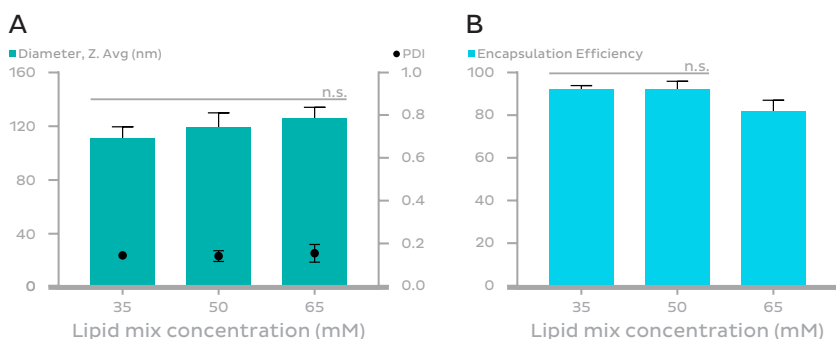


**Formulation Parameters**

Lipid Composition	PNI-ILa:DSPC:Cholesterol:PEG-DMG (50:10:38.5:1.5)
Initial Lipid Concentration	50 mM
N/P	4
Organic Solvent	Ethanol
Aqueous Phase	0.695 mg/mL mRNA in 100 mM sodium acetate
Instrument Setting	2
Downstream Processing	4x dilution with nuclease-free PBS

In some formulations, the initial concentration of lipid can affect the particle size. The effect on EE%, size and PDI for three initial lipid mix concentrations of 35, 50 and 65 mM were compared (**Figure 7**). Other parameters were held constant, particularly the N/P ratio and lipid composition. EE% remained above 80% in all cases, and no significant difference in size, or PDI were observed.

**Figure 7.) Effect of lipid mix concentration on mRNA LNP.** Low volume mRNA-LNPs were formulated with different total lipid concentration between 35 – 65 mM. Size and PDI (A) were measured by dynamic light scattering. Encapsulation efficiency (B) was measured by modified Riobogreen assay. Formulations were prepared in triplicate. Values represent the mean and error bars, the standard deviation. Means grouped by horizontal bars were not significantly different ( $P > 0.05$  by ANOVA (A) and Tukey's multiple comparison test (B)). Error bars not drawn when error is less than the size of the symbol.



#### Formulation Parameters

	Lipid Mix Concentration (mM)		
	35	50	65
Lipid Composition	PNI-ILa:DSPC:Cholesterol:PEG-DMG (50:10:38.5:1.5) mol%		
N/P	4		
Organic Solvent	Ethanol		
Aqueous Phase	0.487 mg/mL mRNA in 100 mM sodium acetate	0.695 mg/mL mRNA in 100 mM sodium acetate	0.904 mg/mL mRNA in 100 mM sodium acetate
Instrument Setting	2		
Downstream Processing	4x dilution with nuclease-free PBS		

## Discussion

We have manufactured lipid nanoparticles encapsulating mRNA at microliter volumes for the purposes of screening materials and formulation parameters, while requiring just 10–25 µg of valuable mRNA and less than 1 mg of novel cationic lipid per formulation. Formulation parameters including PEG content, N/P ratio, and choice of the cationic/ionizable lipid, mRNA length and lipid mix concentration were independently examined for their effect on nanoparticle attributes. Reproducible batch-to-batch encapsulation efficiencies, size and PDI were achieved across all variables, demonstrating how Spark is a robust platform for small-volume production of nucleic acid loaded LNPs. It is important to note, however, that these factors may have a more dramatic effect on biological activity. Hence, further testing with biological end points is necessary. From these results, it is evident that the Spark is ideal for screening novel formulations that use scarce or expensive active pharmaceutical ingredients and formulation excipients.

An examination of PEG content in formulations containing PNI-ILa, revealed an unexpected behavior. It had been demonstrated in the past with formulations containing a different but similar ionizable CL and encapsulating siRNA, that greater PEG quantities in this range led to smaller particles<sup>17</sup>. Here, with mRNA as the payload, the size was unaffected by PEG content. PEG content did however reduce EE% with formulations containing 4 mol% PEG-lipid. This decrease in EE% at higher PEG-lipid was also observed in formulations produced at mL scales on the NanoAssemblr Benchtop (data not shown). It was found that increasing the N/P ratios from 4:1 to 8:1 has minimal effect in encapsulation efficiency. This is expected, the mRNA will be completely encapsulated beyond a threshold N/P ratio.

We have previously shown that mRNA-LNPs produced with the Spark were effective in eliciting exogenous gene expression in rat primary neuronal cultures, and that these formulations can be scaled up for in vivo studies using the larger-volume NanoAssemblr Benchtop instrument while maintaining consistent EE above 90%, even with different mRNA lengths<sup>18</sup>. Hence, once the lead nanoparticle formulations have been selected by

screening using the Spark, they can be scaled up using the NanoAssemblr Benchtop for further optimization and early *in vivo* testing, and the NanoAssemblr Blaze for larger *in vivo* studies or as production demands dictate.

## Conclusion

Screening active ingredients, excipients and formulation conditions are important in the discovery and early development stages in order to streamline future development and rational design of nanomedicines. This work uses the NanoAssemblr Spark's unique ability to quickly and reproducibly formulate mRNA-LNPs at volumes ideal for screening (100 – 250 µL; 10–25 µg of RNA; < 1 mg of novel cationic lipid). The Spark uses proprietary microfluidic mixing technology for the rapid (< 10 seconds), controlled and reproducible manufacturing of nanoparticles. mRNA-LNPs produced using PNI-ILa were in the range of 90 – 140 nm in diameter with PDI below 0.2. The encapsulation efficiencies of mRNA were consistently over 80%. We have determined that formulation parameters such as PEG content above 3% can reduce encapsulation efficiency without detectable changes in particle size or PDI. The Spark is the only system that combines ultra-low volume formulation with reproducible conditions making Spark ideal for screening formulations that use scarce or expensive active pharmaceutical ingredients and/or excipients to inform rational design of nanoparticle drug delivery systems.

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

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

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

precisionnanosystems.com

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