Application note

From Microfluidic Formulation to Payload Quantification: TAMARA and CloudSpec for RNA-LNP Development

Comparative analysis of CloudSpec and RiboGreen assay for RNA payload quantification.

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Abstract

Successful RNA-LNP development depends on both **reproducible formulation** and **reliable characterization**. Among the critical quality attributes of RNA-LNPs, **encapsulation efficiency (EE%)** is essential for evaluating formulation performance. While the RiboGreen assay is widely used for EE% determination, it relies on detergent-mediated nanoparticle lysis prior to total RNA quantification, introducing additional sample preparation and potential variability. **CloudSpec**, based on scatter-free absorbance spectroscopy, provides an alternative approach for direct RNA payload quantification without requiring nanoparticle disruption.

In this collaborative study, **Inside Therapeutics** and **Marama Labs** combined the **TAMARA** microfluidic formulation platform with **CloudSpec** to demonstrate a workflow for RNA-LNP development. SM-102-based polyA-LNPs were formulated by varying the **N/P ratio** and **total flow rate**. Particle size and PDI were characterized by DLS, and RNA payload quantification obtained with CloudSpec was directly compared with the conventional RiboGreen assay.

TAMARA produced **highly reproducible RNA-LNPs** across replicate batches, while CloudSpec generated **free polyA and EE% measurements consistent with the conventional RiboGreen assay**. In addition, CloudSpec quantified **total polyA concentrations that more closely matched the expected formulation input** without requiring nanoparticle lysis.

Introduction

Lipid nanoparticles (LNPs) have become the leading non-viral delivery platform for RNA therapeutics, enabling the successful clinical application of siRNA therapeutics and mRNA-based vaccines. As RNA-based medicines continue to expand into new therapeutic areas, robust formulation processes and reliable analytical characterization are essential for accelerating formulation development, ensuring consistent product quality, and meeting evolving regulatory expectations.

Microfluidic mixing has emerged as a powerful approach for RNA-LNP formulation, in which rapid mixing of the aqueous and organic phases induces flash precipitation and spontaneous self-assembly of lipid nanoparticles. Precise control over key process parameters, including the flow rate ratio (FRR) and total flow rate (TFR), ensures consistent and reproducible LNP production while facilitating formulation optimization. The **TAMARA** microfluidic platform provides highly controlled, reproducible RNA-LNP production across formulation volumes ranging from microliters to milliliters, supporting both high-throughput screening and preclinical development.

Alongside robust formulation, comprehensive characterization is essential throughout RNA-LNP development. One of the key critical quality attributes (CQAs) is **encapsulation efficiency (EE%)**, which describes the proportion of RNA successfully encapsulated within the LNPs relative to the total RNA present. EE% directly influences dose accuracy, formulation performance, and product consistency, making its reliable determination essential during formulation screening, process optimization, and quality control.

The **RiboGreen (RG) assay** is a commonly used method for determining EE% in RNA-LNPs. It is based on a fluorescent dye that selectively binds RNA accessible in solution, enabling quantification of free RNA before nanoparticle disruption and total RNA following detergent-mediated lysis. EE% is then calculated from the difference between total and free RNA relative to the total RNA content. While the assay is simple, sensitive, and well suited for high-throughput screening, accurate total RNA quantification depends on complete nanoparticle disruption and involves multiple sample preparation steps, making the workflow susceptible to potential variability.

To address these limitations, **CloudSpec** uses **scatter-free absorbance spectroscopy (SFA)** to directly quantify RNA payload in intact LNPs. Unlike conventional UV/Vis measurements, which are affected by light scattering from nanoparticles, SFA captures both transmitted and scattered light to determine the true absorbance of the sample, enabling accurate RNA concentration measurements independent of scattering. Combined with fluorescence-based free RNA detection, CloudSpec enables lysis-independent determination of EE% within a single analytical platform, reducing sample preparation while minimizing potential sources of variability.

This collaborative study brings together the **TAMARA** microfluidic formulation platform from **Inside Therapeutics** and the **CloudSpec** RNA payload quantification technology from **Marama Labs** to demonstrate a complementary workflow for RNA-LNP development. The effects of key formulation parameters, including the N/P ratio and TFR, on particle size, PDI, and EE% were evaluated. The analytical performance of CloudSpec was then directly compared with the conventional RiboGreen assay.

Results

1/ Physicochemical characterization of polyA-LNPs

Dynamic light scattering (DLS) was used to characterize the size and polydispersity index (PDI) of SM-102 LNPs encapsulating polyA, formulated using the TAMARA microfluidic platform. Three formulation conditions were evaluated, combining two N/P ratios (6 and 1) with two TFRs, 5 and 10 mL/min. Specifically, formulations were prepared at N/P 6 with TFR 5 and 10 mL/min, and at N/P 1 with TFR 5 mL/min. Each formulation was produced in triplicate to evaluate batch-to-batch reproducibility.

For formulations prepared at an N/P ratio of 6, LNPs exhibited highly consistent physicochemical properties at both TFR settings. Particle sizes were approximately 105 nm with PDI values below 0.20 (~0.13), indicating a narrow size distribution and homogeneous nanoparticle population. The close agreement between replicate batches highlights the excellent reproducibility of the microfluidic formulation process and demonstrates that changing the TFR from 5 to 10 mL/min had minimal impact on particle characteristics under these conditions.

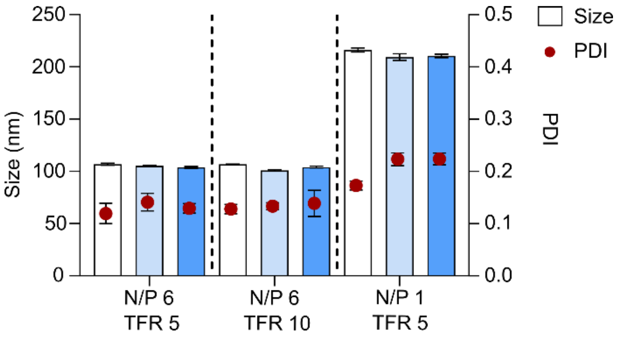

***Figure 1**. Effect of N/P ratio and TFR on LNP size and PDI for SM-102-based, polyA-loaded LNPs. Data are presented as mean ± SD (n = 3).*

In contrast, reducing the N/P ratio to 1 while maintaining a TFR of 5 mL/min resulted in substantially larger particles and PDI. These formulations produced LNPs with an average size of approximately 212 nm and a PDI of around 0.21. The observed increase in particle size and heterogeneity demonstrates the strong influence of N/P ratio on LNP formation and is consistent with the reduced availability of ionizable lipid to drive efficient nanoparticle self-assembly.

Across all conditions tested, the low variability between replicate formulations confirms the robustness of the TAMARA platform for reproducible LNP production.

2/ Payload quantification: CloudSpec vs RiboGreen

polyA quantification was performed using two analytical methods: the conventional RiboGreen (RG) assay and the CloudSpec RiboGreen (CS-RG) assay. Both methods were used to quantify free polyA, total polyA, and encapsulation efficiency (EE%) of SM-102 LNPs prepared under three different formulation conditions described above, allowing direct comparison of their analytical performance.

Both assays produced highly comparable results for free polyA quantification (Figure 2a). Minimal levels of free polyA (<2 µg/mL) were detected for the N/P 6 formulations, whereas substantially higher free RNA concentrations (~20 µg/mL) were measured for the N/P 1 formulation. The close agreement between the two methods demonstrates that the CS-RG assay accurately quantifies unencapsulated polyA and reliably differentiates between formulations with high and low encapsulation performance.

A difference between the two methods was observed for total polyA quantification (Figure 2b). Across all formulation conditions, the CS-RG assay consistently measured higher total polyA concentrations than the conventional RG assay, while preserving the same trends between formulations. Notably, the total polyA concentrations measured by the CS-RG assay were in closer agreement with the theoretical polyA concentration used during formulation (approximately 80 µg/mL), whereas the conventional RG assay consistently reported lower values. Unlike the conventional RG assay, which requires disruption of the LNPs prior to polyA quantification, the CS-RG assay directly measures total polyA without an LNP lysis step. Eliminating this sample preparation step simplifies the workflow and removes a potential source of variability associated with incomplete or inconsistent particle disruption, which may contribute to the lower total RNA recoveries observed with the conventional assay.

Despite the differences in total polyA measurements, the encapsulation efficiencies calculated using both methods were highly comparable (Figure 2c). Both assays reported encapsulation efficiencies approaching 100% for the N/P 6 formulations, while a marked decrease in EE% was observed for the N/P 1 formulation. This demonstrates that both methods reach the same conclusions regarding formulation performance and accurately capture the effect of decreasing the N/P ratio on polyA encapsulation.

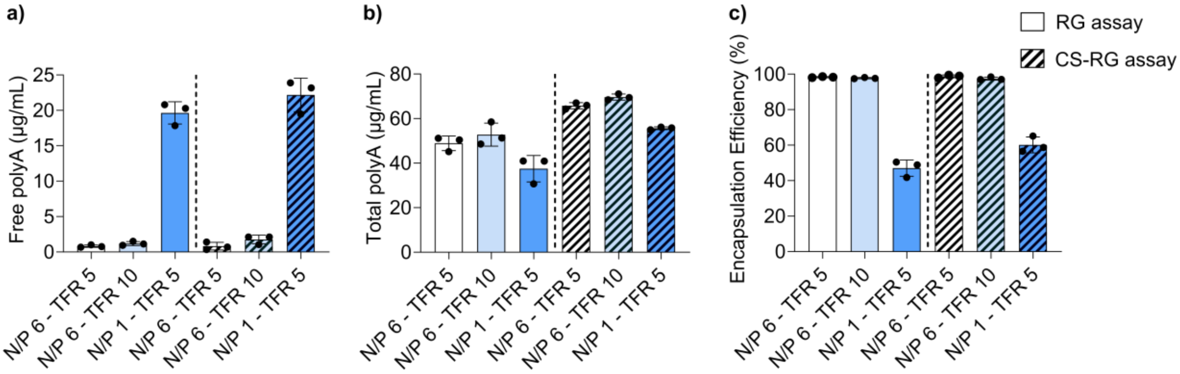

***Figure 2**. Comparison of RNA payload quantification by the RiboGreen assay (RG) and CloudSpec-RiboGreen assay (CS-RG assay). For each analytical method, **(a)** free polyA concentration, **(b)** total polyA concentration, and **(c)** calculated encapsulation efficiency (EE%) are shown for all formulation conditions. Data are presented as mean ± SD (n = 3 independent LNP batches).*

Overall, the CloudSpec assay showed excellent agreement with the conventional RiboGreen assay for free polyA quantification and encapsulation efficiency while providing total polyA measurements that more closely matched the expected polyA input. By eliminating the need for LNP disruption, the CloudSpec workflow reduces sample preparation, minimizes potential sources of assay variability, and enables rapid, streamlined polyA quantification without compromising analytical performance.

Conclusion & discussions

This collaborative study highlights the complementary strengths of reproducible RNA-LNP formulation using TAMARA and robust RNA payload quantification using CloudSpec.

The **TAMARA microfluidic platform** enabled highly reproducible RNA-LNP production across replicate batches while providing precise control over key formulation parameters. The study further demonstrated that the N/P ratio had a significant effect on particle size, PDI, and encapsulation efficiency, whereas increasing the total flow rate had minimal influence under the conditions evaluated. These results highlight the suitability of TAMARA for reproducible formulation, formulation optimization, and high-throughput screening.

CloudSpec showed excellent agreement with the conventional RiboGreen assay for free polyA quantification and encapsulation efficiency, while providing total polyA measurements that more closely matched the expected formulation input without requiring nanoparticle disruption. By eliminating the lysis step, the CloudSpec workflow simplifies sample preparation, reduces assay variability, and enables robust RNA payload quantification.

Together, these complementary technologies support a more reliable approach to RNA-LNP development by pairing highly reproducible formulation with robust payload quantification. This workflow enables confident formulation screening and analytical characterization, facilitating informed decision-making throughout RNA-LNP development.

Materials & methods

1/ polyA-LNP formulation using TAMARA

Polyadenylic acid (polyA)-loaded lipid nanoparticles (LNPs) were formulated using the TAMARA microfluidic platform (Inside Therapeutics, France) with an SM-102-based lipid composition consisting of SM-102/DSPC/cholesterol/PEG2000-DMG at a molar ratio of 50/10/38.5/1.5. PolyA was used as a model RNA because of its commercial availability and well-characterized optical properties. Lipids were dissolved in ethanol as the organic phase, while the aqueous phase consisted of 10 mM citrate buffer (pH 3.0).

Microfluidic mixing was performed using a staggered herringbone mixer (SHM) at a fixed flow rate ratio (FRR) of 3:1. The effects of the N/P ratio (1 and 6) and TFR (5 and 10 mL/min) on RNA-LNP formulation were evaluated, while all other formulation parameters were kept constant. The N/P ratio was adjusted by varying the amount of ionizable lipid while maintaining a constant final polyA concentration of 0.1 µg/µL. Nanoparticles were purified by dialysis prior to physicochemical characterization.

Further details on the formulation procedure can be found in the "RNA-LNP Formulation Protocol" available on the Inside Therapeutics website.

2/ Physicochemical characterization

Particle size and polydispersity index (PDI) were determined by dynamic light scattering (DLS) using a Zetasizer Ultra (Malvern Instrument, Worcestershire, UK). Prior to analysis, 30 µL of LNP suspension was diluted to a final volume of 1 mL with 1X PBS and measured at 25 °C. Three consecutive measurements were acquired for each sample, and the reported values correspond to the mean of the three measurements.

3/ RiboGreen assay for EE%

The Quant-iT RiboGreen assay (ThermoFisher) was performed according to the manufacturer's protocol. The assay was conducted in a flat-bottom black 96-well plate (Thermo Scientific™ Sterilin™). A standard curve was prepared by diluting polyA in 1X TE buffer to final concentrations ranging from 0 to 200 µg/mL. The LNPs samples were diluted to 2 µg/mL in final volume of 100 µL in 1X TE. Then, 100 µL of RiboGreen (diluted 1:200 in 1X TE) was added to both the samples and the standard curve. The plate was incubated in the dark for 5 min, shaking at 100 rpm using an orbital shaker (Benchmark Scientific). After incubation, the fluorescence intensity of non-encapsulated polyA was measured using a VANTAstar Microplate reader (BMG LABTECH) at an excitation wavelength of 485 (±20) nm and an emission wavelength of 535 (±25) nm. Next, 22 µL of 1% (v/v) Triton X-100 was added to each sample, and the plate was incubated for 3 min, shaking at 100 rpm in the plate reader. Following incubation, the fluorescence intensity of total polyA was measured under the same conditions.

4/ CloudSpec analysis for EE%

4.1/ Free RNA quantification using SFAS

Free RNA concentration was quantified using Spectral Fluorescence and Absorbance Spectroscopy (SFAS) on the CloudSpec instrument (Marama Labs, Ireland). PolyA concentration was determined from the fluorescence signal at 560 nm, with the signal at 680 nm used for background correction. Free polyA concentration was calculated using a ratiometric approach relative to a matrix-matched reference standard containing 0.5 µg/mL RNA. The final polyA concentration in the original LNP formulation was obtained by applying the appropriate sample dilution factor.

For analysis, an RG working solution was prepared by diluting the stock reagent 380-fold in 1X TE buffer. The reference standard was prepared by mixing RG working solution with polyA to obtain a final polyA concentration of 0.5 µg/mL in a 95:5 TE/PBS matrix. LNP formulations were diluted to a working concentration of approximately 40 µg/mL polyA, after which 55 µL of sample was mixed with 1045 µL of RG working solution, resulting in a final 50-fold dilution of the original LNP formulation. All samples were prepared to a final volume of 1.1 mL, protected from light, and measured in a 1 cm^2 quartz cuvette using 95:5 TE/PBS as the blank. A fresh baseline was acquired before each measurement.

4.2/ Total RNA quantification using SFAS

Total RNA concentration was quantified directly using the CloudSpec instrument without prior disruption of the lipid nanoparticles. LNP formulations were diluted to a working polyA concentration of 10 µg/mL in 1X PBS, and corresponding empty LNP formulations were prepared at the same dilution to serve as reference samples.

Measurements were performed in a 1 cm^2 quartz cuvette with a 1 mL sample volume. The CloudSpec integrating sphere automatically corrects for optical pathlength modifications caused by light scattering, enabling accurate determination of the absorption coefficient. Extinction and absorption spectra were acquired simultaneously, and the scattering spectrum was obtained from the difference between the two measurements. Total polyA concentration was determined by fitting the spectrum of the polyA-loaded LNPs to a weighted combination of reference spectra from purified polyA and the corresponding empty LNP formulation. The polyA concentration in the original formulation was then calculated by applying the sample dilution factor.

Further details on the CloudSpec methodology can be found in the application note "CloudSpec: Scatter-Free Absorbance for Rapid Payload Quantification of Intact LNPs", available on the Marama Labs website.

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