Skip the mess with dye-free LNP payload quant on Stunner

Introduction

Everyone who's worked with lipid nanoparticles (LNPs) has used a fluorescent dye-based assay to quantify the RNA content and encapsulation efficiency (EE%) of their formulations and production processes. The same amount of people have gotten tired of these assays; with expensive reagents, two separate sets of calibration curves and multiple components to add to each well, they can be time consuming and fiddly.

Enter Stunner (Figure 1). When you measure your particles using Stunner, not only do you get your particle size and PDI, but you also get full quantification of the RNA in your sample. In this app note we'll detail how to use the Stunner to reduce your dye-based assay burden, saving you time, money and hassle in your daily workflows.

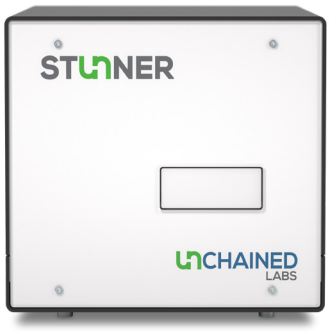


Figure 1: Stunner. The ultimate quantification and sizing tool.

Standard Fluorescent Assays

Typical RNA quantification in LNPs involves using a dye such as RiboGreen or SYBR Gold, which becomes highly fluorescent when it binds to RNA.

EE% assays start by diluting your sample into the working range of the dye. Free RNA is quantified after adding blank buffer, and total RNA is quantified after adding buffer with a surfactant to break down LNPs and release the RNA payload (Figure 2).

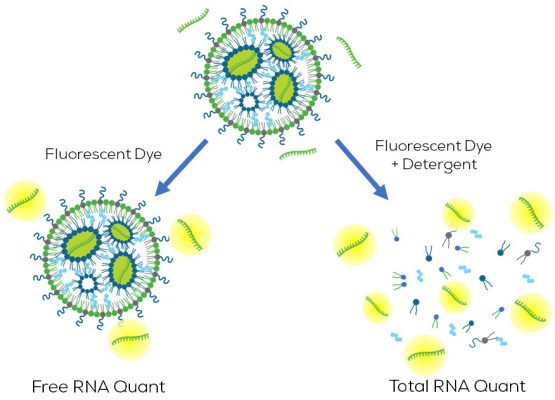


Figure 2: Schematic fluorescent dye-based assays for LNPs.

Don't get any bubbles in there, or you'll have to burst them individually with a needle to get a clean read! Both the surfactant and surfactant-free samples will need their own set of calibration curves and blanks, as the surfactant (commonly Triton X-100) has its own intrinsic fluorescence (Figure 3). The samples then have to be incubated to let the surfactant do its work, and after this the fluorescent dye is added to each well being measured. The samples are incubated again to allow binding of the dye to the RNA, and then you can finally run your measurements, process your calibration curves and get your results. Phew!

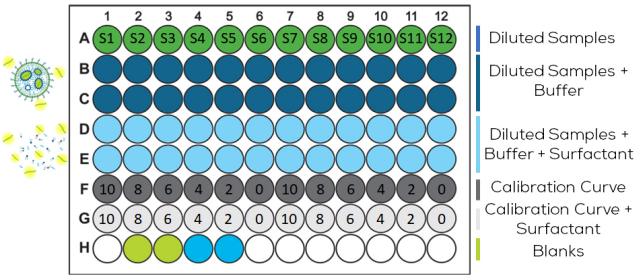


Figure 3: Typical plate layout for a fluorescent dye-based assay.

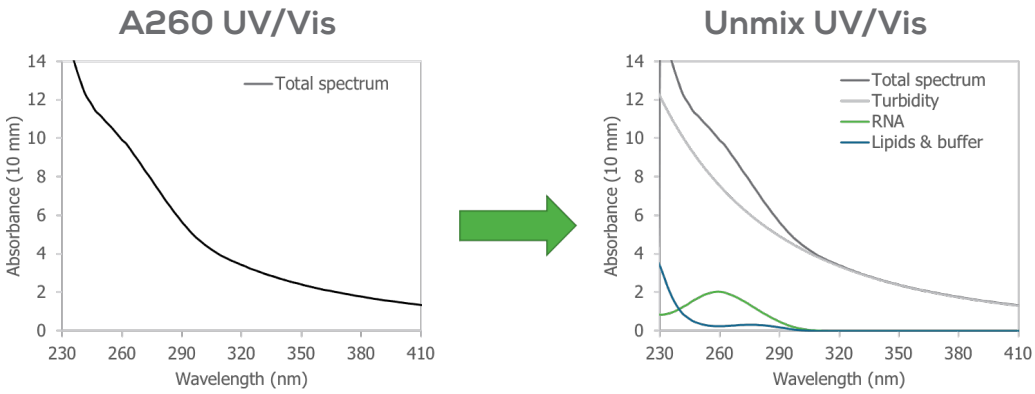


Figure 4: Stunner's RNA-LNP application is able to separate the UV/Vis signals from RNA, lipids, turbidity, and other factors. For this RNA-LNP sample, RNA absorbance (green) measures the total concentration of RNA present in an LNP sample. Turbidity is shown in grey, total absorbance is shown in black, and absorbance of the particle and buffer components in blue.

Total and Free RNA concentrations are calculated, and encapsulation efficiency is given by the equation:

$$\frac{(RNA_{total} - RNA_{free})}{RNA_{total}} \times 100\% = EE\%$$

A 96-well plate allows you to run 12 samples in duplicate, which might take an experienced operator an hour or more, cost over $50 in dye alone, and involve 266 different individual pipetting events - yikes. Want more samples? You'll have to run the whole assay again, calibration curves and all.

The Stunner Solution

Stunner is here to save your day (literally)! Stunner gives you the most sensitive DLS on the market, utilizing cutting edge rotating angle dynamic light scattering (RADLS). A standard LNP analysis uses 7 angles including back-and forward-scatter, giving you much more information on your sample size than previously possible, along with particle count. You also get full UV/Vis quantification of the RNA in your solution, with the absorbance of your RNA separated out from the other particle components by the Unmix algorithm (Figure 4). And all this is in a 96 well plate format input, using the microfluidic Stunner plates which use just 2 µL per sample allowing you to rapidly screen your LNPs for all this information without missing a beat.

This means you can halve your dye-based assay workload – half the samples, half the calibration curves, half the incubation time , and less than half the total time – plus no messing around with bubble-forming surfactants! All you have to do is measure your free RNA by adding your dye to your diluted sample alongside a single set of calibration curves and then do the math using the total RNA data from Stunner. You could even run the assay while Stunner is collecting your data to accelerate your workflow (Tables 1 and 2). EE% is calculated just like before, but with half the hassle and way more data! Check out our SOP in the appendix for a method that will drop the number of individual pipetting events from 266 to 114.

Hybrid method	Data	Total Time (12 samples)	Hands-on Time
Stunner – UV/Vis and RADLS	Size, PDI, Particle Concentration, Total RNA	35 minutes	5 minutes
Reduced Based-RNA Assay	Free RNA	15 minutes	12 minutes
Total	Size, PDI, Particle Concentration, Total RNA, EE%	35 minutes	<20 minutes

Table 1: Estimated time taken for particle analysis using the hybrid Stunner-Dye Assay if run simultaneously.

Standard method	Data	Total Time (12 samples)	Hands-on Time
One-by-one DLS	Size, PDI	60+ minutes	30-60 minutes
Dye-Based Assay	Total RNA/ EE%	45-60 minutes	40 minutes
Total	Size, PDI, Total RNA, EE%	~120 minutes	<70 minutes

Table 2: Estimated time taken for a traditional DLS and fully dye-based assay.

The Process

Screening with PolyA

Let's look at this in action – a series of 3 LNP formulations in duplicate were run on Sunscreen, Unchained Labs' automated solution for high-throughput, microfluidic formulation screening of lipid nanoparticles. Two different reaction buffers were used, 20 mM citrate and 20 mM acetate, to assess which one would be more effective. PolyA was used as the payload as a low-cost RNA analogue. The particles were then dialyzed against 1X PBS.

The DLS data produced by the Stunner highlights differences in particle size and distribution when using different reaction buffers - the acetate buffer produces consistently smaller particles across all three formulations, though this difference is minor in the case of LNP-X.

LNP-Z produces higher quality LNPs in the acetate buffer, with lower PDIs and a Z-average less than half that of those produced in citrate buffer. PDIs for all samples were <0.2, and all particle size averages were <100 nm, with the exception of LNPs -Y and -Z in citrate buffer (Figure 5).

All particle counts for the dialyzed particles were around 9E+11, with slight variations as particle size varies. The clear exception is with LNP-Z, where the particle counts of the samples prepared in citrate buffer are significantly lower, due to the very large particle size (Figure 6).

The concentration data for the samples above, which have a PolyA payload, used a custom RNA analyte based on the Poly A sequence, to get the most accurate data from the Unmix algorithm, unpicking it from the turbidity and interference caused by the lipids

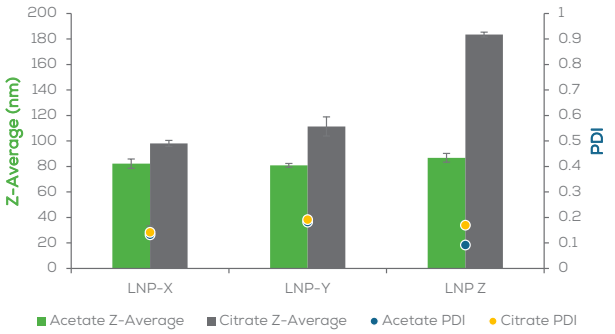


Figure 5: Particle sizes and PDIs of LNPs prepared on Sunscreen. 2 technical repeats.

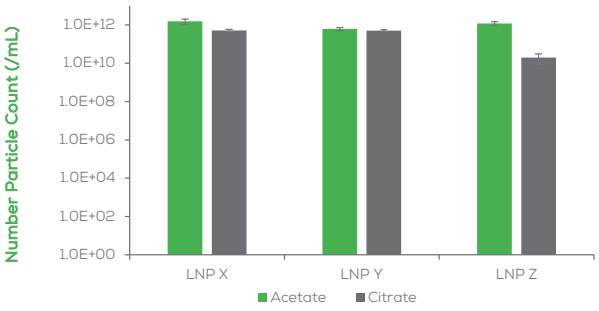


Figure 6: Particle number count of LNPs prepared on Sunscreen. 2 technical repeats..

present (Figure 7). From the total RNA from Stunner, and the free RNA from RiboGreen, you can calculate EE%, using equation 1 as before.

A comparison of the two methods shows very good agreement for total RNA, and EE% values were generally within 1% between the two. Both methods highlighted greater total RNA concentrations for LNP-Z compared to the other formulations, and lower encapsulation efficiencies when the citrate reaction buffer is utilized for this formulation (Figures 7 and 8).

Figure 7: Total PolyA concentrations in LNPs X, Y and Z as measured by Stunner and RiboGreen. 2 technical repeats.

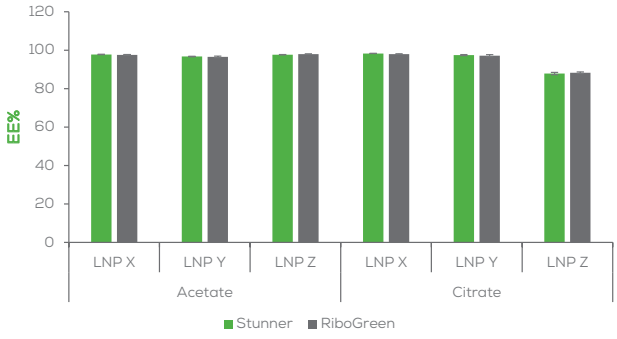


Figure 8: EE% as measured by the Stunner-RiboGreen Hybrid Assay and the Standard RiboGreen assay, 2 technical repeats.

Reliable results so you can relax

Particle Production with FLuc mRNA

The three formulations were run in the best performing buffer (pH 4.00 acetate, 20mM) and were used to encapsulate firefly luciferase RNA (mFLuc), preserving the remaining conditions from the earlier experiments.

The final particles produced were similar to those produced with a PolyA payload. All PDIs were 0.2 or below, indicating narrow particle size distributions (Figure 9).

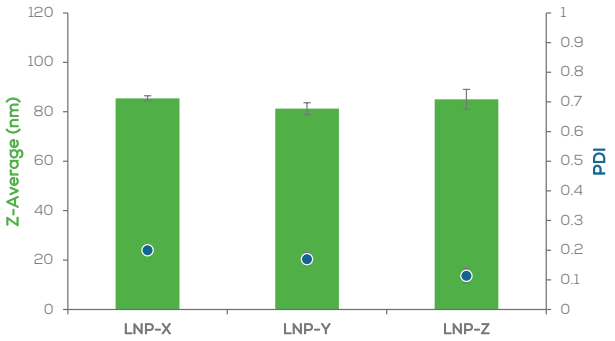


Figure 9: RNA-LNP particle size data. Error bars are SD from triplicate measurements.

Total RNA measurements were again very similar for both RiboGreen and Stunner – the RNA analyte in Stunner's software was set to 2 kb (Figure 10). The coefficient of variation (CV) for the triplicate Stunner measurements was less than 1%, showing excellent precision. The duplicate Ribogreen measurements have a CV of between 0.2 and 5.2%.

EE% was high across the board, with all formulations being above 90% for both methods of measurement (Figure 11). LNP-Z had the lowest EE% at 91%, as measured by the Stunner/ RiboGreen hybrid assay.

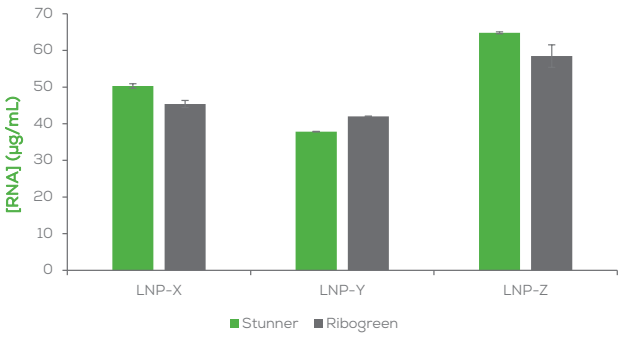


Figure 10: mFLuc mRNA quantification comparison between Ribogreen and Stunner . Error bars are SD from triplicate measurements.

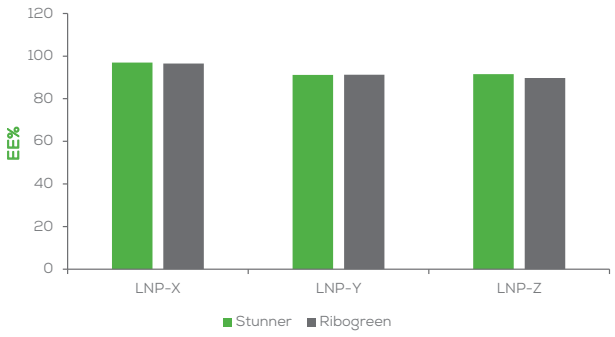


Figure 11: Encapsulation efficiency for LNPs X, Y and Z, as measured by the Stunner Hybrid assay or the standard Ribogreen assay.

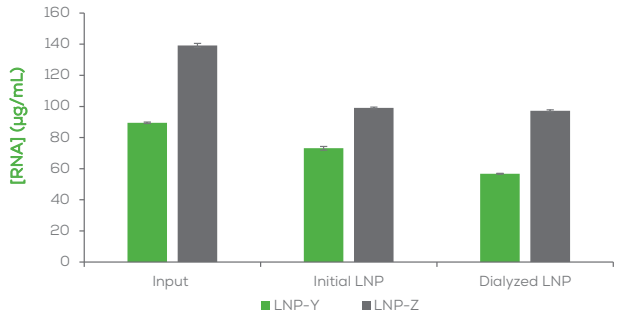


Figure 12: Total RNA in the initial aqueous solution, the LNPs pre-dialysis, and LNPs post-dialysis.

Uniquely, Stunner also allows you to easily keep track of your RNA concentration throughout your production process (Figure 12). When LNP Y and Z are compared for process yield, both LNPs show the expected drop between the input concentration and the initial, undialyzed and undiluted LNP of ~25% due to mixing with the lipid organic phase. However, LNP-Z shows 98% retention of material after dialysis, whereas LNP-Y shows an overall 22% drop. This gives total, final encapsulated yields of 85% for LNP-Z and 71% for LNP-Y, suggesting potential for formulation or process improvements.

Conclusion

Stunner is an unparalleled tool for LNP analysis, with RADLS to tease out the presence of previously invisible aggregates, and UV-Vis to give you your payload information.

So now you can see how much time, money and effort you can save by using Stunner for total RNA quant and how this can replace half of your fluorescent dye assay workflow. Loading 12 samples onto a Stunner plate takes just 5 minutes, compared to significant hands-on time with a typical DLS instrument. The simplified RiboGreen assay allows for free RNA concentration analysis with just 114 total pipetting events, down from 266 for a standard RiboGreen plate, and the plate takes just 15 minutes or less to prepare, compared to more than 45 minutes with a typical assay.

No surfactants, half the samples, and rapid data generation to make high throughput screening a reality for you, without compromising on accuracy, and with more information than ever before!

Appendix

1. LNP Sample Production

Sunscreen conditions for LNP experiments:

Parameter	Setting
Sunny	100X
Total Flow Rate	8000 µL/min
Flow Rate Ratio (Aqueous:Organic)	3:1
Collection Volume	800 µL
Head + Tail Cuts	100 µL

Formulation information:

Parameter	Setting
Lipid Concentration	10 mM
NP Ratio (LNPs X and Y)	6:1
Ionizable Lipid to Nucleic Acid Weight% (LNP-Z)	10%

All samples were dialyzed using 20K MWCO G3 Dialysis Cassettes (Thermo Fisher Scientific) against 1X PBS for 3 hours.

2. Stunner/RiboGreen hybrid assay SOP:

This method is a hybrid RiboGreen and Stunner assay for assessing EE% and RNA content of LNPs and is suitable for analyzing between 1 and 24 samples per 96-well plate.

2.1 Total RNA Quantification with Stunner

Open the Stunner software client and select the "nanoparticles" measurement type. Then select the RNA-LNP app. Input the experimental name and sample placements as per the Stunner software user guide, selecting the most relevant payload on the "edit samples" page. For non-standard RNA constructs, consider making a custom RNA analyte, as covered in the Stunner software user guide. Each sample should be loaded in triplicate for the best results.

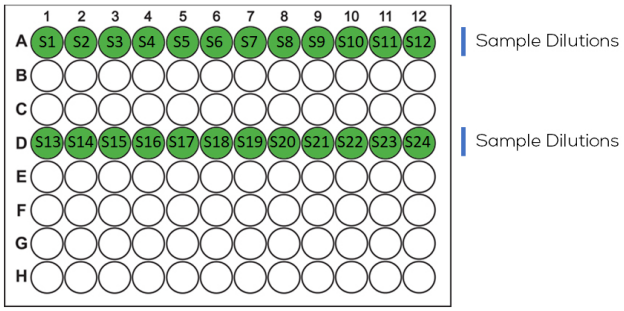
Load 2 µL of the LNP suspensions into the Stunner microwell plate as per the Stunner software client.

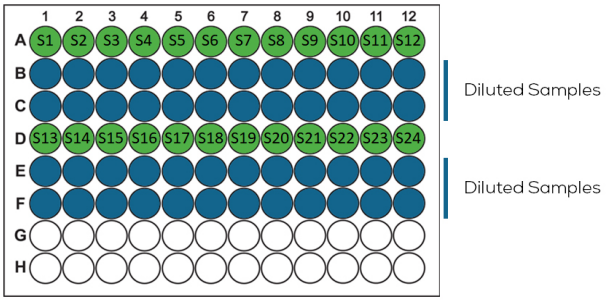
Run the experiment and collect your particle size information and payload concentration from the Stunner Analysis software.

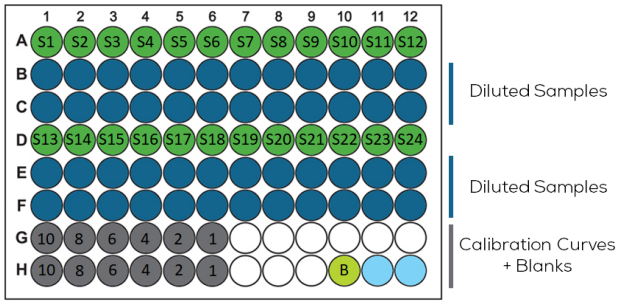
This will provide you with your total RNA content for your samples.

2.2 Free RNA Quantification with RiboGreen

To measure free RNA, dilute 10 µL of your samples in 240 µL of TE buffer in row A, and optionally row D (for 13+ samples), of a black, flat bottomed well plate.







Add 100 µL of your diluted sample into rows B and C, underneath the original sample dilutions (or rows E and F for samples 13-24).

Note: If samples are diluted off-plate, rows A and D can additionally be used as sample measurement wells, increasing plate throughput to 36 samples.

2.3 Calibration Curves and Blanks with RiboGreen

Construct a calibration curve in duplicate in rows G and H – dilute 10, 8, 6, 4, 2 and 0 µL of 20 µg/mL RNA stock to 100 µL in TE Buffer in wells G1-6 and repeat in wells H1-6.

For the blanks, in well H10, add 10 µL of your sample buffer only to 240 µL of TE buffer. Pipette 100 µL of this into wells H11 and H12. These are the sample blanks.

Stock concentrations for RiboGreen standards:

20 µg/mL RNA Stock to Add	TE buffer to add (µL)	Final RNA Conc. (µg/mL)
10	90	1
8	92	0.8
6	94	0.6
4	96	0.4
2	98	0.2
1	99	0.1

2.4 Preparation and Addition of RiboGreen Solution

To calculate the amount of RiboGreen reagent required, add up the total number of wells to be measured and multiply by 100 µL. For example, a full plate as constructed above would require 48 wells for the sample measurements, 12 for the calibration curves, and 2 for the blanks, giving a total of 62 wells, and therefore 6.2 mL of RiboGreen solution. Add 500 µL to account for wastage.

To make up the RiboGreen solution, dilute the RiboGreen dye 100:1 in 1x TE buffer and vortex to mix.

Add 100 µL of the diluted RiboGreen solution, to each well to be measured.

2.5 Sample Measurement

Incubate, protected from light, for 5 minutes at ambient temperature, and measure on a fluorescence plate reader, using the following settings.

Gain and read height will vary depending on your plate reader.

Plate reader settings:

Parameter	Setting
Excitation	485 nm
Emission	528 nm
Optics	Top Read

2.6 Sample Analysis

Subtract the average blank value from all calibration curves and sample wells. Plot the corrected calibration curve, setting the intercept as 0. Use the gradient to calculate the average concentration of each sample on the plate (RNAplate, Equation 1), accounting for the dilution (Equation 2):

1

$$\frac{Sample\ Fluorescence}{Calibration\ Curve\ Gradient} = RNA_{plate}$$

2

$$RNA_{plate}\ X\ 56 = RNA_{free}$$

The EE% can then be assessed using the equation (Equation 2):

3

$$\frac{(RNA_{total} - RNA_{free})}{RNA_{total}}\ x\ 100\% = EE\%$$

Where RNA_{total} is provided by the Stunner data, and RNA_{free} is provided by the RiboGreen assay.

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