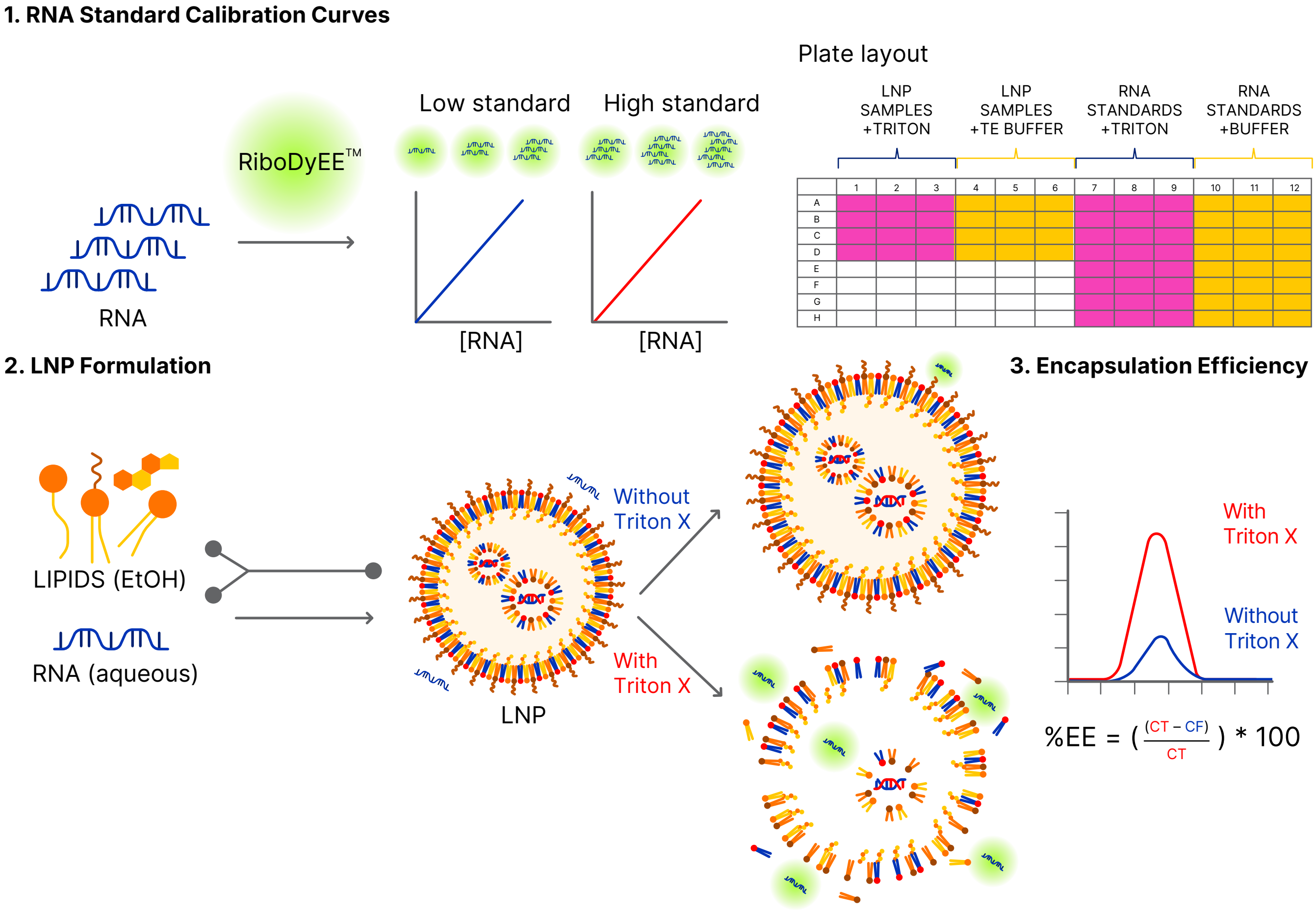
RiboDyEE™: A New Cost-Effective Solution for Robust RNA Encapsulation Efficiency Measurement in Lipid Nanoparticles



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$$\%EE = \left(\frac{(CT - CF)}{CT}\right) * 100$$

Introduction

RNA-based therapeutics represent one of the fastest-growing sectors in biopharmaceuticals, with lipid nanoparticles (LNPs) serving as an essential delivery vehicle.

Following formulation, quantifying the amount of RNA present within the LNP is critical to ensure dosing accuracy and efficiency. RNA encapsulation efficiency (EE) is almost exclusively measured using a fluorescence-based method with a single fluorescent dye (RG). The dye has been adapted into a workflow which enables quantification of LNP EE.

The most followed protocols typically require mixing of the RNA sample with RG, which can then be measured in a fluorescence plate reader and the amount of RNA quantified using a standard curve. While such plate-based fluorescence assays are easy to perform in standard lab setups, there are some notable downfalls that lead to inaccuracies when measuring LNP EE. For example, protocols recommend use of a ribosomal RNA (rRNA) standard that comes with kits when generating standard curves. However, this can lead to inaccuracies in fluorescence readings due to variation between RNA constructs. RG emits fluorescence when it binds to nucleic acids; however, varying RNA constructs can harbor different secondary structures causing parts of the strands to be shielded and hence lower the binding potential of the dye. For example, GC rich regions can exhibit a more rigid structure which could affect dye binding. Additionally, when working with LNPs a detergent such as Triton X-100 is often required to allow 'bursting' of the LNP to release encapsulated RNA; however, these detergents can quench fluorescence signals. Standard operating procedures do not account for this use and hence could lead to inaccuracies, affecting downstream processing and dosing. Current industry standard methods for measuring RNA EE rely on expensive reagents that can represent a significant portion of laboratory budgets, particularly as formulation optimisation and manufacturing scales increase.

Recognizing the need for commercial suppliers to promote best practices for consistent LNP EE assays, we developed RiboDyEE: the first commercially available dye specifically for measuring LNP EE. RiboDyEE can be used as a direct substitute for the industry standard, with an optimized protocol tuned specifically for reproducible and reliable EE measurements. RiboDyEE offers a cost-effective alternative to reduce operational expenses while also being optimized to decrease handling time. Furthermore, we developed a new encapsulation assay format that effectively reduces the need for aliquoting.

Materials and Methods

Reagent	Cat. #	Supplier
RiboDyEE	9000	R&D Systems
Dlin-MC3-DMA	7946	R&D Systems
SM-102	8909	R&D Systems
DMG-PEG2000	7944	R&D Systems
DSPC	7943	R&D Systems
Cholesterol	7945	R&D Systems
Polyadenylic Acid (polyA)		Merck Life Sciences
RG		Fisher Scientific
Tris-HCl buffer		Fisher Scientific
EDTA		Fisher Scientific
Sodium citrate buffer		Fisher Scientific
Absolute ethanol		Fisher Scientific
ALC-0159		Avanti Polar Lipids
ALC-0315		Broadpharm
EZ Cap Firefly Luciferase mRNA		Stratech Scientific

LNP Manufacture

LNP lipid phase consisted of one of the following in absolute ethanol (>99.5%): DSPC, cholesterol, SM-102 and DMG-PEG2000 (ratio of 10:38.5:50:1.5%); DSPC, cholesterol, DLin-MC3-DMA and DMG-PEG2000 (ratio of 10:38.5:50:1.5%); or DSPC, cholesterol, ALC-0315 and ALC-0159 (ratio of 9.4:42.7:46.3:1.6%). The aqueous phase consisted of either poly A or Firefly Luciferase mRNA (Fluc) in citrate buffer 50 mM (pH 5).

LNPs were manufactured on Nova™ IJM Benchtop (Helix Biotech, TN, USA) at a flow rate ratio of 3:1 (aqueous:lipid) and a total flow rate of 15 mL/min. LNPs were purified using size chromatography in Tris 10 mM, pH 7.5 before analysis.

RiboDyEE Reagent Preparation

RiboDyEE was diluted in 1X TE buffer to achieve 200X and 500X solutions to be added in presence or absence of 2%-Triton TE buffer, respectively. 100 µL of these solutions are required for each well.

Encapsulation Efficiency Assay

The assay was performed as described in detail in our RiboDyEE Protocol

In the next section, we provide tips for best practice.

RNA Standard Curves Using RiboDyEE

A high standard curve ranging from 0 to 1000 ng/mL and a low standard curve ranging from 0 to 50 ng/mL (final well volume of 100 µL) should be used to ensure adequate capture of both encapsulated (in the presence of detergent) and unencapsulated (without detergent) RNA respectively. Here, our aqueous phase contained either Firefly Luciferase mRNA or Poly A at both 4 µg/mL and 0.2 µg/mL stock concentrations. Standard curves were added to the plate in triplicate wells and prepared as shown below in Tables 1 and 2.

RNA quantification should be conducted as soon as possible post LNP manufacture. As a useful tip we advise using remaining RNA species from the aqueous phase of formulation to generate standard curves for quantification (making 4 µg/mL and 0.2 µg/mL stocks).

Using the actual RNA species being encapsulated for standard curve preparation, **rather than generic reference standards**, improves accuracy when calculating encapsulation efficiency and mass balance due to differences in fluorescence of different payloads. Generation of **two standard curves** to quantify detergent-included and non-detergent-included wells helps to alleviate any effect on fluorescence measurements.

For best practice we suggest that fresh nucleic acid standard curves are generated each time LNP EE analysis is performed to ensure reliable results.

TABLE 1

Protocol for preparing a high standard curve (in the presence of Triton).

High standard curves

RNA Concentration (ng/mL)	Volume of 4 µg/mL Stock	Volume of TE buffer (µL)	Volume of 2% Triton TE (µL)	Total volume (µL)
1000	50	0	50	100
800	40	10	50	100
600	30	20	50	100
400	20	30	50	100
200	10	40	50	100
100	5	45	50	100
50	2.5	47.4	50	100
0	0	50	50	100

TABLE 2

Protocol for preparing a low standard curve (in the absence of Triton).

Low standard curves

RNA Concentration (ng/mL)	Volume of 0.2 µg/mL Stock (µL)	Volume of TE buffer (µL)	Total volume (µL)
50	50	50	100
40	40	60	100
25	25	75	100
10	10	90	100
5	5	95	100
2.5	2.5	97.5	100
1.25	1.25	98.75	100
0	0	100	100

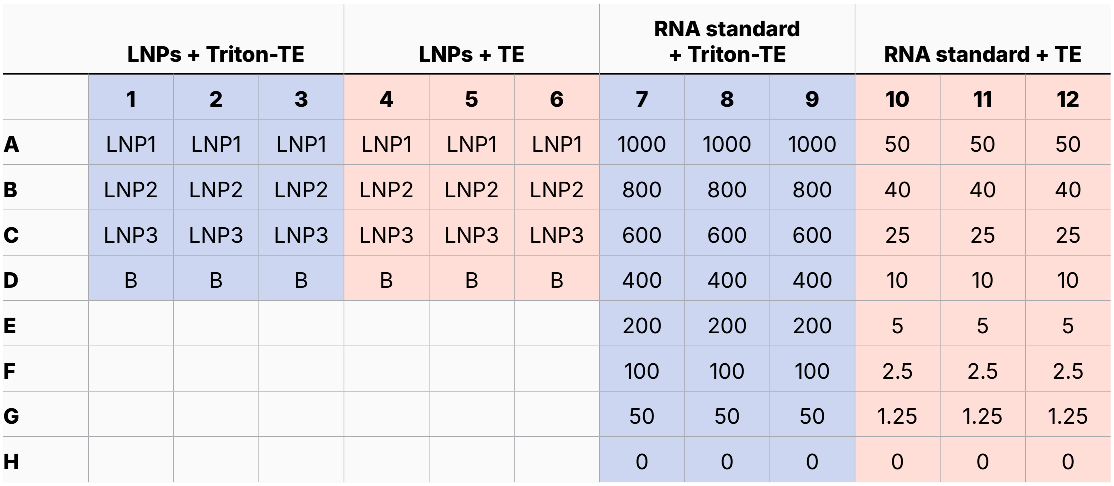
RNA-LNP Sample Preparation and Well Layout

RNA-LNP samples should be diluted with 1X TE buffer to achieve 350 µL at a concentration of 3 µg/mL RNA. For example, for a LNP with a payload concentration of 40 µg/mL, 26 µL LNP should be diluted in 232 µL 1X TE buffer:

$$C1V1 = C2V2$$
$$40 \text{ X } V1 = 3 \text{ X } 350$$
$$V1 = \frac{(3 \text{ x } 350)}{40}$$
$$V1 = 26\ \mu L$$

For plating the LNPs, six wells (volume of 50 µL) of each RNA-LNP sample should be added to the appropriate wells on the plate as shown below. For each RNA-LNP sample, 50 µL 1X TE buffer should be added to three of the wells while 50 µL of 2%-Triton TE buffer is added to the remaining three wells (final volume per well will be 100 µL). A blank should also be prepared using the same dilution as the RNA-LNP sample substituting your LNP for the buffer (for example, 26 µL Tris buffer and 332 µL 1X TE buffer. Example plate layout shown below (Figure 1):

FIGURE 1



	LNPs + Triton-TE			LNPs + TE			RNA standard + Triton-TE			RNA standard + TE		
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	LNP1	LNP1	LNP1	LNP1	LNP1	LNP1	1000	1000	1000	50	50	50
B	LNP2	LNP2	LNP2	LNP2	LNP2	LNP2	800	800	800	40	40	40
C	LNP3	LNP3	LNP3	LNP3	LNP3	LNP3	600	600	600	25	25	25
D	B	B	B	B	B	B	400	400	400	10	10	10
E							200	200	200	5	5	5
F							100	100	100	2.5	2.5	2.5
G							50	50	50	1.25	1.25	1.25
H							0	0	0	0	0	0

Figure 1. Experimental design of a RiboDyEE assay in a 96-well plate, blue wells are prepared with Triton-TE buffer, and pink wells are prepared with TE buffer. Standard curves are plated in Columns 7 – 12 and the rest of the plate is used as needed for LNP samples.

Measuring Assay Readouts

Fluorescence plate readouts were conducting using the GloMax® Discover Microplate Reader using emission filter 500-550 nm and excitation filter Blue 475 nm.

Analytical Validation

All analytical validation calculations were carried out using Microsoft Excel (for ease of experimentation we provide a downloadable excel template for data processing). RiboDyEE curves were monitored for precision, accuracy, LOD, LOQ as well as user variability and inter-day and intra-day variability. Additionally, RiboDyEE was compared to RG using a series of clinically relevant RNA-LNP formulations, where encapsulation efficiency and mass balance were calculated as shown below:

$$\text{Precision} = \left(\frac{\textit{St.Dev of indivdual measurements}}{\textit{mean of individual measurements}}\right) \times 100$$

$$\text{Accuracy} = \left(1 - \frac{\textit{expected-measured}}{\textit{expected}}\right) \times 100$$

$$\text{LOD} = 3.3 \times \left(\frac{\textit{STEYX}}{\textit{slope}}\right)$$

$$\text{LOQ} = 10 \times \left(\frac{\textit{STEYX}}{\textit{slope}}\right)$$

$$\text{Encapsulation Efficiency} = \left(\frac{\textit{TotalRNA-FreeRNA}}{\textit{TotalRNA}}\right) \times 100$$

$$\text{Mass Balance} = \left(\frac{\textit{TotalRNA}}{\textit{TheoreticalRNA}}\right) \times 100$$

Mass Balance Assessment

Complete RNA accounting from input through final product should be reported as mass balance or RNA recovery – allowing for a measure of how much mRNA is remaining in the sample. Often only EE is recorded which does not represent possible mRNA degradation, processing losses or incomplete recovery during the assay. Therefore, it is recommended to include mass balance as an output to help ensure method validity and formulation integrity. This can be implemented into the assay through use of a 'burst' LNP as well as an 'intact' LNP sample, allowing for total RNA present to be compared to RNA input.

Results

Validation - Equivalent Analytical Performance

Three clinically relevant mRNA-LNP formulations were manufactured to show that the RiboDyEE reagent demonstrated equivalent performance in head-to-head experiments when compared to the industry standard RG (n=3). Figure 2 demonstrates that similar encapsulation efficiencies (%EE) and mRNA recovery, represented as mass balance (%MB), were achieved regardless of reagent used. Additionally, Figure 3 and Table 3 demonstrate that the standard curves yielded similar line equations with R2 values being >0.98. Acceptable LOD and LOQ ranges were also achieved for both reagents. RiboDyEE seamlessly quantified encapsulated RNA within mRNA-LNP samples in a reliable and repeatable manner.

FIGURE 2

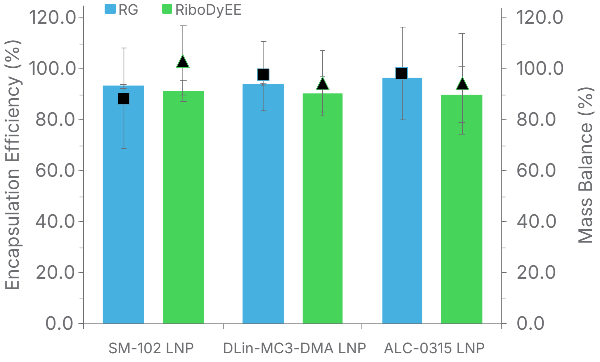


Figure 2. Comparison of RG and RiboDyEE Performance. Encapsulation efficiency (%) is represented by bars, the mass balance is represented by triangles or squares (%). Results are representative of the mean ± standard deviation (n=3).

FIGURE 3

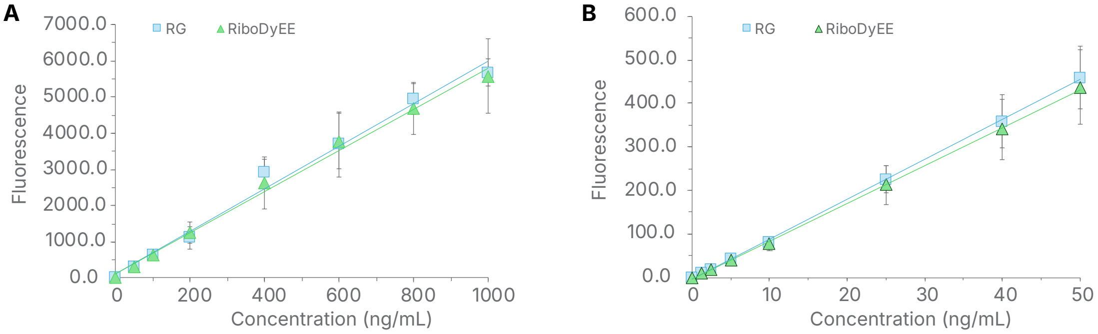


Figure 3. Standard curves generated from RG and RiboDyEE Reagents. Figure 3a represents the higher standard curves ranging 0-1000 ng/mL. Figure 3b represents the lower standard curves ranging 0-50 ng/mL. Results are representative of the mean ± standard deviation (n=3).

TABLE 3

Calibration parameters for RG and RiboDyEE curves.

	RG				RiboDyEE			
	Line Equation	**R2**	**LOD (ng/mL)**	**LOQ (ng/mL)**	**Line Equation**	**R2**	**LOD (ng/mL)**	**LOQ (ng/mL)**
Higher Curve	y = 5.884x + 96.232	0.989	140.1	424.5	y = 5.6995x + 112.02	0.9939	104.0	315.0
Lower Curve	y = 9.1787x - 4.147	0.9996	1.4	4.3	y = 8.716x - 3.7198	0.9995	1.5	4.6

The accuracy of both reagents was analysed at six concentrations across both curves which were above the respective LODs. Here, we were able to demonstrate that accuracy was greater than 87% across all tested conditions, with RiboDyEE being significantly more accurate than RG at 200 ng/mL (Table 4).

Our robust assessment of performance data establishes RiboDyEE as a reliable alternative to the industry standard for RNA quantification.

TABLE 4

Accuracy of RG and RiboDyEE curves at selected concentrations.

	Concentration (ng/mL)					
	2.5	10	50	200	400	1000
RG Accuracy (%)	99.7	93.2	100.9	87.3	119.9	94.9
RiboDyEE Accuracy (%)	104.0	93.9	101.2	100.8	109.9	95.8

Variability: Inter- and Intra- Day Measurements

To further demonstrate the reliability and reproducibility of RiboDyEE, we tested the inter-day variability (Figures 4a and 4b) and intra-day variability (Figures 4c and 4d) of assay measurements.

Inter-day analysis consisted of three independent curves being generated freshly on three separate days (Repeats 1-3) and measured independently. Intra-day analysis consisted of three independent curves generated on the same day but on separate plates (Plates 1-3) and measured independently. Here, we demonstrated that there is minimal intra-day variation, with an increased inter-day variability, resulting in variation to LOD and LOQ limits as well as changes to line equations (Table 5).

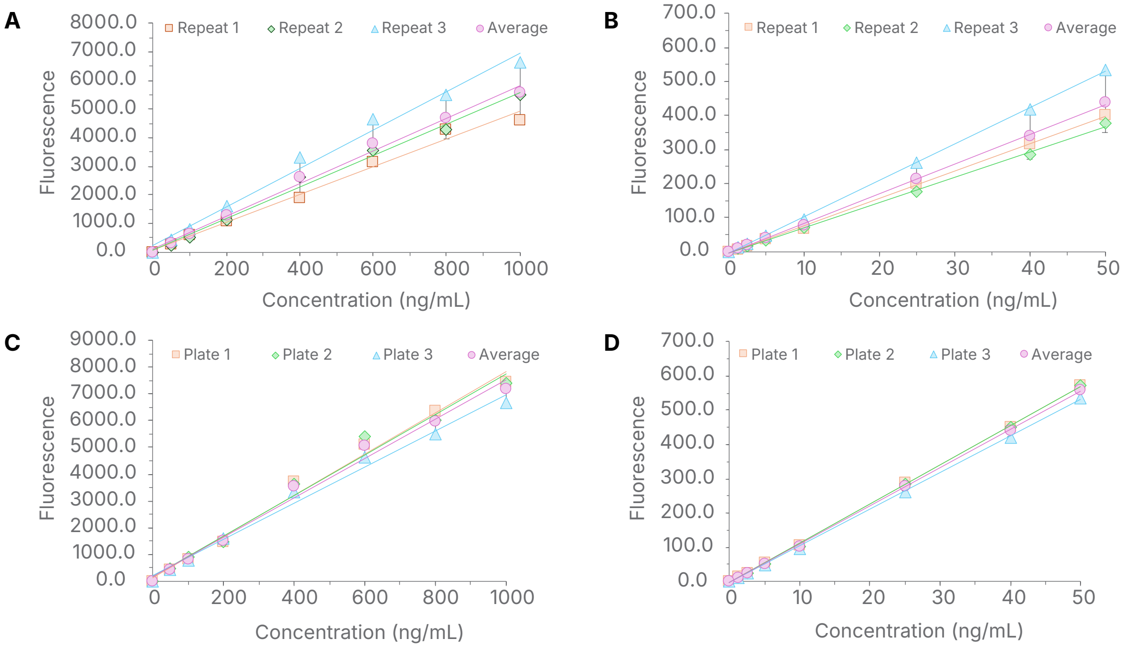


Figure 4. Inter-day and Intra-day variation of mRNA standard curves using RiboDyEE reagent. Inter-day variation of the higher and lower standard curves is represented in Figure 4a and 4b, respectively. Intra-day variation of the higher and lower standard curves is represented in Figure 4c and 4d, respectively.

TABLE 5

Inter-day and Intra-day variation calibration parameters.

		High Curve (0-1000 ng/mL)				Low Curve (0-50 ng/mL)			
		Line Equation	**R^2**	**LOD (ng/ml)**	**LOQ (ng/mL)**	**Line Equation**	**R^2**	**LOD (ng/ml)**	**LOQ (ng/mL)**
Inter-day	Repeat 1	y = 4.8431x + 72.496	0.9883	144.4	437.6	y = 8.0408x - 3.9155	0.9995	1.6	4.8
	Repeat 2	y = 5.5055x + 56.41	0.992	119.2	361.1	y = 7.4106x - 2.8316	0.9986	2.5	7.7
	Repeat 3	y = 6.7499x + 207.14	0.9888	141.5	428.7	y = 10.696x - 4.4123	0.9996	1.3	4.1
	Average	y = 5.6995x + 112.02	0.9939	104.0	315	y = 8.716x - 3.7198	0.9995	1.5	4.6
Intra-day	Plate 1	y = 7.7038x + 126.68	0.9893	138.3	419.2	y = 11.448x - 3.0603	0.9969	1.3	4.1
	Plate 2	y = 7.5791x + 178.73	0.9837	169.0	518.9	y = 11.457x - 4.2348	0.997	1.2	3.6
	Plate 3	y = 6.7499x + 207.14	0.9888	141.5	428.7	y = 10.696x - 4.4123	0.9996	1.3	4.1
	Average	y = 7.3443x + 170.85	0.9882	145.3	440.3	y = 11.201x - 3.9025	0.9997	1.2	3.7

TABLE 6

Calibration parameters of Poly A curves generated by varying users.

	High Curve (0-1000 ng/mL)				Low Curve (0-50 ng/mL)			
	Line Equation	**R^2**	**LOD (ng/mL)**	**LOQ (ng/mL)**	**Line Equation**	**R^2**	**LOD (ng/mL)**	**LOQ (ng/mL)**
Repeat 1	y = 3.5485x + 40.854	0.9916	122.3	370.7	y = 5.1508x - 0.968	0.9997	1.2	3.5
Repeat 2	y = 3.2004x + 57.467	0.9959	85.5	259.2	y = 6.3581x - 2.5818	0.9967	4.0	12.0
Repeat 3	y = 3.0085x + 84.721	0.9935	107.7	326.5	y = 5.4015x - 1.786	0.9992	1.9	5.7
Average	y = 3.2525x + 61.014	0.9947	96.9	293.8	y = 5.6368x - 0.5879	0.9994	1.7	5.1

FIGURE 5

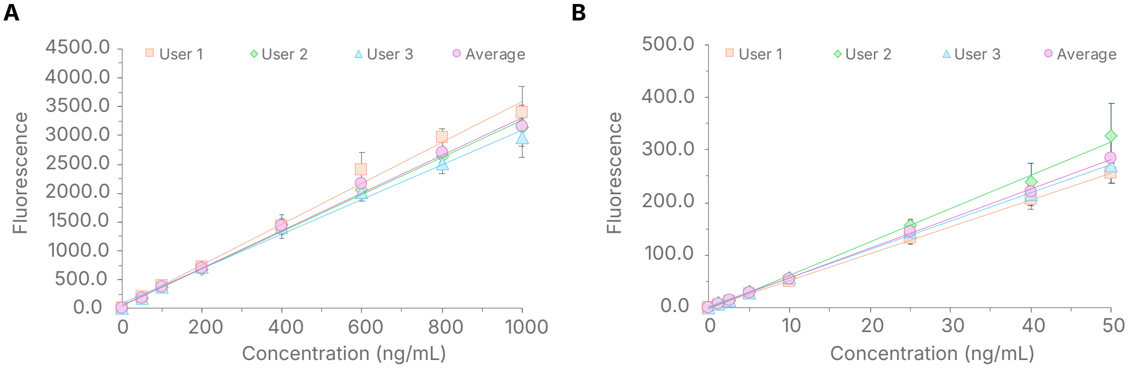


Figure 5. User variability for RiboDyEE. Graphs represent the variability of Poly A high curves (Figure 5a) and low curves (Figure 5b) when generated by different lab users. Data is represented as the mean ± standard deviation (n=3).

We also evaluated user variability shown in Figure 5. We were able to demonstrate that RiboDyEE results in minimal variation of mRNA standard curves across three individual users and that our protocol can reliably be reproduced across users.

Figure 5 and Table 6 demonstrate that the standards curves quantified using the RiboDyEE reagent are highly reproducible and reliable in terms not only of calibration parameters such as R2 values, but also of maintaining similar LOD and LOQ levels throughout testing conditions. Additionally, precision of the curves was monitored as a function of the relative standard deviation (%RSD), allowing us to interpret if the individual points are well clustered around the mean.

Table 7 highlights that precision across the variables described is generally good with %RSD values < 28% and %RSD overall being increased on inter-day curves. For best practice we therefore suggest that fresh nucleic acid standard curves are generated each time LNP EE analysis is performed to ensure reliable results.

Finally, it is also important to highlight the effect of the RNA construct. In Figure 4, mRNA curves display overall higher fluorescence levels compared to poly A curves in Figure 5, highlighting the importance of selecting the correct material when generating the curve, rather than using a generic reference standard. Overall, we were able to demonstrate that variation will increase when RiboDyEE is used across multiple days and best practice would be to generate a standard curve with each individual assay to ensure accurate and reliable quantification.

TABLE 7

%RSD values for standard curves.

		Inter-day	Intra-day	Users
	Concentration	**%RSD**	**%RSD**	**%RSD**
Low Curve	2.5 ng/mL	19.4	4.1	14.0
	10 ng/mL	20.2	4.2	11.6
	50 ng/mL	19.6	3.7	16.5
High Curve	200 ng/mL	22.9	5.2	6.3
	400 ng/mL	27.5	5.7	8.6
	1000 ng/mL	18.5	6.3	11.2

Conclusion

In this study, we validated the performance of RiboDyEE against RG, demonstrating the former as a reliable tool to measure encapsulation efficiencies of RNA-loaded LNPs. RiboDyEE represents a newly validated dye specifically developed as a product for measuring LNP encapsulation under a well-described set of best practices. Additionally, the formulation of RiboDyEE in 120 µL aliquots ensures a stable, convenient and cost-effective solution to quantify RNA encapsulation.

REFERENCES

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2. Schober, G.B. *et al* (2024). Sci. Rep., 14, 2403.

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